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Growth-substrate dependent dechlorination of 1,2-dichloroethane by a homoacetogenic bacterium

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Abstract

A rod shaped, gram positive, non sporulating *Acetobacterium* strain was isolated that dechlorinated 1,2-dichloroethane (1,2-DCA) to ethene at a dechlorination rate of up to 2 nmol Cl^- min^{-1} mg^{-1} of protein in the exponential growth phase with formate (40 mM) as the substrate. Although with other growth substrates such as pyruvate, lactate, H_2/CO_2, and ethanol higher biomass productions were obtained, the dechlorination rate with these substrates was more than 10-fold lower compared with formate growing cells. Neither cell extracts nor autoclaved cells of the isolated *Acetobacterium* strain mediated the dechlorination of 1,2-DCA at significant rates. The addition of 1,2-DCA to the media did not result in increased cell production. No significant differences in corrinoid concentrations could be measured in cells growing on several growth-substrates. However, these measurements indicated that differences in corrinoid structure might cause the different dechlorination activity. The *Acetobacterium* sp. strain gradually lost its dechlorination ability during about 10 transfers in pure culture, probably due to undefined nutritional requirements. 16S rDNA analysis of the isolate revealed a 99.7% similarity with *Acetobacterium wieringae*. However, the type strains of *A. wieringae* and *A. woodii* did not dechlorinate 1,2-DCA.

Introduction

Acetogenic bacteria are strictly anaerobic eubacteria catalyzing the reduction of 2 CO_2 to acetate gaining energy of this process (for a review see e.g. Diekert 1990). During acetogenesis, the transfer of a methyl group to a corrinoid containing protein requires the cobalt atom of the corrinoid to be in an oxidation state of +1. Interestingly, Co(I) corrinoids are known to be able to reduce halogen substituted carbon atoms of halogenated organic compounds, releasing halogen anions (Holliger et al. 1992; Stupperich 1993; Neumann et al. 1996; Glod et al. 1997; Miller et al. 1997; Christiansen et al. 1998; Holliger et al. 1998; Miller et al. 1998; Neumann et al. 2002). Therefore it is not surprising that growing acetogens are able to cometabolically dechlorinate chlorinated organic compounds under specific conditions (Wild et al. 1995). Besides some highly substituted chlorinated compounds such as tetrachloroethene (PCE) and trichloroethene (TCE), even more reduced chlorinated hydrocarbons such as 1,2-DCA may be reductively dehalogenated in cometabolic dechlorination mediated by acetogenic bacteria (Wild et al. 1995). This is probably due to the strong reducing power of the cob(I)alamin that plays a major role in the energy metabolism of homoacetogens. However, a few homoacetogens have been shown to grow with chlorinated methanes as energy substrates (Traunecker et al. 1991; Messmer et al. 1993; Mägli et al. 1996).

Cometabolic conversions of 1,2-DCA have also been observed with pure or mixed cultures of methanogenic bacteria (Bouwer & McCarty 1983; Belay & Daniels 1987; Egli et al. 1987; Van Eekert et al. 1999),

cell suspensions of methanogenic isolates (Holliger et al. 1990, 1992), sulfate reducing bacteria (Egli et al. 1987), and mixed acetogenic bacterial cultures (Wild et al. 1995). Since the dechlorination of 1,2-DCA in acetogenic bacteria is based on cometabolism, conversion rates are several orders of magnitude lower than with dehalorespiring organisms. To date, only *Dehalococcoides ethenogenes* strain 195 has been reported to dehalorespire 1,2-DCA (Maymo-Gatell et al. 1999).

Acetogenic bacteria mostly were reported to dechlorinate chlorinated ethenes and ethanes in undefined mixed cultures (Terzenbach & Blaut 1994; Wild et al. 1995; Bradley 2000). Only one study showed their direct involvement in 1,2-DCA dechlorination (Wild et al. 1995). Characterization of these dechlorinating bacteria can be done more properly by studying appropriate isolates. The latter strategy with methanogenic isolates resulted in a deeper insight in the dechlorination mechanism revealing the involvement of corrinoids and/or coenzyme F_{430} in the 1,2-DCA dechlorination (Holliger et al. 1992).

