Reduction of 3-Chlorobenzoate, 3-Bromobenzoate, and Benzoate to Corresponding Alcohols by *Desulfomicrobium escambiense*, Isolated from a 3-Chlorobenzoate-Dechlorinating Coculture

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An anaerobic bacterial coculture which dechlorinated 3-chlorobenzoate (3CB) to benzoate was obtained by single-colony isolation from an anaerobic bacterial consortium which completely degraded 3CB in defined medium. Of 29 additional halogenated aromatic compounds tested, the coculture removed the *meta* halogen from 2,3- and 2,5-dichlorobenzoate, 3-bromobenzoate (3BB), 5-chlorovanillate (5CV), and 3-chloro-4-hydroxybenzoate. Dechlorinating activity in the coculture required the presence of pyruvate. 5CV was also O-demethoxylated. The coculture contained two cell types: a short, straight gram-negative rod and a long, thin, curved gram-positive rod. The short rod, *Desulfomicrobium escambiense*, was recently isolated and identified as a new sulfate-reducing bacterial species (B. R. Sharak Genthner, S. D. Friedman, and R. Devereux, *Int. J. Syst. Bacteriol.* 47:889–892, 1997; B. R. Sharak Genthner, G. Mundfrom, and R. Devereux, *Arch. Microbiol.* 161:215–219, 1994). *D. escambiense* did not dehalogenate any of the compounds dehalogenated by the coculture, nor did it O-demethoxylate 5CV or vanillate. However, *D. escambiense* reduced 3CB, 3BB, and benzoate to their respective benzyl alcohols. Reduction to alcohols required the presence of pyruvate, which was transformed to acetate, lactate, and succinate in the presence or absence of 3CB, 3BB, or benzoate. Alcohol formation did not occur in pyruvate-sulfate medium. Under these conditions, sulfate was preferentially reduced. Other electron donors that supported the growth of *D. escambiense* during sulfate reduction did not support benzoate reduction to benzyl alcohol.

Reductive dehalogenation is the initial biotransformation step in the anaerobic biodegradation of halogenated aromatic compounds. Although reductive dechlorination of a variety of halogenated aromatic compounds has been reported, isolation of the microorganisms responsible for this biotransformation has been difficult. The first anaerobic reductively dechlorinating bacterial species to be isolated in pure culture was *Desulfomonile tiedjei* (6). *D. tiedjei* has served as the model species for investigating the physiological basis for aromatic reductive dechlorination (13, 15). A few anaerobic bacterial species which dehalogenate chlorinated phenols have recently been isolated and will probably serve as additional models for investigating the reductive dehalogenation reaction (2, 19, 33).

Our laboratory has been studying the reductive dechlorination of 3-chlorobenzoate (3CB) by an anaerobic bacterial coculture. *Desulfomicrobium escambiense*, a new sulfate-reducing bacterial species (21, 22), was isolated from this anaerobic 3CB-dechlorinating coculture (26) in pyruvate medium by serial dilution and single-colony isolation. As a result, it was of interest to determine if *D. escambiense* was responsible for the dechlorination of 3CB in the coculture. In this study, we investigated the dechlorinating capacities of both *D. escambiense* and the coculture from which *D. escambiense* was isolated.

MATERIALS AND METHODS

Anaerobic medium and methods. The anaerobic techniques and defined anaerobic medium (20) were modified as previously described (23, 24). Yeast extract (0.2%) was a medium component only for growth of consortium M35YE (see Table 1). Pyruvate or other supplements were added as indicated. When desired, the N₂-CO₂ gas phase was replaced with H₂-CO₂ (90:10) by flushing sealed tubes or bottles of medium for 5 min before reducing and inoculating them. Cultures containing H₂ were shaken vigorously during incubation. Incubations were carried out at 30°C. Growth was measured as an increase in the optical density at 600 nm.

Source of consortia and isolation of coculture and pure cultures. Consortium M35D was obtained by adapting consortium M35YE (23, 24) to defined medium lacking yeast extract (see Table 1). Consortium M35D was diluted to 10⁻⁹ in defined 3CB medium to derive M35/9. Coculture 3162 was obtained from M35/9 as an isolated colony on defined 3CB roll tubes.

