The Anaerobic Degradation of 3-Chloro-4-Hydroxybenzoate in Freshwater Sediment Proceeds via Either Chlorophenol or Hydroxybenzoate to Phenol and Subsequently to Benzoate

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To study the anaerobic degradation of the chimera 3-chloro-4-hydroxybenzoate (3-Cl,4-OHB), anaerobic freshwater sediment samples from the vicinity of Athens, Ga., were adapted for the transformation of 4-hydroxybenzoate (4-OHB), 3-chlorobenzoate (3-CB), 2-chlorophenol (2-CP), and 2,4-dichlorophenol (2,4-DCP). In nonadapted samples, both 4-OHB (product of aryl dechlorination) and 2-CP (product of aryl decarboxylation) were observed as intermediates in the transformation of 3-Cl,4-OHB to phenol. The accumulated phenol was subsequently transformed to benzoate, an intermediate in the conversion to methane and CO₂. In 4-OHB-adapted samples (i.e., samples adapted for aryl decarboxylation), 2-CP was the first intermediate which was subsequently dechlorinated to phenol. In 3-CB-adapted samples (i.e., samples adapted for *meta*-chlorobenzoate dehalogenation), 3-Cl,4-OHB was stoichiometrically dechlorinated to 4-OHB. In 2-CP-adapted samples (i.e., samples adapted for *ortho*-chlorophenol dehalogenation), 4-OHB was the first major intermediate. Furthermore, 3-CB was not dechlorinated in 2-CP-adapted sediment samples, suggesting the possibility that different 3-Cl,4-OHB dechlorinating systems were induced in the 2-CP- and 3-CB-adapted sediments. Adaptation of sediment samples for dechlorination of 2,4-DCP did not lead to adaptation for dechlorination of 3-Cl,4-OHB. However, 3-Cl,4-OHB was dechlorinated to 4-OHB in our stable, sediment-free 2,4-DCP-dechlorinating enrichment, isolated previously from the same environment. In summary, these results suggest that the history of the sediment determines whether the first step in the anaerobic degradation of 3-Cl,4-OHB is a decarboxylation to 2-CP or a dechlorination to 4-OHB or whether both reactions occur simultaneously. But in all instances, the hydroxyl-substituted benzoic acid was converted via either route to phenol, which was then again transformed to benzoic acid before ring fission occurred.

In anaerobic environments, reductive aryl dechlorination is the initial step in the degradation or mineralization of many chlorinated aromatic compounds. Generally, chlorinated phenols are dehalogenated to phenol (1, 9–12, 14, 19, 20, 22–25, 33, 35, 38), e.g., 2,4-dichlorophenol (2,4-DCP) is usually first dechlorinated to 4-chlorophenol (4-CP). Subsequently, 4-CP is transformed to phenol, which is then carboxylated and dehydroxylated to benzoate (38). Chlorobenzoates are dechlorinated to benzoate (3, 4, 13, 30–32, 34). In both instances, benzoate is the final aromatic intermediate detected prior to the formation of acetate, CO₂, and H₂. In the presence of methanogens, these intermediates are further converted to CO₂ and CH₄ (25, 31, 38).

3-Chloro-4-hydroxybenzoate (3-Cl,4-OHB) contains the structural elements of 3-chlorobenzoate (3-CB), a 4-hydroxybenzoate (4-OHB), and a 2-chlorophenol (2-CP). Cell-free extract of the 3-CB dehalogenating anaerobe *Desulfomonile tiedjei* dechlorinates 3-Cl,4-OHB to 4-OHB (4). In contrast to this organism, cell-free extract of *Clostridium thermoacetatum* grown in the absence of 3-Cl,4-OHB but in the presence of 4-OHB (15, 16) and *Clostridium* sp. strain JW/Z-1 grown in the presence of 4-OHB (unpublished data) decarboxylate 3-Cl,4-OHB and 3-fluoro-4-hydroxybenzoate (3-F,4-OHB) to 2-CP and 2-fluorophenol (2-FP), respectively. Decarboxylation of 3-F,4-OHB to 2-FP has also been observed in a phenol-degrading consortium (26, 28). However, in contrast to *p*-hydroxybenzoate, no detailed studies

have been published on the fate of chlorinated hydroxybenzoates in anaerobic environments. Therefore, we investigated the fate of 3-Cl,4-OHB in a previously studied methanogenic freshwater sediment (19, 20, 35, 38). The question was whether 3-Cl,4-OHB is first dechlorinated to 4-OHB or decarboxylated to 2-CP or whether both reactions occur simultaneously in the sediment. In this paper we present data which indicate that the prior exposure of samples to other chlorinated or hydroxylated compounds with related structural elements determines the transformation pathway of 3-Cl,4-OHB and related compounds.

