

Fraction of Electrons Consumed in Electron Acceptor Reduction and Hydrogen Thresholds as Indicators of Halorespiratory Physiology

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Measurements of the hydrogen consumption threshold and the tracking of electrons transferred to the chlorinated electron acceptor (f_e) reliably detected chlororespiratory physiology in both mixed cultures and pure cultures capable of using tetrachloroethene, *cis*-1,2-dichloroethene, vinyl chloride, 2-chlorophenol, 3-chlorobenzoate, 3-chloro-4-hydroxybenzoate, or 1,2-dichloropropane as an electron acceptor. Hydrogen was consumed to significantly lower threshold concentrations of less than 0.4 ppmv compared with the values obtained for the same cultures without a chlorinated compound as an electron acceptor. The f_e values ranged from 0.63 to 0.7, values which are in good agreement with theoretical calculations based on the thermodynamics of reductive dechlorination as the terminal electron-accepting process. In contrast, a mixed methanogenic culture that cometabolized 3-chlorophenol exhibited a significantly lower f_e value, 0.012.

Halogenated aliphatic and aromatic compounds are major environmental pollutants (17). Since many contaminated sites are anaerobic, stimulation of reductive dehalogenation is a very promising strategy for bioremediating aquifers and sediments contaminated with halogenated compounds. Reductive dechlorination is a cometabolic or respiratory process that is carried out by organochlorine-reducing bacteria (OCRBs). One example of cometabolism is the reductive dechlorination of tetrachloroethene (PCE) by sulfate reducers (6, 46), methanogenic archaea (16, 23), or acetogens (44). The dechlorination rates of the cometabolic processes, however, are low, and their contributions to the dehalogenation reactions observed in natural environments are probably negligible. In contrast, respiratory OCRBs that use chlorinated aliphatic and/or aromatic organic compounds as electron acceptors in energy metabolism and hence for growth are likely to be major contributors to microbially mediated dehalogenation reactions in anaerobic environments. The common characteristic of respiratory organohalogen-reducing bacteria is their ability to couple the energy released during reductive dehalogenation to growth in a process known as halorespiration (or chlororespiration for chlorinated chemicals).

The energetics of the terminal electron-accepting process (TEAP) have been shown to control the minimum H_2 concentration that can be consumed (i.e., the threshold) (2, 8, 9, 32, 33). The H_2 threshold is inversely correlated with changes in Gibbs free energy (ΔG^0) and the electrochemical potential of the H_2 -consuming reaction (Table 1) (8, 32, 33, 52). Lovley and Goodwin (33) have demonstrated that the steady-state H_2 threshold concentrations are controlled by the physiological characteristics of the H_2 -consuming organisms and are independent of the kinetics of the H_2 -producing or H_2 -consuming reactions. This model predicts that the steady-state threshold concentrations of H_2 follow the following order (from least to

most energetically favorable TEAP): acetogenesis > methanogenesis > sulfate reduction (sulfidogenesis) > Fe(III) reduction > Mn(IV) reduction > denitrification. Based on the threshold model, one would predict that chlororespiring OCRBs should consume H_2 so that the threshold concentrations are lower than the threshold concentrations of acetogens, methanogens, and sulfate reducers and probably very close to the threshold concentrations of organisms that exhibit dissimilatory reduction of nitrate to ammonia (ammonifiers).

Another method used to identify the activity of chlororespiring organisms is to determine f_e or f_s . f_e is the fraction of electrons used for energy formation (i.e., the fraction of electrons released during oxidation of the electron donor that are directed toward reduction of the terminal electron acceptor). The fraction of electrons derived from the electron donor that is incorporated into biomass is the synthesis fraction (f_s) (10, 36). Since $f_e + f_s = 1$, and $f_{s(max)}$ is directly related to the maximum growth yield, f_e can be calculated from the maximum growth yield, when it is available. While accurate determinations of growth yields require the use of pure cultures, f_e values can be experimentally determined for pure and mixed cultures. For example, estimated f_e values for chlororespiring OCRBs have been predicted to be very similar to f_e values for ammonifiers according to thermodynamics-based models (20, 21, 36).

Despite the potential importance of chlororespiring OCRBs in reductive dechlorination processes in anaerobic environments, no convenient methods to assay this activity have been developed. So far, the only method which conclusively demonstrates that chlororespiration occurs involves isolating the dechlorinating populations in pure culture and showing that growth with an electron donor that does not support substrate level phosphorylation is dependent on the dechlorination reaction. This approach is time-consuming, since isolating halorespiring OCRBs in pure culture can be tedious. Many of these organisms grow slowly or require additional growth factors, such as amino acids and vitamins (11, 22, 35). For example, despite extensive efforts to isolate vinyl chloride (VC)- and polychlorinated biphenyl-respiring OCRBs, to date no pure cultures that grow in this manner have been obtained.

