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Use of Fluorinated Compounds To Detect Aromatic Metabolites from *m*-Cresol in a Methanogenic Consortium: Evidence for a Demethylation Reaction

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Anaerobic sewage sludge was used to enrich a methanogenic *m*-cresol-degrading consortium. 6-Fluoro-3-methylphenol was synthesized and added to subcultures of the consortium with *m*-cresol. This caused the accumulation of 4-hydroxy-2-methylbenzoic acid. In a separate experiment, the addition of 3-fluorobenzoic acid caused the transient accumulation of 4-hydroxybenzoic acid. Inhibition with bromoethanesulfonic acid caused the accumulation of benzoic acid. Thus, the proposed degradation pathway was *m*-cresol → 4-hydroxy-2-methylbenzoic acid → 4-hydroxybenzoic acid → benzoic acid. The *m*-cresol-degrading consortium was able to convert exogenous 4-hydroxybenzoic acid and benzoic acid to methane. In addition, for each metabolite of *m*-cresol identified, the corresponding fluorinated metabolite was detected, giving the following sequence: 6-fluoro-3-methylphenol → 5-fluoro-4-hydroxy-2-methylbenzoic acid → 3-fluoro-4-hydroxybenzoic acid → 3-fluorobenzoic acid. The second step in each of these pathways is a novel demethylation which was rate limiting. This demethylation reaction would likely facilitate the transformation of the methyl group to methane, which is consistent with the results of a previous study that showed that the methyl carbon of *m*-[*methyl*-^{14}C]cresol was recovered predominantly as [^{14}C]methane (D. J. Roberts, P. M. Fedorak, and S. E. Hrudey, Can. J. Microbiol. 33:335–338, 1987). The final aromatic compound in the proposed route for *m*-cresol metabolism was benzoic acid, and its detection in these cultures merges the pathway for the methanogenic degradation of *m*-cresol with those for the anaerobic metabolism of many phenols.

m-Cresol is one of the most abundant phenols in wastewaters from hydrocarbon processing and coal conversion processes such as coking, gasification, and liquefaction (15, 20, 30). It is also found at creosote-contaminated sites (10, 21). Anaerobic biodegradation of *m*-cresol has been demonstrated under nitrate-reducing (2, 46), sulfate-reducing (32, 42, 43), and methanogenic (7, 15, 34, 35, 42, 43) conditions. As well, Ehrlich et al. (10) and Goerlitz et al. (21) have provided evidence that it is biodegraded in contaminated aquifers by methanogenic consortia.

The study of the anaerobic degradation of *m*-cresol has been hampered by the relatively long acclimation time required to obtain active cultures (7, 33, 42) as well as by the difficulty of maintaining *m*-cresol-degrading cultures, because *m*-cresol degraders were inhibited by the 1 mM sulfide concentration typically used to reduce media for methanogenic cultures (34).

Numerous studies reviewed by Londry and Fedorak (26) have shown that under anaerobic conditions *p*-cresol is degraded by methyl group oxidation, leading to 4-hydroxybenzoic acid as an intermediate. However, the anaerobic metabolism of *m*-cresol is different, and no oxidation of the methyl group has been reported (26). Indeed, Roberts et al. (33) used *m*-[*methyl*-^{14}C]cresol and *p*-[*methyl*-^{14}C]cresol to determine the fates of the methyl carbons in methanogenic consortia. The majority (87%) of the label from *m*-cresol was converted to $^{14}CH_4$, whereas 92% of the label from *p*-[*methyl*-^{14}C]cresol was recovered as $^{14}CO_2$.

Roberts et al. (35) showed that 1 mol of bicarbonate was incorporated into *m*-cresol via a *para*-carboxylation to yield 4-hydroxy-2-methylbenzoic acid. This metabolite was radioactive when cultures were incubated with either *m*-[*methyl*-^{14}C]cresol or [^{14}C]bicarbonate and nonradioactive *m*-cresol. Similarly, Ramanand and Suflita (32) detected 4-hydroxy-2-methylbenzoic acid in their sulfate-reducing culture when bicarbonate was present in the medium. The *para*-carboxylation of *m*-cresol under both methanogenic and sulfate-reducing conditions resembles the *para*-carboxylation of phenol under these conditions (4, 40, 41, 49).

Roberts et al. (35) detected another aromatic metabolite, identified as 2-methylbenzoic acid, that appeared to be a dead-end product, likely originating from the dehydroxylation of 4-hydroxy-2-methylbenzoic acid. Ramanand and Suflita (31) also found trace amounts of 2-methylbenzoic acid in their cultures.

Welch (48) summarized a number of uses of fluorinated compounds in biological studies, including the fact that fluorine can be introduced into a biologically active molecule to block metabolism. Although fluorinated analogs have long been used to study a variety of metabolic processes under aerobic conditions (for a review, see reference 22), they have not been used extensively to study biotransformations that occur under anaerobic conditions. However, Sharak Genthner et al. (40, 41) used fluorophenols to study the degradation of phenol under methanogenic conditions. They found a conversion of 5 or 50 mg of 2- and 3-fluorophenol per liter to 3- and 2-fluorobenzoic acids, respectively, but no transformation of 5 or 50 mg of 4-fluorophenol per liter. They concluded that the pathway for phenol degradation involved an initial *para*-carboxylation followed by dehydroxylation to yield benzoic acid. Londry and Fedorak (27) also used fluorophenols to elucidate the degradation of phenol under methanogenic conditions. 2-Fluorophenol acted as both an inhibitor and an analog in the phenol-degrading cultures,

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leading to the accumulation of 3-fluoro-4-hydroxybenzoic acid, 3-fluorobenzoic acid, 4-hydroxybenzoic acid, and benzoic acid. One of these metabolites, 3-fluorobenzoic acid, caused the transient accumulation of 4-hydroxybenzoic acid and benzoic acid in phenol-degrading cultures. The fluorophenols were effective tools for studying phenol degradation because they inhibited phenol degradation and could also be transformed by these cultures.

The purpose of this investigation was to use fluorinated analogs of phenol and *m*-cresol to help elucidate the pathway of *m*-cresol degradation under methanogenic conditions.

