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Anaerobic Degradation of Halogenated Phenols by Sulfate-Reducing Consortia

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Sulfidogenic consortia enriched from an estuarine sediment were maintained on either 2-, 3-, or 4-chlorophenol as the only source of carbon and energy for over 5 years. The enrichment culture on 4-chlorophenol was the most active and this consortium was selected for further characterization. Utilization of chlorophenol resulted in sulfate depletion corresponding to the values expected for complete mineralization to CO_2. Degradation of 4-chlorophenol was coupled to sulfate reduction, since substrate utilization was dependent on sulfidogenesis and chlorophenol loss did not proceed in the absence of sulfate. Other sulfur oxyanions, sulfite or thiosulfate, also served as electron acceptors for chlorophenol utilization, while carbonate, nitrate, and fumarate did not. The sulfidogenic consortium utilized phenol, 4-bromophenol, and 4-iodophenol in addition to 4-chlorophenol. 4-Fluorophenol, however, did not serve as a substrate. 4-Bromo- and 4-iodophenol were degraded with stoichiometric release of halide, and 4-[^{14}C]bromophenol was mineralized, with 90% of the radiolabel recovered as CO_2.

Contamination of marine and estuarine sediments by anthropogenic halogenated organic compounds such as pesticides, solvents, and other industrial chemicals has been a matter of increased concern. At the high sulfate concentrations found in seawater (20 to 30 mM), sulfate reduction is the dominant process in carbon metabolism in marine sediments (4), and sulfate may be the most important electron acceptor influencing dehalogenation and anaerobic degradation of halogenated compounds. Dehalogenation and degradation of halogenated aromatic compounds by anaerobic bacteria under methanogenic conditions are well established (reviewed in references 16 and 27). For example, degradation of chlorinated phenols and benzoic acids is initiated by reductive dechlorination followed by cleavage of the aromatic ring, and the compounds are ultimately mineralized to CH_4 and CO_2. There is, however, less information on the biodegradation of halogenated aromatic compounds under other reducing conditions.

Although sulfate and other sulfur oxyanions frequently inhibit aryl dehalogenation (1, 8, 17, 26), this is not always the case, as these alternative electron acceptors may indeed support anaerobic degradation of halogenated aromatic compounds. Thermodynamic calculations (5), in fact, indicate that degradation of chlorinated aromatic compounds coupled to sulfate reduction is substantially more favorable than degradation coupled to methane production. Genthner et al. (12) showed that degradation of 4-chlorophenol (4-CP) in an estuarine sediment slurry was stimulated by sulfate. However, upon transfer to fresh medium, sulfate was not required for activity (12). We previously demonstrated that chlorinated phenols and benzoic acids can be degraded under sulfidogenic conditions in both freshwater and estuarine sediments (18, 19, 22). In this paper, we describe the anaerobic metabolism of monochlorinated, monobrominated, and monoiodinated phenols by sulfate-reducing consortia enriched from an estuarine sediment. We also demonstrate that these halogenated phenols are mineralized to CO_2 and that degradation is dependent on sulfate reduction.

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MATERIALS AND METHODS

Source of consortia. Sulfate-reducing enrichment cultures which degrade either 2-CP, 3-CP, or 4-CP were previously established with sediment from the East River, an estuarine intertidal strait in New York City, N.Y. (19). Enrichment cultures were initially established and maintained under both saline (2.4‰ salinity) and freshwater conditions. From the initial enrichment cultures, stable sulfidogenic consortia were obtained by repeated dilution (1:3 to 1:5 at each time) into saline or freshwater medium and by refeeding each dilution with the respective CP isomer. Such consortia were maintained for several years with CP provided as the sole source of carbon and energy and were used for further experiments.

