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Growth of *Dehalococcoides mccartyi* species in an autotrophic consortium producing limited acetate

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Abstract

The dechlorinating *Dehalococcoides mccartyi* species requires acetate as carbon source, but little is known on its growth under acetate limiting conditions. In this study, we observed growth and dechlorination of a *D. mccartyi*-containing mixed consortium in a fixed-carbon-free medium with trichloroethene in the aqueous phase and H₂/CO₂ in the headspace. Around 4 mM formate was produced by day 40, while acetate was constantly below 0.05 mM. Microbial community analysis of the consortium revealed dominance by *D. mccartyi* and *Desulfovibrio* sp. (57 and 22% 16S rRNA gene copies, respectively). From this consortium, *Desulfovibrio* sp. strain F1 was isolated and found to produce formate and acetate (1.2 mM and 48 μM, respectively, by day 24) when cultivated alone in the above mentioned medium without trichloroethene. An established co-culture of strain F1 and *D. mccartyi* strain 195 demonstrated that strain 195 could grow and dechlorinate using acetate produced by strain F1; and that acetate was constantly below 25 μM in the co-culture. To verify that such low level of acetate is utilizable by *D. mccartyi*, we cultivated strain 195 alone under acetate-limiting conditions and found that strain 195 consumed acetate to below detection (5 μM). Based on the acetate consumption and cell yield of *D. mccartyi*, we estimated that on average 1.2×10^8 acetate molecules are needed to supply carbon for one *D. mccartyi* cell. Our study suggests that *Desulfovibrio* may supply a steady but low amount of fixed carbon to dechlorinating bacteria, exhibiting important implications for natural bio-attenuation when fixed carbon is limited.

Keywords: *Dehalococcoides*, Organohalide respiration, Chlorinated ethenes, Bioremediation, Autotrophic growth, Acetate, *Desulfovibrio*

Introduction

Chlorinated ethenes including tetrachloroethene (PCE), trichloroethene (TCE), and their conversion products dichloroethene (DCE) and vinyl chloride (VC) are among the most common groundwater contaminants (Abelson 1990). Substantial laboratory and field studies have been performed on biological reductive dechlorination of chloroethenes and on identifying/characterizing organohalide respiring bacteria. So far, a variety

of anaerobic genera, including *Desulfuromonas*, *Dehalobacter*, *Desulfitobacterium*, *Enterobacter*, and *Sulfurospirillum*, are known to be capable of dechlorinating PCE to DCEs in the presence of electron donors/fixed carbon sources such as lactate, pyruvate, formate, and acetate (Finneran et al. 2002; Holliger et al. 1998; Krumholz et al. 1996; Scholz-Muramatsu et al. 1995; Sharma and McCarty 1996). Reductive dechlorination of chloroethenes has rarely been investigated under autotrophic conditions. It has been reported that *Desulfuromonas* sp. strain BB1 can dechlorinate PCE to *cis*-DCE via consumption of acetate generated from H_2/CO_2 by an acetogen—*Sporomusa ovata* (He et al. 2002).

Further dechlorination of DCEs to ethene has only been described for one genus of bacteria—*Dehalococcoides*, whose genomic sequences have revealed obligate requirements for acetate as carbon source, H_2 as electron donor, and halogenated compounds as electron acceptors (Löffler et al. 2013). The type strain, *Dehalococcoides mccartyi* strain 195, was reported to grow in completely defined mineral salts medium amended with acetate, H_2/CO_2 and TCE (He et al. 2007), and was incapable of dechlorinating TCE in the absence of either acetate or H_2 . To date, the reductive dechlorination of PCE and/or TCE past *cis*-DCE to VC and ethene has not been investigated in an autotrophic consortium via syntrophy. However, in practice, in situ bioremediation is commonly performed by microbial consortia containing dechlorinators and other beneficial microorganisms, and natural bio-attenuation would require autotrophic activity because many chloroethene-contaminated subsurface environments are naturally oligotrophic (Schneidewind et al. 2014; Ramsburg et al. 2004).

When *D. mccartyi* is grown in enriched cultures or established consortia, it commonly utilizes products (e.g., acetate and H_2) generated by fermenting organisms such as *Desulfovibrio*, *Acetobacterium*, and *Clostridium* (He et al. 2007; Ritalahti and Löffler 2004; Richardson et al. 2002; Aulenta et al. 2005; Men et al. 2012). In particular, sulfate-reducing bacteria *Desulfovibrio* are typically found to co-exist with *D. mccartyi* and are metabolically versatile. For example, *Desulfovibrio desulfuricans* can utilize both lactate and pyruvate for energy generation via fermentative pathways in the absence of exogenous terminal electron acceptors, producing acetate, CO_2 , and H_2 , while *Desulfovibrio vulgaris* strain Hildenborough can transform lactate to acetate and H_2 when grown syntrophically with *D. mccartyi* or a H_2 -utilizing methanogen (Men et al. 2012; McInerney et al. 1981; Schink 1997).