MATERIALS AND METHODS

Culture methods. Two types of *m*-cresol-degrading cultures were used in this study. The enrichment cultures consisted of 40 ml of domestic anaerobic sewage sludge (Edmonton Gold Bar Wastewater Treatment Plant, Edmonton, Alberta, Canada) in 40 ml of O_2-free water in sealed 158-ml serum bottles. These were initially challenged with 250 mg of *m*-cresol per liter. After the *m*-cresol was degraded, the substrate was replenished to its original concentration by a draw-and-feed method (14). These cultures were maintained for 18 months by weekly replacements of 8 ml of mineral feed solution (14) containing 2,500 mg of *m*-cresol per liter and 0.5 mM Na_2S. In some experiments, test compounds were added directly to the enrichment cultures. In other experiments, subcultures of the enrichment cultures were used. For these experiments, a single enrichment culture was used to provide a constant inoculum to test the effects of different aromatic compounds, using the serum bottle culture method (13). Prior to inoculation, 5-ml portions of anaerobic medium (13), without 2-methyl-*n*-butyric acid, were added to 58-ml serum bottles that were being flushed with a mixture of 30% CO_2 and 70% N_2 that was scrubbed of O_2 by passage through a heated copper column. The serum bottles were sealed, autoclaved, and cooled, and then the medium was reduced by the addition Na_2S to a final concentration of 0.5 mM. Substrates were prepared separately and were added to a target concentration of 100 mg/liter. Five-milliliter portions of an enrichment culture that had been maintained on *m*-cresol for 37 to 80 weeks were used as inocula for the subculture experiments. Actual substrate concentrations at the time of inoculation were determined by an appropriate analytical method. For each test condition, triplicate cultures were prepared, and for each experiment designed to detect metabolites, parallel sterile controls were incubated and analyzed in the same manner as the test cultures. All cultures were incubated at 37°C in the dark without shaking.

In one experiment, inoculum was taken from a 10-liter, phenol-degrading, methanogenic culture that had been enriched from domestic anaerobic sewage sludge from the same treatment plant. At the time of this work, the phenol-degrading enrichment culture had been maintained for 16 months by daily feedings of 400 mg of phenol per liter.

To aid in the accumulation of metabolites, bromoethanesulfonic acid (BESA) was added to some cultures to a final concentration of 50 mM.

Analytical methods. Methane in the headspace was measured by gas chromatography (GC) (12), and gas volumes were measured with a pressure transducer (33).

Cultures were acidified to pH 2 with 10 M H_2SO_4 and extracted with three 25-ml portions of diethyl ether. The ether extracts were filtered through glass wool and anhydrous sodium sulfate to remove particulate matter and water and then were pooled and concentrated.

The following method was used to analyze the ether extracts and aqueous samples for residual substrates or intermediates. A series 1050 high-performance liquid chromatograph (HPLC) (Hewlett-Packard, Palo Alto, Calif.) with the detector set at 277 nm was used. The mobile phase was a mixture of methanol, water purified through a Milli-Q water purification system (Millipore Corp., Bedford, Mass.), and phosphoric acid (50:50:0.5). The mobile phase was pumped through a reverse-phase column (LiChrospher 100 RP-18; 5 mm, 125 by 4 mm; Hewlett-Packard) at the rate of 1 ml/min.

In some instances, aqueous samples of cultures and controls were analyzed for substrates and metabolites with a Waters M-45 HPLC (Waters Associates, Inc., Milford, Mass.) and a Lambda-Max model 480 LC spectrophotometer (Waters) set at 214 nm. The mobile phase of 0.010 M H_2SO_4 was run through an Aminex HPX-57H column (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) at 0.8 ml/min.

The concentrations of *m*-cresol and phenols in some cultures were determined by a direct aqueous injection GC method, using a 2-m stainless-steel column packed with 5% polymetaphenyl ether coated on Tenax GC (3). The column was housed in a Hewlett-Packard 5790 GC with a flame ionization detector. The column was maintained at 200°C with the carrier gas (N_2) flow at 30 ml/min.

To detect metabolites, ether extracts of cultures were injected into a Hewlett-Packard 5890A GC equipped with a flame ionization detector and a 30-m DB-5 fused silica capillary column (J&W Scientific, Folsom, Calif.). The oven temperature was initially held at 90°C for 4 min and then raised at 8°C/min to 250°C and held for 6 min.

To identify the unknown metabolites, the extracts were analyzed by GC-mass spectrometry (MS). A Hewlett-Packard 5890 series II GC and a Hewlett-Packard 5970 mass selective detector were used with the same oven temperature program described above.

Trimethylsilyl (TMS) derivatives of compounds in culture extracts were prepared with *N,O*-bis(trimethylsilyl)acetamide in acetonitrile by using the manufacturer's instructions (method 5; Pierce Chemicals, Rockford, Ill.). The derivatives were then analyzed by GC-MS and compared with TMS derivatives of known standards silylated the same way.

Studies with ^{14}C-labeled *m*-cresol. An 80-ml enrichment culture was fed 92 mg of *m*-cresol per liter and supplemented with 1.1×10^5 dpm of *m*-[U-*ring*-^{14}C]cresol (radiochemical purity, 98%; specific activity, 1.48 GBq/mmol; Amersham Corp., Arlington Heights, Ill.) and 120 mg of 6-fluoro-3-methylphenol per liter to cause the accumulation of hydroxybenzoic acids and other intermediates. After 2 days of incubation, BESA was added to further enhance the accumulation of metabolites. On several occasions, samples were taken and analyzed by using the Aminex HPLC column. Four-milliliter fractions of the column effluent were collected; these were added to 10 ml of Aqueous Counting Scintillant (Amersham). The amount of radioactivity in each fraction was determined in a model LS 3801 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Chemicals. 4-Fluoro-3-methylphenol, 3-fluoro-2-methylbenzoic acid, 2-, 3-, and 4-fluorophenol, *m*-cresol, and BESA were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. 4-Hydroxybenzoic acid and 2-methylbenzoic acid were purchased from Eastman Organic Chemicals, Rochester, N.Y., and Matheson Coleman & Bell, Norwood,

Ohio, respectively. Phenol and benzoic acid were obtained from Fisher Scientific Co., Fair Lawn, N.J.

4-Hydroxy-2-methylbenzoic acid was synthesized by the method of Cox (9). HPLC analysis showed that the final product contained a small amount of benzoic acid. 3-Fluoro-4-hydroxybenzoic acid was synthesized by the method of Ferguson et al. (16) and purified by a combination of ether extraction and bicarbonate washes of the ether extracts to remove unreacted oximes.

6-Fluoro-3-methylphenol (6-fluoro-*m*-cresol) was synthesized by adapting the method for synthesizing *p*-cresol from 4-aminotoluene (19). Briefly, 2-fluoro-5-methylaniline (Aldrich) was dissolved in hot 35% H_2SO_4 and then cooled and reacted with a solution of sodium nitrite and urea, yielding a diazonium salt. The diazo group was replaced by a hydroxyl group facilitated by the addition of copper(II) nitrate and copper(I) oxide in an aqueous solution. The final product that was extracted into ether and dried, giving a brown liquid product, was essentially pure as determined by HPLC, GC, and GC-MS. The ^1H nuclear magnetic resonance spectrum of the product agreed with that determined by Claudi et al. (8) and showed that a trace amount of ether remained in the preparation.