In this paper, the isolation and characterization of a previously reported (De Wildeman et al. 2002) formate utilizing strain of *Acetobacterium* sp. is described, that dechlorinates 1,2-DCA at relatively high rates compared with other methanogens and acetogens. The 1,2-DCA dechlorination was investigated especially with respect to the requirements for specific growth substrates.

Materials and methods

Chemicals

Chlorinated compounds used were 1,2-dichloroethane (1,2-DCA), trichloroacetic acid (both from Fluka, Buchs, Switzerland), and chloroethane (CA) (standard solution; 2000 μg/ml in methanol; Supelco Inc., Misa group 16, Mix 6, 48799-U, Supelco Park, Bellefonte, PA). Abbreviations of other chlorinated compounds used in this contribution are tetrachloroethene (PCE), trichloroethene (TCE), 1,2-dichloroethene (1,2-DCE) and vinylchloride (VC). Standard gases were calibrated based on a mixture of acetylene, carbon dioxide, carbon monoxide, ethane, ethene and methane, each 1 vol%, in nitrogen (Scott Specialty Gases, Supelco Park, Bellefonte, PA).

Cultivation of organisms

All tests were done in 120 mL serum bottles closed with viton stoppers. The medium used was identical to that used for the isolation of *Dehalospirillum multivorans* (Scholz-Muramatsu et al. 1995). Formate, pyruvate, lactate or ethanol were dosed at 40 mM, while acetate was used at 5 mM. The 1,2-DCA final concentration was 400 μM. CO_2 and/or hydrogen were injected into the headspace (each 300 mbar overpressure) when these compounds served as substrates. The pH of the resulting media varied between 7.40 and 7.65. Growing cultures were incubated at 28 °C and stirred at 180 rpm. In order to monitor growth, optical measurements were taken on a monobeam spectrofotometer with light sensible diodes (Dr. Lange Multi Dioden Array fotometer ISIS 9000). About 1 ml of culture sample was brought into plastic cuvettes with 10 mm path length. Optical density was determined at 595 nm and was compared with sterile media samples.

Isolation using the roll-tube technique

Serum bottles of about 130 ml (150 mm height × 40 mm diameter) were used to grow colonies on agar films at the inner walls of the bottles. 30 ml of a mixture containing (per liter) deionized water (979 ml), basal medium (20 ml), and resazurin (1 ml, 0.2%) were added to the bottles. Formate (40 mM), acetate (5 mM), 0.01% yeast extract, and 3% agar were added.

After inoculation of the bacterial dilution series, 1 ml of a sterile 200 mM 1,2-DCA stock solution in hexadecane was added to the upright tubes.

16S rDNA sequence analysis

Comparison of a stretch of 1465 nucleotides of the 16S rDNA of the isolate with the sequences currently available from the EMBL database (Heidelberg) revealed a 99.7% similarity to the closest relative, *Acetobacterium wieringae* DSM 1991. The analysis was preformed by the BCCMT/LMG Bacteria Collection (Ghent, Belgium). Type strains used for dechlorination experiments were *Acetobacterium wieringae* DSM 1991 and *Acetobacterium woodii* DSM 1030.

GC analysis

Concentrations of 1,2-DCA were determined by analyzing 500 μl headspace samples on a Chrompack CP9000 gas chromatograph equipped with FID set at 200 °C. The GC contained a 30 m Chrompack CP-Sil

5CB column. Nitrogen was used as the carrier gas at a flow rate of 10 ml/min. The oven temperature was kept constant at 80 °C.

Detection of methane, ethene, and ethane was performed on a Shimadzu Gas Chromatograph with FID and a packed column (2 m, Chrompack Porapaq N) under isothermic conditions (80 °C). Ethene, ethane, vinyl chloride, chloroethane and 1,2-DCA had detection limits of about 0.1 μmol/l.

