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Influence of Different Electron Donors and Acceptors on Dehalorespiration of Tetrachloroethene by *Desulfitobacterium frappieri* TCE1

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Strain TCE1, a strictly anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE), was isolated by selective enrichment from a PCE-dechlorinating chemostat mixed culture. Strain TCE1 is a gram-positive, motile, curved rod-shaped organism that is 2 to 4 by 0.6 to 0.8 μm and has approximately six lateral flagella. The pH and temperature optima for growth are 7.2 and 35°C, respectively. On the basis of a comparative 16S rRNA sequence analysis, this bacterium was identified as a new strain of *Desulfitobacterium frappieri*, because it exhibited 99.7% relatedness to the *D. frappieri* type strain, strain PCP-1. Growth with H_2, formate, L-lactate, butyrate, crotonate, or ethanol as the electron donor depends on the availability of an external electron acceptor. Pyruvate and serine can also be used fermentatively. Electron donors (except formate and H_2) are oxidized to acetate and CO_2. When L-lactate is the growth substrate, strain TCE1 can use the following electron acceptors: PCE and TCE (to produce *cis*-1,2-dichloroethene), sulfite and thiosulfate (to produce sulfide), nitrate (to produce nitrite), and fumarate (to produce succinate). Strain TCE1 is not able to reductively dechlorinate 3-chloro-4-hydroxyphenylacetate. The growth yields of the newly isolated bacterium when PCE is the electron acceptor are similar to those obtained for other dehalorespiring anaerobes (e.g., *Desulfitobacterium* sp. strain PCE1 and *Desulfitobacterium hafniense*) and the maximum specific reductive dechlorination rates are 4 to 16 times higher (up to 1.4 μmol of chloride released · min^{-1} · mg of $protein^{-1}$). Dechlorination of PCE and TCE is an inducible process. In PCE-limited chemostat cultures of strain TCE1, dechlorination is strongly inhibited by sulfite but not by other alternative electron acceptors, such as fumarate or nitrate.

Anaerobic microorganisms play a key role in the dehalogenation (4, 8, 11, 29, 43) and eventual mineralization (9, 11, 14, 40) of many chlorinated and fluorinated contaminants. Studies of the physiology and biochemistry of these organisms have revealed that they may transform a wide range of chlorinated aliphatic and aromatic compounds cometabolically (11, 29, 41) or may metabolize them through dehalorespiration, a process in which halogenated compounds (e.g., chlorophenols, chlorobenzoates, and chloroethenes) act as electron acceptors (11, 13, 16, 19, 22, 31, 42, 43). Alternatively, some chlorinated compounds may be used as sources of carbon, electrons, and energy by fermentative bacteria [e.g., (di)chloromethane] or by denitrifying bacteria and phototrophs (e.g., halobenzoates) (15, 23, 40). Recently, Boyle et al. described a *Desulfovibrio* strain (strain TBP-1) which seemed to have the ability to grow with lactate and 2,4,6-tribromophenol via halorespiration (3).

The natural activities of anaerobic dechlorinating populations can result in reduced toxicity and ultimately in complete clean-up of polluted locations (7, 34, 35, 44). However, in some cases dechlorination may cause problems due to the accumulation of more toxic and/or more mobile dechlorination products that are formed biologically from the primary pollutants (e.g., vinyl chloride formed from tetrachloroethene [PCE] dehalogenation). Dechlorinating anaerobic bacteria can also be used for more intensive clean-up of polluted sites if in situ bioremediation procedures and/or bioreactors are used (26, 34, 44). Obviously, the rates and the extents of in situ dechlorination are controlled by various interacting hydrological and (geo)chemical parameters, such as temperature, pH, redox potential, organic matter content, nature of the organic matter, and the availability of various electron donors and acceptors. Dehalorespiring bacteria have been shown to be capable of dechlorination-dependent growth at relatively high rates and hence have great potential for clean-up of soils, aquifers, sediments, and ground- and wastewater streams that are polluted with chlorinated organic compounds. Recently, we used anaerobic dechlorination during full-scale in situ bioremediation of an aquifer contaminated with PCE (34). In this case, subsurface dechlorination was stimulated by injecting an electron donor (methanol), and chloroethenes in extracted groundwater and soil vapor were subsequently removed in a so-called anoxic loop in the soil (34). Laboratory chemostat experiments performed with soil obtained from this location revealed that dehalorespiring bacteria were responsible for PCE and trichloroethene (TCE) dechlorination and that the dechlorinating activities of these bacteria were repressed by nitrate (5 mM) but not by sulfate (5 mM) (12). Similar inhibition of dechlorination of aliphatic and aromatic compounds by alternative electron acceptors has been observed many times in environmental samples and in enrichment cultures and has been attributed to (i) preferential use of alternative acceptors (e.g.,

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nitrate or sulfite) instead of chlorinated compounds as the terminal electron-accepting compounds, (ii) direct inhibition of enzymes involved in dehalogenation, and (iii) competition with nondehalogenating bacteria that can use alternative acceptors and effectively compete for electron donors with dehalorespiring bacteria (8, 24, 27, 29, 36, 38, 39). However, which of these possible causes of inhibition of dehalogenation is most significant in natural environments is not known.

Although some workers have described the influence of alternative electron acceptors (e.g., sulfate and nitrate) on dehalogenation and/or anaerobic degradation of halogenated aromatic compounds in enrichment cultures and mixed cultures (8, 11, 29, 36), only a few laboratory studies of the influence of alternative electron acceptors on dehalorespiration in pure cultures have been described. In one study dechlorination of 3-chlorobenzoate by *Desulfomonile tiedjei* was examined (36), and in another dechlorination of chlorophenol by *Desulfitobacterium dehalogenans* was examined (38, 39). However, virtually no information concerning the effects of alternative electron acceptors on PCE-dechlorinating anaerobes is available.

The aims of this study were (i) to characterize a novel dehalorespiring anaerobe (strain TCE1) that was isolated from an anoxic PCE-dechlorinating bioreactor (12), (ii) to perform a detailed physiological study of dehalorespiration in strain TCE1 and regulation of this process by alternative electron donors and acceptors, and (iii) to carry out a comparative analysis of PCE-dependent growth of strain TCE1 and dehalorespiration in some related *Desulfitobacterium* species. We propose that strain TCE1 described here should be designated a new strain of *Desulfitobacterium frappieri*.

MATERIALS AND METHODS

Organisms, medium, and growth conditions. Anoxic mineral medium was prepared under an N_2-CO_2 (80:20, vol/vol) atmosphere and contained all of the components described previously (12). Electron donors (20 to 100 mM) and electron acceptors (5 to 20 mM) were added from separately autoclaved stock solutions. The final pH of the medium was 7.2 ± 0.1, and bacteria were routinely grown in the dark at 30°C (batch cultures) or 35°C (chemostat cultures). PCE and TCE were filter sterilized (0.2-μm-pore-size PTFE filters; Alltech, Breda, The Netherlands) and were added to batch cultures as solutions in an organic phase (200 to 500 mM chloroethene dissolved in hexadecane) in order to obtain nominal concentrations ranging from 4 to 10 mmol liter^{-1}.

