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Anaerobic Aryl Reductive Dehalogenation of Halobenzoates by Cell Extracts of "*Desulfomonile tiedjei*"

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We studied the transformation of halogenated benzoates by cell extracts of a dehalogenating anaerobe, "*Desulfomonile tiedjei*." We found that cell extracts possessed aryl reductive dehalogenation activity. The activity was heat labile and dependent on the addition of reduced methyl viologen, but not on that of reduced NAD, NADP, flavin mononucleotide, flavin adenine dinucleotide, desulfoviridin, cytochrome c_3, or benzyl viologen. Dehalogenation activity in extracts was stimulated by formate, CO, or H_2, but not by pyruvate plus coenzyme A or by dithionite. The pH and temperature optima for aryl dehalogenation were 8.2 and 35°C, respectively. The rate of dehalogenation was proportional to the amount of protein in the assay mixture. The substrate specificity of aryl dehalogenation activity for various aromatic compounds in "*D. tiedjei*" cell extracts was identical to that of whole cells, except differences were observed in the relative rates of halobenzoate transformation. Dehalogenation was 10-fold greater in "*D. tiedjei*" extracts prepared from cells cultured in the presence of 3-chlorobenzoate, suggesting that the activity was inducible. Aryl reductive dehalogenation in extracts was inhibited by sulfite, sulfide, and thiosulfate, but not sulfate. Experiments with combinations of substrates suggested that cell extracts dehalogenated 3-iodobenzoate more readily than either 3,5-dichlorobenzoate or 3-chlorobenzoate. Dehalogenation activity was found to be membrane associated. This is the first report characterizing aryl dehalogenation activity in cell extracts of an obligate anaerobe.

Aryl reductive dehalogenation is the initial step in the mineralization of many haloaromatic compounds in anoxic habitats. These reactions are biologically catalyzed and have been detected in undefined enrichments, in defined consortia, and with an isolated anaerobic bacterium, "*Desulfomonile tiedjei*" (2, 4, 9, 15, 19, 20). It has been determined that the dechlorination of 3-chlorobenzoate represents a novel type of anaerobic respiration (1a, 15, 19). Several features characterized aryl reductive dehalogenation by these cells. The reaction catalyzed by the defined consortium obeyed Michaelis-Menten kinetics (20), and both types of cultures exhibited a strict specificity for chlorinated benzoates over other haloaromatic substrates (2, 10). Further, dehalogenation occurred preferentially at the *meta* position of these substrates and the reaction was not related to the removal of other aryl substituents (2, 10). These data suggested that dehalogenation was enzyme catalyzed or influenced by a restrictive transport system. In any case, little is known about the biochemistry of anaerobic aryl dehalogenation reactions.

Our objectives were (i) to develop an assay for aryl reductive dehalogenation in cell extracts of "*D. tiedjei*"; (ii) to characterize the cell-free activity; and (iii) to compare the activity in cell extracts with that in whole cells. Dehalogenation in cell extracts of "*D. tiedjei*" exhibited characteristics consistent with an enzyme-catalyzed reaction. Furthermore, extracts exhibited a substrate specificity that was identical to that of whole cells, but the relative rates of halide release were distinctly different.

MATERIALS AND METHODS

Preparation of extracts. "*D. tiedjei*" was grown in a medium containing pyruvate (40 mM), 3-chlorobenzoate (2 mM), and 0.1% yeast extract (1a). Samples of culture fluid were periodically withdrawn and analyzed for halobenzoate disappearance by high-performance liquid chromatography. Late-exponential-phase cultures were harvested by centrifugation at 15,000 × *g* for 35 min at 4°C, and 1 g (wet weight) of cells was suspended in 3 ml of 50 mM Tris buffer, pH 7.7. Cells were broken by French pressure disruption (124 mPa) under a stream of O_2-free N_2. The crude extract was centrifuged at 20,000 × *g* for 15 min at 4°C, and the supernatant was used for dehalogenation assays. The supernatant was further centrifuged at 179,000 × *g* for 1 h at 23°C to sediment membrane fractions in enzyme localization studies. Cultures used for induction experiments were grown either on 40 mM pyruvate alone or with the same concentration of pyruvate and various combinations of the following electron acceptors: 3-chlorobenzoate (2 mM), Na_2SO_4 (10 mM), or $Na_2S_2O_3$ (10 mM).