There are also reports of autotrophic *Desulfovibrio* growth, with at least three confirmed strains. *Desulfovibrio* sp. strain HRM1 (Brysch et al. 1987) and *Desulfovibrio indonesiensis* strain P23 (Fichtel et al. 2012) grow with H_2/CO_2 in the presence of sulfate. However, the volatile fatty acid (VFA) products resulting from their autotrophic growth were not identified. The third strain *Desulfovibrio* sp. strain G11 was claimed to be an autotroph, but no supporting experimental data were presented (McInerney et al. 1981; Dolfig et al. 2008). Mixotrophic growth with H_2/CO_2 and acetate is more

common with *Desulfovibrio* bacteria, e.g., *D. desulfuricans* (Londry and Marais 2003; Mechalias and Rittenberg 1960) and *D. vulgaris* (Peters et al. 1999). Notably, formate was formed as an intermediate when *D. vulgaris* was grown chemolithoheterotrophically with H_2/CO_2 and acetate under sulfate-limiting conditions (Peters et al. 1999). To date, autotrophic sulfate reducers fall into two categories by the mechanisms they utilize to synthesize acetyl-CoA from CO_2 —the reductive citric acid cycle and the reductive CO-dehydrogenase pathway (acetyl-CoA pathway) (Rabus et al. 2006). However, no information is available on autotrophic growth and its VFA products by *Desulfovibrio* autotrophs. The carbon fixing mechanisms that they utilize also remain elusive.

In this study, we characterized an autotrophic dechlorinating consortium (culture EC195) from which *D. mccartyi* strain 195 was isolated. We aimed to determine the actual carbon source for growth of *D. mccartyi* in culture EC195. Acetate utilization patterns by *D. mccartyi* including acetate utilization threshold and cell yield per mole acetate were also investigated.

Materials and methods

Chemicals

Analytical grade chloroethenes including TCE, DCEs, and VC were purchased from Sigma-Aldrich-Fluka (St. Louis, MO) or Supelco (Bellefonte, PA). Ethene was obtained from Alltech Associates, Inc. (Deerfield, IL). Other chemicals used in this study were obtained from Sigma-Aldrich-Fluka (St. Louis, MO). Gases (air, nitrogen, H_2 , and CO_2) were supplied by Praxair, Inc. (Berkeley, CA) and Soxal (Singapore).

Culture growth conditions and cultivation history of culture EC195

Concentrations of volatile compounds such as TCE in this study are presented as nominal concentrations, i.e., total molar amounts in aqueous and gaseous phases divided by the aqueous phase volume. All cultures were prepared in 160 mL serum bottles with 100 mL bicarbonate-buffered medium (containing 30 mM sodium bicarbonate) and 60 mL headspace. All cultures received H_2 (20 mL, or 0.33 atm) in the headspace as electron donor on top of the original N_2/CO_2 headspace (final headspace pressure 1.33 atm), unless specified otherwise. All experimental bottles in this study were incubated in the dark at 30 °C without shaking.

Culture EC195 is a subculture of the parent culture from which *D. mccartyi* strain 195 was isolated (Maymó-Gatell et al. 1997). It was initially cultivated in an anaerobic medium containing activated sludge extract of undefined composition (Maymó-Gatell et al. 1997), before being transferred (2% [v/v]) to defined mineral salts medium spiked with TCE (~ 0.5 mM) as electron acceptor and acetate as carbon source as described previously (He et al. 2003). Acetate was then omitted from the medium recipe for the next 15 transfers while other growth conditions remained the same. The still-actively-dechlorinating culture EC195 was then transferred for another five

times in a modified medium with 50 times higher amount of vitamin B₁₂ (final concentration 50 µg L⁻¹) (He et al. 2007) and then was inoculated into triplicate bottles (3% [v/v]) for dechlorination kinetics studies. The recipe of the final basal medium without TCE or additional fixed carbon amendments was referred to as DCB-1 medium. Due to oxidation of added reductants—L-cysteine, dithiothreitol (DTT), and Na₂S, the DCB-1 medium may contain a trace amount of sulfate (< 0.05 mM detection limit, see below) or other sulfur-containing compounds before inoculation (Chen and Morris 1972).

Pure culture of *D. mccartyi* strain 195 was kindly provided by Dr. Stephen H. Zinder of Cornell University (Ithaca, NY) and was used for all experiments that involved strain 195 in this study. *Desulfovibrio* sp. strain F1 was isolated from culture EC195 and was grown in DCB-1 medium amended with sulfate (2 mM) and H₂ (20 mL, or 0.33 atm) in the headspace, resulting in a H₂/sulfate molar ratio of 4.9, unless specified otherwise.

To establish an autotrophic co-culture, active inocula of *Desulfovibrio* sp. strain F1 (1% [v/v]) and *D. mccartyi* strain 195 (2% [v/v]) were transferred to DCB-1 medium amended with ~ 0.5 mM TCE. Before inoculation, both inocula were rinsed three times under anaerobic conditions with sterilized DCB-1 medium by centrifugation at 10,000×*g* for 15 min and removing the supernatant, in order to minimize the organic compounds carried over into the cultures.