Desulfitobacterium sp. strain PCE1 (13) was isolated previously from an anoxic PCE-dechlorinating batch culture grown with L-lactate as the primary carbon and electron source. *D. dehalogenans* JW/IU-DC1 (= DSM 9161) (38, 39) was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), and *Desulfitobacterium hafniense* DCB-2 (5) was a kind gift from A. J. M. Stams (Department of Microbiology, Wageningen Agricultural University, Wageningen, The Netherlands).

D. frappieri TCE1 has been deposited in the German Collection of Microorganisms and Cell Cultures as strain DSM 12704.

Isolation of PCE- and TCE-dechlorinating strain TCE1. The chloroethene-dechlorinating bacterium strain TCE1 was isolated from dilution series prepared from an anoxic chemostat culture that had been enriched by using soil obtained from a chloroethene-polluted location in Breda, The Netherlands (12). The chemostat enrichment culture was grown at 20°C by using a mixture of formate (10 mM) and glucose (1 mM) as the electron donor. PCE supplied to the chemostat culture at influent concentrations of 100 to 1,000 μmol liter^{-1} was dechlorinated to *cis*-1,2-dichloroethene (*cis*-DCE) and chloroethene (vinyl chloride). Anoxic liquid dilution series were prepared after 6 months of enrichment. Dechlorination of PCE and TCE occurred in tubes diluted as much as 10^{-7}-fold. A second dilution series was prepared from these tubes by using 40-ml serum bottles containing 20 ml of medium solidified with 0.8% agar and supplemented with 20 mM L-lactate and 1 mmol of TCE per liter. Before inoculation, a hexadecane-TCE solution was mixed with agar media, which were kept in the liquid state by incubation in a water bath at 49°C. The dilution series was prepared in the bottles, and immediately after inoculation the agar was solidified by placing the bottles on ice. Dechlorination of TCE to *cis*-DCE occurred in a bottle diluted as much as 10^{10}-fold, which contained two colonies. A pure culture of strain TCE1 was subsequently obtained after these colonies were transferred into liquid lactate-TCE media. The purity of strain TCE1 was checked microscopically by examining growth on anoxic agar plates and by inoculating medium supplemented with 10 mM glucose, on which many anaerobic bacteria, but not strain TCE1, can grow.

Cultivation of dechlorinating bacteria in anoxic chemostats. Chemostats which were constructed of glass, stainless steel, and viton tubing and had working volumes of 500 to 1,500 ml were operated at 35°C. The pH was measured continuously and was maintained at 7.2 ± 0.1 by automatic titration with 2 N NaOH. PCE was added separately with a syringe pump (model sp200i; World Precision Instruments, Inc., Sarasota, Fla.) via a PTFE filter and was injected directly into the culture liquid to obtain nominal concentrations of 5 to 12 mmol liter^{-1}. To avoid possible toxic effects, the actual dissolved chloroethene concentration was kept below 1 mM by stirring (500 to 600 rpm) and by flushing the culture liquid with N_2-CO_2 (80:20, vol/vol) at a flow rate of 1,500 to 3,000 ml h^{-1}.

Electron microscopy. To obtain electron micrographs, we used cells from the exponential growth phase and negatively stained them with uranyl acetate (1%, wt/vol) as described previously for *Desulfitobacterium* sp. strain PCE1 (13).

Phylogenetic analysis. A large fragment of the 16S rRNA gene of strain TCE1 was amplified by PCR by using genomic DNA and universal primers pA (5′-AGAGTTTGATCCTGGCTCAG; *Escherichia coli* positions 8 to 27) and pH (5′-AAGGAGGTGATCCAGCCGCA; *E. coli* positions 1541 to 1522) (17). PCR products were purified with a Prep-A-Gene kit (Bio-Rad, Hercules, Calif.) and were cloned directly into a PCR cloning vector (TA cloning kit; Invitrogen, San Diego, Calif.) as recommended by the manufacturer. Plasmid sequences were determined by using *Taq* DyeDeoxy terminator methods and a model 373A automatic sequencer (Applied Biosystems Inc., Foster City, Calif.). Searches of the sequences in the GenBank database were performed to determine the closest phylogenetic relatives of the new isolate. Retrieved sequences were subjected to pairwise analyses with the newly determined sequence.

Chemical determinations. Sulfide contents were analyzed colorimetrically by using the method of Trüper and Schlegel (37). The concentration of chloride ions in the culture liquid was determined by the colorimetric method of Bergman and Sanik (1). Bacterial dechlorination was considered positive when more than 0.5 mM chloride was produced in the low-chloride culture medium. Uninoculated anoxic media that also contained the chlorinated compounds were used as controls. Organic acid and H_2 contents were analyzed by gas chromatography by using the equipment and conditions which were described previously (12). Chlorinated ethene contents were determined in triplicate by performing headspace analyses by capillary gas chromatography (14).

Other methods. Optical densities at 660 nm were determined with a Biotron 101 colorimeter (Meyvis, Bergen op Zoom, The Netherlands). Protein contents were determined by using the Lowry method and bovine serum albumin as the standard. For cytochrome analysis, dithionite-reduced–minus–air-oxidized difference spectra of cell extracts of strain TCE1 were obtained with a model UV-1601 spectrophotometer (Shimadzu, Den Bosch, The Netherlands) as described previously (13).

Chemicals. All chemicals were obtained from commercial sources, and the highest purity available (more than 98%) was used in each case.

Nucleotide sequence accession number. The 16S rRNA gene sequence of *D. frappieri* TCE1 has been deposited in the GenBank database under accession no. X95972.

RESULTS

Cell morphology and cytological properties of strain TCE1. Strain TCE1 cells were motile curved rods (diameter, 0.6 to 0.8 μm; length, 2 to 4 μm), and each cell had up to six lateral flagella (Fig. 1A). The gram-positive cell wall was revealed by electron micrographs of ultrathin sections of the bacterium (Fig. 1B). The KOH method (with a 3% KOH solution) was used to confirm that strain TCE1 was a gram-positive organism. Endospores were not observed in cells grown either in liquid media or on solidified media. Dithionite-reduced-minus-air-oxidized absorbance spectra of cell extracts indicated that type *c* cytochromes were present (maxima at 421.0, 523.6, and 553.6 nm) (see above). The cytochrome *c* concentration in cells grown in the presence of L-lactate plus PCE was calculated to be ~136 nmol · g of protein^{-1}. Type *b* cytochromes were not detected in extracts of strain TCE1 cells grown in the presence of lactate plus PCE or lactate plus fumarate, although liquid nitrogen spectra should be obtained in order to improve the resolution and to definitively demonstrate whether type *b* cytochromes are present.