Substrate degradation assays. Enrichment cultures maintained on either 2-CP, 3-CP, or 4-CP were used to test for the utilization of different substrates. The cultures were subdivided (4 to 10 ml) into smaller vials (10 ml) in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, Mich.) under an atmosphere of 97% N_2–3% H_2 and then fed the various test substrates at the indicated concentration. CPs and other substrates, including phenol, 4-fluorophenol (4-FP), 4-bromophenol (4-BP), 4-iodophenol (4-IP), 4-chloroaniline, 4-chlorobenzoate, and 4-chlororesorcinol (Aldrich, Milwaukee, Wis., and Sigma Chemical Co., St. Louis, Mo.), were added from deoxygenated stock solutions (25 to 100 mM) made up in 0.1 N NaOH. The flasks were then sealed with Teflon-coated butyl rubber stoppers (Wheaton, Millville, N.J.) and aluminum crimps and incubated without shaking at 30°C in the dark. All cultures were established in duplicate or triplicate with sterile-medium controls. At the indicated time points, samples were collected with sterile, deoxygenated plastic syringes for chemical analyses.

Mineralization of 4-BP. A sulfidogenic culture enriched on 4-CP was used to study the mineralization of 4-BP. Experiments were performed by using 10-ml flasks containing 5 ml of culture that had been fed 275 μM 4-BP and supplemented with 15 nCi of uniformly ring-labelled [^{14}C]4-BP (specific activity, 7.3 mCi/mmol; radiochemical purity, >96% [Sigma Chemical Co.]) to give approximately 35,000 dpm per culture. In replicate cultures not supplemented with [^{14}C]4-BP, the utilization of substrate was monitored by measuring the concentration of 4-BP and bromide in the cultures. At the end of the experiment, the cultures with [^{14}C]4-BP were acidified (pH < 2) with HCl and flushed with argon for 10 min, and the evolved $^{14}CO_2$ was collected in a series of three 0.1 N NaOH 5-ml traps. Then 15 ml of a scintillation fluid (Beckman Ready-safe) was added, and radioactivity was measured with an LS 5000 TD Liquid Scintillation System (Beckman Instruments Inc., Fullerton, Calif.).

Influence of alternative electron acceptors. To test for electron acceptor requirements, 80 ml of the 4-CP-degrading culture was collected by anaerobic centrifugation under 97% N_2–3% H_2, washed once with 50 ml of sulfate-free anaerobic medium, and resuspended in 80 ml of sulfate-free medium. 4-CP was added to an initial concentration of 200 μM, and the culture was divided in the

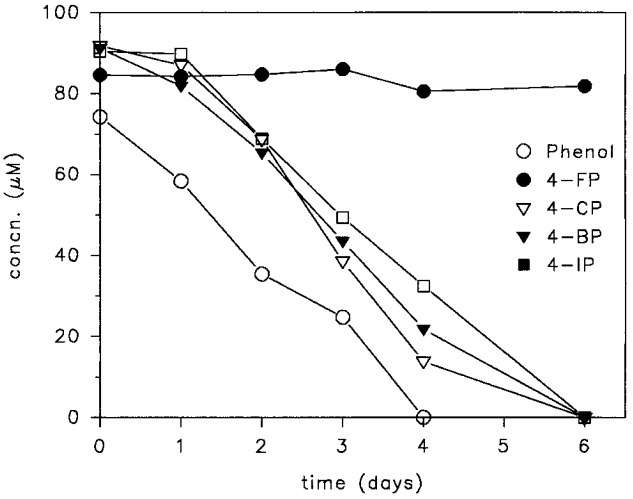

FIG. 1. Degradation of halogenated phenols by a 4-CP-degrading consortium.

anaerobic chamber into aliquots of 5 ml and amended with 10 mM sulfate, 10 mM sulfite, 10 mM thiosulfate, 10 mM nitrate, 10 mM fumarate, or the same volume (100 μl) of water. All cultures were established in duplicate, with sterile medium containing 200 μM 4-CP serving as controls.