Extraction and meaurement of corrinoid concentration

Formate grown *Acetobacterium* cells were harvested in the late logarithmic growth phase by centrifugation at 10000 × g and 4 °C for 20 min. Wet cells were resuspended in 2 ml 10 mM Tris-HCl (pH 7.5) containing 1 mM $MgCl_2$, 10 mg lysozyme and 1 mg DNAse I and were incubated at 37 °C for 60 min. Microscopic study of the suspension revealed broken cells. Cell debris was removed by anoxic centrifugation for 10 min at 10000 × g and 4 °C.

The rate of the abiotic trichloroacetate dechlorination was used to estimate the total amount of corrinoids (Neumann et al. 2002) assuming that vitamin B_{12} was the main corrinoid present. In an anaerobic cuvette, 1 ml of a test buffer was injected via the rubber stopper. The test buffer contained 100 mM TrisHCl at pH 7.5 and 0.5 mM methyl viologen. To this buffer, 10 μl of a 190 mM Ti(III)citrate solution and 10 μl of the cell extract were added. The absorption change at 660 nm wavelength was measured photometrically. The rate of the oxidation of reduced methylviologen was measured and compared to standard series. The reaction was started by the addition of chlorinated compounds (1,1,1-trichloroacetic acid as standard) as electron acceptors.

The protein concentration was determined according to Bradford (1976) with the Bio-Rad Protein Assay using bovine serum albumin as the standard (Bio-Rad, Munchen, Germany).

Results

Isolation of a 1,2-DCA dechlorinating homoacetogen

From unadapted granular methanogenic sludge grown in an anaerobic wastewater treatment plant purifying starch containing water originating from potato industry, different enrichments with 400 μM of 1,2-DCA were performed with pyruvate, lactate, ethanol, formate/acetate, and H_2/CO_2/acetate as the energy- and C-sources. Initially, no yeast extract was used in the liquid media. Cysteine or dithiothreitol (DTT) were used as reducing agents in order to adjust the redox potential for the growth of strictly anaerobic bacteria. Enrichments were initially performed with cysteine as the reducing agent (0.005% w/v). After the fourth transfer, cysteine was replaced by DTT (0.05% w/v). Methanogens were suppressed under these conditions, thus favouring the growth of a non-methanogenic, acetate producing, strictly anaerobic bacterium, reaching an abundance of at least about 95%.

After four transfers, dechlorination activity was only maintained in media containing formate or ethanol. These active cultures were transferred to roll-tubes in dilutions between 1:10 and 1:10000. After 10 days, colonies appeared and were transferred again to media containing formate or ethanol. However, dechlorination activity was only measured in the presence of formate. These cultures dechlorinated 1,2-DCA at a maximum rate of 2 nmol Cl^- min^{-1} mg^{-1} of protein, which closely corresponds with activity measurements in the enrichment cultures. A second roll-tube transfer ensured the purity of the strain.

Morphological and phylogenetic characterization

Phase contrast microscopy of the isolated formate utilizing strain revealed Gram positive rod shaped motile bacteria often occurring in pairs; longer chains were extremely rare. Colonies grown in roll-tubes were smooth, colourless, gel like and reached a diameter of about 1 mm. No spores could be induced in media containing manganese (100 μM) and calcium (1 mM).

A consensus sequence of 1465 nucleotides of the 16S rDNA of the pure strain revealed the following significant similarities (>97%) with its closest relatives belonging to the genus *Acetobacterium*: 99.7% with *A. wieringae* DSM 1991, 99.0% with *A. malicum* DSM 4132, 98.1% with *A. woodii* DSM 1030, 97.6% with *A. carbinolicum* DSM 2925, and 97.6% with *A. fimetarium* DSM 8238.

From this analysis, it could be concluded that the isolate belongs to the genus *Acetobacterium* and is most likely a *A. wieringae* species.