Phylogeny. A large fragment of the 16S rRNA gene of strain TCE1 was amplified by PCR, cloned, and subjected to a sequence analysis. The sequence determined (designated TCE1 clone 2) consisted of 1,616 nucleotides (corresponding to approximately 98% of the complete gene). Sequence database searches revealed that the newly determined sequence was

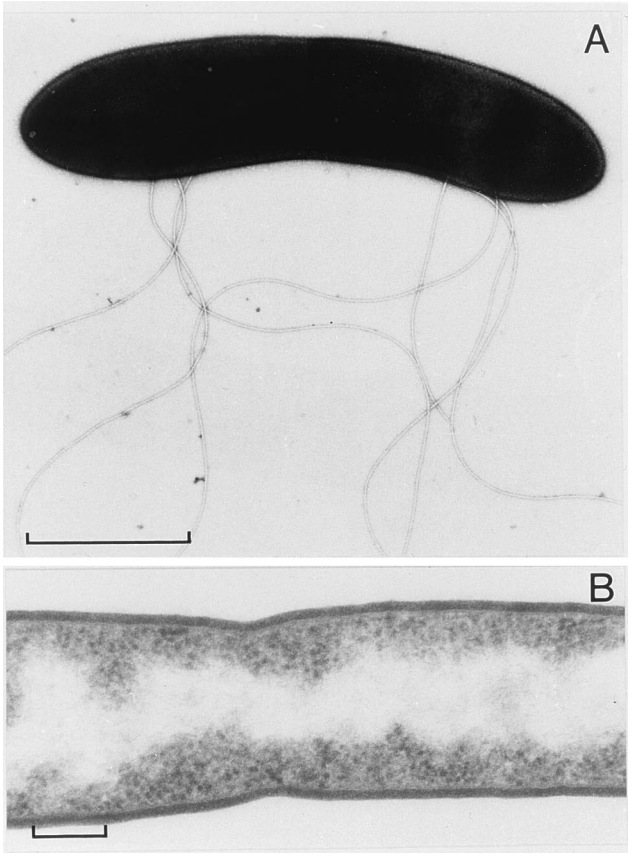

FIG. 1. Electron micrographs of uranyl acetate-stained exponential-phase cells of *D. frappieri* TCE1. (A) Cell with six laterally attached flagella. Bar = 1 μm. (B) Ultrathin section revealing the thick gram-positive cell wall. Bar = 0.25 μm. The morphology of strain TCE1 cells closely resembles that of *Desulfitobacterium* sp. strain PCE1 cells (13).

TABLE 1. Electron donors and acceptors used by strain TCE1 and by some phylogenetically related *Desulfitobacterium* species and a related strain in batch cultures[a]

Electron donor or electron acceptor	Utilization by:			
	Desulfitobacterium sp. strain TCE1	*D. dehalogenans*	*D. hafniense*	*Desulfitobacterium* sp. strain PCE1
Electron donors used with sulfite as the acceptor				
H_2[b]	+	+	−	−
Formate[b]	+	+	+	+
Lactate	+	+	+	+
Pyruvate[c]	+	+	+	+
Butyrate	+	+	+	+
Crotonate	+	−	+	+
Ethanol	+	+	+	+
Serine[c]	+	−	+	+
Electron acceptors used with lactate as the donor				
PCE	+	−	−	+
TCE	+	−	−	−
3-Cl-4OH-PA	−	+	+	+
Nitrate	+	+	+	−
Sulfite	+	+	+	+
Thiosulfate	+	ND[d]	ND	+
Fumarate	+	+	+	+
Fe(III)-EDTA	−	ND	ND	−

[a] Substrates were tested at concentrations of 10 to 50 mM. Growth was judged by examining optical density at 660 nm and acetate or sulfide production.
[b] Acetate (5 mM) was added as a carbon source.
[c] The substrate was also used fermentatively.
[d] ND, not determined.

most closely related to sequences of members of the genus *Desulfitobacterium* and that the type strain of *D. frappieri* (strain PCP-1 [2, 6]) was the closest phylogenetic relative of strain TCE1. Indeed, the 16S rRNA genes of isolate TCE1 and *D. frappieri* PCP-1 (nucleotide sequence accession no. U40078) exhibited 99.7% sequence similarity (corresponding to five mismatched nucleotides and one unmatched nucleotide, respectively). Other desulfitobacteria exhibited significantly lower levels of sequence relatedness to strain TCE1, as follows: *D. hafniense* (accession no. X94975), 96.7%; *Desulfitobacterium* sp. strain PCE1 (X81032), 96.4%; *Desulfitobacterium chlororespirans* (L68528), 95.4%; and *D. dehalogenans* (L28946), 93.9%.

Electron donors and acceptors used by strain TCE1. Strain TCE1 grew fermentatively with pyruvate or serine as the sole substrate. Growth on other substrates depended on the availability of an external electron acceptor (see below). The pH range during growth on pyruvate was 5.7 to 9.5, and the optimum pH (maximum specific growth rate [μ_{max}], 0.2 to 0.3 h^{-1}) was 7.2. The optimum temperature was approximately 35°C. When sulfite was the electron acceptor, strain TCE1 grew with either L-lactate, butyrate, crotonate, pyruvate, serine, formate, H_2, or ethanol as the electron donor (Table 1). Little growth on malate and succinate was observed, and growth was very slow (doubling times, >1 day). No growth was observed in cultures containing sulfite as the electron acceptor and glycine, glutamate, alanine, aspartate, acetate, *n*-valerate, citrate, propionate, methanol, methanol plus acetate, glycerol, triethanolamine, or glucose as the potential electron donor. When L-lactate was used as the electron donor, strain TCE1 grew with PCE, TCE, sulfite, thiosulfate, nitrate, and fumarate as electron acceptors (Table 1). Growth did not occur on L-lactate when sulfate (10 mM), nitrite (1 or 10 mM), O_2 (2% [vol/vol], added in the gas phase), Fe(III)-EDTA (25 mM), 2,4,6-trichlorophenol (100 μM), 3-chloro-4-hydroxyphenylacetate (3-Cl-4OH-PA) (10 mM), *cis*-DCE (4 mmol $liter^{-1}$, dissolved in hexadecane), carbontetrachloride (4 mmol $liter^{-1}$, dissolved in hexadecane), or 1,2-dichloropropane (4 mmol $liter^{-1}$, dissolved in hexadecane) was the electron acceptor.

For comparison, substrate spectra for *Desulfitobacterium* sp. strain PCE1, *D. dehalogenans*, and *D. hafniense* were obtained by using the same medium that was used for strain TCE1 (Table 1). All of the *Desulfitobacterium* species and strains tested grew on formate, L-lactate, pyruvate, butyrate, and ethanol. H_2 was not used by *D. hafniense* and *Desulfitobacterium* sp. strain PCE1, whereas crotonate and serine were not used by *D. dehalogenans*. When L-lactate was the electron donor, all of the strains grew with sulfite, thiosulfate, or fumarate as the electron acceptor. All of the strains except *Desulfitobacterium* sp. strain PCE1 also used nitrate as an electron acceptor. Only strain TCE1 and *Desulfitobacterium* sp. strain PCE1 grew with chloroethenes as electron acceptors. Strain TCE1 was the only bacterium tested that did not grow with 3-Cl-4OH-PA as the electron acceptor.