Dehalogenation assay. We tested dehalogenation in cell extracts in 5-ml crimp-sealed serum vials containing 1 ml of an assay mixture. The assay mixture contained the following: a pH buffer, TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (50 mM), pH 8; a reductant, dithiothreitol, (10 mM); the substrate, 3-chlorobenzoate or 3-iodobenzoate (8 mM); and the electron carrier, methyl viologen (5.4 mM). Serum vials were filled with the assay mixture and sealed with butyl rubber stoppers while inside an anaerobic glovebox (Coy Laboratory Products Inc., Ann Arbor, Mich.). The headspace of the assay vials was then replaced with H_2 gas at 206-kPa overpressure unless otherwise indicated. A H_2 gas phase was used in assays to keep methyl viologen reduced through hydrogenase activity, which was also present in cell extracts. Dehalogenation assays were started by the anoxic addition of 0.2 ml of cell extract (10 to 20 mg of protein). The assay mixtures were incubated at 35°C, anaerobically, in the dark and without agitation unless otherwise indicated. In experiments testing various electron donors, the same assay components listed above were used, except dithiothreitol was omitted and 100

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mM formate, 30 mM pyruvate (plus 2 mM coenzyme A) or 10 mM dithionite was used to reduce methyl viologen under a N_2 headspace. Alternately, the atmosphere in reaction vials was replaced with H_2, CO, or O_2 at 206-kPa overpressure. Cofactors tested for their involvement in aryl dehalogenation included NADH (2 mM), NADPH (2 mM), flavin adenine dinucleotide (1 mM), flavin mononucleotide (1 mM), and either cytochrome c_3 (18 μg) or desulfoviridin (365 μg) from "*D. tiedjei*" (1a). Extracts were heated in a boiling-water bath for 15 min and used as negative controls in the complete assay mixture.

The pH optimum of cell-free dehalogenation activity was determined with 50 mM TES, phosphate, or borate buffer.

The rates of dehalogenation were determined as the rate of formation of the dehalogenated product unless otherwise indicated. The kinetics of dehalogenation were determined from a minimum of five time points for several experiments and were always found to be representative of zero order kinetics for over 30 h and therefore linear for the duration of these experiments.

Other enzyme assays. Hydrogenase, formate dehydrogenase, carbon monoxide dehydrogenase, and pyruvate oxidoreductase were assayed anaerobically by a modification of an existing method (14). The assay conditions (14) were modified by using 50 mM TES (pH 8)–20 mM methyl viologen–10 mM $MgCl_2$. Substrate concentrations used for formate dehydrogenase or pyruvate oxidoreductase were 10 mM formate or 10 mM pyruvate plus 2 mM coenzyme A, respectively. Methyl viologen reduction was measured at 603 nm at room temperature (23°C) in a Beckman DU-65 spectrophotometer.

Analytical. Samples (0.3 ml) from dehalogenation assay mixtures were periodically taken by syringe, acidified with 60 μl of 6 N HCl, and mixed with 0.6 ml of ethyl acetate. These subsamples were then centrifuged for 1 min at 16,000 × *g*, and 0.5 ml of the solvent fraction was transferred to a separate vial. The ethyl acetate was evaporated to dryness under a stream of air, and the residue was taken up in 1 ml of 0.025 N NaOH. The samples were analyzed by high-performance liquid chromatography as described previously (4) and quantitated by comparison with identically prepared external standards.

Protein was measured colorimetrically (18), using bovine serum albumin as the standard.

Chemicals. All aromatic compounds were obtained from Aldrich Chemical Co., Milwaukee, Wis., while the others were obtained from Sigma Chemical Co., St. Louis, Mo. Desulfoviridin and cytochrome c_3 were purified previously from "*D. tiedjei*" (1a).

RESULTS

"*D. tiedjei*" (1a) was found to be the predominant dehalogenating bacterium in a 3-chlorobenzoate-degrading methanogenic consortium (15). We assayed for dehalogenation activity in cell extracts of this sulfate-reducing bacterium. The residual amounts of substrate and dehalogenated products from spent assay mixtures were determined by a solvent extraction method. By using this procedure, the assay components were defined to optimize aryl dehalogenation activity. We tested dehalogenation activity in cell extracts amended with reduced cofactors or proteins known to supply reducing equivalents in sulfate-reducing bacteria (Table 1). Dehalogenation of 3-chlorobenzoate was observed in the complete assay mixture, as evidenced by the loss of the parent substrate and the stoichiometric production of benzoate. Rates of substrate removal were equal to the rates of product formation. The loss of activity in boiled extracts indicated that aryl reductive dehalogenation was heat labile (Table 1). The selective removal of most cofactors or individual proteins had no significant influence on aryl reductive dehalogenation in cell extracts. The only compound that significantly reduced the dehalogenation rate when it was omitted from the complete assay mixture was methyl viologen (Table 1). Therefore, subsequent dehalogenation assays used only this electron carrier. Benzyl viologen could not substitute for methyl viologen in the dehalogenation assays with extracts (data not shown).