Analytical techniques

Chloroethenes and ethene were measured with an Agilent gas chromatograph (GC7890) equipped with a flame ionization detector (GC-FID). Headspace samples (100 µL) that were withdrawn by using a gas-tight glass syringe (model 1725; Hamilton Co., Reno, NV) were separated on a GS-GasPro column (30 m by 0.32 mm; J&W Scientific) as previously described (Ding et al. 2013). Volatile fatty acids (VFAs) including formate and acetate were determined on an Agilent 1260 HPLC system (Agilent, CA, USA) with a UV detector (210 nm). Separation of VFAs was conducted on a Rezex ROA-Organic Acid H⁺ (8%) HPLC column (Phenomenex) at room temperature, with 2.5 mM sulfuric acid as a mobile phase at 0.5 mL min⁻¹ flow rate (10 or 100 µL injection volume). Fluorescence microscopy was performed on a Nikon Eclipse 200 using Syto-9 staining dye (Invitrogen). Scanning electron microscopy was performed as described previously (Ding et al. 2014). In short, cells were harvested by filtration through a 0.2 µm pore size polycarbonate membrane and fixed with 2.5% glutaraldehyde before viewing on a Philips XL 30 scanning electron microscope (FEI, Hillsboro, OR, USA). Sulfate in the culture medium was precipitated first with barium chloride and the resulting suspension was well mixed and measured for absorbance at 420 nm on a Tecan Infinite® 200 PRO microplate reader (Switzerland) (detection limit 0.05 mM). H₂ in the headspace was detected using an Agilent GC7890 equipped with a thermal conductivity detector as described previously (detection limit 8 ppmv) (Bramono et al. 2011).

Molecular analyses

Genomic DNA was extracted from actively growing cultures by using an Ultra Clean Microbial DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's recommendations. Quantitative real-time polymerase chain reaction (qPCR) on *D. mccartyi* and *Desulfovibrio* was performed on an ABI 7500 Fast real-time PCR System (ABI, Foster City, CA) by using QuantiTect SYBR green PCR master mix (QIAGEN, Germany) and primers Dhc-forward/reverse (5'-GGT AAT ACG TAG GGA AGC AAG CG-3' and 5'-CCG GTT AAG CCG GGA AAT T-3') (for *D. mccartyi*) (Holmes et al. 2006) and Dsv691F/826R (5'-CCG TAG ATA TCT GGA GGA ACA TCA G-3' and 5'-ACA TCT AGC ATC CAT CGT TTA CAG C-3') (for *Desulfovibrio*) (Fite et al. 2004). Quantification of total cells in the enrichment cultures by qPCR was performed by targeting the genomic DNA with universal primers 338F and 518R (5'-ACT CCT ACG GGA GGC AGC AG-3' and 5'-ATT ACC GCG GCT GCT GG-3') (Muyzer et al. 1993; Lane 1991).

Construction of 16S rRNA gene clone library, restriction enzyme digestion by *HhaI* and *MspI* (NEB, Ipswich, MA, USA), restriction fragment length polymorphism, and sequencing of representative plasmids were done as described previously (Cheng et al. 2010). The 16S rRNA gene sequences of *Desulfovibrio* sp. strain F1 were deposited in GenBank under the accession numbers KC120814 and KC120815.

PCR amplification and DGGE was performed by using primers 341FGC and 518R (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3' and 5'-ATT ACC GCG GCT GCT GG-3') (Muyzer et al. 1993) as described previously (Cheng et al. 2010).

Results

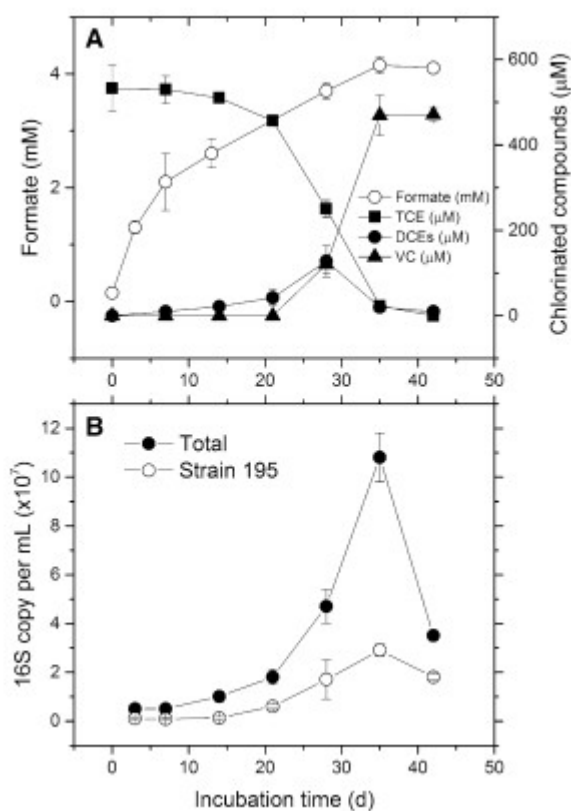
Chloroethene dechlorination by an autotrophic culture EC195

Culture EC195 exhibited autotrophic growth and stable TCE dechlorination activities after being transferred into the fixed-carbon-free DCB-1 medium amended with TCE. A clone library analysis indicated that *D. mccartyi* species (57%) and *Desulfovibrio* species (22%) are the two dominant populations in culture EC195 (Table 1). *Spirochaeta* also occurred in culture EC195, which has commonly been found in other dechlorinating enrichment cultures (Men et al. 2017; Kaufhold et al. 2013).

