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The anaerobic biodegradation of *o*-, *m*- and *p*-cresol by sulfate-reducing bacterial enrichment cultures obtained from a shallow anoxic aquifer

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SUMMARY

Sulfate-reducing bacterial enrichments were obtained from a shallow anoxic aquifer for their ability to metabolize either *o*-, *m*-, or *p*-cresol. GC/MS and simultaneous adaptation experiments suggested that the anaerobic decomposition of *p*-cresol proceeds by the initial oxidation of the aryl methyl group to form *p*-hydroxybenzoic acid. This intermediate was then converted to benzoic acid. Benzoic acid and a hydroxybenzaldehyde were also found in spent culture fluids from an *o*-cresol-degrading enrichment culture. This result, in addition to others, suggested that *o*-cresol may also be anaerobically degraded by the oxidation of the methyl substituent. An alternate pathway for anaerobic *m*-cresol decomposition might exist. Enrichment cultures obtained with either *p*- or *o*-cresol degraded both of these substrates but not *m*-cresol. In contrast, a *m*-cresol enrichment culture did not metabolize the *ortho* or *para* isomers. Anaerobic biodegradation in all enrichment cultures was inhibited by molybdate and oxygen, and was dependent on the presence of sulfate as a terminal electron acceptor. The stoichiometry of sulfate-reduction and substrate depletion by the various enrichment cultures indicated that the parent cresol isomers were completely mineralized. This result was confirmed by the conversion of ^{14}C-labeled *p*-cresol to $^{14}CO_2$. These results help clarify the fate of alkylated aromatic chemicals in anoxic aquifers.

INTRODUCTION

Substituted benzenoid compounds are known pollutants of anoxic subsurface environments, but relatively little information is available on the sus-

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ceptibility of such materials to anaerobic microbial degradation. Alkylated aromatic substrates such as the cresols, xylenols and ethyl phenol isomers have been found as ground water contaminants associated with a variety of land use practices including underground coal gasification [16], creosote waste disposal [6,10] and landfilling [22].

The cresols are the simplest alkyl phenols but different isomers seem to vary in their anaerobic biodegradability and pathways of decomposition. Godsy and co-workers [9] found that *o*-cresol and *m*-cresol degraded while being transported through anoxic portions of a contaminated aquifer, but only the latter was mineralized in laboratory experiments inoculated with contaminated well water. Goerlitz and co-workers [8,10] found field evidence that all cresols were anaerobically biodegraded, but the *ortho* isomer was the most persistent of the isomeric series since it migrated the greatest distance before removal was noted. When laboratory digesters were inoculated with contaminated well water, decomposition of *m*- and *p*-cresol started on day 11 while *o*-cresol disappearance lagged for over 60 days [8]. A similar pattern of selective degradation was noted for cresol isomers in anoxic aquifer slurries held under sulfate-reducing or methanogenic conditions [25]. The *ortho* isomer was the most persistent and the *para* isomer was the most susceptible to anaerobic metabolism under both incubation conditions. The persistence of *o*-cresol and the metabolism of the *meta* and *para* isomers was also noted in denitrifying laboratory columns containing aquifer sediments [20].

While little information is available on the anaerobic biodegradation pathways for *o*- and *m*-cresol, several investigations have probed the fate of the *para* isomer. Studies of *p*-cresol degradation under denitrifying [2,3,28], sulfate-reducing [25] and methanogenic [23] conditions all suggest that the aryl methyl group of this substrate is oxidized to form *p*-hydroxybenzoic acid as a metabolic intermediate. Presumably the mechanism in each case involves the incorporation of water into the methyl substituent by a methylhydroxylase in analogous fashion to that previously shown for an aerobically grown *p*-cresol-degrading pseudomonad [14]. However, when Roberts et al. [21] examined the fates of ^{14}C-methyl labeled isomers in cultures incubated under methanogenic conditions, they found that the methyl group of *p*-cresol was oxidized to CO_2 whereas parallel experiments with *m*-cresol indicated that the majority of the label was found as CH_4. The former result is consistent with the methyl oxidation pathway while the latter suggests a fundamentally different mechanism for *m*-cresol metabolism. Similarly, Tschech and Fuchs [28] found that anaerobic *m*-cresol degradation by a denitrifying pseudomonad seemed not to proceed by methyl group oxidation.

In previous studies [25], we found that cresol isomers were more readily degraded in aquifer samples under sulfate-reducing rather than methanogenic conditions. We suggested a pathway for the anaerobic metabolism of *p*-cresol under sulfate-reducing conditions based on (i) the dependency of the degradation activity on sulfate, (ii) the inhibitory effect of oxygen and molybdate anion on substrate metabolism, (iii) the detection of oxidized intermediates by HPLC co-chromatography and ultraviolet spectral comparison with authentic chemicals, and (iv) the simultaneous adaptation of aquifer microorganisms to the utilization of the proposed metabolic intermediates. The purpose of this study was to enrich and compare the microorganisms responsible for the metabolism of *o*-, *m*-, and *p*-cresol under sulfate-reducing conditions. In addition, we wished to further probe further the pathways for anaerobic bioconversion of these substrates. Results confirm and extend the methyl group oxidation pathway suggested previously for *p*-cresol and preliminary findings suggest that a similar mechanism may also exist for *o*-cresol. However, experiments with the *m*-cresol enrichment suggested that an alternate mechanism for the anaerobic biodegradation of this substrate probably exists.