D. escambiense was obtained by serial dilution of coculture 3162 and single-colony isolation on defined anaerobic pyruvate medium. Anaerobic pyruvate agar plate medium was poured at the laboratory bench, dried in an anaerobic jar under flowing N₂-CO₂ (90:10), sealed in the jar under the gas phase, and transferred into a model 1025 anaerobic chamber (Forma Scientific, Marietta, Ohio) to complete the reduction.

Dehalogenation studies. Dehalogenation studies of coculture 3162 were done with the culture in pyruvate (0.2%) medium as previously described (23, 24). Gray butyl rubber, Teflon-faced stoppers (Wheaton Scientific, Millville, N.J.) were used for 3-chlorotoluene, 3-chlorobenzyl alcohol, and 3-chloro-*o*-, *m*-, or *p*-toluic acid. The gray stoppers were sterilized with ethanol before the cultures were sampled, and they were resealed with silicone rubber sealer (Borden, Inc., Columbus, Ohio) to prevent volatilization. Chemicals were obtained from Aldrich Chemical Co. (Milwaukee, Wis.), except 5-chlorovanillate (5CV; Lancaster Synthesis, Windham, N.H.) and 3-chloro-*o*-, *m*-, or *p*-toluic acid (Pfaltz & Bauer, Waterbury, Conn.). Test compounds were added to 500 µM, except 3-chlorobenzyl alcohol, 3-chlorobenzaldehyde, and 3-chloro-*o*-, *m*-, and *p*-toluic acid, which were added to 100 µM, and 2,4,5-trichlorophenoxyacetic acid and 3-chlorotoluene, which were added to 50 µM. Duplicate cultures were inoculated (1%) with the second transfer of coculture 3162 grown in pyruvate-3CB (800 µM) medium. The ability of coculture 3162 to use H₂ as an electron donor for 3CB dechlorination was determined with the culture in 3CB medium under H₂-CO₂ (90:10) inoculated with the coculture diluted to 10⁻³ prepared in defined medium.

3CB dechlorination by *D. escambiense* was investigated with the culture in pyruvate medium with and without the following supplements: 50% clarified,

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TABLE 1. Characteristics of consortium M35 and its derivatives

Consortium or strain	Source	Characteristics of ^a :			No. of cell types ^b
		3CB	Benzoate	CH ₄	
M35YE	Freshwater sediment	DC	I, d	p	ND ^c
M35D	M35YE	DC	I, d	p	ND
M35/9	M35D	DC	I, d	p	4
Coculture 3162	M35/9	DC	I	—	2
<i>D. escambiense</i>	Coculture 3162	R	R	—	1

^a DC, dechlorinated; I, biodegradative intermediate; d, degraded; p, end product; —, not degraded or produced; R, reduced to the corresponding alcohol.

^b Number of morphologically distinct cell types observed microscopically.

^c ND, not determined.

anaerobic (N₂) ruminal fluid; 20% anaerobic (N₂-CO₂) supernatant from coculture 3162; or nicotinamide (250 µg/liter) plus 1,4-naphthoquinone (100 µg/liter). All supplements were filter sterilized and were chosen based on the known nutritional requirements of *D. tiedjei* (6). Coculture 3162 supernatant fluid was collected from an actively dechlorinating late-logarithmic-phase culture. Dehalogenation by *D. escambiense* of compounds dehalogenated by coculture 3162 was determined with the culture in pyruvate medium supplemented with nicotinamide plus 1,4-naphthoquinone.

Analytical methods. High-performance liquid chromatography (HPLC) samples were filtered (Acrodisc 13CR PTFE; pore size, 0.2 µm) before analysis on a Hewlett-Packard 1090M HPLC apparatus equipped as previously described (24). A 50 mM K₂HPO₄ (pH 3.5)–CH₃CN solvent with 0.2% acetic acid was used in the appropriate ratios (10:90 to 50:50) to optimize the separation of halogenated compounds from dehalogenated products. The pH was adjusted to 3.0 to separate benzoate from 2-iodobenzoate. The wavelengths were optimized for the compounds being analyzed (214 to 280 nm). Acetic acid was deleted from the solvent at λ ≤ 220 nm. Quantification of chlorinated and nonchlorinated aromatic compounds by HPLC and identification by UV spectroscopic and gas chromatographic-mass spectral (GC-MS) analyses (25), determination of sulfate and bromide concentrations in duplicate filtered samples by ion chromatography (22), and quantification of methane by GC (24) were done as previously described. Volatile and nonvolatile fatty acids were quantified by gas chromatography after the preparation of pentafluorobenzyl derivatives (8). Heptanoate was added to each sample as an internal derivatization control (10). The change in free energy was calculated from Gibbs free energy of formation (ΔG^o) values (31, 32).