RESULTS

Characteristics of *m*-cresol-degrading cultures. In 16 of the 23 replicate 80-ml enrichment cultures, the lag times prior to *m*-cresol degradation were between 6 and 9 weeks. However, some cultures required more than 30 weeks to degrade their initial allotment of *m*-cresol. HPLC analyses of the supernatant obtained by the draw-and-feed procedure showed the presence of 4-hydroxy-2-methylbenzoic acid and 2-methylbenzoic acid in *m*-cresol-degrading cultures. There was a transient accumulation of 4-hydroxy-2-methylbenzoic acid in the enrichment cultures which reached a maximum at about 3 days after feeding, when *m*-cresol had been depleted to approximately one-half of its initial concentration. Although the rate of 2-methylbenzoic acid formation during the enrichment of these cultures was not studied, its concentration remained constant over many months while the cultures were being maintained on *m*-cresol by the draw-and-feed method.

An *m*-cresol-degrading enrichment culture was tested for its ability to degrade phenol and 4-hydroxybenzoic and benzoic acids, which are known intermediates of phenol degradation (26, 27). These were tested individually in subcultures without the addition of *m*-cresol. Each of the three compounds was completely metabolized to methane and carbon dioxide within 2 weeks. Whereas *m*-cresol was degraded without a lag to yield 100% ± 2% of the expected volume of methane in 4 days, 4-hydroxybenzoic acid had a 6-day lag, giving 91% ± 1% of the expected methane in 10 days; benzoic acid had a 7-day lag, giving 110% ± 3% of the expected methane in 13 days; and phenol had an 8-day lag, giving 87% ± 2% of the expected methane in 13 days.

After finding that the *m*-cresol-degrading enrichment cultures could degrade phenol and two of its metabolites, a portion of a phenol-degrading enrichment culture was tested for its ability to metabolize *m*-cresol. This culture was initially fed a mixture of 100 mg of phenol and 100 mg of *m*-cresol per liter. The phenol was degraded within a few days, and *m*-cresol degradation began within 2 weeks, yielding methane. This lag time prior to *m*-cresol degradation was much shorter than the 6- to 9-week lag times observed in the enrichment cultures inoculated with unacclimated sewage sludge.

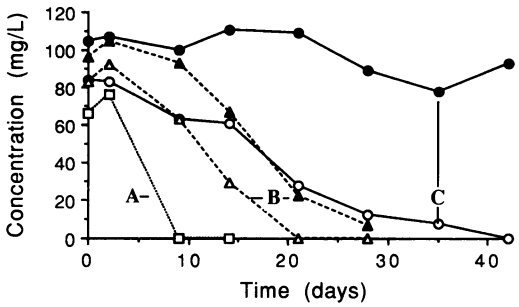

FIG. 1. Removal of phenols from three *m*-cresol-degrading subcultures: A, containing *m*-cresol (□) only; B, containing *m*-cresol (△) and phenol (▲); and C, containing *m*-cresol (○), phenol (●), and 100 mg of 2-fluorophenol per liter (not shown).

Effects of fluorophenols, 3-chlorophenol, and phenol on *m*-cresol degradation. Because the *m*-cresol-degrading enrichment cultures could degrade phenol and two of its metabolites, and because fluorophenols have been shown to be useful analogs for studies of phenol degradation in methanogenic cultures (27, 40), the effects of the three isomers of fluorophenol on *m*-cresol degradation were tested. Neither 2-fluorophenol nor 4-fluorophenol had any effect on *m*-cresol degradation when added at 100 mg/liter, and no fluorophenol transformation products were detected over the 6-week incubation period. Similarly, no transformation products were detected from 3-fluorophenol, but its presence at ≥25 mg/liter completely inhibited the degradation of *m*-cresol, which was present at 100 mg/liter. The observed inhibition by 3-fluorophenol prompted us to test the effect of 3-chlorophenol. At 100 mg/liter, this *meta*-substituted phenol also completely inhibited the metabolism of *m*-cresol. The severe inhibitory effects of 3-fluorophenol and 3-chlorophenol on *m*-cresol degradation indicate the importance of the nature of the substituent in the *meta* position.

Three sets of cultures containing approximately 80 mg of *m*-cresol per liter were used to test the effects of phenol (100 mg/liter) and a mixture of phenol and 2-fluorophenol (100 mg/liter each) on the rate of *m*-cresol degradation. At this concentration, 2-fluorophenol was found to completely inhibit phenol degradation (27). The substrate depletion curves are shown in Fig. 1. In the culture designated A, *m*-cresol was removed from the medium in 9 days. In the culture designated B, which contained phenol and *m*-cresol, the rate of *m*-cresol degradation was slowed, and *m*-cresol was detected in the medium for about 21 days. After 9 days of incubation, the concentration of phenol began to decrease in this culture, and its rate of degradation paralleled that of *m*-cresol. There was virtually no phenol degradation over the 42-day test period in the culture designated C, which contained 2-fluorophenol. The presence of these two phenols slowed the degradation rate of *m*-cresol in culture C.

Effects of fluorinated *m*-cresols on *m*-cresol-degrading cultures. When present at 100 mg/liter, 4-fluoro-3-methylphenol completely inhibited methane production from *m*-cresol. HPLC analyses showed no detectable decrease in *m*-cresol or 4-fluoro-3-methylphenol concentrations and no transformation products.

The addition of 95 mg of 6-fluoro-3-methylphenol per liter to cultures with 90 mg of *m*-cresol per liter slowed *m*-cresol degradation (Fig. 2). In a separate experiment, it was ob-

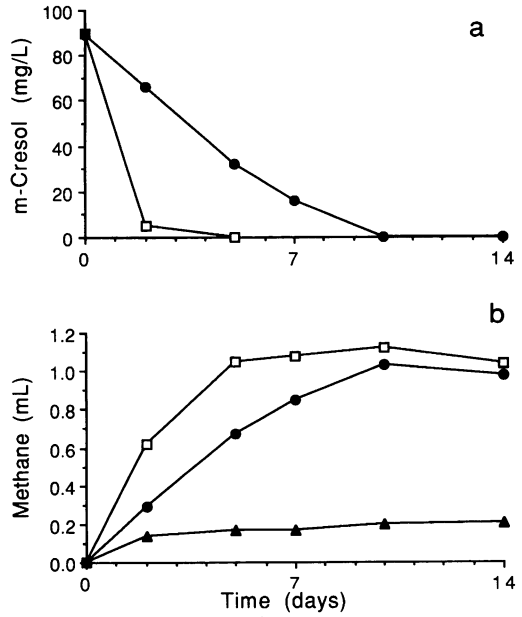

FIG. 2. *m*-Cresol depletion (a) and methane production (b) from *m*-cresol in the absence (□) and presence (●) of 94 mg of 6-fluoro-3-methylphenol per liter. ▲, methane in controls with 6-fluoro-3-methylphenol but no *m*-cresol.