Here we present evidence that both measuring the H_2 threshold and determining f_e provide excellent indications of halorespiratory physiology. The applicability of both methods

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TABLE 1. H_2 threshold concentrations, $\Delta G^{0'}$ values, and redox potentials of different redox couples with H_2 as the electron donor

TEAP	H_2 threshold concn		$\Delta G^{0'}$ (kJ/mol of H_2) ^a	E_0' (V) ^b	Reference(s)
	ppmv	nM			
Acetogenesis	430–4,660	336–3,640	–26.1	–0.28	2, 9
Methanogenesis	6–120	5–95	–33.9	–0.24	8, 9, 32, 33
Sulfate reduction ($SO_4^{2-} \rightarrow HS^-$)	1.3–19	1–15	–38.0	–0.22	8, 9, 33
Fumarate reduction	0.02	0.015	–86.2	+0.032	9
Ammonification ($NO_3^- \rightarrow NH_4^+$)	0.02–0.03	0.015–0.025	–149.9	+0.36	8, 9
Nitrate reduction ($NO_3^- \rightarrow N_2O, N_2$)	<0.06	<0.05	–240	+0.79	33, 34
Fe(III) reduction	0.13–1	0.1–0.8	–228.3	+0.77	8, 33, 34
Chlororespiration	<0.4 ^c	<0.3 ^c	–130 to –187	0.30 to 0.55	This study

^a The $\Delta G^{0'}$ values were obtained from references 13 through 15 and 45 or were calculated from free energies of formation.

^b The redox potentials (E_0') were calculated by using the relationship $E_0' = (\Delta G^{0'}/-0.193) - 414$ (14, 15, 47).

^c Data from this study.

was demonstrated with several pure and mixed cultures. Furthermore, H_2 threshold measurements obtained for mixed cultures that dechlorinated VC to ethene (ETH) indicated that VC-respiring OCRBs were present, despite the fact that we were not able to obtain these organisms in pure culture.

MATERIALS AND METHODS

Chemicals. The following chlorinated compounds were used in this study: PCE, trichloroethene, *cis*-1,2-dichloroethene (*cis*-DCE), 1,2-dichloropropane (1,2-D), 3-chlorobenzoate (3-CBA), 3-chloro-4-hydroxybenzoate (3Cl-4-HBA), 2-chlorophenol (2-CP), 3-chlorophenol (3-CP), and 2,5-dichlorophenol (2,5-DCP), all of which were obtained from Aldrich, Milwaukee, Wis.; and VC, which was obtained from Fluka Chemical Corp., Ronkonkoma, N.Y.

Organisms, medium preparation, and growth conditions. The following pure cultures were used in this study: *Desulfotobacterium chlororespirans* DSM 11544 or ATCC 700175 (27, 39), *Desulfotobacterium* sp. strain Viet1 (29), *Desulfuromonas* sp. strain BB1 (30), *Desulfomonile tiedjei* ATCC 49306 (11, 43), and strain 2CP-C (38), a close relative of strain 2CP-1 (5). The 2,5-DCP-dechlorinating OM enrichment culture was derived from chlorophenol-contaminated soil (38), and the 3-CP-dechlorinating methanogenic enrichment culture was obtained from residential compost in the Lansing area of Michigan (40). Chloroethene-dechlorinating mixed cultures were derived from freshwater river sediments obtained from the King Salmon River in Alaska (culture KS), the Red Cedar River in Michigan (cultures RC), the Au Sable River in Michigan (cultures AuS), and the Pere Marquette River in Michigan (cultures PM) (28, 29). Cultures KS_{1,2-D} and RC_{1,2-D} were enriched for the ability to dechlorinate 1,2-D to propene, and cultures RC, AuS, and PM were enriched for the ability to dechlorinate PCE to ETH. Subcultures of the cultures that dechlorinated PCE to ETH were established after sediment-free, nonmethanogenic cultures had been obtained (29). These subcultures were fed with PCE (20 μ M), *cis*-DCE (26.5 μ M), or VC (41 μ M). Cultures were transferred when approximately 20 μ M of the initial chlorinated compound had been dechlorinated. All subcultures had undergone at least 10 serial transfers (1% [vol/vol] inoculum) at the time that the H_2 threshold measurements were initiated.

Reduced anaerobic mineral medium was prepared as described previously (27, 28, 39). To obtain f_c measurements for cultures of *Desulfotobacterium chlororespirans*, the OM enrichment culture, and the 3-CP-dechlorinating mixed methanogenic culture, the medium described by Sanford et al. (39) was used. The same medium was used to monitor H_2 consumption in cultures of strain 2CP-C. For most other experiments the medium described by Löffler et al. (27, 28) was used; the only exceptions were the experiments in which f_c was measured in strain BB1 and Viet1 cultures. In these experiments, dechlorination was quantified by measuring the increase in the chloride ion concentration, and a chloride-free medium was used. This medium contained (per liter) 0.5 g of $MgSO_4 \cdot 6H_2O$, 0.3 g of NH_4SO_4 , 0.3 g of K_2SO_4 , 0.015 g of $CaSO_4 \cdot H_2O$, 0.05 mg of resazurin, 1 ml of trace element solution A (27), 1 ml of trace element solution B (27), 0.048 g of $Na_2S \cdot 9H_2O$, 0.035 g of L-cysteine, and 2.52 g of $NaHCO_3$. After autoclaving, 2 mM potassium phosphate buffer (pH 7.2) and a vitamin solution (27) were added. Cultures were incubated in 160-ml (nominal capacity) serum bottles containing 100 ml of medium (referred to below as 100-ml cultures). All serum bottles were incubated in the dark at 25°C with the butyl rubber stopper down without shaking. Undiluted halogenated compounds were added to cultures with 5- μ l Hamilton syringes equipped with Chaney adapters (Hamilton, Reno, Nev.) in order to achieve reproducibility. Gaseous VC was added by syringe. Chlorinated benzoates and phenols were added from anaerobic, filter-sterilized stock solutions to final concentrations of 2 and 0.2 mM, respectively. Dechlorination was monitored as described below, and cultures were respiked with the appropriate chlorinated compounds before they were completely depleted. H_2 was

added by syringe at the mixing ratios indicated below. Molar gas concentrations were calculated by using a molar volume of 24.48 liters at 25°C.