Table 1 Summary of a 16 rRNA gene clone library with 92 clones generated with genomic DNA of culture EC195

Bacteria category	Number of clones	Percentage (%)
<i>Dehalococcoides mccartyi</i>	52	56.5
<i>Desulfovibrio</i> sp.	20	21.7
Uncultured bacterium from sludge, or <i>Bacteroides</i> SA-11	6	6.5
<i>Spirochaeta</i>	4	4.3
Anaerobic syntrophic <i>Tepidanaerobacter syntrophicus</i> strain JL (accession number: NR_040966.1)	4	4.3
<i>Clostridium</i> sp. 9B4	4	4.3
(No 16S rRNA gene insert)	2	2.2

Dechlorination of TCE by culture EC195 started with a 15-day lag phase, after which ~ 0.5 mM TCE was dechlorinated to VC and ethene via *cis*-DCE as the intermediate within 35 days (Fig. 1). Formate production was observed in the EC195 culture, reaching ~ 4 mM after 40 days (Fig. 1a). Accompanying growth and dechlorination was the consumption of H_2 which was indicated by decreasing headspace concentrations. The cell densities of *D. mccartyi* and total bacteria in culture EC195 increased ~ 27 -fold and ~ 21 -fold to a maximum of 2.9×10^7 and 1.08×10^8 cells mL^{-1} , respectively (Fig. 1b).

**Fig. 1**

Dechlorination of TCE by culture EC195 in DCB-1 medium without fixed carbon amendments. a Dechlorination of TCE and production of formate by culture EC195. bGrowth profile of total cells versus strain 195 in culture EC195 during dechlorination of TCE to VC and ethene. Ethene (< 0.02 mM) was not shown. Error bars (\pm SD) were shown if not smaller than the symbols. All data points were averaged from triplicate bottles

It was interesting that acetate was not detected throughout the test (detection limit 0.05 mM), although it was reported to be indispensable for *D. mccartyi* species (Löffler et al. 2013). The possibility of formate acting as the carbon source for *D. mccartyi* seemed low, as *D. mccartyi* strain 195 did not grow or dechlorinate when 4 mM formate was added to the DCB-1 medium (data not shown), which was also consistent with the previous report (Maymó-Gatell et al. 1997). Moreover, when strain 195 was cultivated alone in DCB-1 medium with TCE but without fixed carbon, no growth or dechlorination activity was observed and no formate or other VFAs were detected, even after 3-months incubation (data not shown).

Isolation of an autotrophic *Desulfovibrio* sp. strain F1 from culture EC195

Based on the above observations, we hypothesized that the required fixed carbon for *D. mccartyi* and the formate produced were generated autotrophically by one of the dominant species—*Desulfovibrio*, although some low abundance organisms might also produce fixed carbon.

To enrich *Desulfovibrio* which was presumably a sulfate reducer, culture EC195 was transferred in DCB-1 medium amended with 10 mM acetate and 2 mM sulfate, but without TCE. This culture was later subjected to serial dilution. Three repeated serial dilutions until 10^{-8} resulted in a culture with only vibrio-shape cells under fluorescence microscope. After another three rounds of colony picking from agar shakes and immediate serial dilution, an isolate of *Desulfovibrio* was obtained, which was designated strain F1. Cells of strain F1 have a dimension of 1–1.5 μm length and 0.3–0.5 μm width (Fig. S1). Cells are motile with monotrichous flagella (Fig. S1B). A 16S rRNA gene clone library of 80 clones showed a single digestion pattern using either *MspI* or *HhaI* as the restriction enzymes. DGGE analysis on 16S rRNA genes exhibited two bands for strain F1, both of which were affiliated with *Desulfovibrio* after excision and sequencing (Lane 3, Fig. S2). Analysis of 22 clones from the above-mentioned clone library on DGGE gel showed that 15 of them belong to type 1 (top, thicker band, accession number KC120814) and the remaining 7 belong to type 2 (bottom, weaker band, accession number KC120815) (DGGE picture not shown). The closest reported relative of strain F1 is *Desulfovibrio desulfuricans* strain Ser-2 (identical base pairs: type 1—1397/1400, type 2—1399/1400).

Strain F1 was then transferred to DCB-1 medium with 2 mM sulfate but without acetate. Within 15 days of incubation, black precipitates appeared which are typical of metal sulfides, and the headspace pressure of the bottles dropped quickly, indicating the consumption of H_2 . After more than 5 transfers (3% [v/v] inocula) in the same medium, the cultures still showed the same activity and the autotrophic culture has been maintained for more

than 3 years. The possibility that reductant L-cysteine and DTT may have served as the fixed carbon source for *Desulfovibrio* was excluded by observing similar cell growth and formate formation in the L-cysteine- and DTT-free medium where sodium sulfide (Na₂S) served as the only reductant.

Cell growth of strain F1 requires H₂ and sulfate, and was positively correlated to initial sulfate concentrations from 0 to 2 mM (Table 2), a typical characteristic of hydrogenotrophic sulfate reducer ($4 \text{ H}_2 + \text{SO}_4^{2-} + \text{H}^+ \rightarrow \text{HS}^- + 4 \text{ H}_2\text{O}$) (Muyzer and Stams 2008). In set “S0” where no sulfate was added, there was still moderate cell growth (0.17×10^8 16S rRNA gene copy mL⁻¹) which corresponded to ~ 0.03–0.05 mM sulfate if we assume a linear correlation between sulfate concentrations and cell growth. This agreed with previous estimation that sulfate concentration was < 0.05 mM. The highest level of sulfate tested (6 mM for “S4” set in Table 2) caused the pH to reach ~ 8.0 by day 16 and inhibited cell growth, probably through production of sulfide. For experimental sets with initial sulfate of 0.2–2 mM, sulfate concentrations quickly dropped to below the detection limit (0.05 mM) within the first 8 days, while bottles with 6 mM sulfate still contained ~ 2 mM on day 8.