Growth with different electron donors and acceptors. In a batch culture (30°C, pH 7.2) containing L-lactate plus PCE, the μ_{max} of strain TCE1 was 0.078 h^{-1}. Lactate was oxidized to acetate, and PCE was reductively dehalogenated mainly to

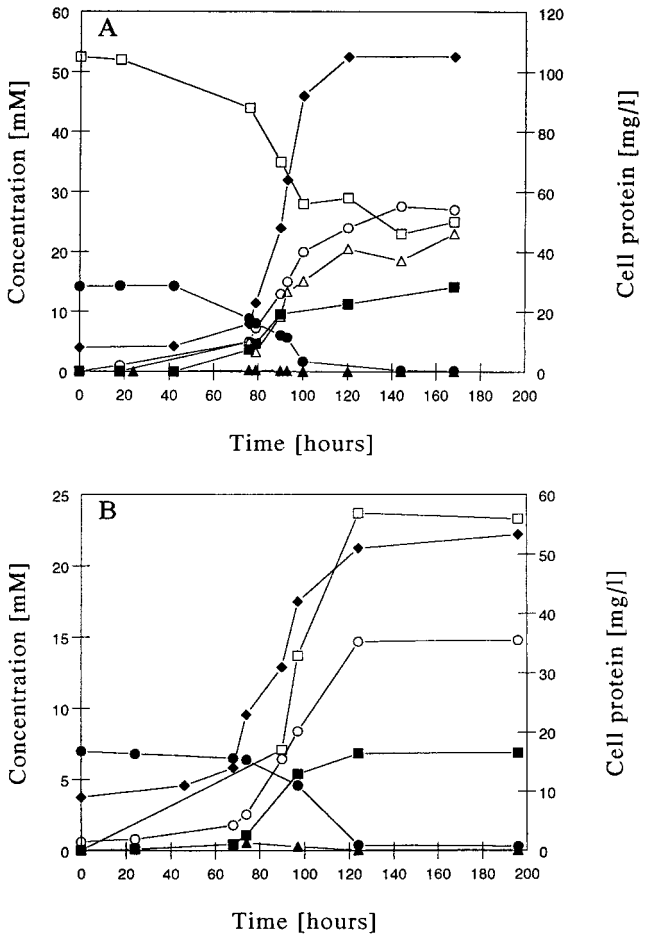

FIG. 2. Product formation during growth of *D. frappieri* TCE1 in batch cultures containing lactate (A) and H_2-CO_2 (B) when PCE was the electron acceptor. Small amounts of 1,1-DCE (<1 mol% of the PCE added [data not shown]) were also produced. (A) Symbols: ●, PCE; ▲, TCE; ■, *cis*-DCE; □, lactate; △, acetate; ○, chloride; ◆, cell protein. (B) Symbols: ●, PCE; ▲, TCE; ■, *cis*-DCE; □, H_2 consumed; ○, chloride; ◆, cell protein.

cis-DCE; 2 mol of chloride was released per mol of *cis*-DCE produced (Fig. 2A). Small amounts of 1,1-DCE and vinyl chloride were also detected (<1 mol% of the PCE added) (data not shown). Dehalorespiration by strain TCE1 in batch cultures was revealed by growth on PCE in the presence of H_2 as the electron donor. Figure 2B shows the consumption of hydrogen during growth in batch culture. The amount of H_2 was measured by determining the combined total amount (liquid and headspace), and the nominal concentration in the culture was ~80 mM. In this case, the calculated μ_{max} (0.034 h^{-1}) was only one-half the μ_{max} obtained during growth in the presence of lactate as the electron donor. Interestingly, the stoichiometries determined in these batch experiments differed from the stoichiometries which were expected on the basis of theory. Instead of stoichiometries of 1:1 and 2:1 for lactate oxidation-PCE reduction and H_2 oxidation-PCE reduction, respectively, we obtained higher values for lactate oxidation-PCE reduction (nearly 2:1) and H_2 oxidation-PCE reduction (1.5:1). Although we do not understand these findings completely, possible explanations are (i) that some of the lactate-acetate was used for assimilation into cell carbon, (ii) that some uncoupling between lactate or H_2 oxidation and PCE reduction occurred (indeed, the efficiency of energy generation with PCE was relatively low [Table 2]), and/or (iii) that some electrons were disposed of on alternative electron acceptors (for example, yeast extract and CO_2) which were present at low concentrations in the medium.

TABLE 2. Efficiencies of energy generation in *D. frappieri* TCE1 when different electron donors and acceptors were used[a]

Electron donor	Electron acceptor	Product	Growth yield (g of protein/mol of e^-)	$\Delta G^{o\prime}$ (kJ/mol of e^-)[b]	Efficiency (mg of protein/kJ)
Lactate	PCE	*cis*-DCE	1.83	−87.0	21
	TCE	*cis*-DCE	2.15	−85.7	25
	Sulfite	Sulfide	1.54	−29.9	51
	Nitrate	Nitrite	1.67	−82.7	20
	Fumarate	Succinate	3.14	−44.2	71
Butyrate	PCE	*cis*-DCE	2.95	−73.7	40
Formate	PCE	*cis*-DCE	1.62	−86.4	19
H_2	PCE	*cis*-DCE	1.93	−85.8	23
Ethanol	PCE	*cis*-DCE	1.54	−83.4	19

[a] The data are data from unique batch experiments.
[b] The $\Delta G^{o\prime}$ values were used only to compare the efficiencies of strain TCE1 when different electron donor-electron acceptor combinations were used. Because the conditions in the batches were the same for all of the combinations, these values can be used. These values should not be used to predict growth in natural habitats.

Lactate, butyrate, and ethanol were oxidized mainly to acetate, but small amounts of propionate, butyrate, formate, and H_2 (<2 mM) were also observed occasionally. During growth with L-lactate as the donor, strain TCE1 reduced sulfite or thiosulfate to sulfide, nitrate to nitrite, and fumarate to succinate. Table 2 shows that the growth yields when sulfite and nitrate were the electron acceptors (1.54 and 1.67 g of cell protein formed per mol of electrons transferred, respectively) were somewhat lower than the growth yields obtained when PCE and TCE were the electron acceptors (1.83 and 2.15 g mol^{-1}, respectively). The highest yield (3.14 g mol^{-1}) was obtained when the organism was grown on lactate plus fumarate (Table 2).

Induction and repression of the dechlorination activity. PCE-dechlorinating capacities were determined with resting cells of strain TCE1 (Fig. 3) and related *Desulfitobacterium* species (data not shown) under nongrowing conditions in phosphate buffer. All resting cell studies were carried out as unique experiments without replication. Cells pregrown on various combinations of electron donors and acceptors were washed and suspended in anoxic isotonic buffer and subsequently were incubated with 10 mM L-lactate and 500 μM PCE (Fig. 3A). Dechlorination rates were determined by a headspace gas chromatography analysis of chloroethenes. From the measurements obtained with strain TCE1 cells grown in the presence of lactate and PCE, relatively high specific rates of dechlorination of PCE to TCE (118 ± 8.1 nmol · min^{-1} · mg of $protein^{-1}$) and of TCE to *cis*-DCE (50 ± 7.9 nmol · min^{-1} · mg of $protein^{-1}$) were calculated. Small amounts of 1,1-DCE (<2 mol%) were also produced. During prolonged incubation, the rate of production of vinyl chloride was very low, 0.0055 nmol · min^{-1} · mg of $protein^{-1}$ (data not shown). The PCE dechlorination rates observed after growth on L-lactate when the electron acceptor was fumarate (0.33 ± 0.5 nmol · min^{-1} · mg of $protein^{-1}$), sulfite (0.096 ± 0.028 nmol · min^{-1} · mg of $protein^{-1}$), or nitrate (<0.01 nmol · min^{-1} · mg of $protein^{-1}$) were less than 1% of the dechlorination rates observed in lactate-PCE-grown cultures. These results indicate that PCE dechlorination activities were not present in strain TCE1 during growth with alternative electron acceptors. However, cells