MATERIALS AND METHODS

Enrichments

Bacteria were enriched from the sulfate-reducing

portion of an anoxic aquifer [25] for their ability to metabolize either *o*-, *m*-, or *p*-cresol. The enrichments were grown in an RAMM mineral salts medium [24] supplemented with 20 mM Na_2SO_4 and the individual cresol isomers as a carbon source. The enrichments were maintained for 2–3 years by making periodic 20–30% inoculum transfers into fresh medium. Either *o*-, *m*- or *p*-cresol at 100–500 μM was added to the enrichments from a concentrated stock solution initially and whenever the substrate was depleted as indicated by HPLC analysis. Enrichment cultures were grown in sealed serum bottles and incubated in the dark at room temperature. Strict anaerobic techniques [17] were employed for all media preparation, culturing and sampling procedures. Once it was reduced, the resazurin indicator in the RAMM medium remained colorless throughout the incubation period. The headspace gas composition of the serum bottles was initially adjusted to 80% N_2/20% CO_2

Characterization of cresol-degrading organisms

To characterize cresol metabolism, the various enrichment cultures were tested for their response to a variety of metabolic inhibitors, alternate electron acceptors and hydrogen. The stoichiometry of sulfate consumption and substrate specificity of the enrichment cultures were also compared. For studies with inhibitors and different electron acceptors, the cells from enrichment cultures were harvested anaerobically by centrifugation (11 000 × *g* for 20 min), washed three times with reduced mineral medium that was not supplemented with sulfate, concentrated 10–50-fold and subdivided into serum bottles while anaerobic conditions were maintained. Substrate depletion of either *o*-, *m*-, or *p*-cresol (75–375 μM) was then measured by HPLC in the absence and presence of the particular treatment. The timescale of the experiments was governed by the speed at which the various enrichment cultures degraded the respective cresol substrates. Generally, experiments were continued until >75% of the initial amendment of electron donor was consumed. The inhibitors included 2.0 mM Na_2MoO_4 or 2-bromoethanesulfonic acid (BESA). The effect of hydrogen or oxygen on cresol metabolism was tested with the enrichments by replacing the headspace of the serum bottles with 80% H_2/20% CO_2 (2 atm) or 100% O_2, respectively.

The influence of various sulfur oxyanions as potential electron acceptors on cresol metabolism was also evaluated with harvested, washed and concentrated (10–25-fold) cells. Cresol metabolism by cells from enrichment cultures was then compared to treatments receiving no electron acceptor or 10 mM of either Na_2SO_4, $Na_2S_2O_3$, or Na_2SO_3. The influence of 10 mM $NaNO_3$ was similarly evaluated.

The substrate specificity and stoichiometry of sulfate consumption by the enrichments was tested with washed cell preparations. Sulfate served as the terminal electron acceptor and each enrichment culture was tested with 200–400 μM of *o*-, *m*- or *p*-cresol. In experiments designed to establish the relationship between sulfate depletion and cresol disappearance, the enrichment cells were concentrated 40–50-fold and the sulfate concentration in the mineral medium was reduced to 500–800 μM. This made it easier to more accurately quantitate the amount of sulfate loss from the medium by HPLC. The amounts of sulfate and cresol in the enrichment cultures were measured at various time points and subsequently plotted against each other on a linear axis. The slope of the linear portion of these curves was used to determine the sulfate consumed per amount of substrate utilized by the cultures. All treatments were performed in duplicate while autoclaved and/or filter-sterilized cultures served as negative controls. No sulfate depletion could be detected in cresol-unamended or autoclaved controls.

Pathways of cresol decomposition

The metabolic fate of *p*-cresol was examined with the culture enriched on this compound with [*ring*-^{14}C]-labeled substrate. The radiopurity of the *p*-cresol was found to be about 43% so it was purified before use by thin layer chromatography (silica gel plates; benzene/dioxane/acetic acid, 90:25:4, v/v). An analysis of the purified *p*-cresol by HPLC revealed only a single peak. Fifteen milliliters of the *p*-cresol enrichment was transferred aseptically to a sterile anaerobic serum bottle and spiked with a mixture of labeled and unlabeled *p*-cresol to give a

final concentration of 115 μM and a total of 3.9 $\times$ 10^6 dpm. Aliquots from the enrichment were periodically centrifuged and analyzed by HPLC. Fractions of the HPLC effluent were collected at 1-min intervals and counted in a liquid scintillation counter (Beckman model LS1801, Fullerton, CA). At the end of the incubation period, an aliquot of the culture was acidified with 1 N HCl to stop residual metabolic activity and to ensure volatilization of $^{14}CO_2$. The bottle was purged with sterile N_2, and the gas was passed through a trap containing 7 ml of Scintiverse (Fisher Scientific, Fairlawn, NJ) to absorb volatile organic compounds and through two additional traps, each containing 6 ml of 1 N NaOH to absorb $^{14}CO_2$. The compounds remaining in the acidified solution after purging are referred to as the acidic fraction. The amount of radioactivity in the various fractions was quantified by liquid scintillation counting.