Experimental determination of f_c and f_e values. f_c values were determined in cultures containing a chlorinated compound and bicarbonate as the only electron acceptors and either H_2 plus acetate (85,000 ppm by volume [ppmv] and 1 mM, respectively; OM enrichment culture), acetate (5 mM; strains BB1 and 2CP-C), lactate (5 mM; *Desulfotobacterium* sp. strain Viet1 and *Desulfotobacterium chlororespirans*), or a volatile fatty acid (VFA) mixture (625 μ M formate, 125 μ M succinate, 125 μ M propionate, and 125 μ M butyrate; methanogenic enrichment culture) as the only electron donor(s). For studies of BB1 and Viet1 cultures, PCE was added by syringe from anaerobic, autoclaved, hexadecane stock solutions (125 μ l PCE in 10 ml of hexadecane per 100-ml culture). The f_e values were determined by linear regression analysis by plotting the amount of electron pairs released during electron donor oxidation as $2[H]$ against the amount of electron pairs used to reduce the chlorinated electron acceptor. f_e was derived from the slope of the regression line. In most cases at least three cultures were used to determine f_e ; the only exceptions were the methanogenic enrichment cultures, which were analyzed in duplicate. The cultures were each started with a 1% (vol/vol) inoculum, and 0.5-ml samples were periodically withdrawn (every 2 to 3 days) and analyzed to determine chloride release and electron donor consumption. Aromatic dechlorination products and VFA were quantified by high-performance liquid chromatography (HPLC), and PCE dechlorination was quantified by determining the amount of chloride ions released into the medium by using the calorimetric assay described below. Electron donor (lactate) consumption in cultures of *Desulfotobacterium chlororespirans* and *Desulfotobacterium* sp. strain Viet1 was examined by measuring the amount of acetate formed during incomplete oxidation of lactate.

Hydrogen threshold measurements. H_2 partial pressures were measured in the headspaces of cultures incubated at 25°C and are expressed below in parts per million by volume (1 ppmv = ca. 0.1 Pa = ca. 10^{-6} atm) according to the convention adapted by Conrad (8). In order to compare our data to the data obtained in other studies, dissolved H_2 concentrations were calculated by using the following equation: H_2 (dissolved) = LP/RT , where H_2 (dissolved) is the aqueous concentration of H_2 (in moles per liter); L is the Ostwald coefficient for H_2 solubility, which is 0.01913 at 25°C (49); R is the universal gas constant (0.0821 liter $\cdot$ atm K^{-1} mol $^{-1}$); P is the pressure (in atmospheres); and T is the kelvin temperature (3, 34). Duplicate 100-ml cultures were inoculated (1%, vol/vol) from dechlorinating cultures that had completely reduced all of the chlorinated electron acceptor present. One set of the duplicate cultures was amended with the individual chlorinated compounds, and the other set did not receive an electron acceptor other than bicarbonate. The concentrations of chlorinated compounds were determined weekly, and the H_2 concentration was measured every third week. Cultures that received a chlorinated compound were spiked with the same chlorinated electron acceptor before it was completely depleted. Values for H_2 thresholds were assessed when the H_2 concentration remained stable (i.e., when there was a less than 5% decrease over a 6-week period). After the H_2 concentrations reached constant threshold values, H_2 (500 to 10,000 ppmv) was added to the cultures, and consumption of this H_2 to a constant threshold value was monitored again. H_2 thresholds in pure cultures (except strain 2CP-C cultures) were determined by using the same protocol, except that acetate was replaced by 20 mM pyruvate. The cultures were grown on pyruvate with and without the appropriate chlorinated electron acceptors. 3-CBA (2 mM), 3Cl-4-HBA (2 mM), and PCE (0.2 mM) were used as electron acceptors for *Desulfomonile tiedjei*, *Desulfotobacterium chlororespirans*, and *Desulfotobacterium* sp. strain Viet1, respectively. All cultures were initiated with 1% (vol/vol) inocula from cultures that had undergone at least three transfers under fermenting or chlororespiring growth conditions. When the initial amount of chlorinated electron acceptor was depleted, the cultures were fed with 2 mM 3-CBA, with 2 mM 3Cl-4-HBA, or with PCE (50 μ l in 5 ml of hexadecane). Strain 2CP-C utilized acetate or H_2 as an electron donor for reductive dechlorination.