Growth and 1,2-DCA conversion of the isolated strain in the presence of different growth substrates

The isolated *Acetobacterium* sp. strain was grown with pyruvate, H_2/CO_2, ethanol, formate, lactate or yeast

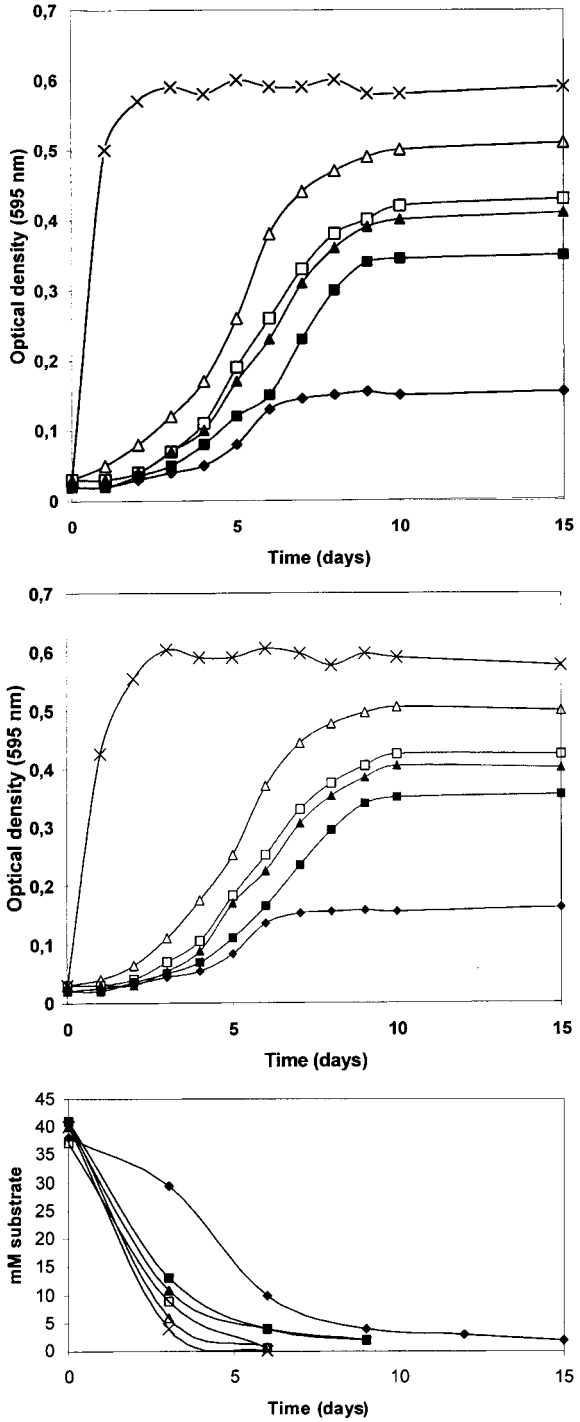

Figure 1. Growth of the isolated *Acetobacterium* sp. strain in media containing formate (40 mM) – acetate (5 mM) – yeast extract (0.1%(w/v)) (×), lactate (40 mM) (△), pyruvate (40 mM) (□), ethanol (40 mM) (▲), H_2/CO_2 (300 mbar overpressure each) – acetate (5 mM) (■), and formate (40 mM) – acetate (5 mM) (♦) in the presence (A) and absence (B) of 400 μM 1,2-DCA. No OD_{595} increase was measured without electron donor. Depletion of substrates is given in Figure 1C.

extract formate in the presence of 400 μM 1,2-DCA (Figure 1A). Almost identical growth curves were measured in the absence of 1,2-DCA (Figure 1B). Corresponding utilization of substrates is given in Figure 1C.