4-CP and sulfate stoichiometry. The depletion of sulfate during CP utilization was measured in triplicate cultures (20-ml volume in Balch tubes sealed with butyl rubber stoppers) that were fed with 100 to 500 μM 4-CP repeatedly over a 3-month period until a total of 2 mM had been consumed. Replicate cultures that were not given CP served as controls for sulfate reduction in order to account for the background organic carbon of the inoculum. At the end of the experiment, the depletion of sulfate and the increase in protein was measured.

Analytical methods. Samples for chemical analyses taken from the cultures were filtered through a 0.45-μm-pore-size filter (Millipore, Bedford, Mass.) and frozen (−20°C) until analyzed. CPs and other aromatic substrates were quantified by high-performance liquid chromatography (HPLC) and UV detection at 280 nm (Beckman System Gold models 126/166; model 231-401 Autosampling Injector [Gilson, Middleton, Wis.]) with a Spherisorb C_{18} column (Supelco Inc., Bellefonte, Pa.) as described previously (17).

Sulfate and halides were analyzed by ion chromatography with conductivity detection on a DX-100 ion chromatograph (Dionex, Sunnyvale, Calif.) equipped with an IonPac AS9 (Dionex) column. A solvent system consisting of 2 mM Na_2CO_3 and 0.75 mM $NaHCO_3$ was used at a flow rate of 2 ml/min with a regenerant of 20 to 25 mN H_2SO_4. Samples were diluted in water when necessary and injected with a 25-μl sample loop. Concentrations were quantified with external standards by using a Chrome-Jet integrator (Spectra-Physics, San Jose, Calif.).

Samples (800 μl) for protein analysis were amended with 10 μl of 10 N NaOH and heated in a water bath (100°C) for 15 min to solubilize proteins. Protein was measured by the Coomassie brilliant blue reaction with a reagent kit (Bio-Rad, Hercules, Calif.) and bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Stoichiometry of CP utilization. We have maintained sulfidogenic enrichment cultures on 2-CP, 3-CP, and 4-CP for over 5 years by repeated feeding of the respective CP and subculturing into fresh medium. The saline enrichment on 4-CP was the most active and was selected for more detailed study. Mineralization of CP coupled to sulfate reduction can be described by the stoichiometric equation as follows: $C_6H_5OCl + 3.25SO_4^{2-} + 4H_2O \rightarrow 6HCO_3^- + 3.25H_2S + 0.5H^+ + Cl^-$. To determine the actual stoichiometry, cultures were repeatedly fed with 4-CP (100 to 500 μM) over a 3-month period and monitored for sulfate depletion. Utilization of 1.9 ± 0.05 mM 4-CP resulted in depletion of 5.1 ± 0.3 mM sulfate (corrected for sulfate loss in background controls [1.3 mM]). This value equals 81% of the sulfate loss expected for complete oxidation of CP to CO_2. Utilization of 4-CP resulted in an increase in the protein concentration of 4.5 μg/ml above that in controls. This corresponds to a conversion of approximately 3% of the carbon to cells, assuming that the increase in cell carbon was equal to the increase in protein concentration (6). Correcting for this carbon conversion value, the measured sulfate loss accounts for 85% of that expected.

TABLE 1. Mineralization of 4-BP by a sulfidogenic consortium

Culture	Recovery of radioactivity (%)[a] in:		
	Culture medium	CO_2	Total
Active	9.6 ± 1.2	91.4 ± 2.1	101.0 ± 3.4
Control	96.9 ± 0.6	1.8 ± 0.2	98.7 ± 0.8

[a] Cultures were fed with 275 μM 4-BP and spiked with uniformly ring-labelled [^{14}C]4-BP, and radioactivity was measured after 30 days of incubation.

Degradation and dehalogenation of 4-halophenols. The 4-CP culture was also capable of degrading other halogenated phenols (Fig. 1). Phenol, 4-CP, 4-BP, and 4-IP (100 μM) were depleted within 6 days at approximately equal rates. 4-FP was not utilized. Utilization of 4-BP and 4-IP yielded stoichiometric release of bromide and iodide, respectively (data not shown). Because of the large background of chloride in the saline sulfidogenic medium, the release of chloride from the small amount of 4-CP utilized could not be determined. Although this result demonstrates that halide is stoichiometrically released during substrate utilization, it does not show when dehalogenation takes place. Furthermore, no metabolites detectable by HPLC were observed during degradation of the halophenols.