served that over the concentration range of 0 to 94 mg of 6-fluoro-3-methylphenol per liter the rate of methane production decreased with increasing concentrations of 6-fluoro-3-methylphenol. A linear regression analysis of the rate of methane production versus the concentration of 6-fluoro-3-methylphenol gave an equation with a slope of −0.0011 (ml of methane/day)/(mg of 6-fluoro-3-methylphenol/liter) and a *y* intercept of 0.13 ml of methane per day. The correlation coefficient for this equation was −0.96. The regression equation predicted that there should be no methane production at 6-fluoro-3-methylphenol concentrations of >120 mg/liter. Indeed, no methane was produced in cultures that received 210 mg of 6-fluoro-3-methylphenol per liter. In addition, no appreciable decrease in the 6-fluoro-3-methylphenol concentration was detected in *m*-cresol-degrading cultures incubated with this analog in the presence or absence of *m*-cresol.

Detection and identification of metabolites from cultures containing 6-fluoro-3-methylphenol. Despite the small changes in the 6-fluoro-3-methylphenol concentrations in cultures, fluorinated metabolites were detected in the extracts from 6-fluoro-3-methylphenol-containing, *m*-cresol-adapted cultures. GC-MS analyses of *N,O*-bis(trimethylsilyl)acetamide-treated extracts from cultures that had received only the fluorinated analog showed two fluorinated transformation products. These metabolites were not detected in the sterile controls. On the basis of a comparison with an authentic standard, one metabolite was identified as 3-fluoro-4-hydroxybenzoic acid. The metabolite had the same HPLC retention time as the standard, and the TMS derivative of the metabolite had the same GC retention time and the same mass spectrum as the TMS derivative of authentic 3-fluoro-4-hydroxybenzoic acid (Fig. 3).

The mass spectrum of the TMS derivative of the second metabolite was virtually identical to that of 3-fluoro-2-methylbenzoic acid. However, the GC retention times of the TMS derivatives of the metabolite and 3-fluoro-2-methylbenzoic acid were not the same. Thus, the metabolite was another isomer of fluoro-2-methylbenzoic acid, and it was presumed to be 5-fluoro-2-methylbenzoic acid (see Discussion for more details).

GC-MS analyses of *N,O*-bis(trimethylsilyl)acetamide-treated extracts from cultures that contained both *m*-cresol and 6-fluoro-3-methylphenol showed the presence of nonfluorinated and fluorinated metabolites that were not found in the sterile controls. On the basis of their GC retention times and comparisons with authentic standards, the nonfluorinated metabolites were identified as 4-hydroxy-2-methylbenzoic acid and 2-methylbenzoic acid, as found in a previous study (35). The fluorinated metabolites found included 3-fluoro-4-hydroxybenzoic acid and the same isomer of fluoro-2-methylbenzoic acid described above. Figure 4 shows the mass spectrum of a TMS derivative of a metabolite having a molecular ion at *m/z* 314. This is 14 mass units greater than the molecular ion of the TMS derivative of 3-fluoro-4-hydroxybenzoic acid (Fig. 3), suggesting that the metabolite was an isomer of a methyl-substituted fluorohydroxybenzoic acid. Similarly, the molecular ion of the metabolite in Fig. 4 is 18 mass units greater that the molecular ion of the TMS derivative of 4-hydroxy-2-methylbenzoic acid (M^+ = 296), suggesting that the metabolite was an isomer of a fluoro-substituted methylhydroxybenzoic acid. Although no standard was available, it was presumed that the metabolite was 5-fluoro-4-hydroxy-2-methylbenzoic acid (see Discussion for more details).

Detection of metabolites in BESA-inhibited cultures. To aid in the detection of other metabolites of *m*-cresol degradation, BESA, a potent inhibitor of methanogenesis, was added to some cultures at the time of inoculation or, in some cases, after 2 days of incubation when approximately 20% of the *m*-cresol had been depleted from the medium. In addition, some cultures were incubated with 6-fluoro-3-methylphenol and *m*-cresol in the presence of BESA.

The addition of BESA to *m*-cresol-degrading cultures, without the fluorinated analog, caused the accumulation of a very small amount of a metabolite that was identified as benzoic acid. The TMS derivative of the metabolite had the same GC retention time and mass spectrum as that of the TMS derivative of authentic benzoic acid (Fig. 5). Also, the presence of BESA caused the accumulation of acetate, but no other fatty acids were detected by the HPLC method with the Aminex column.

When BESA was added at the time of inoculation to *m*-cresol-degrading cultures that contained 100 mg of *m*-cresol and 100 mg of 6-fluoro-3-methylphenol per liter, benzoic acid was again detected. Cultures that were prepared in the same manner but received BESA 2 days after inoculation produced a trace amount of another metabolite. GC-MS analysis of the *N,O*-bis(trimethylsilyl)acetamide-treated extract showed a compound with a weak molecular ion at *m/z* 212. This, along with other characteristic ions, $(M-15)^+$ at *m/z* 197 and the TMS ion at *m/z* 73, suggested that the compound was the TMS derivative of 3-fluorobenzoic acid. However, the trace amount of metabolite present and its poor GC separation from another compound prevented its positive identification.

4-Hydroxybenzoic acid as a metabolite of *m*-cresol. Cultures that received 6-fluoro-3-methylphenol produced a metabolite that was tentatively identified as 5-fluoro-4-hydroxy-2-methylbenzoic acid. The demethylation of this compound would yield 3-fluoro-4-hydroxybenzoic acid, which was positively