The simultaneous adaptation technique [26] and the extraction and identification of intermediates were also used to help elucidate the fate of *o*-, *m*- or *p*-cresol in the various enrichment cultures. For simultaneous adaptation experiments, suspected intermediates were added to washed and concentrated (10-fold) cells from enrichment cultures or to cultures immediately after they exhausted their respective cresol parent substrates. The potential intermediates were added from sterile anaerobic stock solutions to give a final concentration of about 100 μM. In each experiment, the parent cresol was introduced in the same fashion and used for comparison. Disappearance of the cresol and suspected intermediates was monitored by HPLC. Occasionally, samples of culture fluid (1 ml) from the various enrichments were acidified with 6 N HCl and extracted three times with 2 ml of diethyl ether by vigorous hand shaking for about 2 min. The combined ether extracts were then concentrated to about 0.2 ml and 1–3 μl were analyzed by gas chromatography/mass spectrometry (GC/MS).

Analytical procedures

The degradation of substrates, the appearance of intermediates and sulfate depletion were quantitatively monitored by HPLC. When possible, the identity of the intermediates was investigated by GC/MS. For HPLC analysis, samples of culture fluid were periodically taken by syringe and stored at −10°C until analysis. Thawed samples were then centrifuged (Beckman microfuge model B, Palo Alto, CA) at 9000 $\times$ *g* to remove particulates. The reversed phase (C_{18}) separations were performed with HPLC systems that were described previously [5,25].

Isocratic mobile phases were employed and consisted of different ratios of 50 mM acetate buffer (pH 4.5) and acetonitrile. Similarly, the flow rate of the mobile phase and wavelength used to monitor the HPLC eluate differed depending on the compounds being analyzed. A 70:30 buffer/solvent mixture, a flow of 1.5 ml/min and 280 nm was used for the routine analysis of *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and benzoic acid. The same conditions were used for the analysis of *p*-cresol and phenol except the mobile phase composition was changed to 55:45 buffer/solvent mixture. A flow rate of 1.3 ml/min and 280 nm was used for monitoring *o*-cresol, *m*-cresol, *o*-hydroxybenzyl alcohol, and *m*-hydroxybenzyl alcohol. However, a buffer/solvent ratio of 60:40 was used for the former two compounds while a 61.5:38.5 was used for the latter two. The latter mobile phase and HPLC conditions were also employed when *m*-hydroxybenzaldehyde was analyzed, but it was monitored at 265 nm. HPLC conditions for *o*- and *m*-hydroxybenzoic acid included an 82:18 buffer/solvent mixture, 1.5 ml/min flow rate and 300 nm, while 50:50 buffer/solvent ratio, 1.6 ml/min and 260 nm were employed for *o*-hydroxybenzaldehyde.

Sulfate was analyzed by anion-exchange HPLC as previously described [25].

Ether extracts of culture fluids were analyzed with a model 5890 Hewlett-Packard gas chromatograph equipped with a 5970 mass selective detector and a 60m DB-5 fused silica capillary column (1 μm film thickness; 0.32 mm i.d.). The GC/MS was operated with the 59970 MS ChemStation (Hewlett-Packard) computer system. Injections (splitless, 30 s) were made at a temperature of 250°C and an initial column temperature of 60°C. The column

temperature was increased at a rate of 4°C/min to 250°C. Helium served as the carrier gas at a flow rate of 60 ml/min (7.5×10^4 Pa forepressure) and a split of 1:60. The interface column temperature was adjusted to 150°C. The electron impact detector was operated at 70 eV and the scanning rate was about 2/s. The metabolites were identified by comparison of their spectra with authentic standards or by the National Bureau of Standards library spectra supplied with the instrument.

Chemicals

The *p*-[*ring*-^{14}C]-cresol was provided as a gift from Dr. P.H. Pritchard, U.S. EPA, Gulf Breeze, FL. Benzoic acid and *p*-cresol were purchased from the Sigma Chemical Co., St. Louis, MO. *p*-Hydroxybenzoic acid was obtained from Eastman Organic Chemicals (Rochester, NY) while phenol was from Allied Chemical Co. (Morristown, NJ). All other organic chemicals were from Aldrich Chemical Co., Milwaukee, WI. The chemicals were of the highest purity available and were used without further purification.

RESULTS

Pathways of cresol metabolism

A sulfate-reducing bacterial consortium from a shallow anoxic aquifer which degraded *p*-cresol was obtained by enrichment procedures. Attempts to isolate a pure culture sulfate-reducing bacterium capable of degrading *p*-cresol on solid media (roll tubes) were unsuccessful. However, *p*-cresol degradation activity could be transferred to liquid culture when morphologically distinct and isolated colonies were picked from solid media and remixed. Microscopic examination revealed that the enrichment contained at least five morphologically distinct cell types. The cells reliably degraded the enrichment substrate on subsequent reamendments and only occasionally exhibited a lag in biodegradation activity when harvested, washed or transferred.