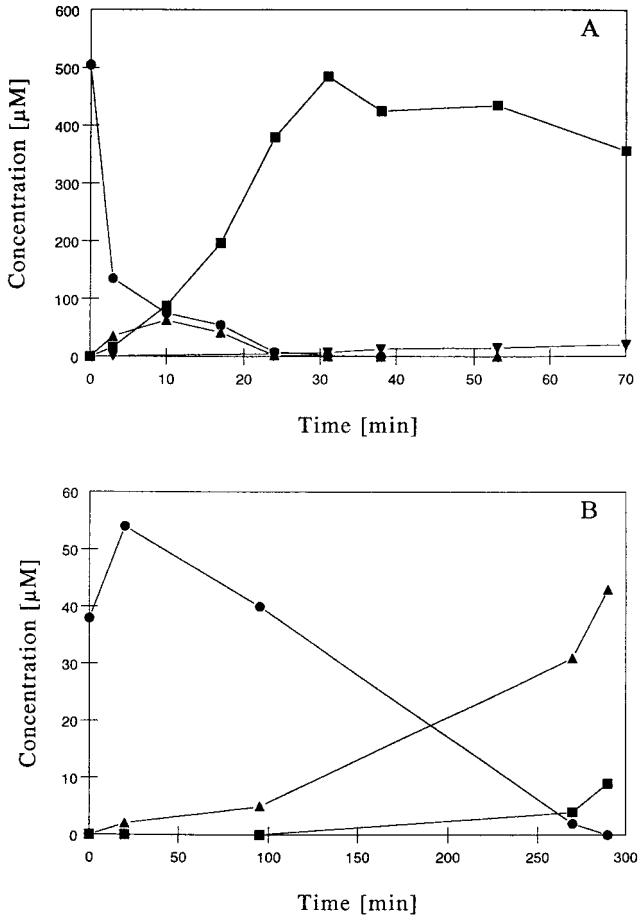

FIG. 3. Time courses for resting cell suspensions of *D. frappieri* TCE1 when lactate was the electron donor and 500 μM PCE (A) or 50 μM CCl_4 (B) was the electron acceptor. (A) Symbols: ●, PCE; ▲, TCE; ■, *cis*-DCE; ▼, 1,1-DCE. (B) Symbols: ●, CCl_4; ▲, $CHCl_3$; ■, CH_2Cl_2.

that were grown fermentatively on pyruvate (i.e., no PCE was present in the medium) also exhibited substantial dechlorination activity (14.7 ± 2.5 nmol · min^{-1} · mg of $protein^{-1}$). Apparently, the presence of PCE is not required for induction of dechlorinating activity. Resting cell suspensions of strain TCE1 grown on L-lactate plus PCE also dechlorinated low concentrations of CCl_4 (rate, 0.040 nmol · min^{-1} · mg of $protein^{-1}$) to $CHCl_3$ and CH_2Cl_2 (Fig. 3B). This chloromethane-dehalogenating activity was probably a cometabolic process, because strain TCE1 was not capable of CCl_4-dependent growth on L-lactate.

For comparison, the PCE dechlorination rates of some other *Desulfitobacterium* species and strains were also determined (data not shown). When *Desulfitobacterium* sp. strain PCE1 was grown on lactate plus PCE, it dechlorinated PCE to TCE at a rate (263 nmol · min^{-1} · mg of $protein^{-1}$) that was even higher than the rate observed for strain TCE1. However, dechlorination of TCE to *trans*-DCE and *cis*-DCE was much slower (1.2 nmol · min^{-1} · mg of $protein^{-1}$). When *Desulfitobacterium* sp. strain PCE1 was grown on L-lactate plus 3-Cl-4OH-PA, PCE dechlorination occurred at a rate of 0.67 nmol · min^{-1} · mg of $protein^{-1}$, which was only 0.25% of the rate observed for cultures grown with PCE as the electron acceptor. *D. hafniense* and *D. dehalogenans* could not grow on L-lactate when PCE was the electron acceptor (Table 1). Nevertheless, cells of both *D. hafniense* and *D. dehalogenans* pregrown on pyruvate or on L-lactate plus 3Cl-4OH-PA reduced some PCE to TCE, although the dechlorination rates for *D. hafniense* (0.033 nmol · min^{-1} · mg of $protein^{-1}$) and *D. dehalogenans* (0.7 nmol · min^{-1} · mg of $protein^{-1}$) were low (data not shown).

Comparison of growth of three *Desulfitobacterium* spp. in chemostat cultures. Cultures of strain TCE1, *Desulfitobacterium* sp. strain PCE1, and *D. hafniense* were grown in chemostats (35°C, pH 7.2) in order to study dehalorespiration under electron acceptor (PCE or 3Cl-4OH-PA)-limited conditions (Table 3). Strain TCE1 steady states were obtained at dilution rates between 0.015 and 0.22 h^{-1} when L-lactate (40 mM) was the electron donor and PCE (5 to 12 mM) was the electron acceptor in the reservoir medium. In a chemostat culture, strain TCE1 formed the same products that it formed in batch cultures (acetate, *cis*-DCE, chloride, and small amounts of TCE and 1,1-DCE). As the dilution rate increased, the dechlorination velocity and the specific dechlorination rate increased to maxima of ~3.2 mmol of Cl^- · $liter^{-1}$ · h^{-1} and ~1.4 μmol of Cl^- · min^{-1} · mg of $protein^{-1}$, respectively. At a dilution rate of 0.33 h^{-1}, strain TCE1 washed out of the culture, and from its rate of washout a μ_{max} of 0.24 h^{-1} was calculated under these growth conditions. The strain TCE1 growth yields obtained in chemostat cultures were the same order of magnitude as the growth yields obtained in batch cultures grown on lactate plus PCE.

For *Desulfitobacterium* sp. strain PCE1 steady states were obtained in chemostats containing different electron donor-electron acceptor combinations in which the electron acceptor was growth limiting (lactate plus PCE, lactate plus 3Cl-4OH-PA, or formate plus 3Cl-4OH-PA) (Table 3). The μ_{max} (range, 0.14 to 0.21 h^{-1}) and maximum dechlorination rates (range, 0.31 to 0.34 μmol of Cl^- · min^{-1} · mg of $protein^{-1}$) obtained for strain PCE1 were lower than the values obtained in chemostats containing strain TCE1. There were no major differences in the growth and dechlorination rates of strain PCE1 when either PCE or 3Cl-4OH-PA was the electron acceptor.

