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Characterization of Anaerobic Dechlorinating Consortia Derived from Aquatic Sediments†

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Four methanogenic consortia which degraded 2-chlorophenol, 3-chlorophenol, 2-chlorobenzoate, and 3-chlorobenzoate, respectively, and one nitrate-reducing consortium which degraded 3-chlorobenzoate were characterized. Degradative activity in these consortia was maintained by laboratory transfer for over 2 years. In the methanogenic consortia, the aromatic ring was dechlorinated before mineralization to methane and carbon dioxide. After dechlorination, the chlorophenol consortia converted phenol to benzoate before mineralization. All methanogenic consortia degraded both phenol and benzoate. The 3-chlorophenol and 3-chlorobenzoate consortia also degraded 2-chlorophenol. No other cross-acclimation to monochlorophenols or monochlorobenzoates was detected in the methanogenic consortia. The consortium which required nitrate for the degradation of 3-chlorobenzoate degraded benzoate and 4-chlorobenzoate anaerobically in the presence of KNO_3, but not in its absence. This consortium also degraded benzoate, but not 3-chlorobenzoate, aerobically.

Anaerobic degradation of chloroaromatic compounds has been investigated in a variety of unadapted inocula, including sewage (2, 3, 8, 16), sediments (8, 9, 16), soils (10, 13), and groundwater aquifers (7, 17). Among the compounds reported to undergo reductive dechlorination followed by anaerobic degradation or transformation are chlorobenzoates (4, 6–9, 16) and chlorophenols (2, 3, 6–8, 10, 13, 17, 18). However, only a few recent studies have characterized the degradation of chlorinated compounds in stable anaerobic microbial communities that were specifically adapted to degrade compounds of this class. Among these were the degradation of these monochlorinated phenolic compounds by sewage sludge adapated to the degradation of individual monochlorophenols (2, 3); the degradation of a variety of haloaromatic compounds by an anaerobic consortium that was able to degrade 3-chlorobenzoate (4, 9, 15, 16, 18); and a pure culture of the anaerobic bacterium responsible for dechlorination of 3-chlorobenzoate in the consortium described above (4, 15). All of the aforementioned cultures were methanogenic.

In a previous study (6) with aquatic sediments used as inocula, we derived anaerobic dechlorinating enrichments which were able to degrade the individual monochlorophenols and monochlorobenzoates. Through subsequent transfer or refeeding of these enrichments, we obtained several stable anaerobic consortia and were able to maintain their degradative activities in the laboratory for over 2 years. In the present study, five of these consortia were selected for more thorough characterization. We also report here the formation of benzoate as an intermediate in the anaerobic degradation of chlorophenols.

MATERIALS AND METHODS

Source of consortia. Anaerobic dechlorinating consortia, described in Table 1, were derived from the primary enrichments described previously (6). Stable consortia were obtained by passing primary enrichments through several laboratory transfers to remove the original sediment.

Anaerobic media and methods. The anaerobic techniques and enrichment media were described previously (6). Cultures were prepared in triplicate. Uninoculated controls and controls lacking the test compound were prepared in duplicate. Incubations were done at 30°C. Defined medium was prepared by deleting yeast extract from the enrichment medium and supplementing it with pyruvate (0.2%), nicotinamide, and 1,4-naphthoquinone, as described previously for strain DCB-1 (K. A. DeWeerd and J. M. Suflita, Abstr. Annu. Meet. Am. Soc. Microbiol., 1988, I108, p. 199).

Anaerobic degradation studies. Samples from the chloroaromatic degradation study were collected in triplicate, and duplicates of each sample were analyzed by high-performance liquid chromatography. Gas samples were collected in triplicate by using a Hamilton gas-tight glass syringe equipped with a Mininert valve (Precision Sampling Corp., Norcross, Ga.). Methane and carbon dioxide were corrected for the gas produced in control cultures. Degradation intermediates were tentatively identified by retention times, UV spectral analyses, and cochromatography with authentic compounds under the two sets of separation parameters described below.