The endproduct of cresol metabolism by this enrichment was investigated with *p*-[*ring*-U-^{14}C]cresol (Fig. 1). HPLC analysis showed that the peak corresponding to the [*ring*-U-^{14}C] parent substrate declined over 8 days of incubation, while more polar peaks with a shorter retention time increased in the culture fluids. After 13 days of incubation, about 87% of the applied radioactivity was trapped in alkali as CO_2, 17% remained in the acidic fraction and 0.9% was trapped as other volatile compounds, to account for slightly more than 100% of the applied label. Therefore it was clear that the parent substrate was mineralized.

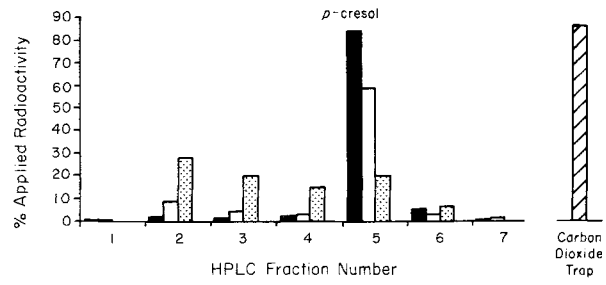

Fig. 1. HPLC analysis of culture fluids from a *p*-cresol-degrading sulfate-reducing bacterial enrichment culture grown in the presence of *p*-[^{14}C]-cresol. The amount of radioactivity in the HPLC effluent was measured on days 0 (■), 5 (□) and 8 (▨). Fractions of the effluent were taken at 1-min intervals. Radioactivity trapped in alkali as CO_2 was also measured on day 13 (▨), the end of the incubation period.

The appearance of HPLC peaks representing more polar metabolites was consistent with the previously suggested pathway for the biodegradation of *p*-cresol in sulfate-reducing aquifer slurries [25]. These peaks could be detected by UV absorbance and by radioactivity. To further investigate the identity of these intermediates, samples of spent culture fluid from experiments with unlabeled *p*-cresol were extracted and analyzed by GC/MS (Fig. 2). Gas chromatographic/mass spectral evidence was obtained for the existence of *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, benzoic acid and phenol as potential intermediates of *p*-cresol. These substances were not detected at the beginning of the experiment, in sterile controls or in the reagents used in the extraction procedure. Each compound was identified on the basis of its GC retention time and the quality of its mass spectrum compared to authentic materials or standard library spectra. In each case the quality of the match was greater than 95%.