TABLE 1. Effect of reduced cofactors on dehalogenation in cell extracts of "*D. tiedjei*"

Incubation condition	Dehalogenation rate ± SD (nmol/min per mg)[a]
Complete mixture	0.80 ± 0.08
Boiled extracts	0.0[b]
Complete mixture:	
Less $NADH_2$	0.72 ± 0.08
Less $NADPH_2$	0.74 ± 0.02
Less $FADH_2$[c]	0.79 ± 0.08
Less $FMNH_2$[c]	0.65 ± 0.08
Less cytochrome c_3	0.65 ± 0.01
Less desulfoviridin	0.61 ± 0.05
Less methyl viologen	0.0[b]

[a] Rate of benzoate formation from 3-chlorobenzoate was determined from two sampling times based on zero order kinetics. The assays had 2.76 mg of protein and were incubated for 8 h, using standard conditions (see text).

[b] Significantly different from complete mixture at 0.001 level; F-test model 1 analysis of variance. All other treatments did not differ from the positive control.

[c] Flavin adenine dinucleotide, and flavin mononucleotide (FAD and FMN) were nonenzymatically reduced by $NADH_2$ and $NADPH_2$ (16).

We compared the ability of cell extracts to reduce methyl viologen with potential electron donors and their effect on dehalogenation (Table 2). Enzyme activities associated with hydrogenase, carbon monooxide dehydrogenase, and formate dehydrogenase were within an order of magnitude of each other, but pyruvate oxidoreductase was about a 100-fold lower (Table 2). All of these dye reducing activities in these extracts were greater than the dehalogenation activities.

TABLE 2. Effect of different electron donors on dehalogenation activity in the presence and absence of methyl viologen (MV)

Donor[a]	Rate of MV reduction ± SD (μmol/min per mg)	Dehalogenation rate ± SD (nmol/min per mg)[b]	
		−MV	+MV
None	ND[c]	0.022 ± 0.003	0.078 ± 0.003
H_2	7.97 ± 0.48	0.046 ± 0.005	0.627 ± 0.021
CO	5.86 ± 1.30	0.066 ± 0.0	0.121 ± 0.012
Formate	3.21[d]	0.028 ± 0.004	0.182 ± 0.007
Pyruvate[e]	0.056 ± 0.004	0.025 ± 0.001	0.062 ± 0.0
O_2	ND	ND	0.0
S_2O_4	ND	ND	0.030 ± 0.001

[a] All assays were conducted under a nitrogen headspace unless otherwise indicated. The amounts of protein were 0.035, 0.355, and 7.1 mg/ml in methyl viologen reduction assys, methyl viologen reduction assay with pyruvate, and dehalogenation assays, respectively. The dehalogenation assays were incubated for 16.5 h, using standard assay components (see text).

[b] Average rate of benzoate formation from 3-chlorobenzoate was determined from two sampling times based on zero order kinetics.

[c] ND, Not determined.

[d] Single determination.

[e] Measured with 2 mM coenzyme A.

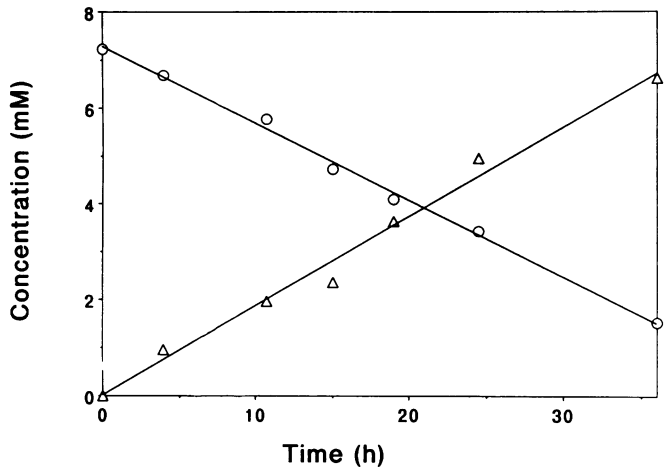

FIG. 1. Time course for dehalogenation of 3-chlorobenzoate (○) to benzoate (△) by "*D. tiedjei*" cell extracts. Dehalogenation assays had 4.42 mg of protein and were incubated with methyl viologen under an H_2 gas phase at 35°C.

A small rate of aryl reductive dehalogenation was observed with these extracts even without methyl viologen, but activity was always much greater in the presence of this compound (Table 2). Dehalogenation was not significantly influenced by the addition of pyruvate plus coenzyme A or dithionite to the assay mixture (Table 2). Dechlorination was also not observed when the cell extracts were exposed to an O_2 atmosphere, which is consistent with results obtained with whole cells of "*D. tiedjei*" (Table 2) (10). Dehalogenation activity was 1.5-, 2.3-, and 8-fold greater in assay mixtures containing CO, formate, or H_2 as an electron donor, respectively, relative to the rates measured under an N_2 atmosphere (Table 2). Therefore, subsequent assays were incubated under a H_2 headspace. A typical time course for the dehalogenation of 3-chlorobenzoate to benzoate with our defined assay mixture containing methyl viologen as the only added electron carrier under an H_2 gas phase is shown (Fig. 1).