RESULTS

Characteristics of the consortia, coculture, and pure cultures. The characteristics of M35YE, the original 3CB-degrading consortium, and its derivatives are given in Table 1. Consortia M35YE, M35D, and M35/9 dechlorinated 3CB, degraded benzoate, and produced methane. Microscopic examination of M35YE and M35D revealed gram-negative cocci and several gram-negative rods of various sizes and shapes. Microscopically, consortium M35/9 was observed to contain four distinct gram-negative cells types, including a thick rod with square ends, a thin curved rod, a coccobacillus (which was often found in pairs), and a long, thin spirillum. Only the thick rod was nonmotile. The first three types were present in approximately equal numbers, whereas the spirillum was much less numerous.

Coculture 3162 obtained from consortium M35D as a single colony on defined 3CB medium contained equal numbers of a coccobacillus and curved rod (Fig. 1) similar to those present in consortium M35D, except that the curved rod stained gram positive in actively dechlorinating cocultures. On anaerobic pyruvate agar plates, coculture 3162 formed small (<1-mm), flat, tan, translucent colonies which were microscopically pure cultures of the coccobacillus. This strain was recently identified as a new sulfate-reducing bacterial species and was designated *Desulfomicrobium escambiense* (22). On pyruvate-sulfate medium, *D. escambiense* colonies were larger (2 mm), circular, entire, convex, smooth, and reddish brown with a black center.

We were unable to isolate the curved rod in anaerobic media containing the following energy sources: 3CB, pyruvate, pyru-

vate-3CB, H₂-CO₂, H₂-3CB, methanol, acetate, vanillate, or 5CV. Supplementing these media with filter-sterilized supernatant (20%) from coculture 3162 did not allow isolation of the curved rod.

Dechlorinating capabilities of coculture 3162. Coculture 3162 dechlorinated 3CB to benzoate in defined 3CB medium but did not subsequently degrade the benzoate formed or produce methane (Table 1). 3CB-dechlorinating activity in the coculture was lost following three serial transfers in defined 3CB medium but was restored after a 5- to 7-week lag by adding either pyruvate or H₂ (Fig. 2). In 3CB-pyruvate medium, the coccobacillus outnumbered the curved rod (~100:1), while in 3CB-H₂ medium, the numbers of coccobacilli were greatly diminished, resulting in the two cell types being present in equal numbers.

In addition to 3CB, coculture 3162 removed the *meta*-halogen from 2,3- and 2,5-dichlorobenzoate, 3-bromobenzoate (3BB), 5CV, and 3-chloro-4-hydroxybenzoate (Fig. 3A). The appropriate concentration of dehalogenated product was detected (Fig. 3B), and the identity of the product was confirmed by UV spectroscopy. Very little 3BB was debrominated after 4 months, but both benzoate (Fig. 3B) and free bromide ions (53.3 µM) were detected at the appropriate concentrations. Vanillate, formed by dechlorination of 5CV, was subsequently O-demethylated to protocatechuic acid, which was not further degraded. The concentration of 3-chlorobenzyl alcohol declined slightly (20 µM), and an intermediate with the retention time of benzyl alcohol was detected, but its low concentration (<10 µM) did not permit confirmation by UV spectroscopy.

Coculture 3162 did not dehalogenate the following: 2- and

FIG. 1. Phase-contrast photomicrograph of coculture 3162 in defined pyruvate-3CB medium. a, Unidentified curved rod; b, paired cells of *D. escambiense*; c, single cell of *D. escambiense*. Bar, 10 µm.

FIG. 2. Restoration of 3CB-dechlorinating activity in coculture 3162 by addition of pyruvate (A) or H_2 (B).