MATERIALS AND METHODS

Source of samples and growth medium. The sediment sample was collected from a freshwater pond site, Sandy Creek Nature Center, near Athens, Ga. (19), on 7 May 1991. Nonchlorinated aromatic compounds, 1 mmol/liter, or 0.5 mmol of chlorinated aromatic compounds per liter was added to nonadapted sediment. After the added compound was totally transformed (which required different times for the individual compounds [see Results]; 21), the various samples were called adapted sediments. The sample preparation was the same as described before (19). The pH of the samples was adjusted to 6.9 to 7.1 at the beginning of the experiment; at the end of the experiment, the samples usually exhibited a pH within approximately 0.3 of the starting pH.

The 2,4-DCP-dechlorinating enrichment (35) was obtained from a 2,4-DCP-degrading sediment sample taken from the

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same site (38). The medium for growing the 2,4-DCP-dechlorinating enrichment was the same as for strain JW/Z-1, except 40 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) was used as a buffer and the pH of the medium was 8.2 to 8.4. Yeast extract (0.5% [wt/vol]) was used as the main carbon and energy source. Usually a 2% (vol/vol) inoculum was used for starting a new culture.

The 4-OHB-decarboxylating bacterium, strain JW/Z-1, was grown as described before (37) except that 1% (wt/vol) yeast extract, 0.1% (wt/vol) glycine, and 0.1% (wt/vol) L-lysine were used as carbon sources. New cultures were started with a 2.5% (vol/vol) inoculum from the preculture.

All tests were conducted in duplicate. Since duplicate samples exhibited similar transformation patterns but sometimes with slight shifts in time, only one was selected for presentation. The incubation temperature was 30°C.

Analysis of aromatic compounds. Chlorinated and nonchlorinated aromatic compounds were separated and quantified by high-pressure liquid chromatography as described before (37), except that for the analysis of 2,4-DCP and 4-CP the mobile phase was methanol-deionized water-acetic acid (60:39.8:0.2 [vol/vol]). For the analysis of the other aromatic compounds, the ratio of the solvents was changed to 35:64.7:0.33.

The formation of 4-OHB in the 2,4-DCP-dechlorinating enrichment when supplemented with 3-Cl,4-OHB was confirmed by cochromatography with the three different mobile phases compounded of methanol-deionized water-acetic acid in a ratio of (i) 35:64.7:0.33, (ii) 15:84.6:0.43, and (iii) 7:92.5:0.47. By using these mobile phases, the corresponding retention times of 4-OHB were 3.66, 8.58, and 14.45 min, respectively. The sum of the remaining 3-Cl,4-OHB and the produced 4-OHB in grown culture was close to 100% of the added 3-Cl,4-OHB. Final confirmation of the product 4-OHB was obtained by a combined gas chromatography-mass spectrometry analysis as described before (38).

RESULTS AND DISCUSSION

3-Cl,4-OHB transformation in nonadapted freshwater sediment. The decarboxylation product 2-CP accumulated slowly during the first 9 days of incubation of freshly collected sediments supplemented with 1 mmol of 3-Cl,4-OHB per liter. During the following 7 days, the rate of disappearance of 3-Cl,4-OHB increased to $0.51 \mu\text{mol ml}^{-1} \text{ day}^{-1}$ (maximal rate, calculated from the last three points in Fig. 1A); 2-CP (0.24 mM) and 4-OHB (0.45 mM) were formed as the two major intermediates (Fig. 1A and B), indicating that both transformations of 3-Cl,4-OHB, the decarboxylation to 4-CP and the dechlorination to 4-OHB, occurred at the same time in the sediment.