Mineralization of 4-BP. To verify that the halogenated phenols were mineralized under sulfate-reducing conditions, we examined the evolution of $^{14}CO_2$ from [^{14}C]4-BP. A concentration of 275 ± 3 μM 4-BP was depleted within 30 days, with concomitant release of 228 ± 10 μM bromide. The results in Table 1 demonstrate that [^{14}C]4-BP was mineralized, with over 90% of the radiolabel recovered as CO_2.

Requirement of sulfur oxyanions for 4-CP degradation. To determine whether sulfate was required for CP utilization, we tested the effect of a selection of electron acceptors. As seen in Fig. 2, 200 μM 4-CP was stable for over 30 days in the absence of an inorganic electron acceptor (other than carbonate in the mineral salts medium) but was utilized within 15 days when 10 mM sulfate was added. 4-CP was also utilized in the presence

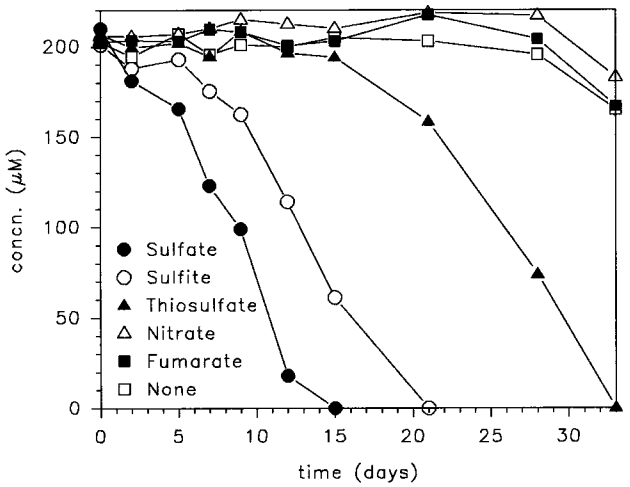

FIG. 2. Requirement of electron acceptors for 4-CP degradation.

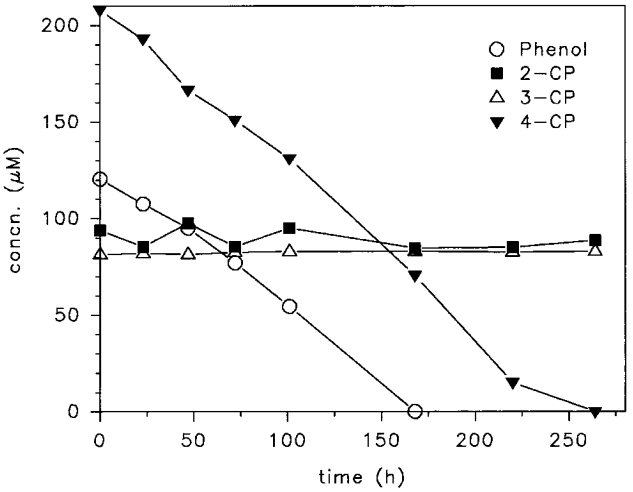

FIG. 3. Degradation of monochlorinated phenols and phenol by a 4-CP-utilizing consortium.

of sulfite and thiosulfate after a lag time of 5 and 15 days, respectively, but no utilization was observed in cultures amended with 10 mM nitrate or fumarate. The small loss of substrate observed after 28 days was also observed in sterile controls and was presumably due to volatilization. Thus, CP utilization is dependent on the presence of sulfate or other sulfur oxyanions. This further supported an earlier study in which we demonstrated that molybdate, a specific inhibitor of sulfate reduction, completely inhibited CP metabolism as well as sulfate reduction (19).

