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Characterization of Hydrogenase and Reductive Dehalogenase Activities of *Dehalococcoides ethenogenes* Strain 195

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Dehalococcoides ethenogenes **strain 195 reductively dechlorinates tetrachloroethene (PCE) and trichloroethene (TCE) to vinyl chloride and ethene using H_2 as an electron donor. PCE- and TCE-reductive dehalogenase (RD) activities were mainly membrane associated, whereas only about 20% of the hydrogenase activity was membrane associated. Experiments with methyl viologen (MV) were consistent with a periplasmic location for the RDs or a component feeding electrons to them. The protonophore uncoupler tetrachlorosalicylanilide did not inhibit reductive dechlorination in cells incubated with H_2 and PCE and partially restored activity in cells incubated with the ATPase inhibitor *N*,*N*′-dicyclohexylcarbodiimide. Benzyl viologen or diquat ($E^{o\prime} \approx -360$ mV) supported reductive dechlorination of PCE or TCE at rates comparable to MV (−450 mV) in cell extracts.**

Dehalococcoides ethenogenes strain 195 was the first organism to be isolated that is capable of dechlorinating tetrachloroethene (PCE) and trichloroethene (TCE) past dichloroethene (DCE) (12) to vinyl chloride (VC) and ethene (11–13). It uses H_2 as an electron donor, lacks a peptidoglycan cell wall (12), and is phylogenetically affiliated with the *Chloroflexi* (green nonsulfur bacteria) phylum (1, 4). Biochemical studies of reductive dechlorination in pure cultures of *D. ethenogenes* strain 195 are hampered by the poor growth yields attributed to its requirement for undefined growth factors from mixed cultures (11, 12, 14). The mixed methanol-PCE-yeast extract culture from which *D. ethenogenes* was isolated can be grown in relatively large amounts (3), and Magnuson et al. (10) purified from it a PCE-reductive dehalogenase (PCE-RD) dechlorinating PCE to TCE and a TCE-reductive dehalogenase (TCE-RD) dechlorinating TCE and DCEs to VC and ethene. Inhibition by alkyl iodides indicated that each enzyme contained corrinoid cofactor, consistent with the high vitamin B_{12} requirement for growth of strain 195 (10, 12). Using the N-terminal sequence of the TCE-RD, the gene encoding it (*tceA*) was isolated from DNA from a pure culture of *D. ethenogenes* (9), demonstrating that the purified TCE-RD was indeed from *D. ethenogenes*. Like the PCE-RD from *Sulfurospirillum multivorans* (18) and the *ortho*-chlorophenol-RD from *Desulfitobacterium dehalogenans* (20), the deduced protein sequence of *tceA* contains a putative twin arginine transport (TAT) signal, suggesting a periplasmic location, and *tceA* is adjacent to a gene encoding a small hydrophobic polypeptide (*tceB*) presumed to be a membrane anchor. More recently, membrane-associated chlorobenzene-reductive dehalogenase activities were characterized for *Dehalococcoides* strain CBDB1 (6). Here we describe studies of the location and activities of RDs and hydrogenase in whole cells and cell extracts of *D. ethenogenes*.

Preparation of whole cells, cell extracts, and membranes and assay of reductive dehalogenase and hydrogenase activities. *D. ethenogenes* strain 195 was grown as described by Maymó-Gatell et al. (12), typically in 1.2-liter bottles containing 500 ml of medium. Whole-cell suspensions were prepared by anaerobically washing the cells twice by centrifugation at 34,540 × *g* for 25 min and resuspending the pellet in a buffer containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7, 150 mM NaCl, and either 2 mM *dl*-dithiothreitol or 1 mM Ti(III) citrate. The final volume was about 1/10 of the original volume. *dl*-Dithiothreitol, initially used as a reducing agent, appeared to be inhibitory to reductive dehalogenase activity (data not shown), after which Ti(III) was used. Cell extracts were prepared by passing the cell suspension through a French pressure cell at ca. 6,000 lb/in^2 (42 MPa) and centrifuging the crude extract anaerobically at 13,000 × *g* for 10 min. To prepare the membrane fraction, the cell extract was centrifuged anaerobically for 1 h at 104,000 × *g*.