Cell extract activity exhibited a temperature optimum for dehalogenation at 35°C and a pH optimum at 8.2 (Fig. 2). The rate of dehalogenation and the specific activity were proportional to the amount of protein added to the assay mixture (Fig. 3). However, the specific activity dropped when the assay protein content exceeded 5 mg/ml (Fig. 3).

The substrate specificity of the cell extracts was investigated with a variety of haloaromatic compounds (Table 3). The removal rate of different halogens from the *meta* position of halobenzoates was proportional to the size (atomic radius) of the halogen. The fastest rate of dehalogenation was measured with 3-iodobenzoate, while 3-fluorobenzoate was not metabolized (Table 3). These results differ from whole-cell studies of "*D. tiedjei*" which indicated that the removal of aryl chloride groups was faster than that of other halogen substituents (10). However, like the whole cells, extracts of "*D. tiedjei*" were unable to transform the *ortho* or *para* isomers of chlorobenzoate (Table 3) (2, 10), but could dehalogenate all iodobenzoates (10) and exhibited a preference for the *meta* isomer (Table 3) (2). Dehalogenation was also observed for other haloaromatic compounds which possessed a halogen *meta* to the carboxyl group (Table 3). The presence of an additional aryl substituent generally retarded the rate of *meta* halogen removal (Table 3). Other

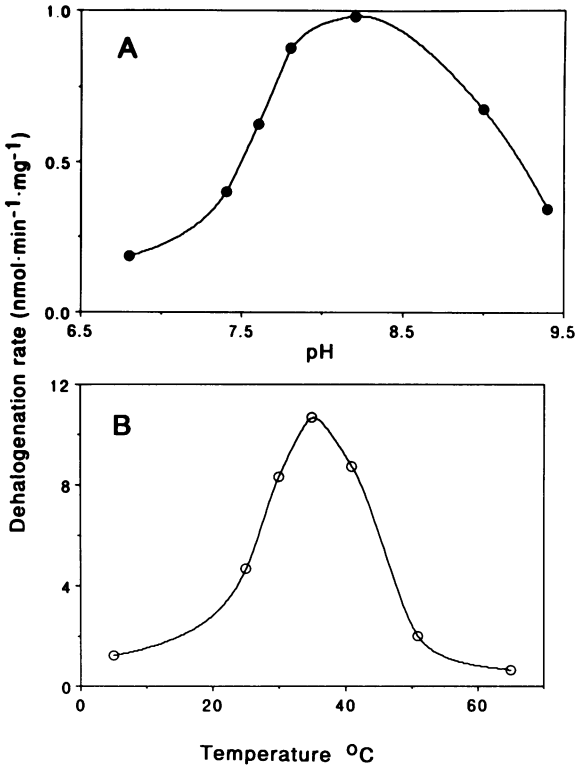

FIG. 2. Effect of pH (A) and temperature (B) on dehalogenation activity by extracts of "*D. tiedjei*." Average rates of benzoate formation from 3-chlorobenzoate (A) or 3-iodobenzoate (B) are presented as the mean of duplicate determinations (standard deviation, <5%) and were determined from two sampling times based on zero order kinetics. Dehalogenation assays for panel A or B had 7.1 or 3.5 mg of protein and were incubated for 10 or 7.9 h, respectively. A comparable temperature profile was obtained when 3-chlorobenzoate was the assay substrate (data not shown).

haloaromatic compounds not containing an aryl carboxyl group were not dehalogenated by cell extracts (Table 3).

We also compared the ability of cell extracts to catalyze dehalogenation reactions when "*D. tiedjei*" was grown under a variety of conditions. Extracts from cells which were grown in the absence of 3-chlorobenzoate exhibited no dechlorination activity in this experiment. A repeat of this experiment with a longer incubation period resulted in trace dechlorination (0.03 nmol/min per mg) from these extracts (data not shown). However, when 3-chlorobenzoate was included in the growth medium, extract dechlorination activity was increased by an order of magnitude (data not shown). However, deiodination activity of extracts from cells grown with 3-chlorobenzoate had only twice the activity of extracts from cells grown without the halogenated substrate (Fig. 4). Dehalogenation in assay mixtures containing both active and inactive cell extracts suggested that no inhibitory substances prevented activity in the latter types of extracts (Fig. 4).

"*D. tiedjei*" is a sulfate-reducing bacterium (1a), and dehalogenation by this organism is inhibited with various sulfur oxyanions (10). We therefore tested the ability of cell extracts to dehalogenate in the presence of a variety of sulfur compounds. Sulfate additions to the assay mixtures did not inhibit dehalogenation. However, NaCl, sulfide, sulfite, and