Figure 2 depicts the dechlorination of 400 μM 1,2-DCA of the isolated strain in the presence of different growth-substrates such as pyruvate, H_2/CO_2, ethanol, and formate. This dechlorination performance is expressed as the amount of ethene produced from 1,2-DCA via a dichloroelimination. No other possible intermediates such as chloroethane or vinylchloride were detected. Although pyruvate, lactate, H_2/CO_2, and ethanol grown cultures reached a final OD_{595} value of 0.3 to 0.5 after 8 days (Figure 1), ethene formation by the isolated strain did never exceed 16 μM after 8 weeks, corresponding to a total conversion of at most 4% of the initial 1,2-DCA (Figure 2). In contrast, with formate as electron donor, 50% of the initial 1,2-DCA was converted to ethene (200 μM) after 8 weeks, at an average degradation rate of 0.2 nmol Cl^- min^-1 $mg^{-1}_{protein}$. The conversion rate reached almost 2 nmol Cl^- min^{-1} $mg^{-1}_{protein}$ during the exponential growth phase between days 3 and 7 (Figure 2). This dechlorination rate was calculated from protein increase measurements during growth of the isolate. The dechlorination rate slowly decreased in the stationary phase of growth. After 8 weeks, media reduced with DTT and containing pyruvate, H_2/CO_2, or ethanol only showed less than 8% compared with the total ethene production of formate media. During the first week of incubation, dechlorination by the latter active cultures growing on formate accounted for only 0.0025% of overall metabolism based on acetogenesis. Adding 0.1% (w/v) yeast extract to culture media containing 40 mM formate and 5 mM acetate, enhanced the growth rate and final OD_{595} (Figure 1), however, the dechlorination was completely suppressed (Figure 2). Neither significant ethene production from 400 μM 1,2-DCA could be measured when the type strains of *Acetobacterium wieringae* and *Acetobacterium woodii* were inoculated (10 vol%). These results are included in Figure 2 and illustrate the difference between these type strains and the isolated *Acetobacterium* strain of this study. All dechlorination tests with the different *Acetobacterium* strains were started from a 10 vol% transfer of a pre-culture grown on formate with OD_{595} in the range of 0.15–0.18. Hence, initial biomass concentration measurements had OD_{595} of about 0.02.

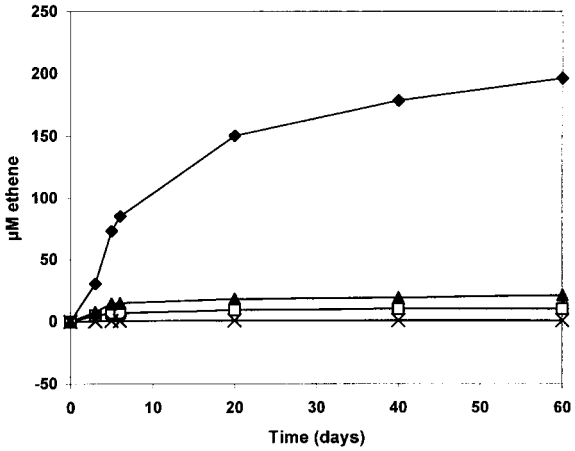

Figure 2. Ethene production from 400 μM 1,2-DCA by the isolated *Acetobacterium* sp. strain growing on formate (40 mM) plus acetate (5 mM) (♦), ethanol (40 mM) (▲), pyruvate (40 mM), or lactate (40 mM), or H_2/CO_2 (300 mbar each) (□); ethene production from 400 μM 1,2-DCA by the type strains *Acetobacterium wieringae* DSM 1991 and *A. woodii* DSM 1030 grown on formate (40 mM) and acetate (5 mM), and by the isolate growing on formate (40 mM), acetate (5 mM) and yeast extract (0.1% w/v) (×).

Biotic versus abiotic dechlorination

In order to discriminate between abiotic and cometabolic conversion, experiments were performed with living formate growing *Acetobacterium* sp. cells, with autoclaved formate grown cells and without cells (Figure 3). Within the first 3 days, autoclaved cells and sterile media produced 9% and 5%, respectively, of the amount of ethene produced by living cells. Slow degradation continued with autoclaved cells, ending up in the formation of 13% of the ethene produced by living cells after 60 days. The abiotic ethene formation ceased when about 1.5 μmol of ethene were produced (Figure 3). The corrinoid concentrations (expressed in nmol corrinoid per gram cell dry weight) in media containing different growth substrates were decreasing as follows: H_2/CO_2 (340 nmol/g), formate (320 nmol/g), pyruvate (240 nmol/g), lactate (210 nmol/g) and ethanol (190 nmol/g).