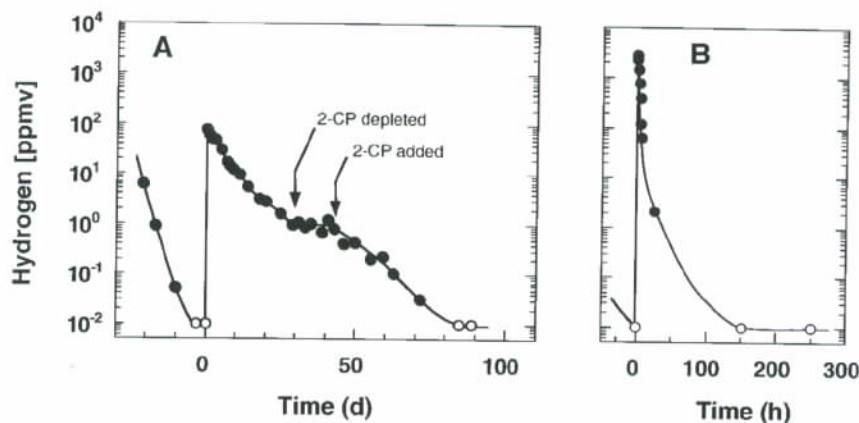


FIG. 1. H_2 consumption by strain 2CP-C with 2-CP as the electron acceptor and H_2 as the electron donor. (A) Two freshly inoculated cultures containing 0.2 mM acetate were fed 2-CP. After the H_2 concentration was less than 0.01 ppmv, the cultures were fed 100 ppmv of H_2 . 2-CP was respiked when it was depleted, except for the 14-day period indicated by the arrows. (B) Triplicate cultures were initially fed 2 mM acetate and 2-CP. After 4 mM 2-CP had been added, all of the acetate was consumed and the H_2 threshold concentrations were less than 0.01 ppmv. The cultures were then fed 2-CP and 2,500 ppmv of H_2 , and H_2 consumption was monitored again. Note the logarithmic scale of the y axis. The detection limit for H_2 was 0.01 ppmv, and the open circles indicate data that could not be quantified due to the limitations of the detection method. The data were averaged, and the variability between cultures was less than 20%. No H_2 consumption was observed in cultures that did not receive 2-CP.

mination of 2-CP. This strain was grown with acetate (0.2 or 2 mM) and 0.2 mM 2-CP, and the culture was respiked with 2-CP (0.2 mM) when the 2-CP was depleted. When constant H_2 threshold concentrations were reached, the cultures were spiked with 100 or 2,500 ppmv of H_2 , and the consumption of H_2 was monitored again.

Analytical methods. All volatile chlorinated aliphatic compound contents were measured in the headspace of 100-ml cultures at 25°C and were analyzed by using a gas chromatograph and direct (splitless) injection (28). Gas-tight 0.25-ml syringes (Hamilton) with Teflon push-button valves were used to withdraw and inject 0.2-ml gas samples (19).

H_2 contents were measured with a Hewlett-Packard gas chromatograph (model series 6890) equipped with a reduction gas detector (Trace Analytical, Menlo Park, Calif.). Headspace samples (3 ml) were injected into a 1-ml gas sampling loop and were separated with a molecular sieve analytical column (MS 5A; 0.79 m by 0.3175 cm; 60/80 mesh; Trace Analytical) at an oven temperature of 40°C. Ultra-high-purity nitrogen (99.999% pure; AGA Gas, Inc., Maumee, Ohio) was used as the carrier gas at a flow rate of 27.5 ml min⁻¹ after it was passed through a catalytic combustion converter (Trace Analytical) to remove traces of H_2 . The detection limit for H_2 under these conditions was 0.01 ppmv (ca. 0.001 Pa). Samples containing 50 to 500 ppmv (ca. 5 to 50 Pa) of H_2 were diluted in H_2 -free dinitrogen immediately before they were injected, or the volume of the gas sampling loop was reduced to 0.25 ml. H_2 concentrations greater than 500 ppmv (ca. 50 Pa) were determined by using a thermal conductivity detector as described previously (28). Analyses of aromatic compounds and VFAs were performed by HPLC (27, 39). Chloride release was measured by a colorimetric assay (1).

RESULTS

H_2 threshold. To test the H_2 threshold concept, we evaluated the minimum H_2 contents obtained in the presence and in the absence of various chlorinated electron acceptors with a variety of pure and mixed cultures. H_2 was repeatedly consumed until the concentration was less than 0.01 ppmv by strain 2CP-C as long as 2-CP was present regardless of whether acetate or H_2 was added at high or low concentrations (Fig. 1). In contrast, cultures that had dechlorinated all of the 2-CP to phenol (all of the chlorinated electron acceptor was reduced) did not consume H_2 at all. Therefore, the fate of the chlorinated electron acceptor was closely monitored in all other experiments to ensure that the consumption of H_2 was never limited by electron acceptor availability. *Desulfotobacterium chlororespirans* and *Desulfotobacterium* sp. strain Viet1, which are known to be chlororespiring microorganisms (27, 29, 39; unpublished data), reduced the H_2 threshold concentrations to 0.27 ± 0.06 ppmv ($n = 4$) and 0.4 ± 0.13 ppmv ($n = 6$), respectively, when they were grown with 3Cl-4-HBA or PCE as

an electron acceptor. *Desulfomonile tiedjei* DCB-1, another chlororespirer (37), reduced the H_2 concentration below the detection limit (0.01 ppmv) when it was grown with 3-CBA as the electron acceptor; this concentration was considerably less than the 0.7 ppmv previously reported for DCB-1 (12). In contrast, the same cultures exhibited significantly higher H_2 threshold concentrations (range, 100 to 400 ppmv) when they were grown with pyruvate fermentation in the absence of a chlorinated compound.