Reductive dechlorination assays were similar to that used by Magnuson et al. (10) in which dechlorination products were assayed via gas chromatography after incubation with a reducing agent, typically methyl viologen (MV). Dechlorination assays were performed in 8-ml crimp-top vials with a final liquid volume of 1 ml. Titanium(III) citrate (23) was added as a reducing agent to a concentration of 8.3 mM when indicated. Addition of Ti(III) citrate led to more-reproducible activities but also to a low background dechlorination activity (8 to 26% of activity with MV), which was subtracted when other potential electron donors were added. Artificial electron donors were added to a final concentration of 1 mM. After incubation for a given time at 34°C, dechlorination products were assayed by gas chromatography (13).

Hydrogenase activity was determined spectrophotometrically as the reduction of artificial electron acceptors in 1.5-ml anaerobic glass cuvettes (liquid volume, 0.7 ml) sealed with rubber stoppers and incubated at room temperature (ca.

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TABLE 1. Maximum rates of PCE- and TCE-reductive dehalogenase and hydrogenase activity by whole cells and crude extracts[a]

Redox-active compound	Redox potential (mV)	PCE-RD activity (nmol min^{-1} mg of $protein^{-1}$)[b]		TCE-RD activity (nmol min^{-1} mg of $protein^{-1}$)		Hydrogenase activity (μmol min^{-1} mg of $protein^{-1}$)	
		Whole cells	Crude extracts	Whole cells	Crude extracts	Whole cells	Crude extracts
H_2	−414	5.0 ± 2.8	4.0 ± 3.8	19.0 ± 2.0	12.5 ± 1.0		
BV	−360	2.2 ± 8.4	13.7 ± 1.6	22.3 ± 4.9	11.1 ± 0.9	8.36 ± 2.91	8.84 ± 3.25
Diquat	−361	35.2 ± 9.9	56.5 ± 7.3	9.3 ± 0.2	9.1 ± 0.3	6.74 ± 0.76	7.24 ± 4.84
MV	−440	25.4 ± 9.1	32.2 ± 32.6	23.0 ± 1.2	35.4 ± 6.6	1.22 ± 0.21	1.79 ± 0.21
Ethyl viologen	−480	44.2 ± 23.5	74.7 ± 10.7	24.1 ± 4.3	15.6 ± 2.0	1.29 ± 0.50	1.58 ± 0.81
Ferredoxin	−432	ND	0	ND	0	ND	ND
NADH	−320	ND	0	ND	0	ND	ND
AQDS	−184	I	I	2.5 ± 0.2	7.3 ± 1.6	4.21 ± 1.07	2.68 ± 1.29
PQQ	+80	3.5 ± 0.7	5.0 ± 3.0	8.0 ± 0.6	6.6 ± 3.5	ND	ND
Phenazine methosulfonate	+80	I	I	I	I	1.15 ± 1.99	1.33 ± 2.30
Phenosafranine	−252	I	I	I	I	0	0

[a] All rates are relative to the N_2 (no redox-active compound) control, which was set to 0. PCE- and TCE-reductive dechlorination activities are expressed as rates of PCE and TCE loss, respectively, as determined by the appearance of products of reductive dechlorination. Inhibitory indicates rates lower than the no electron donor (N_2) control. Redox-active compounds were added at concentrations of 1 mM for reductive dehalogenase or 200 μM for hydrogenase activity assays. Approximately 8.3 mM titanium(III) citrate was added to all reductive dechlorination assays. Indicated are averages ± standard deviations of results from at least two separate experiments.

[b] ND, not determined; 0, rate was not measurably different from the N_2 control; I, inhibitory.

22°C). The following wavelengths were used: 450 nm for anthraquinone 2,6-disulfonic acid (AQDS) (ε = 1.8 mM^{-1} cm^{-1}), 578 nm for MV (ε = 9.7 mM^{-1} cm^{-1}) and ethyl viologen (ε = 10 mM^{-1} cm^{-1}), 546 nm for benzyl viologen (BV) (ε = 9.75 mM^{-1} cm^{-1}), 366 nm for phenazine methosulfate (ε = 7.9 mM^{-1} cm^{-1}), 519 nm for phenosafranine, and 760 nm for diquat (ε = 2.9 mM^{-1} cm^{-1}) (5, 15).