Table 2 Effects of H₂, sulfate, and acetate on cell growth and formate production on day 24 by *Desulfovibrio* sp. strain F1

Set	Starting condition			Measured value on Day 24	
	H ₂ (mL)	Sulfate (mM)	Acetate (mM)	16S rRNA gene copy ($\times 10^8$ mL ⁻¹)	Formate (mM)
H0	0	2	0	0.72 (± 0.02)	0.15 (± 0.02)
H1	10	2	0	5.2 (± 0.6)	2.88 (± 0.12)
H2	30	2	0	4.9 (± 0.3)	8.95 (± 0.14)
H3	50	2	0	4.5 (± 1.2)	8.79 (± 0.08)
S0	30	0	0	0.17 (± 0.01)	0.78 (± 0.05)
S1	30	0.2	0	1.1 (± 0.3)	4.30 (± 0.13)
S2	30	0.6	0	1.9 (± 0.1)	9.83 (± 0.30)
S3	30	2	0	5.5 (± 0.1)	9.01 (± 0.28)
S4	30	6	0	5.8 (± 1.3)	3.46 (± 0.26)
AS–	30	0	1	0.21 (± 0.03)	0.95 (± 0.04)
AS+	30	2	1	6.3 (± 0.1)	9.63 (± 0.26)

Culture volume and headspace volume were 100 and 60 mL in all bottles. Measured values are averaged from triplicates with standard deviation indicated in brackets

For sets having 0 mM sulfate added (S0, AS–), there might be < 0.05 mM sulfate present in the beginning of the cultivation due to oxidation of sulfur-containing reductants in DCB-1 medium

Formate production by strain F1 was also dependent on H₂ and sulfate (Table 2). The highest formate concentration (9.83 ± 0.30 mM) was observed on day 24 in bottles supplied with 30 mL (0.5 atm) H₂ and 0.6 mM sulfate. Acetate, which was thought to stimulate cell growth as described by Brysch et al. (1987), did not have any significant impact on cell growth or formate production, suggesting that acetate possibly does not serve as carbon source or electron donor for strain F1 under the tested conditions (set “AS–” and

“AS+” in Table 2). Mass balances of the “H1” bottles [10 mL (0.16 atm) H₂ and 2 mM sulfate] showed that on day 16, 2.8 ± 0.2 mL H₂ was left of the initial 10 mL, while 1.66 ± 0.4 mM formate was formed and sulfate was reduced from 2–< 0.05 mM. The consumed 7.2 mL H₂ (equivalent aqueous concentration 9.8 mM) is close to the theoretical H₂ consumption of 9.7 mM (assuming that formate production requires one molecule of H₂ and sulfate reduction requires four).

Desulfovibrio sp. strain F1 produces trace amounts of acetate

Although strain F1 has been confirmed to grow autotrophically, its relationship with *D. mccartyi* in culture EC195 remained unclear, because acetate was not detected in the culture liquid of strain F1 with the initial acetate detection protocol using HPLC-UV (detection limit: 0.05 mM). Acetate was again measured after decreasing the detection limit to 5 μ M by increasing the HPLC injection volume from 10 to 100 μ L. Strain F1 was then grown in DCB-1 medium without sulfate amendment and with 30 mL (0.5 atm) H₂ (identical condition as “S0” set in Table 2). Using the revised acetate detection method, we did observe production of acetate which reached 48 ± 3 μ M by day 42, together with a formate concentration of 1.2 ± 0.1 mM (Fig. 2).

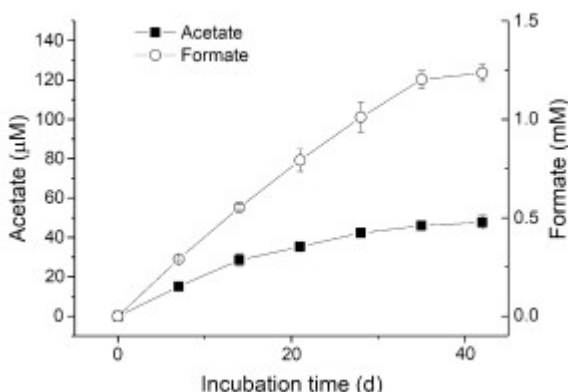


Fig. 2

Production of trace amounts of acetate by *Desulfovibrio* sp. strain F1 in DCB-1 medium without fixed carbon amendments. Cultures were grown in 30 mL medium with 40 mL headspace, and were inoculated from parent cultures that had been maintained in DCB-1 medium for at least five transfers. H₂ (30 mL) was added as electron donor. All data points were averaged from triplicate bottles

Chloroethene dechlorination by an autotrophic co-culture of strain F1 and strain 195

A co-culture of strain 195 (2% [v/v]) and strain F1 (1% [v/v]) was established to verify whether strain F1 supplies the required carbon source (acetate) for the growth and dechlorination of strain 195 in the DCB-1 medium without fixed carbon amendment. This co-culture was transferred in DCB-1 medium amended with H₂ and TCE at least five times, and constantly showed TCE dechlorination and VFA production activities.