Aerobic degradation. For the aerobic degradation study, the enrichment medium was prepared and dispensed aerobically and modified by replacing the bicarbonate buffer with a phosphate buffer (0.1 M, pH 7.0). A sterile, cotton-stoppered syringe (20 ml, 18-gauge needle) inserted through the serum stopper allowed diffusion of oxygen and minimized loss of the chloroaromatic compounds. Cultures were shaken during incubation. All other methods used were as described for the anaerobic degradation studies.

Dilution assay. The five consortia were diluted (10^{-1} to 10^{-9}) in anaerobic enrichment medium (90 ml) containing either the chlorophenol (500 μM) or the chlorobenzoate (800 μM) to which they were adapted. Samples were analyzed by high-performance liquid chromatography. Phenol-degrading anaerobes in the 2-chlorophenol consortium were enumer-

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TABLE 1. Description of consortia

Enrichment substrate	Consortium type	Inoculum source	Inoculum characteristics[a]	Primary enrichment condition[b]
2-Chlorobenzoate	Methanogenic	Escambia River site B	F, D	B
3-Chlorobenzoate	Methanogenic	Escambia River site A	F, U	B
2-Chlorophenol	Methanogenic	Escambia River site A	F, U	M
3-Chlorophenol	Methanogenic	Bayou Chico	E, D	B
3-Chlorobenzoate (KNO_3-requiring)	Nitrate	Bayou Chico	E, D	N

[a] Abbreviations: F, Freshwater; E, estuarine; D, downstream from industrial effluent; U, upstream from industrial effluent.
[b] Abbreviations: B, 1 mM bromoethane sulfonic acid added; M, methanogenic; N, 15 mM KNO_3 added.

ated by diluting them (10^{-1} to 10^{-9}) in enrichment medium with two concentrations of phenol, i.e., 500 μM and 4 mM.

Analytical methods. High-performance liquid chromatographic samples from the degradation studies were prepared and analyzed as described previously (6). Duplicate samples that were used to tentatively identify compounds detected during the degradation study were filtered (nylon membrane; diameter, 25 mm; pore size, 0.2 μm; Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) and analyzed on an octadecylsilane-Hypersil (microbore, C-18; 2.5 cm; inner diameter, 2 mm) column (Hewlett-Packard Co., Palo Alto, Calif.) in a high-performance liquid chromatograph (1090M; Hewlett-Packard) equipped with a workstation (HP79994A; Hewlett-Packard). UV spectral data were obtained by using a diode array detector (200 to 600 nm). A methanol-H_2O solvent (25:75 to 40:60) acidified with phosphoric acid (pH 3.0) was used (1.0 ml/min, 40°C). Acetonitrile was added (1:1) to enhance the recovery of phenol and chlorophenols.

Methane and CO_2 were determined by using a gas chromatograph (model 5840A; Hewlett-Packard) equipped with a thermoconductivity detector and a Carbosieve SII column (10 by 1/8 in. [25.4 by 0.32 cm]; stainless steel; 100/120; Supelco, Inc., Bellefonte, Pa.) with helium carrier gas (30 ml/min) at 180°C.

RESULTS

Degradation of the chloroaromatic compounds in methanogenic consortia. Degradation of the four chloroaromatic compounds in methanogenic consortia is shown in Fig. 1. As the 2-chlorophenol and 3-chlorophenol concentrations declined, a compound with the retention time of phenol was detected in amounts accounting for 38 and 46% of the added chlorophenols, respectively (Fig. 1A and B). As the amount of this compound decreased, a second compound appeared which had a retention time similar to that of benzoate and accounted for 57 and 70%, respectively, of the 2-chlorophenol and 3-chlorophenol added. In Fig. 1 the sum of the micromoles of chlorophenol, phenol, and benzoate at each datum point before benzoate degradation began accounted