4CB; 2- and 4BB; 2-, 3-, and 4-fluorobenzoate; 2-, 3-, and 4-iodobenzoate; 3,4- and 3,5-dichlorobenzoate; 2-, 3-, and 4-chlorophenol; 3- and 5-chlorosalicylate (3- and 5-chloro-2-hydroxybenzoate); 3-chlorotoluene (3-chloromethylbenzene); 3-chloro-(*o*, *m*, or *p*)-toluic acid [3-chloro-(*o*, *m*, or *p*)-methyl-

FIG. 3. Dechlorination of *meta*-halogenated benzoate derivatives by coculture 3162 (A) with formation of appropriate nonhalogenated products (B). Abbreviations: 2,3 DCB, 2,3-dichlorobenzoate; 2,5 DCB, 2,5-dichlorobenzoate; 3C4HB, 3-chloro-4-hydroxybenzoate; 4HB, 4-hydroxybenzoate.

FIG. 4. Reduction of 3CB to 3-chlorobenzyl alcohol (A) and corresponding fermentation of pyruvate (B) by *D. escambiense* with the formation of acetate, lactate, and succinate.

benzoate]; 3-chlorocinnamate (3-chloro-phenylpropenoic acid); and 2,4,5-trichlorophenoxyacetic acid.

After 4 months, we did not detect 3-chlorobenzaldehyde in either cocultures or uninoculated controls containing 3-chlorobenzaldehyde as the target compound. This suggested that abiotic loss of 3-chlorobenzaldehyde had taken place. We did not detect 3-chlorobenzoate, 3-chlorobenzyl alcohol, benzaldehyde, or benzoate as a product of this abiotic loss.

Reduction of halogenated aromatic acids to aromatic alcohols by *D. escambiense*. *D. escambiense* was tested for its ability to dehalogenate the six compounds dehalogenated by coculture 3162. Only 3CB and 3BB concentrations were observed to decrease (~50%) in pyruvate medium. Benzoate was not detected, but intermediates formed in the 3CB and 3BB cultures were identified as 3-chloro- and 3-bromobenzyl alcohol, respectively, by comparing GC retention times and UV and mass spectra to those of authentic standards. *D. escambiense* did not O-demethylate 5CV or vanillate.

The ability of *D. escambiense* to reduce 3CB, 3BB, and benzoate to the corresponding alcohols was further examined. All three compounds were stoichiometrically reduced at similar rates to their respective alcohols in defined pyruvate medium, with pyruvate being required for this reduction; however, the reduction and fermentation were incomplete (data not shown). Approximately 50% of the aromatic acid and 30% of the pyruvate remained in the medium after fermentation and growth had ceased, as shown for 3CB and pyruvate in Fig. 4. Pyruvate was fermented to acetate, lactate, and succinate in the presence (Fig. 4) or absence of 3CB, with the aromatic substrate having little effect on the concentrations and ratios of fermentation products (data not shown). Reduction and degradation curves for aromatic substrates and pyruvate, respectively, were similar. Both were rapidly metabolized in the initial 1 to 2 weeks, with rates decreasing considerably thereafter.

Replacing pyruvate with ethanol, formate, lactate, or H_2 ,

TABLE 2. Reduction of benzoic acid to benzyl alcohol during reduction of pyruvate (18.2 mM) to succinate by *D. escambienne*

Substrate(s)	Maximum OD (600 nm) ^a	Concn of ^b :		
		BA (μ M)	Bz Alc (μ M)	Succinate (mM)
Pyruvate	0.02	NA ^c	NA	6.6
Pyruvate-BA	0.02	495	235	4.5
Lactate-BA	0	850	0	0
Ethanol-BA	0	866	0	0
Formate-BA	0	851	0	0
H ₂ -CO ₂ -BA	0	840	0	0
Pyruvate-BA-SO ₄ ²⁻	0.058	810	0	0
Pyruvate-SO ₄ ²⁻	0.059	NA	NA	0
Benzoate	0	806	0	0

^a OD, optical density.^b BA, benzoic acid; Bz Alc, benzyl alcohol.^c NA, not applicable.

which support the growth of *D. escambienne* during sulfate reduction (21, 22), did not support the reduction of benzoate to benzyl alcohol (Table 2). In pyruvate-sulfate and pyruvate-benzoate-sulfate media, pyruvate was fermented to acetate and lactate but not succinate, and SO₄²⁻ was reduced; however, benzyl alcohol was not formed (Table 2). Adding sulfate to pyruvate or pyruvate-benzoate medium increased cell yields, as estimated by the maximum optical density attained, but the addition of benzoate to pyruvate or pyruvate-sulfate medium did not increase cell yields (Table 2). Thus, reduction of aromatic acids to aromatic alcohols did not result in a significant increase in energy to the cell.