In DCB-1 medium amended with TCE, the co-culture dechlorinated ~ 0.62 mM TCE to VC and ethene within 20 days (Fig. 3a), exhibiting a similar but faster dechlorination profile compared with culture EC195. On day 20, strain 195 and strain F1 reached cell densities of 4.5×10^8 and 2.3×10^7 16S rRNA gene copies mL⁻¹, respectively, representing 126-fold and 39-fold increases compared to the cell densities upon inoculation (Fig. 3b). Cell densities of strain F1 reached its maximum on day 3 and remained stagnant through the remainder of the experiment. Accompanying the TCE dechlorination, formate accumulated to 1.2 mM in the DCB-1 medium while acetate peaked at day 3 (24 μ M) and then slowly dropped to below 5 μ M (Fig. 3c). Therefore, in this autotrophic co-culture, *Desulfovibrio* sp. strain F1 fixes the inorganic carbon to support the growth and dechlorination of strain 195 in the form of acetate.

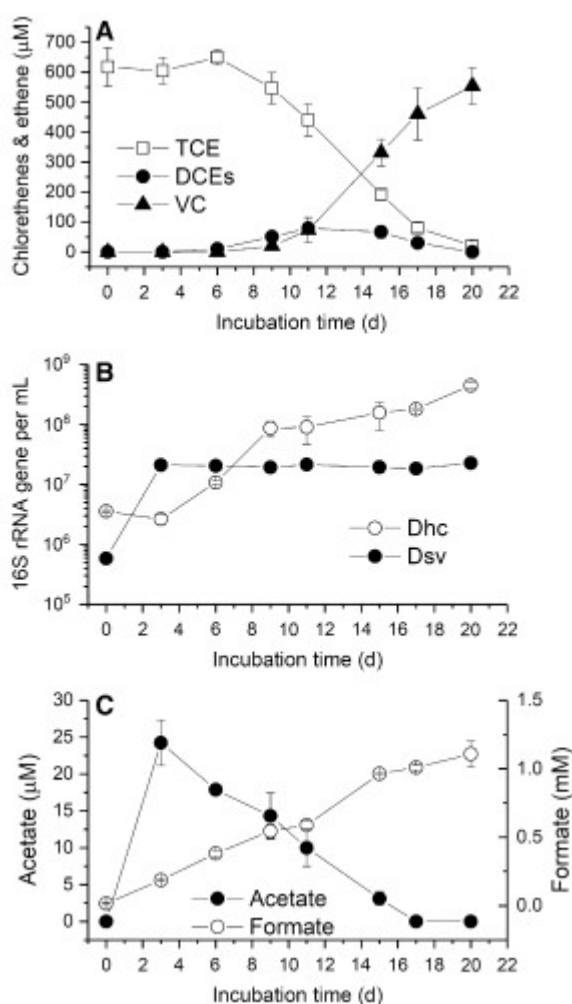


Fig. 3

Dechlorination of TCE by an established autotrophic co-culture in DCB-1 medium without fixed carbon amendments. a Dechlorination of TCE by the co-culture. b Growth profile of *D. mccartyi* and *Desulfovibrio* cells during dechlorination of TCE (Dhc: *D. mccartyi* strain 195, Dsv: *Desulfovibrio* sp. strain F1). c Production of acetate and formate during TCE dechlorination by the co-culture. Ethene (< 0.02 mM) was not shown. Error bars (\pm SD) were shown if not smaller than the symbols. All data points were averaged from triplicate bottles

Acetate utilization threshold by *D. mccartyi* strain 195

To verify that the low level acetate ($< 50 \mu\text{M}$) is indeed utilizable for *D. mccartyi*, we grew *D. mccartyi* strain 195 in various acetate concentrations and monitored acetate consumption during dechlorination of $\sim 0.63 \text{ mM}$ TCE (Fig. 4). When initial acetate concentrations were $157 \pm 9 \mu\text{M}$ or higher (Fig. 4a, b), strain 195 consumed $93 \pm 7 \mu\text{M}$ acetate, producing VC and ethene (0.148 mol of acetate uptake per mole of TCE dechlorinated), with final cell densities of $4.8 \times 10^8 \text{ mL}^{-1}$ (Fig. 4f). For initial acetate concentrations of $72 \pm 2 \mu\text{M}$ or lower (Fig. 4c, d), the growth of strain 195 became limited and nearly all the acetate was consumed to a level below $5 \mu\text{M}$. The extent of TCE dechlorination was also lower than those with higher acetate concentrations. No dechlorination activity was observed if no acetate was provided (Fig. 4e). In control bottles with $303 \pm 7 \mu\text{M}$ acetate but without inocula, no change in acetate concentration was observed throughout the experimental period (data not shown). Initial acetate concentrations higher than $157 \mu\text{M}$ (up to 10 mM tested) had no enhanced effect on TCE dechlorination rates/extents (data not shown). Under acetate limiting conditions (72 and $45 \mu\text{M}$), the final cell densities achieved were significantly lower than those when acetate was abundant (157 and $292 \mu\text{M}$), and were positively correlated with the amount of acetate consumed (Fig. 4f). On day 8 when all acetate was consumed to below $5 \mu\text{M}$, there was still TCE left that was further converted to VC under acetate limiting conditions (Fig. 4c, d), showing that dechlorination continued after cell growth ceased.