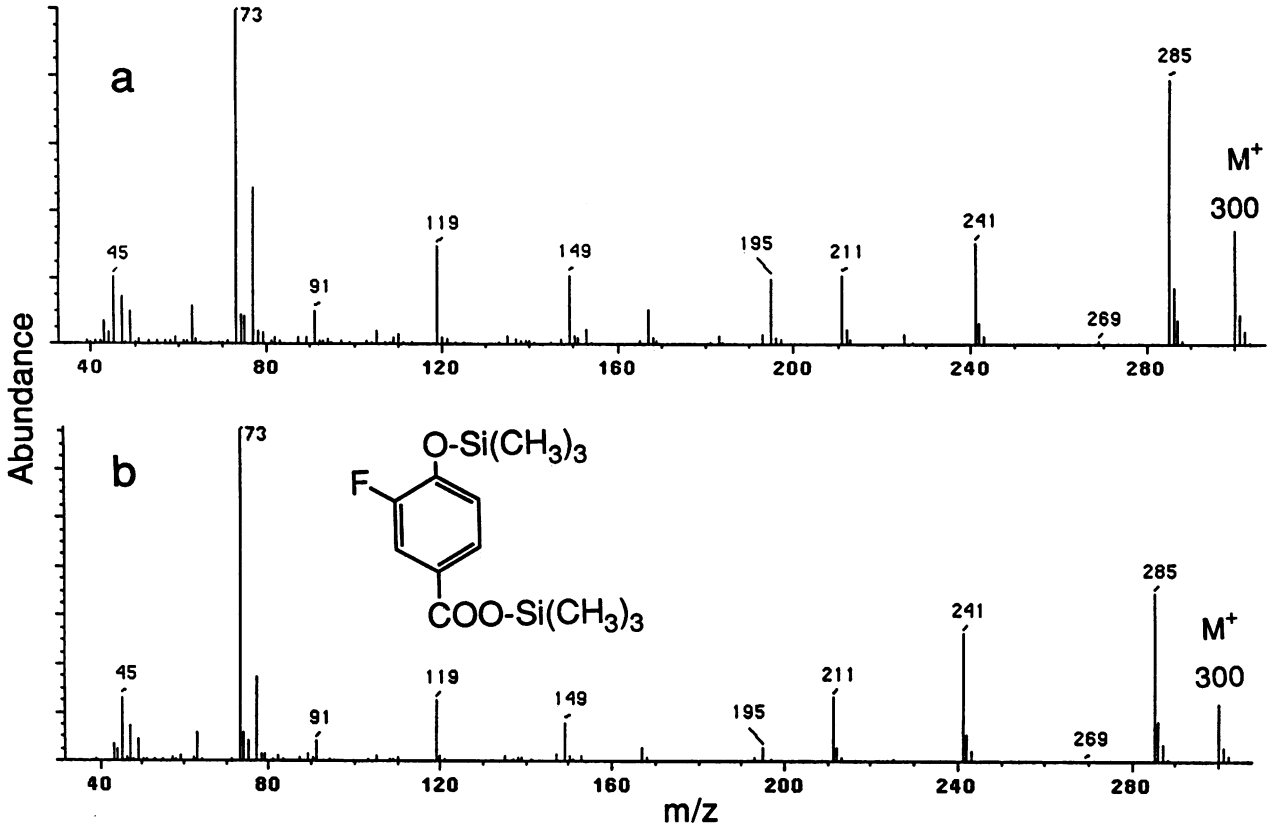

FIG. 3. From GC-MS analyses, mass spectra of a TMS-derivatized metabolite from 6-fluoro-3-methylphenol (a) and of the TMS derivative of 3-fluoro-4-hydroxybenzoic acid (b).

identified in the methanogenic consortium. The detection of benzoic acid in *m*-cresol-degrading cultures suggested that 4-hydroxy-2-methylbenzoic acid could be demethylated to 4-hydroxybenzoic acid, which was subsequently dehydroxylated to give benzoic acid. However, 4-hydroxybenzoic acid was not detected in any of the above-mentioned cultures. Thus, three approaches were tried to determine whether 4-hydroxybenzoic acid was an intermediate in *m*-cresol degradation.

First, ^{14}C-labeled *m*-cresol was used in an attempt to detect ^{14}C-labeled 4-hydroxybenzoic acid in cultures inhibited by 6-fluoro-3-methylphenol and BESA. HPLC analysis with the Aminex column showed that, over a 5-day incubation period, the concentration of *m*-cresol decreased, as did the amount of radioactivity in the fractions collected from the *m*-cresol peak. At the time of inoculation, the amount of radioactivity associated with the *m*-cresol peak corresponded to 1.1×10^5 dpm in the 80-ml culture. After 5 days of incubation, the amount of radioactivity associated with this peak decreased to 5.7×10^4 dpm. During this time, there was also an increase in the peak area of a radioactive compound that eluted at the same retention time (80 min) as

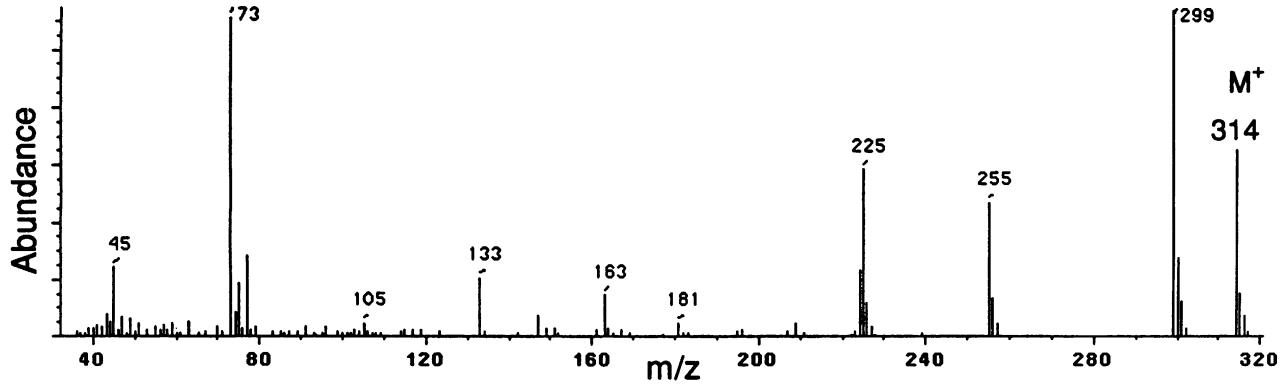

FIG. 4. From GC-MS analyses, the mass spectrum of the TMS derivative of a metabolite presumed to be 5-fluoro-4-hydroxy-2-methylbenzoic acid.