The formed 4-OHB was transformed to phenol without a lag time (Fig. 1A and B). This observation is in agreement with the fact that 4-OHB-induced cultures of strain JW/Z-1, which was isolated from the same location (37), decarboxylates both 4-OHB and 3-Cl,4-OHB. The dehalogenation of 2-CP to phenol occurred also without a lag, while 3-Cl,4-OHB was still transformed (Fig. 1A and B). One possible explanation for this observation is that the low amount of 2-CP (10 to 80 μM) accumulated during the first 9 days of incubation induced the dechlorination activity. However, another possibility is that 3-Cl,4-OHB induced 2-CP dechlorination, because 3-Cl,4-OHB contains (with respect to the hydroxy group) an *ortho*-chlorine and, therefore, can be

FIG. 1. Degradation of 3-Cl,4-OHB in nonadapted sediments. (A and B) Without addition of acetate ([B] note the enlarged scale for the formation of the intermediates); (C) addition of 20 mmol of sodium acetate per liter. $\square$, 3-Cl,4-OHB; $\blacksquare$, 2-CP; $\bullet$, 4-OHB; $\circ$, phenol; $\blacktriangle$, benzoate.

regarded as an analog of 2-CP. The simultaneous accumulation of 4-OHB and the decrease of 2-CP between 8.5 and 14 days (Fig. 1B) further indicated the possibility that both halogenated compounds, 2-CP and 3-Cl,4-OHB, were transformed by the same bacterium. To distinguish between the two possibilities, purified dechlorinating cultures are required.

After about 20 days of lag time, the accumulated phenol was further degraded. As in previous observations (14), benzoate was not detected unless the samples were supplemented with acetate (Fig. 1C). Similar responses were observed in sediment samples supplemented with 20 mM sodium acetate and 0.7 mM bromoethane sulfonic acid (the methanogenesis inhibitor) (data not shown). At this concentration methane production in the samples was inhibited only 5 to 10% in comparison to the samples supplemented with 20 mmol of acetate per liter, which might explain why the benzoate degradation was only inhibited partially. About 40 to 50% more methane was produced in samples with acetate added than in the samples with no acetate added. Presumably, acetate inhibited the benzoate degradation in a manner similar to that observed in a methanogenic syntrophic co-

FIG. 2. Transformation and/or degradation of 3-Cl,4-OHB in 4-OHB-adapted sediments. (A) Without addition of sodium acetate and bromoethane sulfonate; (B) in the presence of 20 mM sodium acetate and 1.4 mM bromoethane sulfonate. □, 3-Cl,4-OHB; ■, 2-CP; ○, phenol; ▲, benzoate. The initial phenol concentration is due to carryover from the adaptation to 4-OHB transformation. Concentrations of up to 2 mM phenol did not have a detectable inhibitory effect on the dechlorination of 4-CP (35) and 2,4-DCP and the decarboxylation of 4-OHB and 3-Cl,4-OHB (37; unpublished data).

culture (7). Furthermore, in the acetate-supplemented samples (Fig. 1C), the disappearance of 3-Cl,4-OHB was faster (2.5 days less), and only 2-CP (1 mM) was detected, indicating that the decarboxylation of 3-Cl,4-OHB was the predominant reaction. This observation is consistent with the behavior of the pure culture of the decarboxylating bacterium, strain JW/Z-1, which decarboxylates 3-Cl,4-OHB to 2-CP. Its growth is stimulated by the addition of acetate and sodium ion (37; unpublished data). Since this strain was isolated from the same location, this or similar organisms are assumed to be present in the sediment samples. Presumably, dechlorinating bacteria were still present but not active enough, because 3-Cl,4-OHB was nearly stoichiometrically decarboxylated to 2-CP and no 4-OHB (product of dechlorination) was detected (Fig. 1C). In a bioremediation application, this could lead to an unwanted situation. The addition of one compound, acetate, to a sediment could cause an increased transformation of a hazardous compound, 3-Cl,4-OHB, or similar compounds but inhibit the further mineralization of a key intermediate such as benzoate.