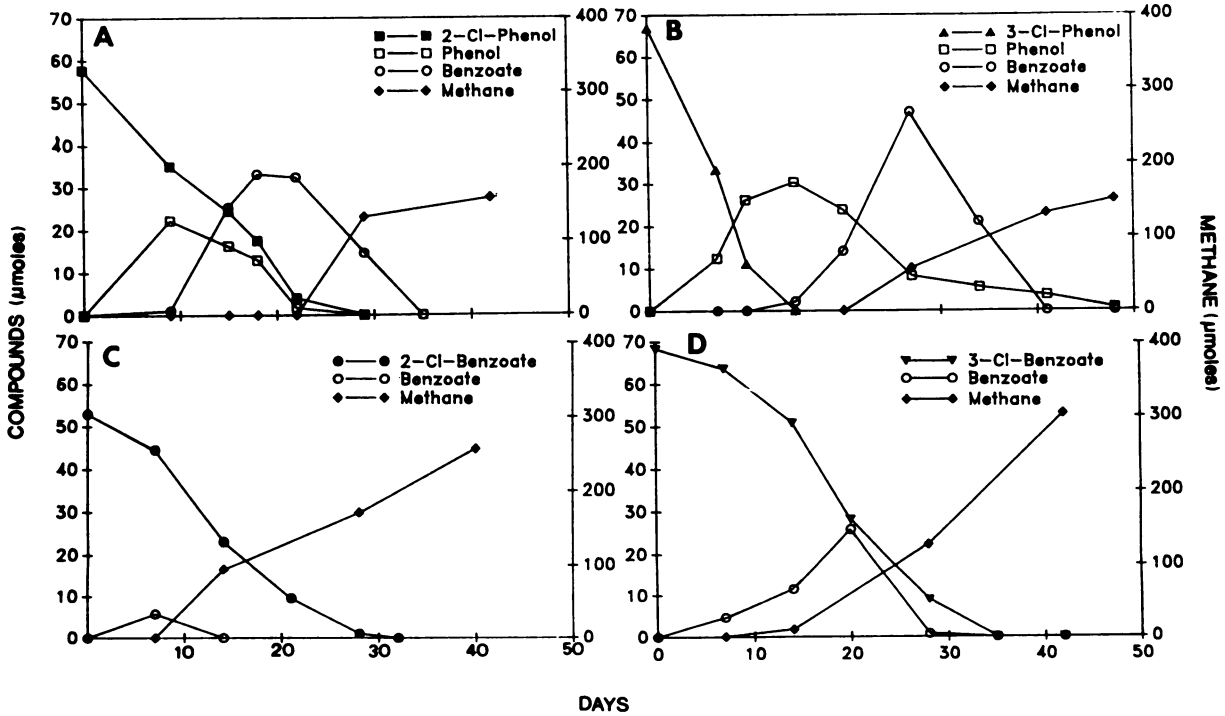

FIG. 1. Degradation of chloroaromatic compounds by methanogenic consortia. (A) 2-Chlorophenol degradation in an Escambia River site A consortium. (B) 3-Chlorophenol degradation in a Bayou Chico consortium. (C) 2-Chlorobenzoate degradation in Escambia River site B consortium. (D) 3-Chlorobenzoate degradation in Escambia River site A consortium. Each datum point is the mean of triplicate cultures.

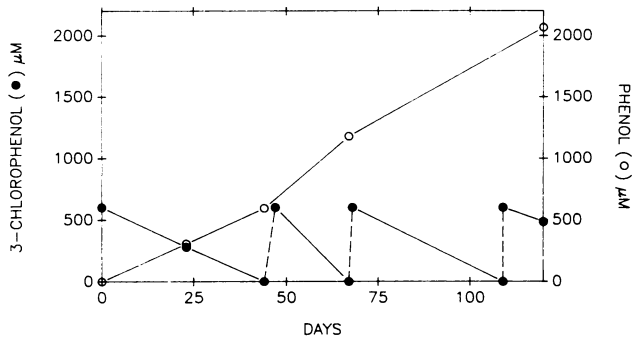

FIG. 2. Reductive dechlorination of 3-chlorophenol in a defined medium containing 0.2% pyruvate. Dashed lines indicate the readdition of 3-chlorophenol.