Hydrogenase activity in cells and cell extracts. H_2 is the only electron donor known to support growth of *D. ethenogenes*, and hydrogenase activity was tested as the reduction of potential electron acceptors by whole-cell suspensions and by crude extracts. As summarized in Table 1, AQDS, BV, diquat, ethyl viologen, MV, and phenazine methosulfate were reduced by whole cells and crude extracts when H_2 was present. Carbon monoxide also supported similar rates of MV reduction by whole cells (data not shown), indicating the presence of a carbon monoxide dehydrogenase. Activities in whole cells and crude extracts were similar for the reduction of MV (Table 1), which is considered unable to permeate through lipid bilayers (7), indicating that a site at which electrons could be donated from a hydrogenase was located on the outside of the cytoplasmic membrane.

Reductive dehalogenase activity of cell extracts with reduced MV as the electron donor. We tested the reductive dechlorination of chlorinated ethenes by crude extracts of *D. ethenogenes* with MV (ca. 4 mM) as the electron donor, since other reductive dehalogenases utilize this electron donor (2, 17, 20). PCE, TCE, *cis*-DCE, and 1,1-DCE were dechlorinated by crude extracts at rates of 0.15, 0.30, 0.16, and 0.45 μmol of chlorinated ethene min^{-1} mg of $protein^{-1}$, respectively. *trans*-DCE and VC were dechlorinated at much lower rates of 0.03 and <0.005 μmol of chlorinated ethene min^{-1} mg of $protein^{-1}$, respectively, similar to results with the growing culture (11). PCE was dechlorinated to VC and minor accumulations of *cis*-DCE and TCE, while TCE dechlorination resulted in major accumulation of *cis*-DCE (data not shown), similar to results with growing cultures (11). Activities of PCE and TCE dehalogenases in whole cells treated with reduced MV were similar to activities in cell extracts (Table 1), indicating that sites donating electrons to them were located outside the cytoplasmic membrane.

Localization of the hydrogenase and reductive dehalogenases. Only ca. 20% of the hydrogenase activity from *D. ethenogenes* cells that were lysed by relatively gentle treatment with a French press was found in the membrane fraction, whereas ca. 80% of TCE and PCE reductive dehalogenase activities were associated with the membrane fraction (data not shown), similar to findings for a mixed culture of strain 195 (10) and for *Dehalococcoides* strain CBDB1 (6). The cell membrane fraction was capable of reductive dehalogenation of PCE using H_2 as the electron donor at a rate nearly equal to that of the crude extracts (Fig. 1), whereas essentially no reductive dehalogenation was detected in the soluble fraction and addition of that fraction did not stimulate reductive dehalogenation by the membrane fraction. These results are consistent with the membrane containing all of the components needed for electron

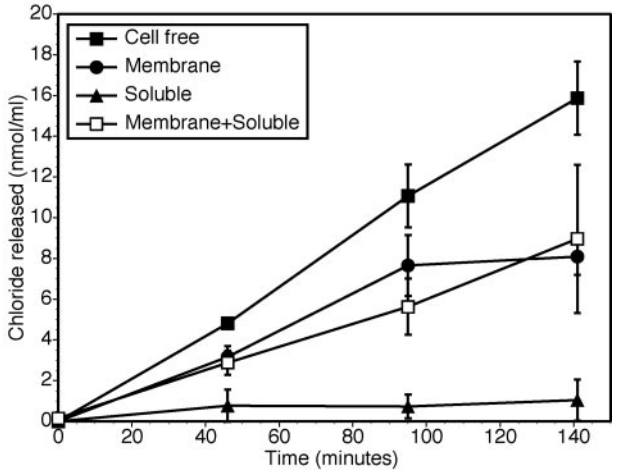

FIG. 1. Reductive dechlorination of PCE using H_2 as the electron donor by different subcellular fractions of *D. ethenogenes* strain 195.

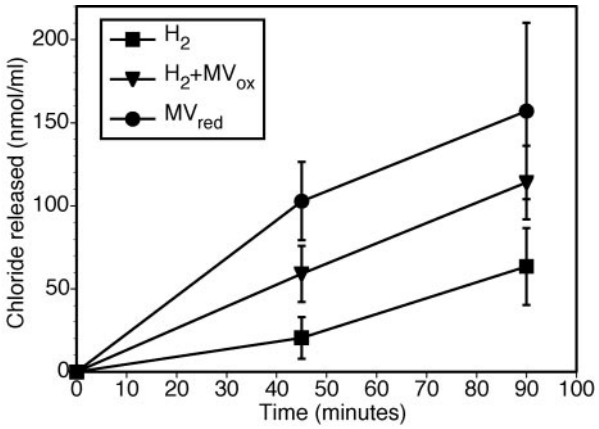

FIG. 2. Reductive dechlorination of PCE by whole cells of *D. ethenogenes* strain 195 when provided with H_2, reduced MV, or oxidized MV in the presence of H_2 as electron donors.