Chemostat experiments performed with *D. hafniense* grown on L-lactate plus 3Cl-4OH-PA revealed that this bacterium has the lowest dechlorination capacity of the *Desulfitobacterium* strains tested in this study (Table 3). The protein yields obtained after dechlorination-dependent growth of *D. hafniense* were similar for all three dehalorespiring bacteria tested.

Influence of different electron acceptors on PCE dechlorination. The influence of alternative electron acceptors on PCE reduction was studied by injecting either sulfite, fumarate, or nitrate directly into chemostat steady-state cultures of strain TCE1 grown on 40 mM L-lactate under PCE-limiting conditions (10 mM PCE) (Fig. 4). The dilution rate was adjusted to 0.05 h^{-1} in all cases. When nitrate was injected to a final concentration of 2 mM, only a slight decrease in PCE dechlorination was observed, as determined by a transient increase in the amount of residual PCE, but PCE dechlorination recovered within the first hour (Fig. 4A). Nitrate disappeared from the culture vessel (Fig. 4B), and an increase in the amount of cell biomass was observed (Fig. 4A). These observations demonstrate that strain TCE1 used PCE and nitrate simultaneously as electron acceptors for growth on lactate. Likewise, PCE reduction was not affected after 2 mM fumarate was added to a PCE-limited steady-state culture of strain TCE1 (Fig. 4C). The accumulation of succinate (Fig. 4D) and the increase in biomass (Fig. 4C) showed that in such cultures PCE and fumarate were consumed simultaneously.

In contrast, PCE dechlorination was strongly inhibited by sulfite. Adding 2 mM sulfite to a PCE-limited steady-state

TABLE 3. Analysis of steady-state chemostat cultures of strain TCE1, *Desulfitobacterium* sp. strain PCE1, and *D. hafniense* grown under PCE- or 3Cl-4OH-PA-limiting conditions in the presence of excess lactate or formate as the electron donor

Organism	μ_{max} (h^{-1})	Steady-state dilution rate (h^{-1})	Substrates consumed (mM)				Products formed (mM)								Protein concn (mg $liter^{-1}$)	Growth yield[b]	Dechlorination velocity[c]	Specific rate of dechlorination[d]
			Lactate	PCE	Formate	3-Cl-4OH-PA	Acetate	Butyrate	TCE	*cis*-DCE	1,1-DCE	*trans*-DCE	4OH-PA[a]	Chloride				
Strain TCE1	0.24	0.015	14.1	10.9			15.2		0.002	10.8	0.04			23.0	69	1.5	0.34	0.08
		0.05	16.1	12.5			15.8		ND[e]	ND	ND			21.0	71	1.7	1.1	0.25
		0.062	17.9	8.8			24.0		ND	ND	ND			20.3	104	2.6	1.2	0.2
		0.071	15.7	9.7			13.1		0.04	9.6	0.04			17.6	64	1.8	1.2	0.33
		0.081	12.8	7.7			17.9		ND	ND	ND			15.3	56	1.9	1.2	0.37
		0.223	9.6	4.8			9.1		ND	ND	ND			14.3	39	1.4	3.2	1.4
Desulfitobacterium sp. strain PCE1	0.14	0.02	26.6	27.7			26.0	0.6	25.3	1.3		0.8		26	60	1.7	0.52	0.14
		0.04	11.2	10.6			15.5	0	8.9	0.9		0.6		11	48	2.2	0.44	0.15
		0.06	20.3	13.4			11.2	0.1	11.1	1.4		0.8		12	55	2.3	0.72	0.22
		0.08	14.3	17.5			11.1	0	15.7	1.1		0.7		14	62	2.2	1.1	0.31
	0.14	0.02	26.0			10.1	32.4	0.8					9.5	11	81	3.7	0.23	0.05
		0.05	15.0			10.1	10.1	0.2					9.2	10	66	3.3	0.5	0.13
		0.11	19.0			10.3	8.3	0.2					9.6	11	60	2.8	1.2	0.34
	0.21	0.03			28.2	18.7	0.4	0					17.5	22	78	1.8	0.66	0.14
		0.06			25.2	18.7	0	0.2					17.1	21	71	1.7	1.3	0.3
		0.09			26.3	18.3	0	0.1					17.3	17	80	2.4	1.5	0.32
D. hafniense	0.052	0.009	35.0			8.0	36.0						ND	7.8	64	4.1	0.07	0.02
		0.03	16.6			8.0	16.2						7.6	8.0	48	3.0	0.22	0.09

[a] 4OH-PA, 4-hydroxyphenylacetate.
[b] Expressed in grams of protein formed per mole of electrons transferred.
[c] Expressed in millimoles of Cl^- released per liter per hour.
[d] Expressed in micromoles of Cl^- released per minute per milligram of protein.
[e] ND, not determined.