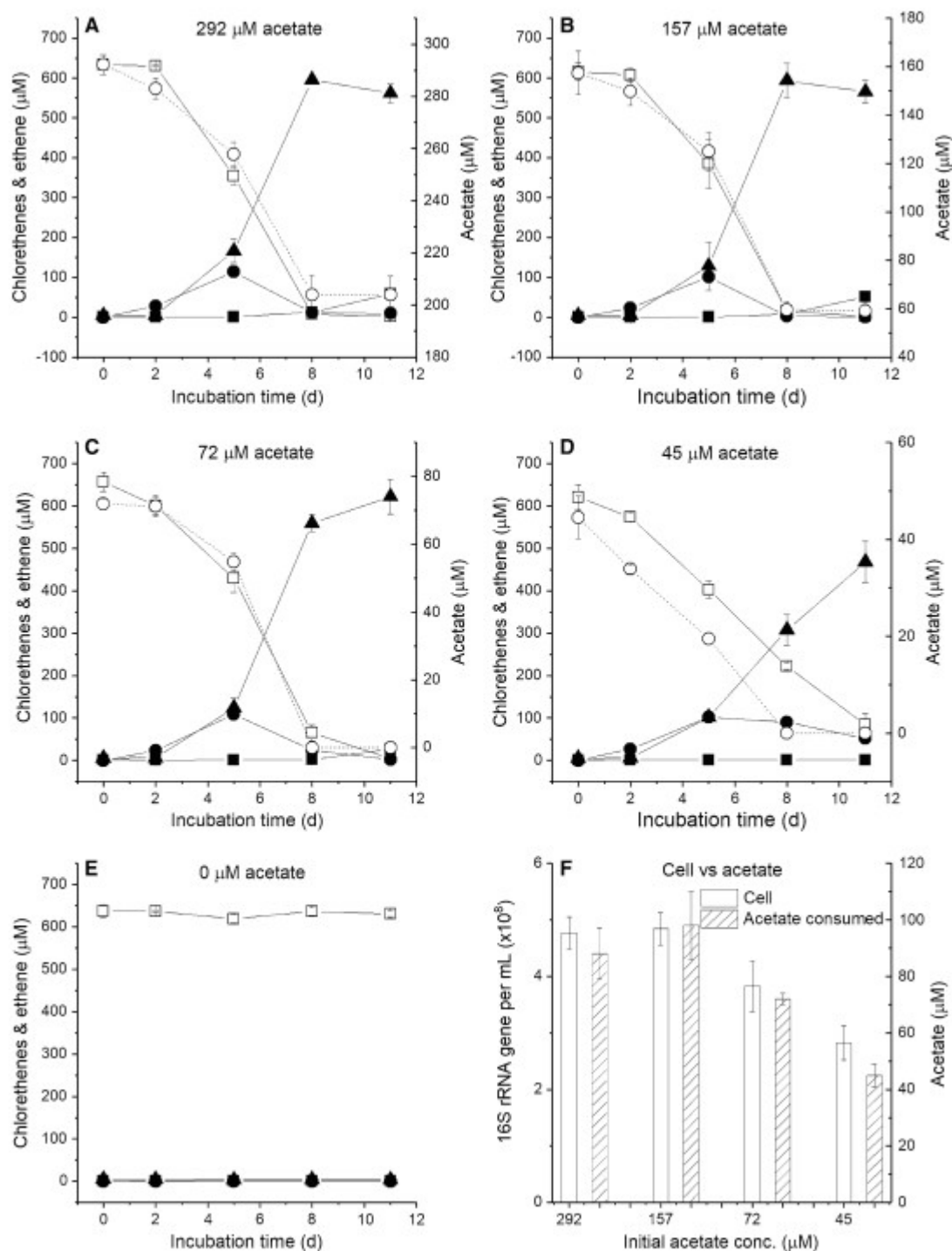


Fig. 4

Comparison of *D. mccartyi* strain 195 grown with various concentrations of acetate. a-e Dechlorination kinetics and acetate utilization profiles of *D. mccartyi* strain 195. Initial acetate concentrations are 292 μM , 157 μM , 72 μM , 45 μM , and 0 μM , respectively for (a-e). Acetate concentrations in (e) never exceeded detection limit and therefore are not shown. Open circles—acetate; open squares—TCE; closed circles—DCEs; closed triangles—VC; closed squares—ethene. f Final cell density and acetate

consumption by strain 195 at conditions described in (a-e). All data points are averages of triplicate bottles

Discussion

Desulfovibrio sp. strain F1 was isolated in this study, an autotrophic sulfate reducer joining the already described autotrophic *Desulfovibrio* sp. strains HRM1, P23 (sharing 88% 16S rRNA gene sequence identity with strain F1), and G11 (sharing 97% 16S rRNA gene sequence identity with strain F1) (McInerney et al. 1981; Brysch et al. 1987; Fichtel et al. 2012). Strain F1 can generate formate and a small amount of acetate from consumption of H_2 and CO_2 . Generation of formate from H_2/CO_2 by *Desulfovibrio* strains has been reported previously (Peters et al. 1999), but only chemolithoheterotrophically (i.e., with acetate as fixed carbon source). It was suggested that possessing a formate dehydrogenase is the prerequisite for producing formate during chemolithotrophic growth on H_2 (Peters et al. 1999). This is consistent with enzymatic findings of *Desulfovibrio desulfuricans* which does contain active formate dehydrogenases (Costa et al. 1997). *Desulfovibrio* sp. strain HRM1 was reported to grow autotrophically (Brysch et al. 1987), but it is not yet known which VFAs are produced during its carbon fixation process.