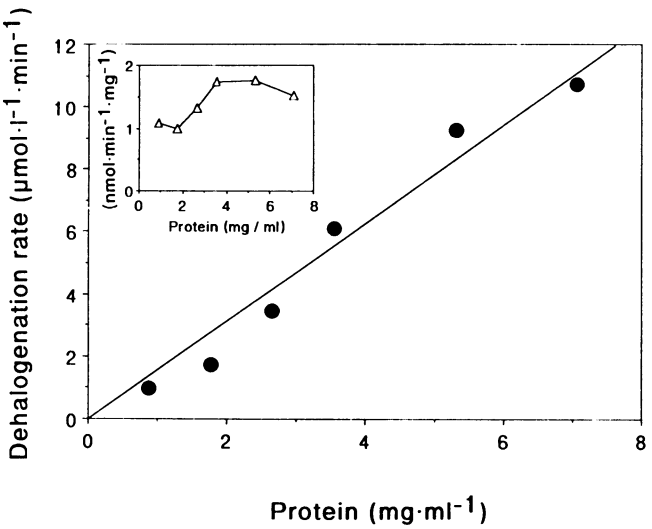

FIG. 3. Effect of increasing cell extract protein content on rate (●) and specific activity (inset; △) of aryl dehalogenation. Average rates of benzoate formation from duplicates (standard deviation, <5%) were determined from two sampling times based on zero order kinetics. Assays were incubated for 13.1 h, using standard conditions (see text).

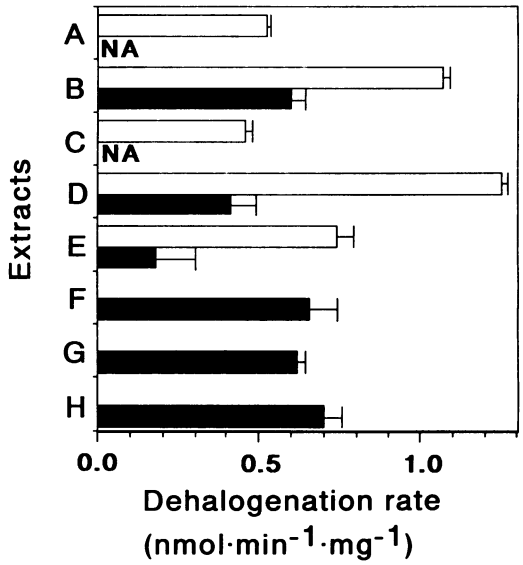

FIG. 4. Aryl dehalogenation activity present in extracts from "*D. tiedjei*" cultured on pyruvate (A), pyruvate plus 3-chlorobenzoate (B), pyruvate plus sulfate (C), pyruvate plus 3-chlorobenzoate and sulfate (D), pyruvate plus 3-chlorobenzoate and thiosulfate (E), a 1:1 mixture of extracts A and B (F), a 1:1 mixture of extracts B and C (G), and a 1:1 mixture of extracts B and D (H). Columns represent average rates of benzoate formation from 3-chlorobenzoate (solid) or 3-iodobenzoate (open) by extracts which were determined from two sampling times based on zero order kinetics. Error bars indicate the standard deviation of duplicates; NA signifies no activity. The specific activity of extracts F to H was based on the protein content of extract B alone and only determined for the dehalogenation of 3-chlorobenzoate.

thiosulfate reduced dehalogenation rates to 74, 46, 16, and 11% of the sulfur-free control, respectively (Fig. 5).

Early studies with a dehalogenating consortium containing "*D. tiedjei*" showed that the metabolism of 3-chlorobenzoate was competitively inhibited by 3,5-dichlorobenzoate (20). Presumably this inhibition could occur at either a substrate transport site or an enzyme active site. We therefore tested these compounds and 3-iodobenzoate as competing substrates in cell extracts. 3,5-Dichlorobenzoate and 3-iodobenzoate significantly reduced the rate of 3-chlorobenzoate dehalogenation, but the reverse was not true (Table 4). Similarly, 3-iodobenzoate reduced the 3,5-dichlorobenzoate dehalogenation rate, but not vice versa (Table 4). These results are consistent with whole-cell experiments (20) and suggest that the inhibition patterns observed with these substrates are independent of transport processes. Dehalogenation activity was also found to partition with the membrane fraction after ultracentrifugation (Table 5).

TABLE 3. Substrate specificity of dehalogenation in cell extracts of "*D. tiedjei*"

Substrate	Product	Dehalogenation rate ± SD (nmol/min per mg)[a]
3-Fluorobenzoate	None	0.0
3-Chlorobenzoate	Benzoate	0.615 ± 0.031
3-Bromobenzoate	Benzoate	0.683 ± 0.029
3-Iodobenzoate	Benzoate	2.80 ± 0.060
2-Chlorobenzoate	None	0.0
4-Chlorobenzoate	None	0.0
2,5-Dichlorobenzoate	2-Chlorobenzoate	0.336 ± 0.013
3,4-Dichlorobenzoate	4-Chlorobenzoate	0.431 ± 0.058
3,5-Dichlorobenzoate	3-Chlorobenzoate, benzoate	0.611[b] ± 0.018
3-Chloro-4-hydroxybenzoate	4-Hydroxybenzoate	0.473 ± 0.017
3-Chloro-4-methylbenzoate	4-Methylbenzoate	0.297 ± 0.011
3-Chlorophenol	None	0.0
3-Chlorocinnamate	None	0.0
2-Iodobenzoate[c]	Benzoate	0.306 ± 0.103
3-Iodobenzoate[c]	Benzoate	1.50 ± 0.074
4-Iodobenzoate[c]	Benzoate	0.623 ± 0.049

[a] Values are the average rate of product formation determined from triplicate with six sampling points. Assays had 4.42 mg of protein and were incubated for 20 h, using standard conditions (see text).