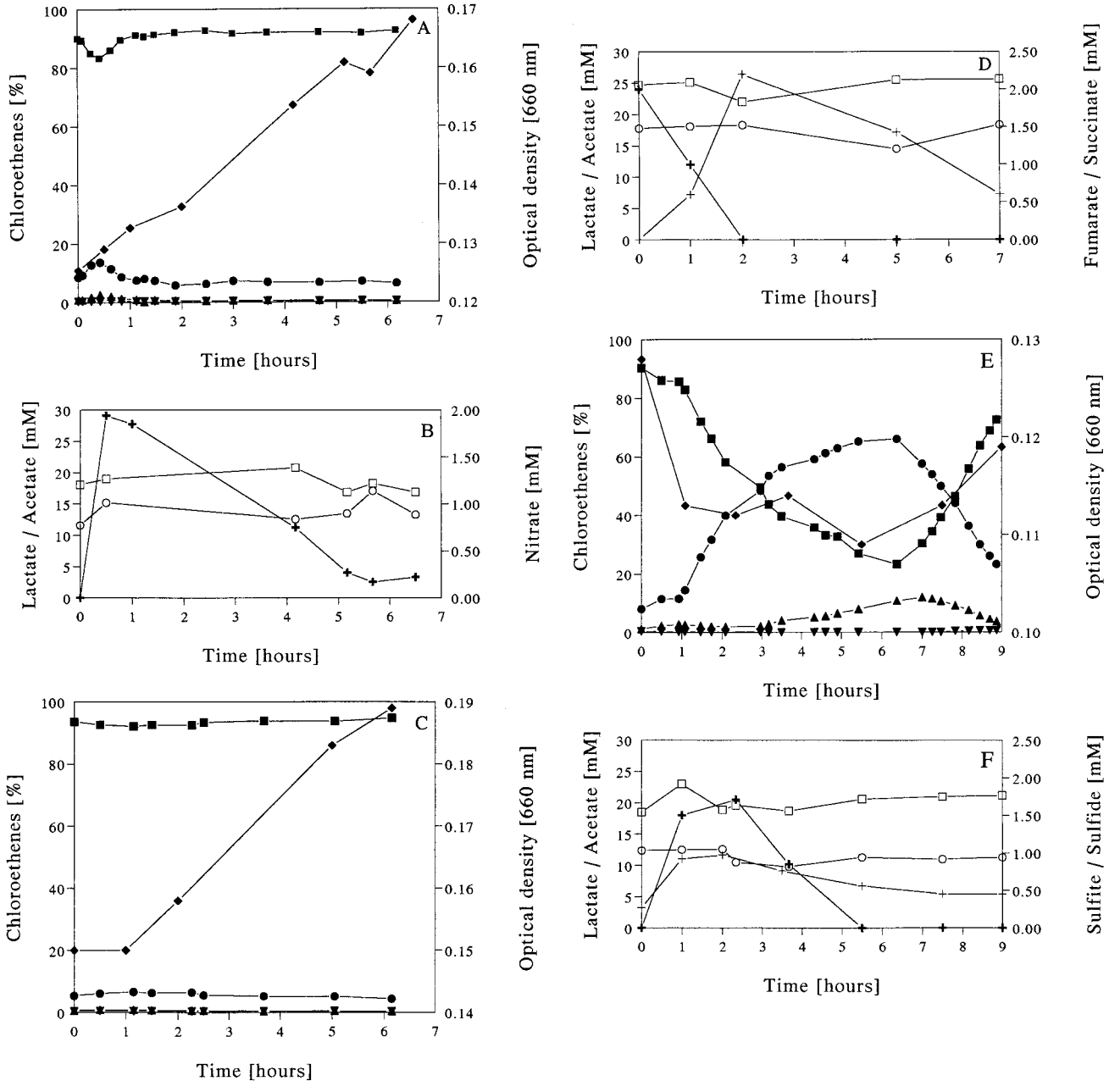

FIG. 4. Effects of different electron acceptors on PCE dehalogenation after direct injection of 2 mM nitrate (A and B), 2 mM fumarate (C and D), or 2 mM sulfite (E and F) into steady-state chemostat cultures of *D. frappieri* TCE1 grown under PCE limitation conditions (10 mM = 100%). The electron donor was lactate (40 mM), and the dilution rate was 0.05 h^{-1} in all cases. The steady-state residual concentration of PCE was ~0.8 mM (=8%). (A, C, and E). Symbols: ●, PCE; ▲, TCE; ■, *cis*-DCE; ▼, 1,1-DCE; ◆, optical density at 660 nm. (B, D, and F) Symbols: □, lactate; ○, acetate; +, added electron acceptor (nitrate in panel B, fumarate in panel D, and sulfite in panel F); +, reduced product of added electron acceptor (succinate in panel D and sulfide in panel F).

culture of strain TCE1 resulted in an immediate accumulation of PCE and wash-out of the dechlorination products *cis*-DCE (Fig. 4E) and chloride. The data for sulfite consumption and sulfide accumulation (Fig. 4F) revealed that sulfite was reduced by strain TCE1. Nevertheless, slight decreases in optical density at 660 nm and acetate concentrations indicated that growth was inhibited in the presence of sulfite. When all of the sulfite was consumed, dechlorination resumed, and the culture returned to its initial steady state (Fig. 4E). The growth and PCE dechlorination responses of strain TCE1 to the simultaneous availability of multiple electron acceptors were also studied by adding nitrate, fumarate, and sulfite (2 mM each) to the reservoir medium. Subsequently, medium containing these electron acceptors was used to gradually replace the medium used for PCE-limited chemostat cultivation of strain TCE1 (dilution rate, 0.05 h^{-1}; PCE concentration, 5 mM; lactate concentration, 40 mM). Dechlorination was not affected by the presence of the three electron acceptors. After five volume changes, a steady state was obtained in which strain TCE1 reduced more than 90% of the PCE along with all of the

fumarate, nitrate, and sulfite. In contrast, dehalorespiration was completely blocked when the reservoir lactate concentration was reduced from 40 to 10 mM and the concentrations of nitrate, fumarate, and sulfite were increased to 10 mM, which made lactate the growth-limiting substrate (data not shown).

DISCUSSION

In this paper we describe strain TCE1, a novel strictly anaerobic bacterium that couples oxidation of H_2 and various organic substrates to reductive dechlorination of PCE and TCE. Analysis of the 16S rRNA revealed that strain TCE1 is a member of the genus *Desulfitobacterium* and is closely related to the pentachlorophenol-dehalogenating organism *D. frappieri* PCP-1. The level of 16S rRNA relatedness (99.7%) clearly indicates that strain TCE1 is a new strain of *D. frappieri*. Currently, the genus *Desulfitobacterium* contains several species whose members can dechlorinate halophenolic compounds, including *D. dehalogenans* (38, 39), *D. chlororespirans* (30), *D. frappieri* (2, 6), and *D. hafniense* (5), as well as *Desulfitobacterium* sp. strain PCE-S (27, 28) and *Desulfitobacterium* sp. strain PCE1 (13). Only one of these organisms, strain PCE1, is also able to dechlorinate PCE by reductive dehalogenation, forming mainly TCE and small amounts of *cis*-DCE and *trans*-DCE. Strain TCE1 appears to be the first *Desulfitobacterium* strain that is not able to grow with chloroaromatic compounds (e.g., chlorophenols and chlorophenylacetate) as electron acceptors (which clearly differentiates this strain from the *D. frappieri* type strain, strain PCP-1), and it is the third strain of the genus *Desulfitobacterium*, besides *Desulfitobacterium* sp. strain PCE1 (which uses PCE [13]) and *Desulfitobacterium* sp. strain PCE-S (which uses PCE and TCE [27]), that can use chlorinated ethenes as electron acceptors. In addition to the differences in substrate specificity, *D. frappieri* TCE1 is a motile, non-spore-forming organism, which clearly distinguishes it from *D. frappieri* PCP-1 (2). *Desulfitobacterium* sp. strain PCE-S as described by Miller et al. (28) will also probably be designated a *D. frappieri* strain in the future; however, the sequence of strain PCE-S 16S rRNA has not been deposited in a database, and therefore, the relatedness between strains PCE-S and TCE1 cannot be determined at this time. The ability to metabolize chlorinated compounds appears to be particularly pronounced in the *Clostridium-Bacillus* subphylum of the gram-positive bacteria. All members of the genus *Desulfitobacterium*, which have been isolated from different ecosystems (soils, compost soils, lake sediments, aquifers, and bioreactors) at geographically distant locations, have been shown to be capable of reductive dechlorination of chlorinated ethenes and/or chlorinated phenolic compounds (8, 11, 29, 43). Furthermore, the PCE-dechlorinating organism *Dehalobacter restrictus* and the dichloromethane-utilizing organism *Dehalobacterium formicoaceticum* are phylogenetically closely related to the genus *Desulfitobacterium* (16, 23, 32) within the *Clostridium-Bacillus* subphylum.