Adding 50% ruminal fluid, 20% filter-sterilized supernatant from coculture 3162, or nicotinamide plus 1,4-naphthoquinone did not support the dehalogenation of 3CB or 3BB by *D. escambienne* in defined pyruvate medium. As above, 3CB and 3BB were reduced to their respective alcohols. Benzyl alcohol was not detected in the coculture during growth in pyruvate-3CB medium.

DISCUSSION

The isolation of coculture 3162 in pyruvate-3CB medium and subsequent attempts to further purify the two members of this coculture were based in part on *D. tiedjei* physiology (5, 6, 27, 28). Although we did not succeed in isolating the dechlorinating species in pure culture, we were able to characterize the dehalogenating ability of the coculture and to isolate one of the bacterial strains in the coculture, which was characterized as to its ability to biotransform 3CB, 3BB, and benzoate.

The dehalogenating ability of coculture 3162 was more limited than that of pure cultures of *D. tiedjei*, which dehalogenated the *ortho*, *meta*, and *para* isomers of BB and iodobenzoate as well as 3CB (3). Like *D. tiedjei*, coculture 3162 dechlorinated *meta*-chlorinated benzoates and their derivatives but was more restricted in not dehalogenating 2- or 4BB, iodobenzoate isomers, or some dichlorobenzoates containing a *meta*-substituted chlorine. Our data indicate that a hydroxyl at position 2 of the benzene ring interfered with reductive dechlorination by coculture 3162 at the *meta* position while a hydroxyl or methoxyl group at position 3 or 4, respectively, did not. In contrast, a chloro group at position 2 or 5 did not interfere with *meta* reductive dechlorination, whereas its presence at position 3 or 4 did. Thus, there was an interaction between substituent type and position on the ability of the coculture to dechlorinate at the *meta* position.

In addition, coculture 3162, like *D. tiedjei*, could use pyruvate or H₂ (4, 6) as a source of reducing equivalents for dehalogenation. It is our conclusion that, as a member of the coculture, *D. escambienne* ferments pyruvate, providing reducing equivalents for dehalogenation via "interspecies hydrogen transfer" (12). Growth and dechlorination of 3CB by coculture 3162 in 3CB-H₂ medium indicates that H₂ can also serve as a direct source of electrons for the dehalogenation of 3CB. It is apparent from the decline in the number of coccobacilli, i.e., *D. escambienne* cell types, when the coculture is grown on H₂ rather than pyruvate that *D. escambienne* grows more efficiently with pyruvate than with H₂. Since acetate is a major product of pyruvate fermentation, this may be a result of less efficient production of acetate as the carbon source for *D. escambienne* cell growth when the coculture is grown in 3CB-H₂ medium. Failure to observe a similar relative decline in the numbers of curved rods suggests that the source of energy for the growth of the curved rod is similar regardless of whether pyruvate or H₂ is present. These observations, in combination with our observation that a pure culture of *D. escambienne* does not dechlorinate 3CB, suggest that the curved rod is responsible for dehalogenation by the coculture. They also suggest that either *D. escambienne* or the curved rod is an acetogen, forming acetate for cell carbon directly from the reduction of carbon dioxide (34).

Although DeWeerd et al. (5) indicated that there was no apparent relationship between dechlorination and O-demethylation, it was interesting that both *D. tiedjei* and coculture 3162 O-demethylated a chlorobenzoate derivative bearing a *meta* methoxyl group.

In contrast to the coculture, a pure culture of *D. escambienne* failed to dehalogenate any benzoate derivatives transformed by the coculture or to O-demethylate 5CV or vanillate. These data suggest that the curved rod in the coculture is responsible for both dechlorination and O-demethylation. Attempts to isolate the curved rod have not been successful, despite the use of a variety of media and supplements; however, all potential combinations of supplements have not been exhausted. It is possible that isolation of *D. escambienne* on pyruvate medium selected for a nondehalogenating strain; however, reisolation of *D. escambienne* on pyruvate-3CB medium also produced nondechlorinating strains.