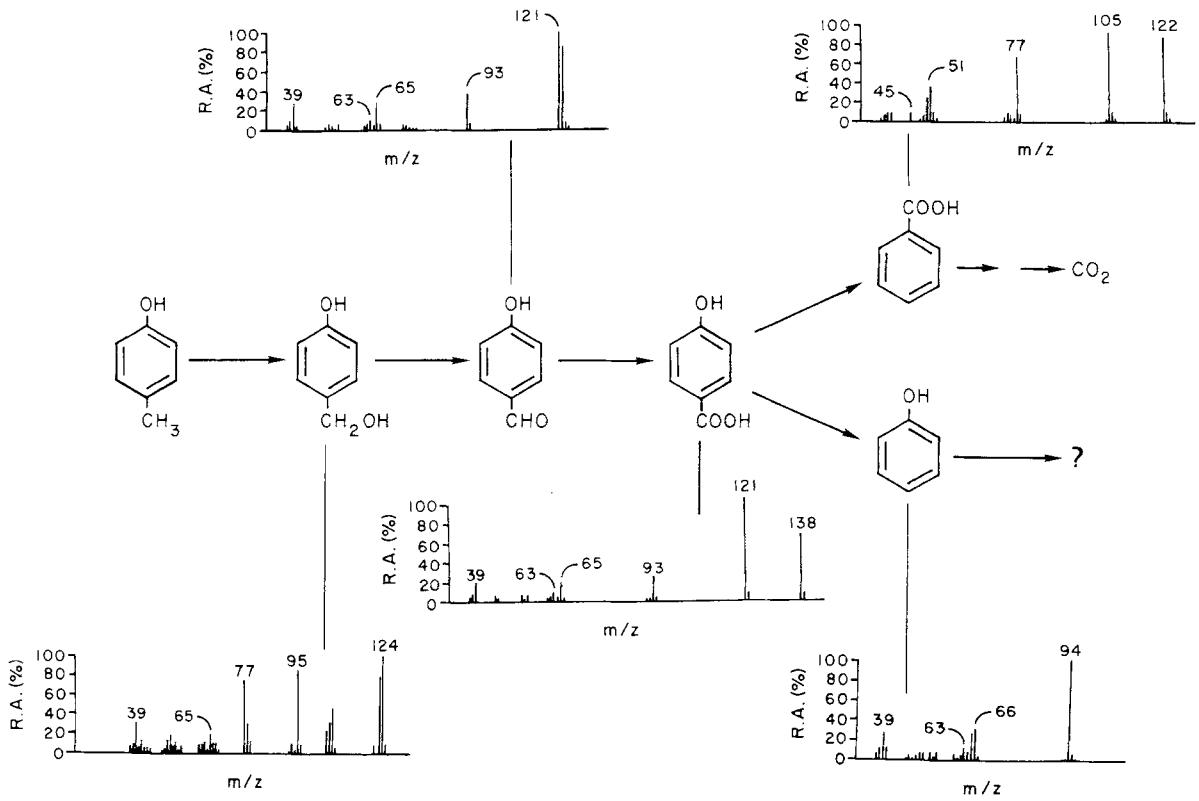

Fig. 2. Proposed pathway for the anaerobic decomposition of *p*-cresol by a sulfate-reducing bacterial enrichment culture and the mass spectral evidence for the suggested intermediates.

However, Fig. 2 also suggests that the pathway diverges after *p*-hydroxybenzoic acid to form benzoic acid and phenol. Several lines of evidence suggest, but do not conclusively prove, that the benzoate pathway is probably the major route of decomposition used by these organisms. First, in simultaneous adaptation studies with the *p*-cresol-degrading enrichment, benzoic acid was utilized at a rate of 17.9 μM/day without a lag. This rate was faster than that measured for the parent substrate or *p*-hydroxybenzoic acid (Table 1). When phenol was tested, the rate of metabolism was hardly different from 0 for over 6 days (Table 1). However, with further incubation, a faster rate of phenol decomposition was observed such that after 25 days the initial amendment of 100 μM phenol was reduced to 18 μM.

Further, when *p*-hydroxybenzoic acid was incubated with a more dilute culture in the absence or presence of equimolar concentrations of phenol, the

Table 1

The simultaneous adaptation of sulfate-reducing bacterial enrichments to potential cresol intermediates

Test compound	Rate of substrate degradation (μM/day ± 95% C.I.)	
	p-cresol enrichment[a]	*o*-cresol enrichment[a]
Cresol	11.5 ± 7	1.9 ± 0.5
Hydroxybenzyl alcohol	n.d.[b]	15.1 ± 6.6
Hydroxybenzaldehyde	n.d.	0.7 ± 0.4
Hydroxybenzoic acid	6.8 ± 1.9	0.5 ± 0.3
Benzoic acid	17.9 ± 12.5	1.7 ± 0.6
Phenol	1.8 ± 1.5	0.5 ± 0.6

Intermediates with a *para* configuration were used for the *p*-cresol enrichment while *ortho*-substituted compounds were used for the *o*-cresol enrichment.

[a] Sterile controls were uniformly unable to transform the test compounds.

[b] n.d. = not determined.

latter compound was not immediately degraded and had no significant influence on the degradation of *p*-hydroxybenzoic acid (2–3.4 μM/day). However, *p*-hydroxybenzoic acid metabolism was blocked when benzoic acid instead of phenol was added to the incubation mixture. Benzoic acid was metabolized without a lag regardless of whether *p*-hydroxybenzoic acid was also in the incubation mixture.

A different sulfate-reducing bacterial community which metabolized *o*-cresol was obtained from the same aquifer by enrichment culture. This enrichment culture was slower-growing than the *p*-cresol enrichment culture and typically exhibited slow rates of substrate removal. Only occasionally were the cells able to metabolize the enrichment substrate without a lag when transferred or harvested. However, substrate reamendments were generally degraded immediately. Typically, we were only able to add the *o*-cresol at a maximum concentration of 300 μM. Higher concentrations inhibited degradation.

An analysis of the culture fluids of the *o*-cresol enrichment culture by GC/MS revealed two potential intermediates. One chromatographic peak was identified as *o*-hydroxybenzaldehyde on the basis of its retention time and similarity to a library spectrum of this compound over other isomeric configurations (Fig. 3). Another peak which appeared in the total ion chromatogram was chromatographically and spectrometrically identical to benzoic acid (data not shown). Neither peak was detected at the start of the experiment, in sterile controls or in reagents used in the extraction procedure. The mass spectra suggest that *o*-cresol can also be metabolized anaerobically by methyl group oxidation.

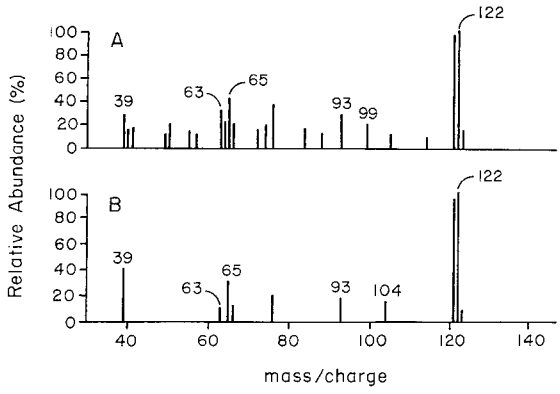

Fig. 3. (A) Mass spectrum of a proposed metabolite detected in the culture fluids of a bacterial enrichment culture degrading *o*-cresol under sulfate-reducing conditions. (B) A library spectrum of *o*-hydroxybenzaldehyde.