We also monitored the H_2 concentrations in several acetogenic and nonmethanogenic mixed cultures that dechlorinated different chloroethenes or 1,2-D. The absence of methanogenic archaea was confirmed by the lack of methane production from methanogenic substrates, and no archaeal 16S ribosomal DNA could be amplified by PCR from genomic DNA isolated from these cultures (29). Reductive dechlorination in all of the mixed cultures tested was supported by H_2 , and in all of the mixed cultures H_2 was consumed to threshold concentrations below 0.4 ppmv in the presence of individual chlorinated aliphatic compounds (Table 2). Interestingly, the three VC-dechlorinating cultures also consumed H_2 to concentrations of 0.19 to 0.21 ppmv, indicating that VC may be used as a terminal electron acceptor. In the absence of a chlorinated compound as the electron acceptor, the same cultures consumed H_2 to threshold concentrations ranging from 250 to 440 ppmv, indicating that acetogenesis was the TEAP. Formation of acetate from H_2 - CO_2 in all of the mixed cultures was confirmed by HPLC analyses. Only the KS_{1,2-D} enrichment culture reduced the H_2 concentration to about 120 ppmv when it was grown acetogenically without 1,2-D, but this value was still 2 orders of magnitude higher than the threshold value observed for the same culture grown in the presence of the chlorinated electron acceptor.

Determination of f_e values. f_e values were determined experimentally by using several pure and mixed cultures capable of carrying out at least one reductive dechlorination reaction (Fig. 2). The total amounts of electron donor oxidized and electron acceptor reduced in replicate cultures varied less than 25%, and the differences in f_e values determined for identical cultures were less than 5%. The f_e values for the chlororespiring organisms *Desulfotobacterium chlororespirans*, *Desulfotobac-*

TABLE 2. H₂ thresholds exhibited by cultures in the presence or in the absence of chlorinated aliphatic compounds supplied as electron acceptors

Culture	Compound ^a	H ₂ concn (ppmv) ^b	
		In the absence of chlorinated electron acceptor ^c	In the presence of chlorinated electron acceptor ^d
KS	1,2-D	117.3 ± 37.1 (7) ^e	0.30 ± 0.14 (15)
RC	1,2-D	343.2 ± 62.7 (4)	0.25 ± 0.17 (23)
PM	PCE	319.3 ± 112.4 (3)	0.21 ± 0.16 (5)
PM	<i>cis</i> -DCE	438.7 ± 84.7 (3)	0.24 ± 0.07 (6)
PM	VC	379.3 ± 117.3 (3)	0.20 ± 0.05 (7)
AuS	PCE	318.8 ± 85.7 (5)	0.21 ± 0.16 (11)
AuS	<i>cis</i> -DCE	368.3 ± 88.3 (4)	0.34 ± 0.25 (6)
AuS	VC	253.2 ± 60.9 (4)	0.19 ± 0.13 (8)
RC	PCE	334.1 ± 24.5 (7)	0.16 ± 0.07 (10)
RC	<i>cis</i> -DCE	413.3 ± 107 (3)	0.21 ± 0.08 (9)
RC	VC	369 ± 61.7 (3)	0.21 ± 0.11 (13)

^a Compound with which the culture was enriched.^b H₂ concentrations were monitored until stable threshold values were obtained. When no further decrease in the H₂ concentration was observed, a culture was fed H₂ (500 to 10,000 ppmv), and the consumption of H₂ to a constant threshold value was monitored again. Two or three threshold concentrations were obtained per culture.^c Bicarbonate was supplied as the only available electron acceptor, and initially 5 ml (83,000 ppmv) of H₂ was added as the electron donor.^d Cultures were grown in a bicarbonate-buffered medium in the presence of individual chlorinated aliphatic compounds and 5 ml (83,000 ppmv) of H₂ as the electron donor. Headspace analyses to monitor dechlorination were performed weekly, and the chlorinated compounds were respiked before they were completely depleted.^e Mean ± standard deviation. The numbers in parentheses are the numbers of replicate analyses.

terium sp. strain Viet1, *Desulfuromonas* sp. strain BB1, and strain 2CP-C were 0.67, 0.63, 0.66, and 0.64, respectively. An *f_c* value of 0.65 was obtained for the 2,5-DCP-dechlorinating OM enrichment culture. No consumption of acetate was observed in the OM culture, indicating that acetate was not used as an electron donor for 2,5-DCP dechlorination. No loss of H₂ was observed in autoclaved controls, indicating that the consumption that occurred prior to dechlorination was biologically mediated (Fig. 2). *f_c* values of 0.6 to 0.7 were also obtained for enrichment cultures derived from a contaminated aquifer in which PCE was the terminal electron acceptor. These enrichment cultures yielded a pure culture capable of using PCE as the electron acceptor. The mixed methanogenic enrichment culture that cometabolized 3-CP, in contrast, exhibited *f_c* values of less than 0.015. This culture was transferred several times with no increase in the *f_c* value.

DISCUSSION

Obtaining proof of the chlororespiratory physiology of OCRBs has often involved a long and laborious set of experiments. After we examined several pure and mixed dechlorinating cultures, it became evident that H₂ threshold concentrations and *f_c* values are excellent indicators of the presence and activity of chlororespiratory OCRBs.