for the amount of chlorophenol added initially. The first and second compounds cochromatographed with authentic phenol and benzoate, respectively, under the separation conditions described previously (6). By using duplicate samples and the separation conditions described above, these two compounds again cochromatographed with authentic phenol and benzoate, respectively. Under these conditions, the UV spectra of these two compounds matched those of authentic phenol and benzoate, respectively. These compounds were not observed in control cultures without added 2-chlorophenol. Methane, in excess of that produced in control cultures lacking the chlorophenols, was detected after the chlorophenols and phenol were below detectable limits and benzoate began to decline. Taking into account the carboxyl group fixed during the conversion of phenol to benzoate, methane accounted for approximately one-third of the added carbon, while carbon dioxide accounted for the remaining aromatic carbon.

The 3-chlorophenol consortium was also able to grow and dechlorinate 3-chlorophenol in a defined medium containing pyruvate as a potential electron donor and carbon source (Fig. 2). 3-Chlorophenol decreased to undetectable limits following four sequential additions over a period of 120 days. However, a compound which cochromatographed with and had the retention time and UV spectrum of phenol was present at concentrations which accounted for the 3-chlorophenol added to the culture. At 2.5 months of incubation, pyruvate was below detectable limits. This phenomenon was observed in subsequent laboratory transfers of this culture. None of the other four consortia degraded their respective chloroaromatic compounds in this defined medium.

An intermediate which was present in the 2-chlorobenzoate consortium at a low level on day 7 (Fig. 1C) was tentatively idenified as benzoate by cochromatography and UV spectral analysis, as described above. This compound was detected at higher levels in the 3-chlorobenzoate consortium (Fig. 1D). Methane was produced in higher amounts than it was in the chlorophenol consortia, accounting for approximately two-thirds of the aromatic carbon, while the remaining aromatic carbon could be accounted for by carbon dioxide.

Characterization of methanogenic consortia. The degradative characteristics of the methanogenic consortia are summarized in Table 2. All four methanogenic consortia degraded phenol and benzoate. The 3-chlorophenol and 3-chlorobenzoate consortia also degraded 2-chlorophenol. No other cross-acclimations to chloroaromatic compounds were observed. None of the methanogenic consortia degraded their respective chloroaromatic compounds under aerobic conditions.

TABLE 2. Degradative activities of anaerobic dechlorinating consortia

Consortium type	Degradative activities[a]							
	Phenols				Benzoates			
	Phenol	2-Cl	3-Cl	4-Cl	Benzoate	2-Cl	3-Cl	4-Cl
2-Chlorophenol	+	+	−	−	+	−	−	−
3-Chlorophenol	+	+	+	−	+	−	−	−
2-Chlorobenzoate	+	−	−	−	+	+	−	−
3-Chlorobenzoate	+	+	−	−	+	−	+	−
3-Chlorobenzoate (KNO_3-requiring)[b]	−	−	−	−	+[c]	−	+[b]	+[b]

[a] Phenol and chlorophenols were present at 500 μM; benzoate and chlorobenzoates were present at 800 μM. Symbols: +, degradation observed; −, no degradation observed after 3 months of incubation.
[b] Also present was 15 mM KNO_3.
[c] KNO_3 was required for anaerobic degradation.

Dechlorinating anaerobes (bacteria per milliliter) were estimated by using the dilution assay described above. Beyond a dilution of 10^{-5}, the 2-chlorophenol, 3-chlorophenol, and 3-chlorobenzoate consortia failed to degrade their respective chloroaromatic compounds after 3 months of incubation, indicating the presence of greater than 10^5 and less than 10^6 dechlorinating bacteria per ml. In contrast, the 2-chlorobenzoate consortium contained greater than 10^7 and less than 10^8 dechlorinating bacteria per ml. Substantial concentrations (>400 μM) of a compound tentatively identified as benzoate by retention time and cochromatography were detected in the 10^{-7} dilution. This method also indicated that phenol-degrading anaerobic bacteria were present at greater than 10^9 per ml in this consortium. In these dilution cultures, phenol was converted to benzoate before mineralization.