Growth of strain TCE1 with H_2 or formate as the electron donor was coupled to reduction of PCE. Because oxidation of these substrates does not yield ATP through substrate level phosphorylation, it may be assumed that strain TCE1 grows by means of dehalorespiration. In addition to the few chloroethene-degrading desulfitobacteria, *Dehalobacter restrictus* (16), *Dehalospirillum multivorans* (31), *Dehalococcoides ethenogenes* 195 (25), and *Desulfuromonas chloroethenica* (18, 19) are also strict anaerobes that can use PCE as an electron acceptor. Based on the reduction of PCE to *cis*-DCE and the relatively broad spectrum of electron donors and acceptors used, strain TCE1 metabolism resembles the versatile metabolism of *Desulfitobacterium* sp. strain PCE-S (27) and *Dehalospirillum multivorans* (31). In the present study we confirmed that *D. dehalogenans* and *D. hafniense* are also able to grow by means of reductive dehalogenation (5, 39), as we observed dechlorination-dependent growth of these strains with lactate as the electron donor and 3Cl-4OH-PA as the electron acceptor (Table 1). The substrate utilization pattern observed for *D. dehalogenans* corresponded well to the pattern described by Utkin et al. (38, 39). However, the growth of *D. hafniense* on formate, lactate, and butyrate which we observed (Table 1) was not consistent with the results obtained by Christiansen and Ahring (5), who found that pyruvate and tryptophan were the only substrates that support growth of this organism.

The growth yields determined for growth of strain TCE1 on lactate with different terminal electron acceptors revealed that the efficiency of energy conservation with PCE or TCE was within the range of values found with nonchlorinated electron acceptors and decreased (from 3.1 to 1.5 g of protein per mol of electrons) in the following order: fumarate > TCE > PCE > nitrate > sulfite (Table 2). Interestingly, this sequence does not correlate with the amounts of free energy available from oxidation of lactate to acetate with these electron acceptors. Table 2 clearly shows that the free energy that can be obtained from the reduction of PCE (and TCE) is considerably greater than the free energy that can be obtained during the reduction of fumarate, yet the protein yield is lower. This leads to the suggestion that either some part of the process is uncoupled from energy conservation or an energy input is needed (for example, for transport processes, detoxification reactions, or reversed electron transport) for metabolism of the chloroethenes to occur.

A comparison of the maximum observed dechlorination rates of different dehalogenating bacteria (in batch and/or chemostat cultures) demonstrated the outstanding potential of strain TCE1 for use in bioremediation (Table 3). The very high dechlorination rate obtained (1.4 μmol of chloride released per min per mg of cell protein at the dilution rate used) is the highest rate of dechlorination of PCE described so far. The dechlorination activities of other species which have been reported are all significantly lower, as follows: *Desulfitobacterium* sp. strain PCE1, slightly more than 0.3 μmol of $Cl^- \cdot min^{-1} \cdot$ mg of $protein^{-1}$); *Dehalospirillum multivorans*, 0.05 μmol of $Cl^- \cdot min^{-1} \cdot$ mg of $protein^{-1}$ (31); *Dehalococcoides ethenogenes* 195, 0.07 μmol of $Cl^- \cdot min^{-1} \cdot$ mg of $protein^{-1}$ (25); and *Dehalobacterium formicoaceticum*, 0.1 μmol of $Cl^- \cdot min^{-1} \cdot$ mg of $protein^{-1}$ on dichloromethane (23). Only with the type strain of *D. frappieri* (strain PCP-1) has a comparable activity been found for *ortho* dechlorination of 2,3,5-trichlorophenol ($\sim$1.2 μmol of $Cl^- \cdot min^{-1} \cdot$ mg of cell $protein^{-1}$) (6).

When we examined the influence of alternative electron acceptors, we found that a low concentration (2 mM) of nitrate or fumarate did not have a negative effect on the rate of PCE dechlorination. Both nitrate and fumarate were used simultaneously with PCE as the limiting electron acceptor. However, sulfite (2 mM) suppressed the rate of PCE dehalogenation by strain TCE1. Addition of this alternative electron acceptor resulted in a short-term decrease in the growth rate of this strain, but the growth rate increased again after the sulfite was completely consumed (when PCE was being used again). When a mixture of the three electron donors (2 mM nitrate, 2 mM fumarate, and 2 mM sulfite) was added in the presence of excess lactate (40 mM) and a limiting concentration of PCE (10 mM), PCE dechlorination carried out by strain TCE1 was not suppressed. In contrast, PCE dechlorination was completely blocked under lactate-limiting conditions (10 mM lactate) and when there was excess electron acceptor (10 mM

nitrate, 10 mM fumarate, 10 mM sulfite, and 10 mM PCE). This indicates that the relative availability of electron donors and acceptors in the environment may be more important than the actual concentrations of the compounds. Little information is available concerning regulation in dechlorinating bacteria of the use of electron acceptors if they are present in various combinations.

Townsend and Suflita (36) described the influence of sulfur oxyanions on the reductive dehalogenation of 3-chlorobenzoate by *Desulfomonile tiedjei*. Dehalogenation of 3-chlorobenzoate was greatly reduced after 5 mM sulfate, 5 mM sulfite, or 5 mM thiosulfate was added, whereas 5 mM nitrate had no influence on the dehalogenation activity. Only at sulfate concentrations less than 1 mM did the authors observe no significant negative influence on the dehalogenation process in this bacterium (36). Townsend and Suflita suggested that the sulfur oxyanions tested were used as preferred electron acceptors and repressed the expression of reductive dehalogenases in *Desulfomonile tiedjei* (36). Our observations indicate that a similar situation may occur during reductive alkyl dehalogenation by strain TCE1.

Moreover, in a recent study of the PCE dehalogenases in cell extracts of *Desulfitobacterium* sp. strain PCE-S and *Dehalospirillum multivorans*, inhibition of the enzyme activity was observed following addition of 1 mM of sulfite (27). Since neither of these organisms can use sulfite as an alternative electron acceptor (because they lack a sulfite reductase), Miller et al. proposed that inhibition of PCE reduction by sulfite is due to binding of this inhibitor to the cobalt of a corrinoid which is the prosthetic group of the dechlorinating enzyme involved (27). Similar results were reported by Magnuson et al. (24), who observed complete inhibition of a PCE-reductive dehalogenase (51 kDa) and a TCE-reductive dehalogenase (61 kDa) isolated from *Dehalococcoides ethenogenes* 195 after 2 mM sodium sulfite was added. Neither of these enzymes was inhibited by 2 mM sulfate, 2 mM sulfide, or 2 mM selenate. Magnuson et al. also suggested that the inhibition could be due to a reaction between the inhibitor and the metal centers (possibly iron-sulfur clusters) of cofactors of the dehalogenases (24).

The findings described above may help explain our observations with strain TCE1, because the dehalogenase activity of this strain was completely suppressed by 2 mM sulfite in chemostat experiments under PCE-limiting conditions. Strain TCE1 actually was washed out from the chemostat when sulfite was added. After all of the sulfite was removed, PCE was used immediately again in chemostat cultures of strain TCE1, suggesting that the dehalogenases involved may have been reversibly inhibited by interactions of sulfite with prosthetic groups of the enzymes like the interactions proposed for other dehalogenating bacteria (24, 27). To obtain a better understanding of the inhibitory effects on dehalogenation by *D. frappieri* TCE1, the enzymes involved need to be isolated and characterized in future work.

In general, further elucidation of the biochemical mechanisms of dehalorespiration is necessary in order to completely understand energy conservation in the various anaerobic dehalogenating bacteria that have been described (11, 16, 21, 27, 28, 31, 36, 43). This is particularly important in order to gain sufficient control over dehalogenation processes based on the activities of such anaerobes when they are used in situ or off site for the treatment of halogen-contaminated soil or water (7, 10, 20, 26, 33, 44).

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