Interestingly, *D. escambienne* was found to reduce 3CB, 3BB, and benzoate to their corresponding alcohols without dechlorination; this probably occurred via a two-step reduction of acid to aldehyde followed by aldehyde to alcohol. This biotransformation required pyruvate, which is fermented by *D. escambienne* (22) to acetate, lactate, and succinate (21) and was presumably the source of reducing equivalents for the reduction of the aromatic acid since the time courses for the fermentation of pyruvate and reduction of the aromatic acids were similar. Succinate formation in sulfate-reducing bacteria (11, 16, 18), and probably in *D. escambienne* as well, occurs via a partial reverse tricarboxylic acid cycle. Thus, NAD and flavin adenine dinucleotide (FAD) are likely to be the electron carriers involved in the reduction of the aromatic acids. This conclusion is substantiated by the failure of ethanol, formate, lactate, and H₂, which are not fermented but serve as electron donors for *D. escambienne*, to support the reduction of benzoate to benzyl alcohol.

Failure of *D. escambienne* to form benzyl alcohol or succinate during sulfate reduction indicates that the latter is the preferred reductive mechanism, probably because it is energetically more favorable than either succinate formation (−65.9 kJ/mol of pyruvate utilized) or benzoate reduction (−16.4 kJ/mol of H₂ utilized), producing −85.3 kJ/mol of pyru-

vate utilized and -38.0 kJ/mol of H_2 utilized, respectively (31, 32). The small change in the free energy available from the reduction of benzoate to benzyl alcohol explains the lack of a significant increase in *D. escambiense* cell yields when benzoate was added to the various media.

Detection of stoichiometric amounts of benzoate from 3CB during dechlorination by the coculture indicates that the aromatic alcohol is probably not being formed in the coculture. Preferential utilization of the most energetically favorable terminal electron sink during interspecies hydrogen transfer explains our observation; i.e., with a limited number of reducing equivalents, the most efficient electron sink will be utilized. 3CB reductive dechlorination results in a change in free energy of -125 kJ/mol of H_2 (7) compared with -16.4 kJ/mol of H_2 for the reduction of benzoate to benzyl alcohol.

Although we are the first to report the anaerobic reduction of benzoic acids to benzyl alcohols, anaerobic sediment enrichments (14) were previously observed to oxidize an aromatic aldehyde to its corresponding carboxylic acid, accompanied by partial reduction of the aldehyde to its corresponding aromatic alcohol. The authors concluded that formation of the alcohol and acid were metabolically related, i.e., that the alcohol was formed as a result of using reducing equivalents generated from oxidation to an acid. A methanogenic consortium that transforms phenol to benzoate was reported to oxidize 2-hydroxybenzyl alcohol to 2-hydroxybenzoic acid (1); however, the authors were unable to determine the specific electron acceptor. Given sulfate as the electron acceptor, benzaldehydes were oxidized to benzoic acids by three strains of *Desulfovibrio* (35), and a new *Desulfotomaculum* species completely degraded benzyl alcohol (9).

More recently, *Desulfovibrio desulfuricans* was shown to be capable of bidirectional transformation of aromatic aldehydes under nitrate-dissimilating conditions, forming benzoates and benzyl alcohols (17). In the presence of lactate, benzaldehyde was reduced primarily to benzyl alcohol, but in its absence, benzoate was the primary product. Thus, the product formed was dependent upon the availability of reducing equivalents. In contrast to *D. desulfuricans* (17), *D. escambiense* used benzoic acids as electron acceptors, forming benzyl alcohols, and used reducing equivalents generated from pyruvate fermentation but not directly from lactate. Thus, the requirement for pyruvate during both dehalogenation by the coculture and alcohol formation by *D. escambiense* pure cultures is probably a result of pyruvate being the source of reducing equivalents for these two reductive biotransformations.

D. escambiense and *D. tiedjei* share several characteristics, including being sulfate-reducing bacteria, being isolated from a 3CB-dechlorinating methanogenic consortium (27, 30), and having the ability to ferment pyruvate to form acetate, lactate, and succinate as fermentation products (28, 29). However, *D. escambiense*, in contrast to *D. tiedjei*, does not appear to be able to independently dehalogenate aromatic compounds. Since the curved rod in coculture with *D. escambiense* is apparently responsible for or involved in the dechlorination of 3CB, attempts to isolate the curved rod continue and 16S rRNA analysis of coculture 3162 are under way to place the curved rod phylogenetically without isolation.

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