Potential intermediates (50–100 μM) possessing an *ortho* substitution pattern were also tested in simultaneous adaptation experiments. Table 1 shows that the *o*-cresol enrichment culture could degrade the corresponding hydroxybenzyl alcohol, hydroxybenzaldehyde, hydroxybenzoic acid and benzoic acid without a lag. However, the rate of hydroxybenzaldehyde and hydroxybenzoic acid loss was very slow and phenol degradation by these cells was not significantly different from zero.

In support of the above findings, when washed cell preparations from the *o*-cresol enrichment culture were given *o*-hydroxybenzyl alcohol and assayed by HPLC, a peak which was spectrophotometrically identical to and co-chromatographed with *o*-hydroxybenzoic acid accumulated to over 80% of that theoretically possible (Fig. 4). The accumulated product eventually degraded upon further incubation.

A sulfate-reducing enrichment of aquifer bacteria was also obtained with *m*-cresol as a sole carbon

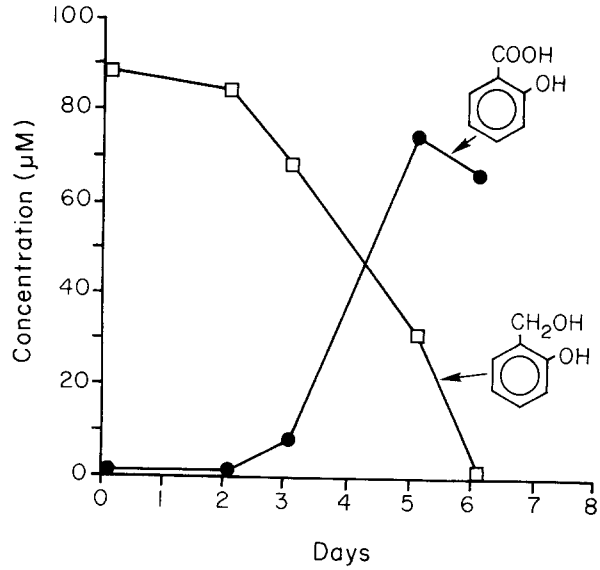

Fig. 4. The utilization of *o*-hydroxybenzyl alcohol and the production of *o*-hydroxybenzoic acid by a bacterial enrichment culture capable of degrading *o*-cresol under sulfate-reducing conditions.

source. Degradation of *m*-cresol by the enrichment was slow and we frequently observed a lag of weeks to months with the enrichment substrate when the bacteria were transferred, harvested, or reamended with substrate. Consequently, simultaneous adaptation experiments could not be performed. Generally, concentrations of substrate were kept at about 100 μM to avoid inhibitory effects.

A comparison of the substrate specificities of the enrichments revealed that the *m*-cresol enrichment was able to metabolize only the *m*-cresol isomer (Table 2). However, the two other enrichment cultures exhibited some degradation of both the *o*- and *p*-cresol substrates but not *m*-cresol.

Characterization of cresol metabolism

In order to characterize anaerobic cresol degradation activity, the stoichiometries of cresol metabolism and sulfate reduction were compared. Cresol depletion was concomitant with sulfate loss in each enrichment. Experimentally determined values of 3.3 ± 2.7, 3.4 ± 1.4 and 3.2 ± 0.8 mol of sulfate were consumed by the various enrichments per mol of *p*-, *o*- and *m*-cresol respectively. These values are similar to the value of 3.4 previously estimated for *p*-cresol-degrading aquifer slurries and accounts for almost 80% of the theoretical (4.25) stoichiometry [25].

To further characterize the enrichment cultures, the influence of a variety of treatments and alternate electron acceptors on cresol metabolism was evaluated. Table 3 shows that when molybdate was used as an inhibitor of sulfate reduction [27], the rate of cresol degradation by two enrichment cultures was inhibited to the point where little or no parent substrate was transformed.

However, an inhibitor of methanogenesis, BESA [11], had much less influence on the same enrichment cultures. When the headspace of the incubation vessels was replaced with a mixture of 80% H_2/20% CO_2, the rate of cresol metabolism was reduced, but not entirely inhibited. However, when the headspace of the vessels was replaced with oxygen, the biodegradation of cresol was eliminated.

Similar rate estimations with the *m*-cresol enrichment were not possible because of the extensive lag period. However, following a 45-day incubation, the initial amendment of *m*-cresol (89 μM) was reduced by 50%, 20% and 10% in enrichments receiving sulfate, BESA and H_2 treatments, respectively. No substrate depletion was detected when the *m*-cresol enrichment culture was treated with molybdate or oxygen.