[b] Rate of 3-chlorobenzoate formation.

[c] Assays had 3.5 mg of protein and were incubated for 30 h, using standard conditions (see text).

DISCUSSION

The reductive dehalogenation of aryl halides appears to be a major fate process for many haloaromatic chemicals in various anoxic habitats (9). Ironically, the only reports of cell-free systems capable of catalyzing such reactions have been obtained with extracts from facultative bacteria or proteins purified from bovine thyroid glands (1, 5, 6). The only isolated anaerobic bacterium possessing this activity is "*D. tiedjei*" (1a, 10, 15). Recent improvements in the culture of this bacterium have facilitated the study of such bioconversions (1a).

Aryl reductive dehalogenation by cell extracts was dependent on the dye methyl viologen, which had the lowest redox potential (−424 mV) of the cofactors tested in our experiments. This suggests that the physiological electron donor for aryl reductive dehalogenation reactions in "*D. tiedjei*" may be a low redox carrier protein such as a ferredoxin or flavodoxin. These proteins may be inactivated during the preparation of cell extracts in some experiments, but a

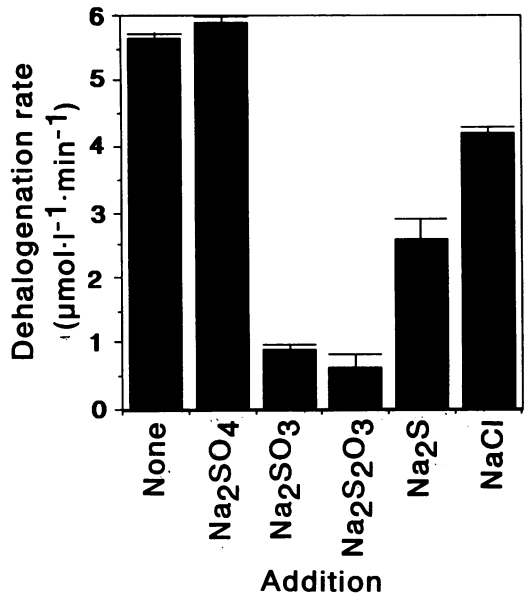

FIG. 5. Effect of various sulfur compounds and NaCl on the dehalogenation activity in extracts of "*D. tiedjei*." Columns represent average rates of benzoate formation from 3-chlorobenzoate. Error bars show the standard deviation of duplicates determined from two sampling times based on zero order kinetics. All treatments except Na_2SO_4 addition were significantly different (0.05 level) from the control (no addition) by F-test from a model 1 analysis of variance. The assays had 4.4 mg of protein and were incubated for 24 h, using standard conditions (see text). The concentration of sulfur-containing compounds was 20 mM; that of NaCl was 40 mM.

fraction may remain active in other experiments since trace dehalogenation activity was observed in some assays performed without methyl viologen. Other investigators have identified a soluble flavoprotein deiodinase which required flavin mononucleotide and an E_h of −412 mV for complete activity (5), whereas the reductive dechlorination of chlorohydroquinones occurred under aerobic and anaerobic conditions and required ascorbic acid for maximum activity (1).

The cell extracts of "*D. tiedjei*" possessed many enzyme activities capable of supplying electrons for aryl dehalogenation, and their specific activities were similar to those of found in *Desulfosarcina variabilis* 3be13 (14). However, the dehalogenation rates differed considerably with the various potential electron donors, suggesting that other electron-accepting reactions are simultaneously occurring in the assay mixtures. Varied dehalogenation rates may be partially due to the evolution of H_2 gas from hydrogenase and reduced methyl viologen since this reaction would likely compete with a dehalogenase for electron transfer. H_2 uptake by extracts would likely predominate under an H_2 gas phase due to a large mass action effect, but H_2 evolution from reduced methyl viologen is more probable with the other electron donors. Using a gas chromatograph equipped with a mercury vapor detector, we found that substantial H_2 concentrations were evolved from chemically reduced methyl viologen by extracts in this assay under an N_2 gas phase (data not shown). This may explain why H_2 gas was the best source of electrons in the assay and why dehalogenation activity was not stimulated with dithionite-reduced methyl viologen. However, the effect of dithionite on aryl dehalogenation may be similar to that of sulfite or thiosulfate. The inability of dithionite to stimulate aryl reductive