Gram stains revealed that the methanogenic consortia which degraded 2-chlorobenzoate, 2-chlorophenol, and 3-chlorobenzoate contained mixtures of gram-negative rods. Extremely long cells, suggestive of *Methanospirillum hungatei*, were evident. The 3-chlorophenol methanogenic consortium contained large, gram-positive, irregularly shaped cocci mixed with gram-negative rods.

Characterization of nitrate-requiring consortium. The consortium which required nitrate for degradation of 3-chlorobenzoate also degraded benzoate and 4-chlorobenzoate anaerobically in the presence of nitrate, but not in its absence (Table 2). Phenol was not degraded under these conditions. Benzoate was degraded aerobically in the presence or absence of nitrate, while 3-chlorobenzoate was not degraded aerobically under either condition. When diluted to 10^{-7}, this consortium was able to degrade 3-chlorobenzoate in the presence of nitrate. At higher dilutions degradation was not observed, indicating the presence of greater than 10^7 and less than 10^8 anaerobic bacteria per ml that were able to degrade 3-chlorobenzoate. The majority of cells were small, gram-negative rods, but a few gram-positive rods and cocci were also present.

DISCUSSION

The detection of substantial quantities of the appropriate nonchlorinated compound, i.e., phenol or benzoate, in all four methanogenic consortia suggests that the chloroaromatic compounds underwent reductive dechlorination be-

fore degradation of the aromatic structure, as reported previously for 2-chlorophenol (3), 4-chlorophenol (17), and 3-chlorobenzoate (7, 9, 16). Boyd and Shelton (2) assumed that reductive dechlorination is the initial step in the degradation of monochlorophenols, but did not actually detect nonchlorinated intermediates.

We were able to maintain 2-chlorobenzoate degradation in five consortia for more than 2 years. The high concentrations of benzoate detected in the dilution assay cultures of the 2-chlorobenzoate consortium suggest that 2-chlorobenzoate undergoes reductive dechlorination, as reported previously for 3-chlorobenzoate (16). The early detection (day 14) of methane, which arises from degradation of the aromatic structure, may be the result of rapid benzoate turnover and could account for the low concentration of benzoate detected. Anaerobic degradation of 2-chlorobenzoate has not been reported previously; therefore, this is the first report of anaerobic 2-chlorobenzoate dechlorination and degradation.

Since the sum of the amounts of chlorophenol, phenol, and benzoate detected in the chlorophenol-degrading consortia over time accounted for the amount of chlorophenol added initially, phenol was apparently converted to benzoate before degradation of the aromatic structure. The presence of benzoate in the enrichments from which these chlorophenol-degrading consortia were derived was noted previously (6). The substantial quantities of benzoate detected in our chlorophenol consortia suggest that conversion of phenol to benzoate is a major route in the catabolic pathway of 2-chlorophenol and 3-chlorophenol in these consortia. The formation of benzoate from phenol and CO_2 by sewage sludge organisms in the presence of H_2 was recently reported by Knoll and Winter (11). Hydrogen has been reported to inhibit degradation of benzoate by a syntrophic anaerobe (12), suggesting the involvement of a similar anaerobic bacterial species. Benzoate was detected in our consortia at significant concentrations without added H_2. Prior to the observations of Knoll and Winter (11), benzoate was not considered an intermediate in the metabolic pathway for anaerobic phenol degradation. We are the first to report the formation of benzoate as an intermediate in the anaerobic degradation of chlorophenols.

Our data indicate that the dechlorinated aromatic structure was subsequently degraded to carbon dioxide and methane, as was reported previously for the monochlorophenols (2, 17) and 3-chlorobenzoate (8, 9, 16). Complete degradation of benzoate (11) and phenol (5) to carbon dioxide and methane in anaerobic mixed cultures is described by the following equations, respectively:

$$4C_7H_6O_2 + 18H_2O \rightarrow 15CH_4 + 13CO_2 \quad (1)$$

$$4C_6H_6O + 16H_2O \rightarrow 14CH_4 + 10CO_2. \quad (2)$$

However, reductive dechlorination would require 1 mol of hydrogen per mol of monochloroaromatic compound (9, 16), which, under conditions of limited hydrogen, would be unavailable for methane production. This would decrease methane and increase carbon dioxide by 1 mol each, resulting in the following questions:

$$\text{(chlorobenzoates): } 4\,ClC_7H_5O_2 + 20H_2O \rightarrow 14CH_4 + 14CO_2 + 4HCl \quad (3)$$

$$\text{(chlorophenols): } 4ClC_6H_5O + 18H_2O \rightarrow 13CH_4 + 11CO_2 + 4HCl. \quad (4)$$