The inhibition of cresol metabolism by BESA (Table 3) at first suggested the involvement of methanogenic bacteria in the enrichment. Separate experiments with the *p*- and *o*-cresol enrichments

Table 2

A comparison of the substrate specificity of bacterial communities enriched for their ability to degrade either *o*-, *m*- or *p*-cresol under sulfate-reducing conditions

Test substrate	Substrate remaining (%)[a]		
	p-cresol enrichment	*o*-cresol enrichment	*m*-cresol enrichment
o-Cresol	70	37	100
m-Cresol	94	98	58
p-Cresol	0	45	95

[a] Percent of initial substrate amendment at the end of a 27-, 42- and 45-day incubation of the *p*-, *o*- and *m*-cresol enrichments, respectively.

Table 3

Effect of various treatments on the degradation of cresols by sulfate-reducing bacterial enrichments

Treatment	Biodegradation rate (μM/day ± 95% C.I.)[a]	
	p-cresol enrichment	*o*-cresol enrichment
Sulfate	23.5 ± 7.7	1.9 ± 0.6
BESA	5.3 ± 2.2	1.8 ± 1.5
Molybdate	0.6 ± 1.4	0.2 ± 0.1
Hydrogen/carbon dioxide	13.5 ± 8.5	1.0 ± 0.5
Oxygen	0.9 ± 0.9	0.0

The enrichments were assayed with their respective growth substrates.

[a] The rate was calculated over a 45- and 14-day incubation period for the *o*- and *p*-cresol enrichment, respectively.

failed to show an increase in methane production over substrate-unamended controls. This proved to be true regardless of attempts to inhibit sulfate reduction with molybdate in an effort to shift the flow of carbon toward methanogenesis (data not shown).

Experiments were also performed to evaluate the influence of terminal electron acceptors on cresol metabolism by the enrichment cultures. Table 4 shows that cresol degradation by two enrichments was largely dependent on the presence of sulfate. Without an electron acceptor in the incubation mixture, no depletion of the cresols was detected. Sulfur oxyanions like sulfite and thiosulfate were generally poor substitutes for sulfate in these enrichments. The notable exception in this respect is the *p*-cresol enrichment which exhibited some activity with thiosulfate as an electron acceptor. As already noted in Table 3, when oxygen was introduced as a terminal electron acceptor, cresol metabolism ceased. Parallel experiments with the *m*-cresol enrichment indicated that no substrate depletion was evident with any treatment other than sulfate as a terminal electron acceptor.

When nitrate was evaluated as a potential terminal electron acceptor, a slow rate of cresol removal was noted in the *p*-cresol, but not in the *o*-cresol enrichment. Interestingly, the redox indicator always turned pink with increasing incubation time when the former enrichment was amended with nitrate. The influence of nitrate was not evaluated with the *m*-cresol enrichment.

Table 4

Effect of potential electron acceptors on the degradation of cresols by sulfate-reducing bacterial enrichments.

Electron acceptor	Biodegradation rate (μM/day ± 95% C.I.)[a]	
	p-cresol enrichment	*o*-cresol enrichment
None	1.9 ± 3.7	0.6 ± 0.9
Sulfate	23.5 ± 7.7	1.9 ± 0.6
Sulfite	1.0 ± 2.0	0.1 ± 0.5
Thiosulfate	19.8 ± 4.6	0.1 ± 0.3
Nitrate	3.0 ± 1.1	0.8 ± 1.2

The enrichments were assayed with their respective growth substrates.

[a] The rate was calculated over a 45- and 17-day incubation period for the *o*- and *p*-cresol enrichment, respectively.

DISCUSSION

Results with the *p*-cresol enrichment are consistent and extend the previously proposed anaerobic biodegradation pathway for this compound which suggested that the aryl methyl group of the substrate was oxidized under anaerobic conditions [2,3,21,23,25]. Mass spectral evidence was obtained for the corresponding *para*-substituted aromatic alcohol, aldehyde, and acid. Similarly, mass spectral analysis of spent enrichment fluids and simultaneous adaptation experiments allow us to suggest that the next likely step in the anaerobic biodegradation pathway for *p*-cresol is conversion of the hydroxybenzoic acid intermediate to benzoic acid. Benzoic acid can arise in this pathway by reductive dehydroxylation of the *p*-hydroxybenzoic acid, or through the decarboxylation of the aromatic acid to form phenol which may then be carboyxlated. Both routes are known to be catalyzed by anaerobic bacteria metabolizing aromatic substrates [19,29]. Since the enrichment was simultaneously adapted to the utilization of benzoic acid, but not phenol, the former reaction mechanism is considered more likely. However, it is important to point out the interpretational limits of simultaneous adaptation information. A substrate may be degraded without a lag period, even though it is not an intermediate in a metabolic pathway, if the requisite enzymes for metabolism are constitutively produced. Conversely, a compound may be a reaction intermediate and yet not be readily metabolized by whole cell preparations due to permeability barriers or other reasons. The resulting benzoic acid is presumably reductively metabolized by ring saturation as reviewed by several authors [1,7,30,31]. However, our experiments cannot eliminate a completely separate pathway for *p*-hydroxybenzoic acid utilization involving ring saturation reactions prior to the removal of the aryl substituents [12].