This requirement for sulfate reduction has not been previously demonstrated for degradation of chlorinated aromatic compounds. In general, substrate utilization and, particularly, reductive dechlorination are partially or completely inhibited by sulfur oxyanions (2, 8, 10, 13, 17, 24, 26), possibly because of competition for reducing equivalents (8). Our enrichment approach differs in that sulfate is provided in excess to promote a sulfate-reducing consortium from the onset, which may explain the different observations in other studies. Thus far, we have been unable to detect the production of phenol in halophenol-degrading cultures, and hence we have no direct evidence that reductive dehalogenation serves as the initial step under sulfate-reducing conditions. Phenol was, however, readily degraded without any lag by the CP-utilizing cultures. This is consistent with, but not proof of, its role as an intermediate. Reductive dechlorination of di-CPs in the presence of sulfate has been demonstrated, but dependency of sulfate was not investigated (19, 24).

Substrate specificity. Figure 3 shows the utilization of phenol, 2-CP, 3-CP, and 4-CP by the consortium enriched on 4-CP. The consortium degraded 200 μM 4-CP within 11 days but did not utilize either 2-CP or 3-CP. As in previous experiments, phenol was readily utilized. In addition, 4-CP and phenol were metabolized when provided in a mixture of all four compounds (data not shown). A similar isomer specificity was observed with the consortia enriched on 2-CP and 3-CP (data not shown). Although the original sediment inoculum had the capacity to utilize all three mono-CP isomers (19), once the consortia were acclimated to a single isomer, they were specific to that substrate. Preliminary work with genus- and group-specific hybridization probes (7) suggests that phylogenetically distinct consortia were enriched on the three mono-CP isomers (32).

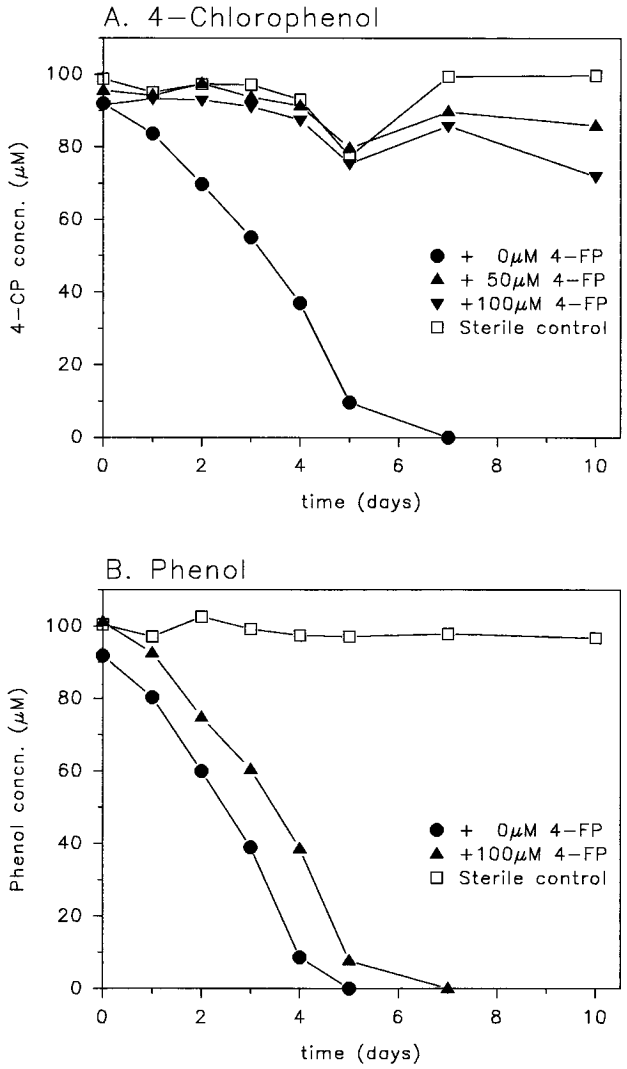

FIG. 4. Effect of 4-FP on degradation of 4-CP (A) and phenol (B).