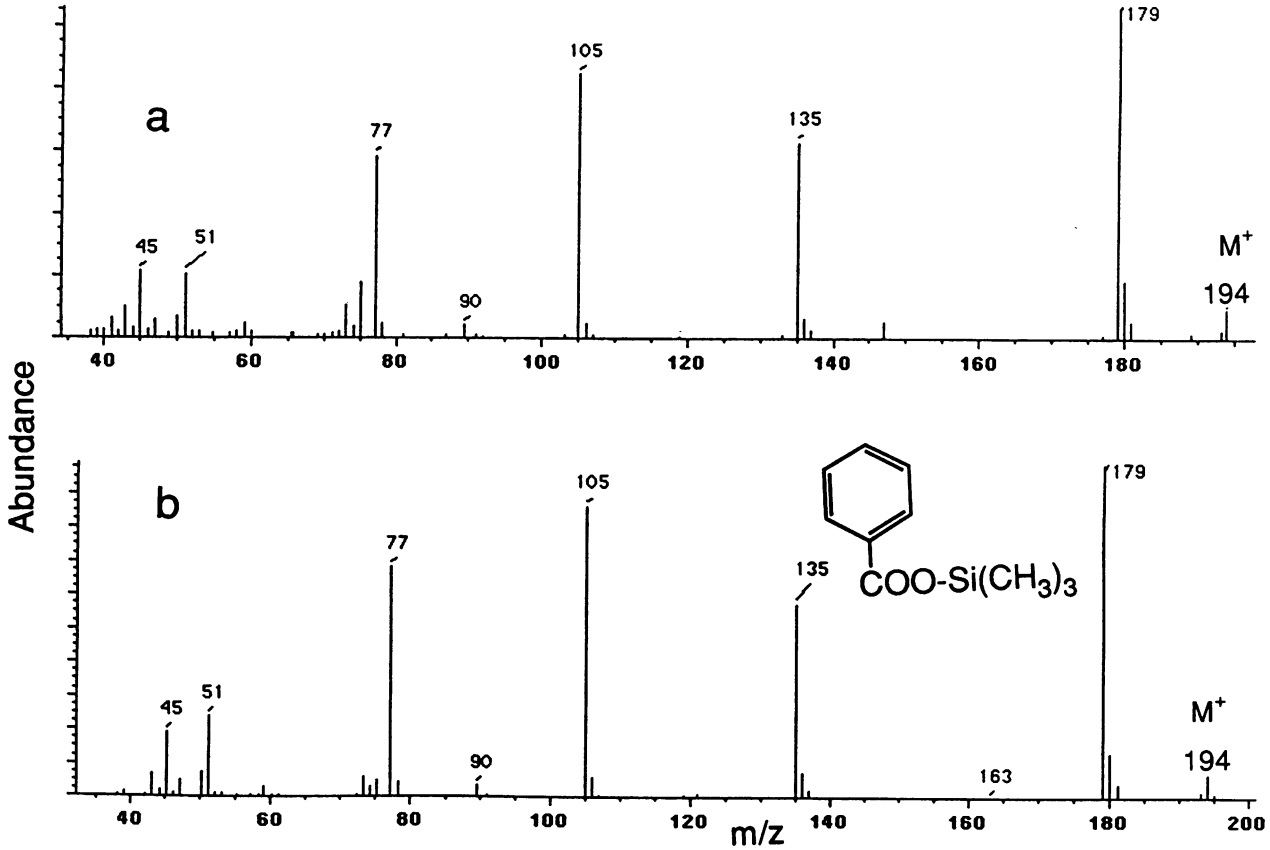

FIG. 5. From GC-MS analyses, mass spectra of a TMS-derivatized metabolite from a BESA-inhibited, *m*-cresol-degrading culture (a) and of the TMS derivative of benzoic acid (b).

4-hydroxybenzoic acid. After 5 days of incubation, the amount of radioactivity in this peak corresponded 3.4×10^4 dpm in the 80-ml culture. Thus, approximately 64% of the decrease in radioactivity from the *m*-cresol peak was present in the peak corresponding to 4-hydroxybenzoic acid. Radioactive acetic acid was also detected, and it accounted for 4% of the amount of *m*-cresol transformed over the 5-day period.

Second, 100 mg of 3-fluorobenzoic acid per liter was added to an 80-ml *m*-cresol-degrading enrichment culture that was fed 100 mg of *m*-cresol per liter. In a previous study (27), 3-fluorobenzoic acid caused the accumulation of 4-hydroxybenzoic acid in a phenol-degrading methanogenic consortium. On the basis of reverse-phase HPLC analyses of the *m*-cresol-degrading culture, there were transient accumulations of 4-hydroxy-2-methylbenzoic and 4-hydroxybenzoic acids over two feeding cycles. The former metabolite reached a maximum concentration of 12 mg/liter, whereas the latter reached a concentration of 1 mg/liter.

Third, an *m*-cresol-degrading enrichment culture was fed 80 mg of *m*-cresol per liter and was supplemented with 80 mg of 4-hydroxybenzoic acid per liter. The concentrations of these two compounds were monitored by HPLC analyses, and it was observed that 4-hydroxybenzoic acid was preferentially consumed (Fig. 6). The results of these three experiments clearly indicate that the 4-hydroxybenzoic acid is an intermediate in the *m*-cresol biodegradation pathway.

DISCUSSION

The 6- to 9-week lag times required to obtain active *m*-cresol-degrading anaerobic cultures are typical of those found in other studies. For example, lag times of 42 to 63 days (34) and 46 to 90 days (42) were observed under methanogenic conditions, and a lag time of 43 days (42) was observed under sulfate-reducing conditions. Interestingly, when the methanogenic phenol-degrading enrichment culture was fed a mixture of phenol and *m*-cresol, it rather quickly adapted to *m*-cresol degradation within 2 weeks after the depletion of the phenol. Fedorak (11) observed that

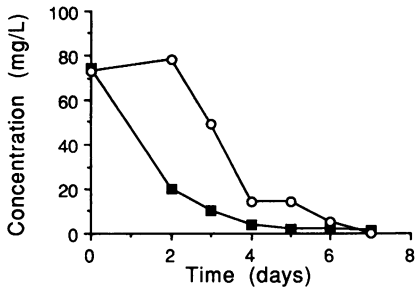

FIG. 6. Depletion of *m*-cresol and 4-hydroxybenzoic acid from an *m*-cresol-degrading enrichment culture fed *m*-cresol (○) and 4-hydroxybenzoic acid (■).