The initial *p*-cresol intermediates are the same ones expected from the aerobic decomposition of this substrate [4,13,15]. However, in our studies, the addition of oxygen eliminated substrate depletion by the enrichment. In addition, the culture fluids contained the redox indicator resazurin and no trace of pink coloration was detected. Clearly the aerobic and anaerobic reactions differ with respect to the nature of the oxidants. In aerobic metabolism, oxygen or water can serve as co-reactants for the initial oxidation of the aryl methyl group. Presumably, only water can fulfill this role under anaerobic conditions.

A similar metabolic pathway may be proposed for the anaerobic biodegradation of *o*-cresol. Hydroxybenzaldehyde and benzoic acid were detected by GC/MS as possible metabolic intermediates. The simultaneous adaptation of the *o*-cresol enrichment to partially oxidized potential intermediates in such a pathway, particularly *o*-hydroxybenzyl alcohol, lends support to this suggestion (Table 1). Lastly, the almost stoichiometric conversion of the *o*-hydroxybenzyl alcohol to *o*-hydroxybenzoic acid (Fig. 4) also argues for an aryl methyl group hydroxylation mechanism for the anaerobic destruction of *o*-cresol. Confirmation of this suggested pathway awaits further experimentation.

A pathway for the anaerobic metabolism of *m*-cresol was not indicated by the experiments presented here. However, if the pathways for the anaerobic metabolism of *o*- and *p*-cresol are similar, a comparison of the substrate specificities of the three enrichments (Table 2) suggests that the pathway for *m*-cresol decomposition may possibly be quite different from the other two isomers.

Our experiments also indicate that the cresol isomers were ultimately mineralized by the respective sulfate-reducing enrichments. This is conclusively shown by the ability of the *p*-cresol enrichment to convert ^{14}C-labeled parent substrate to $^{14}CO_2$ (Fig. 1). If *o*- and *m*-cresol were also largely mineralized by the enrichments the stoichiometry of substrate depletion and sulfate reduction for the reactions should be similar. The measured values were similar to each other and about 80% of the theoretical stoichiometry. The deviation from theoretical can potentially be explained by alternate fates for the cresol carbon. Possibilities include cresol metabolism that was not linked to sulfate reduction (i.e. methanogenesis) or substrate utilization for microbial growth. No methane production by the enrichment cultures could be detected and the latter reason was, of course, the basis for obtaining the enrichment cultures. Therefore it is unlikely that methanogenic bacteria are active in the enrichment cultures. These results notwithstanding, the stoichiometry of sulfate consumption by the enrichment cultures suggests that the parent cresol isomers were largely mineralized by the respective enrichment cultures.

The characterization of the various enrichment cultures indicated that substrate metabolism in each was clearly linked to sulfate-reduction. First, sulfate consumption by the enrichment cultures was concomitant with cresol decomposition and closely agreed with the theoretical stoichiometry, as noted above. In addition, all enrichment cultures were severely inhibited in the presence of the molybdate. The effect of this sulfate-reduction inhibitor was much larger than the influence of BESA. In addition, anaerobic biodegradation activity was absent without an electron acceptor amendment to the cultures or if they were placed in the presence of oxygen. For the most part, biodegradation was strictly dependent on the presence of sulfate. No significant change in sulfate content was detected at the end of the incubation period in experiments where the electron donor was not consumed. However, it is interesting that the *p*-cresol enrichment culture could partially consume its substrate when nitrate was provided instead of sulfate as a terminal electron acceptor (Table 4) and that the culture fluid turned pink only when nitrate was available to the cells. The pink color was probably due to the oxidation of resazurin by some of the products of denitrification (e.g. nitrous oxide) as has been recently described [18]. It may be that one more denitrifying bacteria exist as part of the *p*-cresol-degrading enrichment culture.

Our inability to isolate in pure culture a sulfate-reducing bacterium capable of *p*-cresol mineralization suggests that the enrichment organisms form a

complex consortium based on the syntrophic utilization of this substrate. A similar syntrophic association has been described for a denitrifying co-culture [3]. In the latter example, one bacterium in the co-culture oxidized *p*-cresol to *p*-hydroxybenzoic acid, yielding energy through electron transport but no net carbon for growth. The other co-culture organism was able to mineralize the latter intermediate and provide carbon for the growth of both organisms [3]. Our finding that hydrogen inhibited *p*-cresol metabolism by the enrichment cells suggests that interspecies hydrogen transfer may be necessary and part of the basis for the syntrophism.

The experiments reported here help to clarify the fate of simple alkylated aromatic compounds as influenced by bacteria indigenous to a shallow anoxic aquifer. To our knowledge, this is the first report to suggest a pathway for the anaerobic metabolism of *o*-cresol. The pathways for both *o*- and *p*-cresol seem to converge on benzoic acid, which is a central intermediate formed during the anaerobic biodegradation of many aromatic substrates [1,7,30,31]. While there is little doubt that *m*-cresol was also biodegraded in our experiments, the metabolic pathway for this substrate is still in question. It also remains to be seen whether the reaction mechanisms discussed here can be extrapolated to more complicated alkylated aromatic pollutants. Future experiments are planned to address these topics.

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