We also tested the utilization of selected di-CPs and nonhalogenated phenolic compounds, as well as other chlorinated aromatic substrate analogs, by the 4-CP-utilizing consortium. An additional chlorine substituent completely inhibited metabolism, e.g., 2,4- and 3,4-di-CP were not degraded (data not shown); also, substrate analogs with a chlorine substituent in the *para* position, such as 4-chlororesorcinol, 4-chlorobenzoate, and 4-chloroaniline, were not utilized by the 4-CP-degrading consortium.

Effect of 4-FP on 4-CP utilization. Since 4-FP was not utilized by the 4-CP-degrading consortium (Fig. 1), we examined whether it might serve as a selective inhibitor. When 4-FP (50 or 100 μM) was added to a 4-CP-utilizing culture, degradation of 4-CP was completely inhibited (Fig. 4A). 4-FP had no effect, however, on the utilization of phenol (Fig. 4B). This suggests that 4-FP may serve to competitively inhibit an initial dehalogenation step.

If reductive dechlorination is the initial step in CP metabolism, we would have expected the dechlorination of 2,4- and 3,4-di-CP with accumulation of 2-CP and 3-CP, respectively. Since no transformation of these di-CPs was observed, it leaves open the possibility that either the second chlorine substituent

inhibited *para* dechlorination or reductive aryl dechlorination is not the initial step in sulfidogenic CP metabolism.

Several sulfate-reducing bacteria can degrade aromatic compounds, including phenol, benzoate, catechol, and aniline, and utilize these as sole sources of carbon and energy (3, 14, 25, 29, 34, 35). Transformation of chlorinated aromatic compounds by these isolates, however, has not been reported. The sulfidogenic bacterium *Desulfomonile tiedjei* DCB-1 reductively removes the chlorine from 3-chlorobenzoate and some CPs (9, 30). Interestingly, although benzoate is degraded by the bacterium with sulfate or thiosulfate as the electron acceptor (9), 3-chlorobenzoate or CPs are not mineralized. Similarly, the recently described *Desulfitobacterium dehalogenans* JW/IU-DC1 was reported to dechlorinate *ortho*-chlorinated phenols but does not utilize the product phenol (36). The 4-CP-utilizing culture described here thus appears different in that both phenol and CP are degraded. The dependency on sulfate reduction and inhibition by molybdate (19) suggests that sulfate-reducing bacteria may be directly responsible for CP degradation, although the possibility of syntrophic associations cannot be ruled out.

We have demonstrated CP- and chlorobenzoate-degrading activity by sulfidogenic consortia enriched from other sediments as well. Utilization of all three mono-CPs coupled to sulfate reduction occurred in both estuarine and freshwater sediments of the Hudson River (18). Sulfidogenic degradation of 3-chlorobenzoate was also found in cultures of freshwater sediment from the Hudson River and the Nile (18, 22). Recently, Drzyzga et al. (11) showed that a sulfidogenic consortium enriched from an anaerobic lake sediment degraded 2- and 4-fluorobenzoate. These results suggest that sulfate-reducing consortia capable of haloaromatic degradation may be widely distributed.

Sulfidogenesis is the major terminal electron-accepting process during degradation of organic matter in marine sediments and is therefore central to carbon turnover (4, 20, 21, 31). Halogenated organic compounds, such as pesticides, solvents, and other industrial chemicals, are common contaminants in estuarine and marine environments, and their biodegradability under sulfate-reducing conditions is therefore important in determining their fate in marine sediments. However, a wide variety of halogenated compounds are also produced biologically, and in fact the marine environment is a rich source of natural halogenated compounds, including phenols, acyclics, terpenes, indoles, and acetylenes (15, 23, 28, 33). It is reasonable, therefore, to expect that haloaromatic-utilizing sulfate reducers would be found in marine sediments.

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