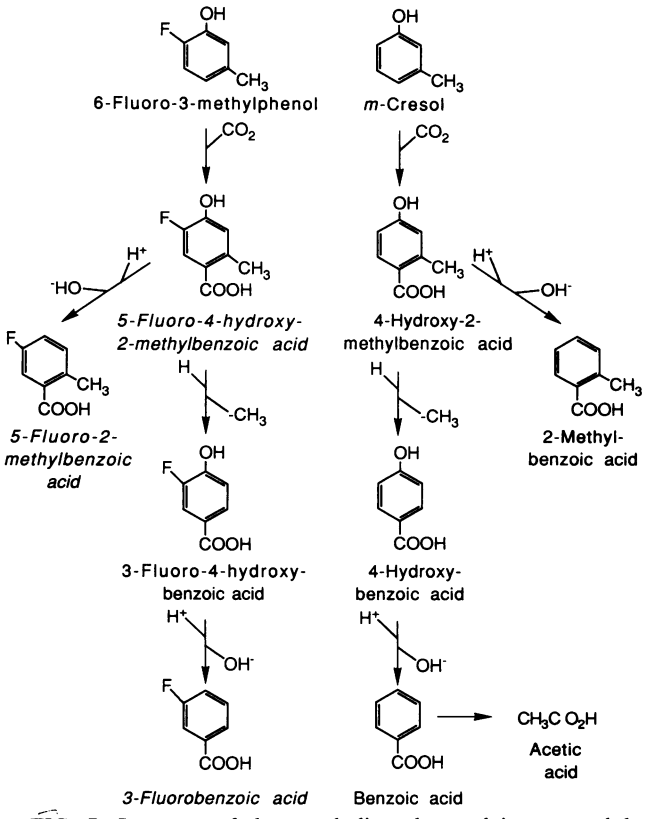

FIG. 7. Summary of the metabolites detected in *m*-cresol-degrading methanogenic cultures grown under different conditions and the proposed pathways leading to the formation of these metabolites. All but four metabolites were positively identified by GC-MS; the three exceptions are printed in italics. No authentic standards were available to confirm the proposed isomeric structures of 5-fluoro-4-hydroxy-2-methylbenzoic acid and 5-fluoro-2-methylbenzoic acid. The metabolite that was likely 3-fluorobenzoic acid was not present in sufficient quantity for positive identification. 4-Hydroxybenzoic acid was identified on the basis of its HPLC retention times, using two different columns.

m-cresol-degrading activity occurred about 20 days sooner in methanogenic sewage sludge enrichment cultures given a mixture of phenol and *m*-cresol compared with enrichment cultures that received only *m*-cresol.

The *m*-cresol-degrading cultures described in this study were not tested for their ability to degrade 2-methylbenzoic acid, because repeated attempts in our laboratory (35) failed to find this activity in *m*-cresol-degrading methanogenic consortia. However, Londry and Fedorak (28) recently demonstrated that the microbial population in fresh anaerobic sewage sludge samples could degrade 2-methylbenzoic acid after lag times of 12 to 36 weeks.

Under methanogenic conditions, the initial transformation of phenol and *m*-cresol is ring carboxylation at the position *para* to the hydroxyl group (for a review, see reference 26). Phenols with a fluorine atom in their *para* position cannot be carboxylated, and their presence inhibits transformation of an unfluorinated phenol. For instance, previous studies (27, 40) showed that 4-fluorophenol completely inhibited phenol degradation, and this study showed that 4-fluoro-3-methylphenol completely inhibited *m*-cresol degradation.

The proposed pathway for *m*-cresol degradation under methanogenic conditions is shown on the right side of Fig. 7. Each of the metabolites shown was detected and identified by one or more of the analytical methods used. None of the metabolites shown in Fig. 7 was found in sterile controls. The ease with which 4-hydroxy-2-methylbenzoic acid could be detected as a transient intermediate indicated that the *para*-carboxylation of *m*-cresol was not the rate-limiting step in these cultures. Rather, the subsequent step, the metabolism of the 4-hydroxy-2-methylbenzoic acid, appeared to be rate limiting. This step appears to involve a demethylation yielding 4-hydroxybenzoic acid, which was the most elusive intermediate. It was first found when radioactive *m*-cresol was used in cultures that contained 6-fluoro-3-methylphenol and BESA to inhibit *m*-cresol degradation. 4-Hydroxybenzoic acid was preferentially degraded in an enrichment culture that received both *m*-cresol and 4-hydroxybenzoic acid. In subcultures, it was degraded with a shorter lag time than benzoic acid or phenol. The same sequence of degradation was observed in methanogenic *m*-cresol-degrading enrichment cultures from a river sediment (24). In addition, 4-hydroxybenzoic acid was found in cultures that were supplemented with 3-fluorobenzoic acid. Dehydroxylation of 4-hydroxybenzoic acid would yield benzoic acid, which is a common intermediate in the anaerobic metabolism of many phenols (26).

Surprisingly, the lag times for the *m*-cresol-degrading culture to use the intermediates 4-hydroxybenzoic acid and benzoic acid added individually without *m*-cresol in the medium were longer than that required to use *m*-cresol. These lag times were 6, 7, and <1 day, respectively. When *m*-cresol was present in the medium with 4-hydroxybenzoic acid, the metabolite was consumed more rapidly than *m*-cresol (Fig. 6). These results suggest that the transport of 4-hydroxybenzoic acid into the microbial cell is facilitated by the presence of *m*-cresol. Experiments with a mixture of benzoic acid and *m*-cresol in *m*-cresol-degrading cultures were not done.

The left side of Fig. 7 shows the parallel transformation of 6-fluoro-3-methylphenol, a fluorinated analog of *m*-cresol. For each metabolite of *m*-cresol, a corresponding fluorinated metabolite was detected. In some instances, authentic standards were not available so the metabolites could not be positively identified. 5-Fluoro-4-hydroxy-2-methylbenzoic acid is an example of one such compound. The mass spectrum of the TMS derivative of this metabolite (Fig. 4) is consistent with it being a fluorohydroxymethylbenzoic acid. The proposed isomer would arise from the *para*-carboxylation of 6-fluoro-3-methylphenol. Subsequent demethylation of 5-fluoro-4-hydroxy-2-methylbenzoic acid (as was observed for 4-hydroxy-2-methylbenzoic acid) would yield 3-fluoro-4-hydroxybenzoic acid, which was positively identified by comparison with an authentic standard.

Among the metabolites detected was 2-methylbenzoic acid, which has been described as a dead-end product (35). This compound would result from the dehydroxylation of 4-hydroxy-2-methylbenzoic acid. With 6-fluoro-3-methylphenol in the *m*-cresol-degrading cultures, a fluorinated methylbenzoic acid was observed. Although no corresponding standard was available, the identity of this metabolite was most likely 5-fluoro-2-methylbenzoic acid, which would arise from the dehydroxylation of the tentatively identified 5-fluoro-4-hydroxy-2-methylbenzoic acid (Fig. 7).

The *m*-cresol-degrading culture had dehydroxylating activity which produced benzoic acid from 4-hydroxybenzoic acid, and the formation of 2-methylbenzoic acid was likely the result of a premature dehydroxylation. Because 4-hydroxy-2-methylbenzoic acid transiently accumulated in

these cultures, it likely resided long enough to be susceptible to a nonspecific dehydroxylation yielding 2-methylbenzoic acid.