The H₂ threshold concentrations depend on the thermodynamics of the H₂-consuming step or, more explicitly, the thermodynamics of the terminal electron-accepting reaction (9, 33, 52). Due to this relationship the characteristic steady-state H₂ threshold concentration indicates which electron acceptor is used in the TEAP (Table 1). According to the threshold model, one would predict that chlororespiring OCRBs consume H₂ to levels below those the threshold concentrations

observed for acetogens, methanogens, and sulfidogens. Our results confirm the validity of this hypothesis that reductive dechlorination is the TEAP. For example, due to the thermodynamic restrictions of CO₂ reduction to acetate, acetogenesis cannot explain the consumption of H₂ to the very low concentrations measured in the presence of the chlorinated compounds. Indeed, this is consistent with the observations of Yang and McCarty (50), who found that acetogenesis (and methanogenesis) stopped and dechlorination continued in the presence of *cis*-DCE once the H₂ concentration was less than 11 nM (14 ppmv). The results of Conrad (8) show that TEAPs with highly negative Gibbs free energies (i.e., TEAPs with ferric iron or nitrate used as the electron acceptor) exhibit H₂ threshold values similar to those of chlororespiring cultures even though there are different amounts of free energy ($\Delta G'$) available from the individual terminal electron-accepting reactions. The reasons for this phenomenon are unclear. In the absence of interfering TEAPs, like ferric iron reduction or nitrate reduction, however, it is reasonable to expect that H₂ threshold measurements can reliably indicate that chlororespiratory reductive dechlorination is the dominant TEAP. The possible exceptions are microbial populations, such as *Desulfuromonas* sp. strain TT4B and BB1 populations, which do not use H₂ as an electron donor for reductive dechlorination (24, 30, 31). The relative importance of these types of microorganisms in the environment with regard to the H₂ threshold, however, has yet to be established.

Factors other than the thermodynamics of the terminal electron-accepting reaction can influence the apparent H₂ threshold concentrations. In this study we used basal salts mineral medium, and the sources of H₂ were well-defined. Besides gaseous H₂, acetate was the only possible source of H₂ in the cultures. Oxidation of acetate with concomitant formation of H₂ in the absence of electron acceptors has been observed in acetate-oxidizing methanogenic cocultures (25, 41, 53). Therefore, if organisms with such a capability were present in our cultures, the H₂ threshold concentrations reported here are overestimates of the actual threshold concentrations. Gas leakage through the septum was also of some concern, since the average H₂ mixing ratio in the troposphere is 0.56 ppmv (7), and even higher values are generally obtained in microbiological laboratories in which H₂ is used for medium preparation and analytical applications. Therefore, the low H₂ concentrations (<0.5 ppmv) measured for chlororespiring OCRBs cannot be explained by H₂ leakage out of the serum bottles. In contrast, one can argue that the actual threshold concentrations might be even lower due to leakage of atmospheric H₂ into the culture bottles. H₂ threshold concentrations are also temperature dependent (25, 51, 53). Lee and Zinder (25) demonstrated that there was a 250-fold increase in the H₂ partial pressure in a thermophilic methanogenic coculture over a 60°C temperature range. All H₂ threshold concentrations in our study were measured by using cultures that were incubated at 25°C. Hence, the *in situ* H₂ concentrations might be lower than the values reported here.

Another important consideration is that the H₂ threshold concentrations are approached asymptotically (Fig. 1). The time required until a steady-state threshold concentration is reached depends on the kinetics of the H₂-consuming reactions, and an initial analysis indicated that the H₂ consumption kinetics for different chlororespiring organisms can be quite different (unpublished results). The rates of H₂ consumption do not influence the final H₂ threshold concentrations; however, it is important to verify that the H₂ concentrations measured are the end points of H₂ consumption.