TABLE 4. Effect of a competing substrate on aryl dehalogenation reactions in cell extracts "*D. tiedjei*"

Competing substrate		Dehalogenation rate ± SD of substrate 1 (μmol/liter per min)[a]
1	2	
3-Chlorobenzoate	None	3.19[b] ± 0.23
3-Chlorobenzoate	3,5-Dichlorobenzoate	0.90 ± 0.11[c]
3,5-Dichlorobenzoate	None	2.54[d] ± 0.11
3,5-Dichlorobenzoate	3-Chlorobenzoate	2.16 ± 0.38[e]
3-Chlorobenzoate	None	2.70[f] ± 0.09
3-Chlorobenzoate	3-Iodobenzoate	1.49 ± 0.10[c]
3-Iodobenzoate	None	5.95[g] ± 0.43
3-Iodobenzoate	3-Chlorobenzoate	5.71 ± 0.28[e]
3,5-Dichlorobenzoate	None	2.70[h] ± 0.29
3,5-Dichlorobenzoate	3-Iodobenzoate	1.85 ± 0.13[c]
3-Iodobenzoate	None	5.26[b] ± 0.36
3-Iodobenzoate	3,5-Dichlorobenzoate	4.63 ± 0.26[e]

[a] Mean of triplicate determinations.
[b] Average rate of benzoate formation. All assays had 3.5 mg of protein and 4 mM concentrations of substrates and were incubated for 30 h, using standard conditions (see text). Dehalogenation rates were determined from the average of triplicate rates from six sampling times.
[c] Significantly different at the 0.05 level from a reaction mixture not containing the competing substrate; F-test model 1 analysis of variance.
[d] Average rate of 3,5-dichlorobenzoate removal.
[e] Not significantly different.
[f] Average rate of 3-chlorobenzoate removal.
[g] Average rate of 3-iodobenzoate removal.
[h] Average rate of 3-chlorobenzoate formation.

dehalogenation was similar to the reductive dehalogenation of chlorinated methanes (8), but differed from the dehalogenation of DDT and iodotyrosine (3, 5). Unlike its inhibitory effect on alkyl dehalogenation reactions (3, 12), CO stimulated the reductive removal of aryl halides.

Aryl dehalogenation activity in "*D. tiedjei*" extracts was inactivated by heat, exhibited pH and temperature optima, and was proportional to the amount of protein in assay mixtures. These characteristics are all consistent with a biologically catalyzed reaction. Cell extracts remove aryl halides at pH values much lower than raney alloys which abiotically catalyze similar reactions under alkaline conditions (21). The temperature optimum for cell extract dehalogenation was the same as the optimal growth temperature of "*D. tiedjei*" (1a). The drop in specific activity at higher protein contents may be due to a methyl viologen limitation since an optimum methyl viologen/protein ratio of 0.09 (mg/mg) was found in other experiments (data not shown).

TABLE 5. Localization of aryl dehalogenation activity in extracts of "*D. tiedjei*"

Extract fraction	Dehalogenation activity[a]	
	Rate (μmol/liter per min)	Sp act (nmol/min per mg)
Crude extract	3.005 ± 0.177[b]	0.425 ± 0.018
Supernatant[c]	0.355 ± 0.021	0.058 ± 0.003
Pellet[c]	1.515 ± 0.092	0.881 ± 0.038

[a] Average rate of benzoate formation from 3-chlorobenzoate was determined from two sampling times based on zero order kinetics. Vials were incubated with 7.1, 6.2, or 1.7 mg of protein of crude extract, supernatant, or pellet, respectively. The dehalogenation assays were incubated for 9.5 h at 35°C, using standard assay components (see text).
[b] Standard deviation of duplicates.
[c] Following ultracentrifugation of the crude extract at 179,000 × *g* for 1 h.

The similarity of substrate specificities by extracts to those from whole cells for the exclusive metabolism of halobenzoates (2, 10) suggests that the specificity of this reaction was not governed by a restrictive transport process, but the involvement of an enzyme.

Excluding fluorobenzoate, aryl reductive dehalogenation by "*D. tiedjei*" extracts was fastest with the largest halogens. The relative rates of halogen removal by whole cells (2, 10) may differ from cell extracts due to difficulties in cellular transport of these substrates to an enzyme active site. The *meta* isomers of both chloro- and iodobenzoates were dehalogenated by extracts more rapidly compared with the other positional isomers, a finding consistent with previous studies with either consortial or "*D. tiedjei*" cells (2, 10). The cell extracts dechlorinated only the *meta* isomer of chlorobenzoate, but all iodobenzoate isomers were deiodinated. These results agree with previous findings with consortial cells (2), but contrast with others that used "*D. tiedjei*" cells (10).

Cell extracts, like consortial cells, dechlorinated dichlorobenzoates and *meta* chlorobenzoates containing a *para* hydroxyl or methyl group more slowly than monochlorobenzoate (2, 20). The multiply substituted *meta*-halobenzoates may be dehalogenated more slowly perhaps due to steric hinderance at an enzyme active site.