4-OHB-adapted sediments. The lag time for decarboxylation of 4-OHB in nonadapted sediments was about 0.5 days, and it took an additional 3.5 days to transform 1 mM 4-OHB to phenol. In the 4-OHB-adapted sediments (see Materials and Methods) the decarboxylation of about 1 mM 3-Cl,4-OHB to 2-CP occurred without a lag time within 2.5 days (Fig. 2A). Presumably, the organism(s) responsible for this decarboxylation reaction is similar to the isolate JW/Z-1, which decarboxylates both 4-OHB and 3-Cl,4-OHB when

FIG. 3. Transformation and/or degradation of 3-Cl,4-OHB (A), 3-CB (B), and 2-CP (C) in 3-CB-adapted sediments. □, 3-Cl,4-OHB; ■, 2-CP; ●, 4-OHB; ○, phenol; ▲, benzoate; ×, 3-CB.

induced by either 4-OHB or 3-Cl,4-OHB. Dehalogenation of the accumulated 2-CP to phenol was observed after a lag of about 5 days (Fig. 2A). The phenol formed was further transformed after an additional 15 days of lag time. Subsequently, trace amounts of benzoate were observed as an intermediate (Fig. 2A). Addition of bromoethane sulfonic acid (1.4 mM) did not change the transformation pattern of 3-Cl,4-OHB, but the transformation of phenol to benzoate was delayed and benzoate accumulated (Fig. 2B), again because of the inhibition of methanogenesis and the accumulation of hydrogen which presumably inhibited the thermodynamically unfavorable ring cleavage of benzoate. Dechlorination of 3-Cl,4-OHB was not observed at measurable rates in the 4-OHB-adapted sediment. This was as expected, since dechlorination of 3-Cl,4-OHB in the nonadapted sediment was detected only after about 9 days of incubation, and 3-Cl,4-OHB disappeared within 2.5 days in the 4-OHB-adapted sediments (Fig. 1B, 2A, and 2B). In conclusion, in 4-OHB-adapted sediments, the only effective transformation reaction of 3-Cl,4-OHB was the decarboxylation to 2-CP.

3-CB-adapted sediment. It took about 50 days of incubation to adapt sediment to dehalogenate 3-CB, i.e., to degrade 1 mmol of 3-CB per liter (data not shown). By using this adapted sediment, 3-Cl,4-OHB was transformed to 4-OHB

FIG. 4. Transformation and/or degradation of 3-Cl,4-OHB (A), 2-CP (B), and 2,4-DCP (C) in 2-CP-adapted sediments. $\square$, 3-Cl,4-OHB; $\blacksquare$, 2-CP; $\bullet$, 4-OHB; $\circ$, phenol; $\blacktriangle$, benzoate; $\triangle$, 4-CP; $+$, 2,4-DCP. The initial phenol concentration is due to carryover from the adaptation to 2-CP transformation. (Also see the legend to Fig. 2.)

without a lag and the rate of transformation increased with time (Fig. 3A). On the basis of the work of Dolfig and Tiedje (5, 6, 21), it is speculated that the increasing dechlorinating rate (Fig. 3A and B) was due to growth of the dechlorinating bacterium. In parallel incubated samples, 3-CB was degraded as rapidly as 3-Cl,4-OHB (Fig. 3B). Thus, it is possible that the same bacterium (enzyme system) dechlorinates 3-CB and 3-Cl,4-OHB. This speculation is consistent with the reported substrate specificity of cell-free extract from the 3-CB-dehalogenating *D. tiedjei* DCB-1 (4).

In 3-CB-adapted sediments, added 3-F,4-OHB and 4-OHB were decarboxylated to 2-FP and phenol, respectively, after a lag of 2 days (data not shown). Dechlorination of 2-CP was observed after a lag of 9 days (Fig. 3C). This indicates that the bacterium or enzyme which dechlorinates 3-CB and 3-Cl,4-OHB does not dehalogenate either 2-CP or 3-F,4-OHB.

2-CP-adapted sediment. In nonadapted sediments it took 28 days to dechlorinate 1 mM 2-CP to phenol (with a lag time of 14 days). In the 2-CP-adapted sediment, 3-Cl,4-OHB and 2,4-DCP were both dechlorinated without a lag (Fig. 4A and

FIG. 5. Transformation of 3-Cl,4-OHB (A), 2-CP (B), and 2,4-DCP (C) in 2,4-DCP-adapted sediment. $\square$, 3-Cl,4-OHB; $\blacksquare$, 2-CP; $\bullet$, 4-OHB; $\circ$, phenol; $\blacktriangle$, benzoate; $\triangle$, 4-CP; $+$, 2,4-DCP. The initial 4-CP concentration is due to carryover from the adaptation to 2,4-DCP. 4-CP was omitted from panels A and B since it was not dechlorinated within the experiment. Concentrations below 1 mM 4-CP did not have a significant effect on the transformation of 2,4-DCP and benzoate and had only a minor inhibitory effect on the decarboxylation of 4-OHB (35, 37; unpublished data). However, 4-CP has a strong effect on the phenol transformation to benzoate (38).