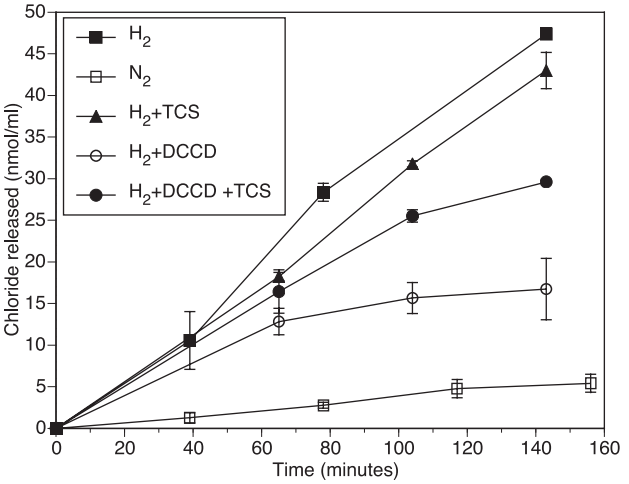

FIG. 3. The effect of the ATPase inhibitor DCCD and the protonophore TCS on reductive dechlorination of PCE by whole-cell suspensions of *D. ethenogenes* strain 195. Assays were performed with H_2 as the electron donor (except the negative control, which had an N_2 headspace) and PCE as the electron acceptor. TCS (12.5 μM) and DCCD (100 μM) were added as indicated. Error bars represent standard deviations of duplicate results.

transport between H_2 and PCE. Similar results were obtained for TCE-reductive dehalogenation (data not shown).

Effect of MV on reductive dechlorination by whole cells. Reduced MV, an artificial low-potential electron donor, supported reductive dehalogenation of PCE by whole cells at rates considerably higher than the natural electron donor H_2 (Table 1 and Fig. 2). When oxidized MV was added to cells incubated with H_2 and PCE, the suspension turned purple, indicating reduction of the MV by hydrogenase, and the rate of PCE-reductive dechlorination was approximately double that in the presence of H_2 alone, indicating that exogenous MV carried electrons more rapidly than the endogenous electron transport chain.

Experimental data support localization of dehalogenases in *D. ethenogenes* strain 195 on the outside of the cytoplasmic membrane, as reduced MV, considered unable to permeate through lipid bilayers (7), could support reductive dechlorination of PCE and TCE by whole cells. This localization is in agreement with the presence of a predicted TAT signal on the TCE-RD and other putative RD genes from *D. ethenogenes* strain 195 (21), but it should be mentioned that MV could donate electrons to a point in the electron transport chain that is upstream of the dehalogenases.

Effect of TCS and DCCD on reductive dechlorination by whole cells. Tetrachlorosalicylanilide (TCS) is a protonophore uncoupler that has been shown to function under anaerobic conditions under which other protonophores, such as nitroaromatics, are metabolized (15). TCS abolished PCE dechlorination by *S. multivorans* (15), indicating that a proton motive force (PMF) was needed for reductive dehalogenation in that organism. In the case of *D. ethenogenes*, 12.5 μM TCS, a concentration inhibitory to *S. multivorans*, did not inhibit PCE-reductive dechlorination in resting cell suspension (Fig. 3) and was, in some experiments, slightly stimulatory (data not shown). A PMF is apparently not needed for reductive dechlorination in *D. ethenogenes*. Further evidence that a PMF is not needed for dechlorination is that cell extracts and membrane preparations with no PMF present catalyzed reductive dehalogenation of chloroethenes using H_2 as an electron donor at rates comparable to whole cells.