The results of this study suggest that chlororespiring OCRBs

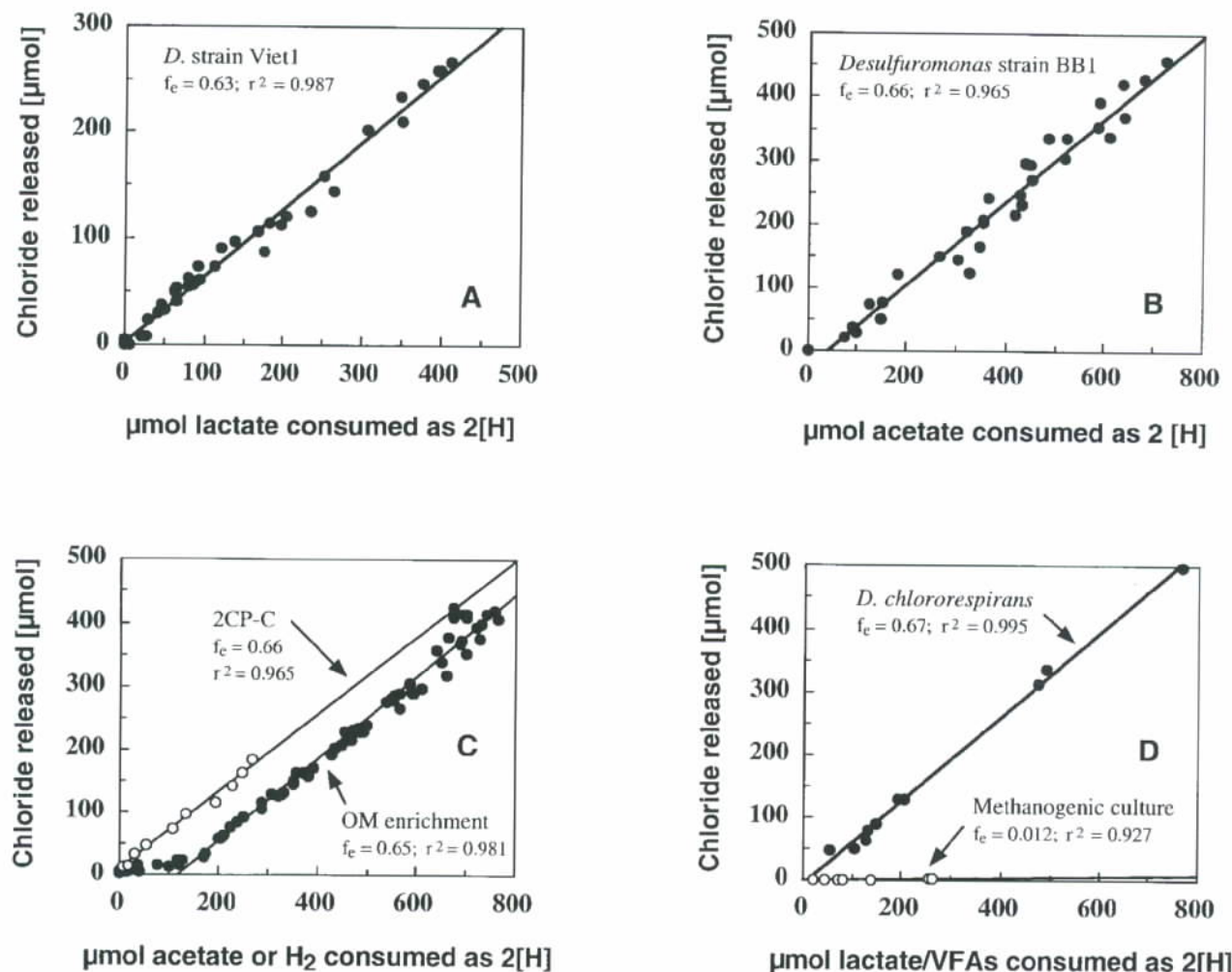


FIG. 2. Graphical determination of f_e values for dechlorinating cultures, in which the amounts of reducing equivalents (2[H]) generated during oxidation of the electron donor were plotted against the amounts of electron acceptor reduced. The f_e is indicated by the slope of the regression line. (A) *Desulfuromonas* sp. strain BB1 (acetate plus PCE). (B) *Desulfitobacterium* sp. strain Viet1 (lactate plus PCE). (C) OM enrichment culture (H₂ plus 2,5-DCP) and strain 2CP-C (acetate plus 2-CP). (D) *Desulfitobacterium chlororespirans* (lactate plus 3Cl-4-HBA) and methanogenic enrichment culture (VFA mixture and 3-CP).

are excellent competitors for H₂ in anaerobic environments and may be the predominant hydrogenotrophic organisms in environments contaminated with chlorinated compounds. Due to the favorable thermodynamics of reductive dechlorination reactions (Table 1), chlororespirers can maintain concentrations below the concentrations that would allow sulfate reduction or methanogenesis. Hence, chlororespirers can outcompete methanogens and sulfidogens for H₂ and should dominate anaerobic environments as long as reductive dechlorination is not limited by the concentration or bioavailability of the chlorinated electron acceptor. As evidence of this we diluted out methanogens and sulfidogens over time from chlororespiring enrichment cultures. Similar observations were made in a continuous stirred-tank reactor fed with benzoate and *cis*-DCE, in which dechlorination accounted for 42% of the electrons from the electron donor when the steady-state H₂ concentration was only 2 nM (2.6 ppmv) and below the threshold concentration observed for methanogenesis (11 nM) (50).

In field studies, Lovley et al. (34) have demonstrated that H₂ threshold concentrations can be used to determine the dominant TEAP in anaerobic subsurface environments. This ap-

proach should also be applicable to establishing the presence and activity of chlororespiring OCRBs in situ if nitrate and ferric iron are not present. However, H₂ threshold data cannot predict the extent of reductive dechlorination. For example, incomplete halo-respiratory dechlorination of PCE to *cis*-DCE by a hydrogenotrophic dechlorinator results in low H₂ threshold concentrations similar to those observed after complete halo-respiratory dechlorination to ETH.

Each reductive dechlorination step in the sequential dechlorination of PCE to ETH is associated with a considerable change in free energy, which ranges from -139 to -173 kJ mol⁻¹ when H₂ is the electron donor. Theoretically, each individual step could support chlororespiration, although to date no anaerobic organisms that grow at the expense of VC have been described and only one organism that uses *cis*-DCE has been described (35). This is unfortunate, since VC accumulation in contaminated groundwater as a result of reductive dechlorination of PCE is a major concern due to the toxic nature of this compound. The H₂ threshold data presented in this paper strongly suggest that VC-respiring organisms are present in river sediments. These findings are encouraging and imply

TABLE 3. Comparison of measured f_e values and f_e values calculated from yield data for different chlororespiring pure cultures

Culture	Electron acceptor	Measured f_s^a	Calculated f_e^b	Measured f_e^c	Reference
<i>Dehalobacter restrictus</i> PER-K23	PCE	0.27	0.73	ND ^d	22
<i>Dehalobacter</i> sp. strain TEA	PCE	0.04	0.96	ND	48
<i>Dehalospirillum multivorans</i>	PCE	0.20	0.80	ND	42
<i>Desulfuromonas ethenica</i> TT4B	PCE	0.03	0.97	ND	24
<i>Desulfuromonas</i> sp. strain BB1	PCE	0.07	0.93	0.67	30
<i>Dehalococcoides ethenogenes</i> 195	PCE	0.46	0.54	ND	35
<i>Desulfotobacterium</i> sp. strain PCE1	PCE	0.22	0.78	ND	18
<i>Desulfotobacterium chlororespirans</i> Co23	3Cl-4-HBA	0.29	0.71	0.67	39
<i>Desulfomonile tiedjei</i> DCB-1	3-CBA	0.24	0.76	ND	12
Strain 2CP-C	2-CP	0.32	0.68	0.66	5

^a f_s values were calculated from reported yield data for each culture grown under chlororespiring conditions. The molecular formula for cells used was $C_5H_7O_2N$.