C). However, 3-CB was not transformed (data not shown). Therefore, the 3-Cl,4-OHB-dechlorinating system in the 3-CB-adapted sediment might be different from the one in the 2-CP-adapted sediment. Furthermore, neither 3-chlorophenol nor 2-FP was dehalogenated by the 2-CP-adapted sediment (data not shown).

2,4-DCP-adapted sediment and enrichment. In the non-adapted sediments 18 days were required to dechlorinate 0.5 mM 2,4-DCP to 4-CP with a lag of 4 days. In the 2,4-DCP-adapted sediment samples, neither 3-Cl,4-OHB (Fig. 5A) nor 3-CB (data not shown) was dechlorinated.

However, when 1 mmol of 3-Cl,4-OHB per liter was added to our 2,4-DCP-dechlorinating enrichment culture (35), in the presence or absence of 1 mM 2,4-DCP, dechlorination of 3-Cl,4-OHB occurred and about 0.15 mM 4-OHB was produced in the culture supplemented with 2,4-DCP. When 2 mmol of 3-Cl,4-OHB per liter was added, 0.45 and 1.24 mM

FIG. 6. Proposed random pathway of degradation of 3-Cl,4-OHB in anaerobic freshwater sediment from the Sandy Creek Nature Center pond near Athens, Ga.

4-OHB was formed in the presence and absence of 1 mM 2,4-DCP, respectively. The added 2,4-DCP was concomitantly dechlorinated to 4-CP at both 3-Cl,4-OHB concentrations. This observation was surprising since the 2,4-DCP-dechlorinating enrichment culture, so far, exhibited a very narrow substrate specificity. Among the chlorophenols the culture only dechlorinated 2,4-DCP and 2,4,6-trichlorophenol, and, e.g., 2,4-dichlorobenzoate was not transformed (2).

The 2-CP- and the 2,4-DCP-adapted sediments dechlorinated 2-CP and 2,4-DCP (Fig. 4B and C and 5B and C). However, the relative rates of dechlorinations were significantly different, indicating again the possibility of the existence of two dechlorinating systems for 2,4-DCP in this sediment.

The sediment, adapted to dechlorinate 3-Cl,4-OHB, could dechlorinate 2-CP and 2,4-DCP (data not shown), and the relative rates of the 2-CP and 2,4-DCP dechlorinations were similar to the one observed in 2-CP-adapted sediments (Fig. 4B and C). One possible reason for this is that one dechlorinating activity, induced by either 2-CP or 3-Cl,4-OHB, was responsible for the dechlorination of 2-CP, 2,4-DCP, and 3-Cl,4-OHB.

Suggested pathway for 3-Cl,4-OHB degradation. From the above results we postulate a random pathway of anaerobic degradation of 3-Cl,4-OHB in freshwater sediments (Fig. 6). 3-Cl,4-OHB could either first be decarboxylated to 2-CP and then dechlorinated to phenol or first be dechlorinated to 4-OHB and then decarboxylated to phenol, or both reactions could occur at the same time. Both paths lead to phenol, which is then again transformed (dehydroxylated and carboxylated) to benzoate before ring fission occurs. Which of the two suggested routes is utilized in a given sediment will depend on the history of that sediment, i.e., whether aryl-decarboxylation or aryl-dechlorination had been induced in the sediment as a result of prior contaminations or the presence of other contaminants. The obtained results suggest that, in the analyzed sediment, at least two different organisms (or enzymes) can dechlorinate 3-Cl,4-OHB to 4-OHB. It was somewhat unexpected to find that in both routes 3-Cl,4-OHB, a benzoate derivative, was first converted to phenol, which again was then transformed (dehydroxylated and carboxylated) to benzoate. The assumption that phenol is transformed in all experiments to benzoate is based on the results of previous experiments (8, 17, 18, 27–29, 36, 38) and on the accumulation of benzoate when its degradation was inhibited by the addition of acetate (Fig. 1C) or indirectly by the inhibition of methanogenesis (Fig. 2B), a

required reaction to keep the concentration of H_2 in the sediment low enough to favor benzoate degradation.

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