DCCD (*N*,*N*′-dicyclohexylcarbodiimide) inhibits ATP synthetase activity (22) by attaching to an aspartate involved in proton transport by the F_0 unit; however, DCCD is also a nonspecific cross-linking agent and can inhibit other cellular functions. Addition of 100 μM DCCD inhibited PCE dechlorination by resting cells within 60 min (Fig. 3). If the inhibition of dechlorination by DCCD was due to the inhibition of ATPase function, one likely mechanism for the inhibition of dechlorination would be that because protons could no longer enter the cell through the ATPase, the PMF would build up to a point where it inhibited its own production by electron transport (respiratory coupling). If this was the case, addition of a protonophore should reverse the inhibition. Indeed, addition of TCS partially relieved the inhibition of DCCD-treated cells (Fig. 3).

Utilization of electron donors for reductive dehalogenation. Several redox-active compounds were tested for the ability to serve as electron donors for reductive dechlorination of PCE and TCE by whole cells and crude extracts of *D. ethenogenes* strain 195. The specific rates of the PCE and TCE dehalogenase activities in crude extracts and whole cells in the presence of artificial electron donors are presented in Table 1.

Titanium(III) citrate, which, when added to extracts, stabilized reductive dehalogenase activity, was found to donate electrons for reductive dechlorination of either PCE or TCE at relatively low rates without the addition of another redox-active compound, similar to results obtained with the PCE-reductive dehalogenase from *S. multivorans* (16). This basal-level activity with Ti(III) allowed us to identify inhibitory effects of redox-active compounds. The reduced viologen dyes MV, ethyl viologen, diquat, and BV stimulated reductive dechlorination of PCE, whereas reduced AQDS, phenosafranine, and phenazine methosulfate could not support reductive dechlorination of PCE. Instead, AQDS, phenosafranine, and phenazine methosulfate appeared to inhibit reductive dechlo-

rination. Results with TCE as the electron acceptor were similar, except that AQDS stimulated reductive dechlorination. Neither of the natural electron donors (reduced spinach ferredoxin and NADH) stimulated PCE- or TCE-reductive dehalogenation.

Similar to results for the PCE-RD from *S. multivorans* (15), the only redox-active compounds that supported dechlorination of PCE and TCE at high rates in *D. ethenogenes* extracts were viologen dyes. However, diquat and BV, with oxidation-reduction potentials near −360 mV, supported reductive dechlorination in extracts at rates comparable to MV (−450 mV) and ethyl viologen (−480 mV), in contrast to *S. multivorans* extracts, in which diquat and BV supported rates of <5% of those with the other two viologens. BV also supported high rates of reductive dechlorination of chlorobenzenes in *Dehalococcoides* strain CBDB1 (6), further supporting differences in electron transport between *Dehalococcoides* and *Sulfurospirillum*.

Of particular interest as electron donors are quinones, especially in light of our finding that *D. ethenogenes* membrane preparations did not require cytoplasmic constituents for electron transport from hydrogenase to the RDs. AQDS, a soluble analogue of menaquinone, gave somewhat anomalous results, stimulating TCE-reductive dechlorination but partially inhibiting PCE dechlorination. This result was obtained several times with different cell preparations and points to differences between the two enzymes. Another quinone electron donor we examined was pyrroloquinoline quinone (PQQ). Reduced PQQ (+80 mV) supported reductive dechlorination of PCE and TCE at levels above controls with no electron donor added (Table 1), and this result was repeated three times. 3-Hydroxy-L-tyrosine, a precursor in the biosynthesis of PQQ, could not act as an electron donor for reductive dechlorination (data not shown). The model for reductive dechlorination proposed by Miller et al. (15) and Van de Pas et al. (20) includes two electron donors for the reduction of the organochlorine at the active site: one of high (~0 mV) potential and the other of low (~−400 mV) potential to reduce Co(II) to Co(I). In our experiments, the Ti(III) added as a reducing agent could have provided the low-potential electron and the quinone high-potential electrons. The menaquinone inhibitor 2-heptyl-4-hydroxy-quinoline-*N*-oxide (HOQNO) (24) inhibited reductive dechlorination in *Dehalobacter restrictus* (19) and *Desulfomonile tiedjei* DCB-1 (8) but not in *S. multivorans* (15). Concentrations of HOQNO up to 35 nmol/mg of protein had no significant effect on the rate of PCE or TCE dechlorination by *D. ethenogenes* resting cell suspensions using H_2 as the electron donor (data not presented), indicating that if a quinone is involved in electron transport in *D. ethenogenes*, it is not likely to be menaquinone. For example, Louie and Mohn (8) obtained evidence of an unusual quinone involved in reductive dechlorination by *D. tiedjei*.

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