^b f_e values were calculated from the relationship $f_e = 1 - f_s$.

^c f_e values were measured as described in Materials and Methods.

^d ND, not determined.

that chloroethene-contaminated anaerobic sites could be completely detoxified through stimulation of chlororespiring OCRBs.

Chlororespiring cultures consistently have f_e values between 0.63 and 0.71, which are considerably lower than the f_e values obtained for sulfidogenic cultures (0.84 to 0.94) (47) or methanogenic cultures (0.95) (10). Chlororespiring cultures, however, have higher f_e values than aerobic cultures (0.57) (21) or denitrifying cultures (0.54) (4) that utilize the same electron donor, acetate. Energetically, this is sensible since the change in Gibbs free energy (ΔG^0) for chlororespiration is between the values for sulfate reduction and denitrification (Table 1). The ΔG^0 for chlororespiration is in the same range as the energy available from dissimilatory nitrate reduction to ammonia. In fact, the f_e values that have been measured for ammonifiers are around 0.6 (4) and are slightly lower than the f_e values determined in this study for chlororespiring OCRBs. This verifies that there is a thermodynamic basis to f_e , with more energetically favorable electron-accepting reactions having lower f_e values, as described by McCarty (36). The expectation is that the f_e increases according to the following sequence of electron acceptors: $O_2 < NO_3^- < \text{chlorinated compounds} < SO_4^{2-} < CO_2$.

The f_e values determined for chlororespiring cultures indicate that the reductive dechlorination reactions are indeed respiratory TEAPs, since other possible mechanisms coupling this process to growth would not exhibit similar f_e values. For example, it is possible that some microorganisms fortuitously catalyze reductive dechlorination reactions (cometabolism) and channel a small fraction of the available electrons from the electron donor toward reductive dechlorination without gaining energy from this process. Cometabolic dechlorination, however, is usually characterized by a low f_e value in methanogenic and acetogenic systems. This low f_e value associated with fortuitous cometabolic reactions occurs because no electron transport or proton translocation forming a proton motive force to drive membrane-associated ATP formation occurs. Since only respiratory processes have such mechanisms for ATP generation, less energy would be available for growth in an organism lacking this ability. Therefore, the energy that could be derived from reductive dechlorination would be lost (as heat), and the microbial growth yields (f_s) would be smaller. Table 3 shows f_s values calculated from yield data obtained for various pure cultures by utilizing chlorinated compounds as sole terminal electron acceptors. The f_e was calculated ($1 - f_s$) for each culture, and it is evident that most of the values are in agreement with the measured f_e values for chlo-

rorespiratory OCRBs reported in this study. For example, there is good agreement between the measured f_e values for strains 2CP-C (0.66) and Co23 (0.67) and the f_e values calculated from yield data (0.68 and 0.71, respectively). The exceptions to this agreement are the f_e values calculated from the yield data obtained for *Desulfuromonas* sp. strain BB1, *Desulfuromonas ethenica* TT4B, and *Dehalobacter* sp. strain TEA (Table 3), which are considerably higher than would be predicted. It has been shown, however, that *Desulfuromonas* sp. strain BB1, a close relative of *Desulfuromonas ethenica* TT4B, has a measured f_e of 0.66 (Fig. 2). There is also some inconsistency between the closely related organisms *Dehalobacter* sp. strain TEA ($f_e = 0.96$) and *Dehalobacter restrictus* PER-K23 ($f_e = 0.73$). This disagreement in the data has some reasonable explanations. Measurement of H_2 can be difficult, or other potential electron donors (e.g., yeast extract) were not included in the calculation; therefore, a complete accounting of the mass balance was potentially affected. Another concern is that some of the yield data were determined after growth had stopped and the stationary phase was reached. Preliminary studies with strain BB1 have shown that electron donor oxidation and reductive dechlorination continue in the stationary phase (30). This creates an uncoupling of growth from chlororespiration and leads to underestimation of the maximum growth yield (and therefore of f_s) and overestimation of f_e , depending on when the growth yields were determined. Hence, $f_{s(\max)}$ should be determined during the exponential growth phase. Generally, chlororespiring OCRBs are grown with the chlorinated electron acceptor and the electron donor present in excess. Under substrate-sufficient conditions consumption of an energy source and complete absence of growth have been observed, indicating that the energy generated in catabolic reactions (reductive dechlorination of PCE) was uncoupled from anabolism (26). As a result, f_e values calculated from yield data (f_s) are overestimates. Hence, it is recommended that f_e be measured as described in this study.

In conclusion, our findings suggest that f_e values and H_2 threshold concentrations may be used to help predict whether chlororespiration is the predominant TEAP in pure and mixed cultures of OCRBs. When combined, the two values can distinguish anaerobic cometabolic dechlorination processes from chlororespiration. Since electron transport mechanisms of OCRBs have not been well-elucidated, further research should focus on identifying components of the respiratory chain in order to conclusively link f_e values and H_2 threshold concentrations to halo-respiratory processes.

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