Boyd and Shelton (2) reported a 1:1 ratio of CH_4:CO_2 for 2-chlorophenol and 4-chlorophenol, which is similar to the ratio expected (1:1.2) from Equation 4. We detected a CH_4:CO_2 ratio greater than 1 in the chlorobenzoate consortia and less than 1 in the chlorophenol consortia. Although our ratios do not agree with those expected from Equations 3 and 4, they were consistent in detecting greater methane production by the chlorobenzoate consortia. The reducing equivalents available from yeast extract may explain the discrepancy.

Methane production in our chlorophenol consortia was delayed when compared with that in our chlorobenzoate-degrading consortia. Similarly, Boyd et al. (3) have noted that 3-chlorophenol initially inhibits methane production in unacclimated sludge. These observations suggest that either chlorophenols or phenol inhibit methanogenesis.

We estimated the anaerobic, chloroaromatic-degrading bacteria (bacteria per milliliter) to be present at greater than 10^5 in three of our consortia and at greater than 10^7 in two consortia. Shelton and Tiedje (15) have reported that strain DCB-1 is present in a 3-chlorobenzoate consortium at greater than 10^6 bacteria per ml. Interestingly, in the 2-chlorophenol consortium, phenol-degrading anaerobic bacteria were apparently present in much higher numbers ($>10^9$ bacteria per ml) than were the dechlorinating anaerobes ($<10^6$ bacteria per ml).

Since benzoate was detected during chlorophenol degradation, its degradation by the chlorophenol consortia was expected, but degradation of phenol by the chlorobenzoate consortia was unexpected, as these were transferred for several months with no exposure to phenol. Although the degradation of benzoate and phenol may be related in some manner, the presence of distinct phenol- and benzoate-degrading bacteria species in our consortia cannot be discounted.

The ability of the 3-chlorophenol and 3-chlorobenzoate consortia to degrade 2-chlorophenol was the only cross-acclimation to chloroaromatic compounds observed in our study. The 2-chlorophenol consortium did not have reciprocal degradative activities. By contrast, Boyd and Shelton (2) have reported that sludge acclimated to 2-chlorophenol or 3-chlorophenol also degraded 4-chlorophenol and sludge acclimated to 4-chlorophenol degraded all three monochlorophenols. It was concluded that a mixture of the bacterial populations was present in the sludges and accounted for the ability to degrade all three monochlorophenols. Boyd and Shelton (2) did not report on the ability of these chlorophenol consortia to degrade chlorobenzoates. DeWeerd et al. (4) have reported that their 3-chlorobenzoate consortium dechlorinated 2,4,5-trichlorophenoxyacetate but did not degrade any other monochlorophenols (4, 18). However, a 3-chlorobenzoate-dechlorinating strain (DCB-1) isolated from this consortium was not responsible for 2,4,5-trichlorophenoxyacetate degradation.

To the best of our knowledge, this is the first report of chlorobenzoate degradation under nitrate-reducing conditions. Strains of facultative anaerobes which grow on benzoate aerobically, and anaerobically under nitrate-reducing conditions, have been shown to degrade fluorobenzoates under nitrate-reducing conditions (1, 14, 19). The nitrate requirement for anaerobic 3-chlorobenzoate degradation by our consortium suggests the presence of similar bacterial strains. However, the fluorobenzoate-degrading facultative anaerobes neither grow with nor degrade chlorobenzoates. It is possible that a symbiotic relationship between an anaerobic dechlorinating bacterium and a bacterial species that is able to degrade benzoate under nitrate-reducing conditions is responsible for 3-chlorobenzoate degradation in this consortium.

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