Previously, several studies attempted to identify metabolites in the anaerobic degradation of *m*-cresol (32–34, 36, 43, 46). Some of these investigations (32, 36, 43) addressed the possibility of methyl group oxidation leading to a carboxylic acid. This mechanism has been observed for *p*-cresol under nitrate-reducing (6), sulfate-reducing (43), iron-reducing (29), and methanogenic (39) conditions and for *o*-cresol under sulfate-reducing conditions (43). To date, there has been no evidence for methyl group oxidation during *m*-cresol degradation.

The proposed pathway for *m*-cresol degradation shown in Fig. 7 includes the demethylation of 4-hydroxy-2-methylbenzoic acid to give 4-hydroxybenzoic acid. This is a novel mechanism for the anaerobic degradation of aromatic compounds in which a C—C bond is broken. However, demethylations of hopanes in petroleum reservoirs have been attributed to microbial action over geological time (37, 45, 47). In these cases, C—C bonds are broken, but the mechanism of this biotransformation has not been elucidated nor have laboratory biodegradation studies produced demethylated hopanes (45).

In contrast, there are numerous reports of anaerobic demethylation of methoxy groups in which a C—O bond is broken. For example, the demethylation of several hydroxymethoxybenzoic acids was demonstrated with mixed cultures of rat intestinal microflora (38). Bache and Pfennig (1) showed that three strains of *Acetobacterium woodii* grew on 10 different methoxy-aromatic compounds, and they concluded that the de- or transmethoxylation of these substrates occurred inside the cell and that methanol does not become available to other bacteria. Kaiser and Hanselmann (23) also observed this transformation of methoxybenzoic acids in a methanogenic consortium. Under nitrate-reducing conditions, *Pseudomonas* sp. strain PN-1 demethylated vanillate, ferulate, guaiacol, 3,4,5-trimethoxycinnamate, and 3,4,5-trimethoxybenzoate (44). Frazer and Young (18) reported the demethylation of *O*-[*methyl*-^{14}C]vanillic acid by an anaerobic acetogen that yielded $^{14}CO_2$ and $^{14}CH_3COOH$.

The mechanism of the methyl group removal from 4-hydroxy-2-methylbenzoic acid (Fig. 7) and the fate of the methyl group were not determined. However, it is likely that the methyl group was transformed to methane, because in a previous study (33) with *m*-[*methyl*-^{14}C]cresol, 87% of the label was recovered as $^{14}CH_4$. In addition, Kaiser and Hanselmann (23) concluded that the methyl groups of syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid) and 3,4,5-trimethoxybenzoic acid were converted to methane prior to ring cleavage in cultures inoculated with anaerobic mud.

From studies with a methanogenic consortium, Fina and Fiskin (17) demonstrated that the ^{14}C-labeled carboxyl carbon of benzoic acid was released as $^{14}CO_2$. Using similar culture conditions, Keith et al. (25) concluded that after ring reduction and cleavage the carboxyl group of benzoic acid became the carboxyl group of acetate, which was subsequently released as carbon dioxide. The *para*-carboxylation of *m*-cresol with $^{14}CO_2$ also yielded acetate with the carboxyl group labeled (35). On the basis of the last observation and the appearance of $^{14}CH_4$ from *m*-[*methyl*-^{14}C]cresol, Roberts et al. (35) hypothesized that ring fission occurred between C-1 and C-2 of 4-hydroxy-2-methylbenzoic acid, producing a C_8 carboxylic acid that underwent β-oxidation. The pathway given in Fig. 7 is much different from the earlier hypothesis (35), but the fates of the methyl and carboxyl carbons of 4-hydroxy-2-methylbenzoic acid would most likely be methane and the carboxyl group in acetate, respectively, as found previously (35).

o-Cresol has been shown to undergo *para*-carboxylation under methanogenic (5) conditions and by a pure culture under nitrate-reducing conditions (36) to form 4-hydroxy-3-methylbenzoic acid. In contrast to the demethylation of 4-hydroxy-2-methylbenzoic acid from *m*-cresol that we have observed, 4-hydroxy-3-methylbenzoic acid was dehydroxylated and 3-methylbenzoic acid was detected in the methanogenic consortium (5), and the coenzyme A thioester of 3-methylbenzoic acid was observed in the nitrate-reducing culture (36). The fate of the methyl carbon of *o*-cresol was not specifically addressed in the two investigations cited.

Our results also showed that consortia enriched on either phenol or *m*-cresol can convert the other compound to methane, but only after a lag period of several days. For example, when phenol was the only substrate given to an *m*-cresol-degrading enrichment, methane was observed after an 8-day lag time. Similarly, when *m*-cresol was the only substrate given to a phenol-degrading enrichment, *m*-cresol degradation began after a 2-week lag period. Also, when both substrates were added to the *m*-cresol-degrading consortium (culture B, Fig. 1), *m*-cresol degradation was slowed and there was a lag before phenol degradation began. In cultures that received 2-fluorophenol, phenol, and *m*-cresol (culture C, Fig. 1), phenol degradation ceased but *m*-cresol degradation occurred at a rate that was slower than observed in the other cultures, presumably because of the constant high phenol concentration. These results suggest that different microorganisms are responsible for the *m*-cresol- and phenol-degrading activities in these consortia and that they survive during the long-term maintenance of the consortia with the other substrate. This survival may be facilitated by the formation of 4-hydroxybenzoic acid and benzoic acid as intermediates in both the phenol and proposed *m*-cresol degradation pathways.

There are some striking similarities between the metabolism of *m*-cresol under methanogenic conditions (35) and that under sulfate-reducing conditions (31, 32). For example, 4-hydroxy-2-methylbenzoic and 2-methylbenzoic acids were found under both conditions. Also, Ramanand and Suflita (32) reported that the addition of 4-hydroxybenzoic acid to their sulfate-reducing consortium inhibited *m*-cresol metabolism such that the former compound was metabolized prior to the latter. This is consistent with the results obtained from our methanogenic consortium (Fig. 6). Thus, it is quite possible that the novel demethylation reported here may also occur under sulfate-reducing conditions.

The use of the fluorinated compounds proved to be very valuable in the elucidation of a pathway for *m*-cresol degradation under methanogenic conditions. 6-Fluoro-3-methylphenol was especially useful because the rate of *m*-cresol degradation could be controlled by the concentration of the fluorinated analog supplemented to the medium. In addition, for each metabolite of *m*-cresol identified, the corresponding fluorinated metabolite was detected. These methods revealed a novel demethylation reaction that transformed 4-hydroxy-2-methylbenzoic acid to 4-hydroxybenzoic acid. The detection of the latter metabolite was facilitated by the addition of 3-fluorobenzoic acid to the culture. The final aromatic compound in the proposed route for *m*-cresol metabolism was benzoic acid, and its detection in these cultures merges the pathway for the methanogenic degradation of *m*-cresol with those for the anaerobic metabolism of many phenols.

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