The deiodination rates from extracts A through E (Fig. 4) varied less than an order of magnitude, unlike dechlorination activities from the same extracts. This result is probably due to the longer assay incubation times used with this substrate and the generally faster rates of deiodination relative to dechlorination (Table 3). To confirm this suspicion, the experiment was repeated, using a longer incubation period, and a trace amount of dechlorination was detected in extracts from cells not previously exposed to the haloaromatic compound. However, dechlorination activity was again found to be at least 10-fold higher in extracts obtained from cells grown in the presence of 3-chlorobenzoate (data not shown). These results suggest that aryl dehalogenation is not simply catalyzed by reduced cofactors present in "*D. tiedjei*" extracts, but is an inducible reaction. The dechlorination activity in *Rhodococcus chlorophenolicus* was also found to be an inducible activity (1). Unlike the dehalogenation of alkyl compounds, which occurs in minutes with reduced metal hemes and corrinoids (7, 8), aryl reductive dehalogenation did not occur in assay mixtures containing up to 50 mM reduced hemin or cyanocobalamin over a 30-h period (data not shown).

"*D. tiedjei*," formerly called strain DCB-1, was described as an sulfidogen by Stevens et al. based on the stimulation in growth by sulfite and thiosulfate, but an apparent inhibition with sulfate (18). This bacterium has recently been characterized as a new genus and species of sulfate-reducing bacteria based on its physiology, phylogeny, and biochemical characteristics (1a). The bacterium is able to reduce sulfate, sulfite, and thiosulfate with formate, H_2, pyruvate, methoxybenzoates, and benzoate as electron donors (1a). The addition of sulfate, thiosulfate, and sulfite were shown previously to have a negative effect on dehalogenation by growing cultures of "*D. tiedjei*" (10). However, in recent experiments, we found sulfite and thiosulfate, but not sulfate, inhibited dehalogenation by washed cell suspensions of "*D. tiedjei*" under nongrowth conditions (K. A. DeWeerd, F. Concannon, and J. M. Suflita, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, Q-53, p. 297). The addition of sulfite or thiosulfate also inhibited aryl dehalogenation by "*D. tiedjei*" extracts. The reduction of these sulfur oxyanions by bisulfite reductase (desulfoviridin) in "*D. tiedjei*" extracts (1a, 18) could potentially compete with aryl dehalogenation for reducing equivalents in the assay. Sulfate had no effect on dehalogenation in extracts, possibly because the reduction of sulfate does not occur directly as with sulfite or thiosulfate but proceeds only after an initial activation step forming adenosine-5′-phosphosulfate (13). It is unlikely that this activation could occur in extracts since ATP and pyrophosphatase were not included in the assay mixture and cells were not cultured under sulfate-reducing conditions. The inhibition by sulfite, thiosulfate, and sulfide could also be due to the reactivity of these compounds with sulfhydryl groups on proteins (13) or flavins in flavoproteins (11) which may be possibly involved in aryl dehalogenation (5). The inhibition of dehalogenation by NaCl is probably due to the chloride ion since 40 mM sodium was also present in Na_2SO_4, which had no effect on the activity, and either NH_4Cl or KCl also inhibited dehalogenation by cell extracts (data not shown).

Extracts of "*D. tiedjei*" dechlorinated 3,5-dichlorobenzoate almost completely to 3-chlorobenzoate before the latter metabolite was consumed, and significant amounts of benzoate were measured (data not shown). The same results were obtained with consortial cells (20), reinforcing the competitive inhibition explanation for catalysis, rather than inhibition at a substrate transport site. The latter could not likely be a substantive factor in cell extracts. The competitive inhibition among 3-iodobenzoate, 3-chlorobenzoate, and 3,5-dichlorobenzoate for dehalogenation suggests that their transformation occurs at the same enzymatic site, with the preference for 3-iodobenzoate being greater than 3,5-dichlorobenzoate, which in turn is greater than 3-chlorobenzoate.

The majority of aryl dehalogenation activity in extracts was found to partition largely with the membrane fraction. In addition, the activity was not extracted from the membrane with low detergent concentrations, but required ratios of 0.5 mg of detergent to 1 mg of protein for optimum extraction (data not shown). These results suggest that this is an integral membrane protein. The iodotyrosine deiodination activity was found in both soluble and particulate fractions (5), whereas the majority of chlorohydroxyquinone dechlorination activity was soluble (1).

Since reduced hemin and cyanocobalamin failed to catalyze aryl dehalogenation of halobenzoates, these compounds appear to be more resistant to chemically mediated reductive dehalogenation than nonaromatic compounds (7, 8, 22). The development of an assay for aryl reductive dehalogenation and the characterization of activities in extracts of "*D. tiedjei*" should be helpful for the eventual purification of an aryl reductive dehalogenase.

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