Desulfovibrio sp. strain F1 is thus the first instance of a *Desulfovibrio* strain reported to produce formate and acetate during autotrophic growth. Formate production was proportional to the initial H_2 concentrations in the presence of 2 mM sulfate (~ 0.49 mmol formate per mmol H_2) (set H0-H2, Table 2) which is higher than that of *Acetobacterium* strains (0.083 mmol formate per mmol H_2) (Peters et al. 1999). However, formate concentrations increased continually for at least 40 days during growth of *Desulfovibrio* sp. strain F1, while in the study by Peters et al. formate was only produced transiently (formate concentration peaked before day 20 and then dropped significantly). Moreover, formate production by strain F1 was not completely suppressed when sulfate was as high as 6 mM (Table 2), while *Desulfovibrio vulgaris* only produced formate under sulfate-limiting conditions (sulfate 0.5 mM) (Peters et al. 1999). These results suggest that the carbon fixation system or gene regulation system in strain F1 may be different from those of previously reported autotrophic sulfate reducers.

Formate is an important intermediate in the acetyl-CoA carbon fixation pathway which is produced in the first step by formate dehydrogenase (Rabus et al. 2006; Müller 2003). Given that strain F1 produced high concentrations of formate, it likely utilizes the acetyl-CoA pathway as the carbon fixing mechanism. The accumulation of formate may be due to the low efficiency of key enzymes in the pathway of forming the methyl branch of acetyl-CoA, which can create a bottleneck for converting formate (Müller 2003). The production of acetate is also intriguing. Previously, autotrophic sulfate reducers utilizing the acetyl-CoA pathway would convert CO_2 to acetyl-CoA for anabolism without production of acetate, a key difference from acetogens that gain energy by producing acetate via the acetyl-CoA pathway (Drake et al. 2006). However we show that strain F1, as a sulfate

reducer, produces a trace amount of acetate (up to 50 μM) via autotrophic growth. In future we will try to confirm which autotrophic pathway strain F1 utilizes and to identify key functional enzymes for formate/acetate production, for which the genome sequence of strain F1 should be acquired.

Acetate is an important factor when implementing bioaugmentation processes with *Dehalococcoides* in situ, especially in oligotrophic areas. In this study, we have characterized the acetate utilization profiles by *D. mccartyi* strain 195. Acetate assimilation is proportional to the final cell density of *Dehalococcoides* (Fig. 4f), in its role for producing biomass. Consumption of 93 μM acetate was observed when achieving a cell density of $4.8 \times 10^8 \text{ mL}^{-1}$ in strain 195 culture, corresponding to an average of 1.2×10^8 acetate molecules required per cell for the carbon element. The value is close to the calculated value of 1.6×10^8 acetate molecules required per cell for the carbon element, assuming a cell dry weight of $1.2 \times 10^{-14} \text{ g}$ per cell (Löffler et al. 2013) and cell chemical composition $\text{C}_5\text{H}_7\text{O}_2\text{N}$. As for the acetate uptake threshold, strain 195 amended with limited acetate decreased the acetate concentration to less than 5 μM . This is consistent with the findings by Wei and Finneran (2013) who found that low acetate concentrations had no limiting effects on TCE dechlorination rates. The lowest acetate concentration tested was 500 μM in Wei and Finneran's study, while it is 45 μM in this study (Fig. 4). This low acetate utilization threshold is a desirable feature for bioremediation sites, because low concentrations of consistently released acetate sources (either chemical or biological) can be used to provide sufficient fixed carbon to sustain *Dehalococcoides* while at the same time limiting the growth of other fast-growing acetate-consuming microbes. We observed that although cell growth stopped after acetate was consumed, dechlorination continued as shown in Fig. 4c, d. This was confirmed by the discrepancy between *Dehalococcoides* cell yields in the mixed culture EC195 (Fig. 1b) and in strain 195 (Fig. 4f), as the former probably suffered from acetate limitation. Continued dechlorination after acetate depletion is expected as acetate does not limit enzymatic activity of reductive dehalogenases which rely on presence of live cells as well as sufficient electron donors and acceptors (H_2 and TCE in this case). Although methanogens compete with *Dehalococcoides* for acetate, they typically utilize acetate down to 7–70 μM (Jetten et al. 1992) while our study demonstrates that *Dehalococcoides* can use < 5 μM acetate and that a co-culture of *Dehalococcoides*/*Desulfovibrio* strain F1 typically accumulated acetate up to only 25 μM . A continuous supply of μM -level acetate by *Desulfovibrio* thus could favor the growth of *Dehalococcoides*.

In all, this work suggests that *Dehalococcoides* species together with autotrophic *Desulfovibrio* strains could play a significant role in the in situ dechlorination of chloroethenes under lithoautotrophic conditions. Insights gained through the study of this autotrophic dehalogenation system will help to develop more effective engineering approaches for bioremediation of halogenated compounds in the contaminated sites.

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