During the first 3 enrichment steps, addition of cysteine (0.005% w/v) as the only reductant resulted in slow production of ethene but also lead to H_2S production. In order to outcompete non-dechlorinating sulfate-reducing bacteria that could be enriched in the culture, DTT (0.05%) was chosen as an alternative but strong reducing agent substituting cysteine that was routinely used. It was observed that 0.05% (w/v) DTT stimulated both the dechlorination activity and growth rate of the *Acetobacterium* sp. Moreover, methanogenic activity disappeared in the presence of DTT, indicating that methanogens were not involved in the dechlorination and might have been inhibited by DTT.

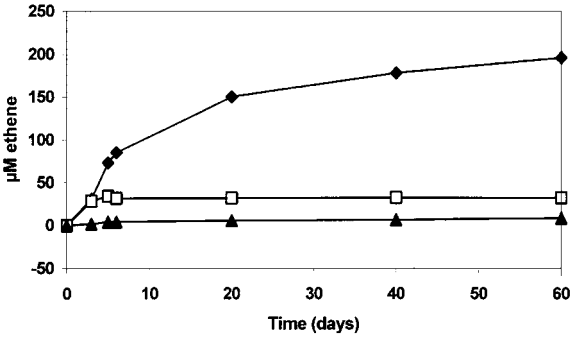

Figure 3. Ethene production from 400 μM 1,2-DCA by the *Acetobacterium* sp. isolate in formate (40 mM) + acetate (5 mM) + DTT (0.05 w/w%) medium (i) after inoculation with 10 vol% of a formate grown culture (40 mM formate and 5 mM acetate initial concentrations, final OD_{600} of 0.15) (♦), (ii) in the presence of *Acetobacterium* sp. cells that were killed (80 °C, 10 min) after growing in formate medium (also 40 mM fomate and 5 mM acetate initial, final OD_{600} of 0.15) (□), and (iii) in the absence of bacteria (▲).

Discussion

Although the reductive dechlorination of highly chlorinated ethanes and ethenes is known to proceed via cometabolic conversions by anaerobic bacteria (Vogel et al. 1987), lower chlorinated ethanes and ethenes appear to be far less susceptible to reduction reactions, if at all. Especially methanogens and acetogens containing high levels of corrinoids and cofactors are known to carry out these slow cometabolic dechlorination reactions. Pure cultures of methanogens were studied with respect to their ability to cometabolically dechlorinate 1,2-DCA. In cell extracts of *Methanosarcina barkeri* grown with methanol (Holliger et al. 1990), dechlorination rates of at most 0.07 nmol Cl^- min^{-1} $mg^{-1}_{protein}$ were reported. Depending on the growth substrate used, different dechlorination rates for the bacterial cell suspensions obtained from one species were observed, however, the maximum and minimum dechlorination rates only differed by a factor of two.

In this study with a novel homoacetogenic isolate, the influence of different growth substrates was much higher. Formate supported the cometabolic conversion of 1,2-DCA at a rate of up to 2 nmol Cl^- min^{-1} $mg^{-1}_{protein}$, while alternative substrates did not significantly support the dechlorination of this compound,

even though the cells grew up to by far higher cell densities with these substrates as compared to formate. Furthermore, yeast extract added to formate media supported very fast growth of the *Acetobacterium*sp. strain, which was also observed for the type strain of *A. wieringae* (Braun & Gottschalk 1982), but the bacteria did no longer dechlorinate 1,2-DCA in these rich nutritional conditions. In the most active cultures containing formate as sole electron source (without yeast extract), dechlorination proceeded during the stationary phase of the cells, indicating that endogenous metabolism or products of formate degradation might sustain 1,2-DCA reduction.

Corrinoid concentrations under different growth conditions were not clearly related to dechlorination activities. However, formate and H_2/CO_2 grown cells, containing the highest and almost similar corrinoid concentrations of 320 and 340 nmol per gram cell dry weight respectively, suggest that the dechlorination activity might be due to qualitative differences in corrinoid structure. Further qualitative determination tests on the corrinoids produced under different cell-growth conditions, might confirm this suggestion.

The presence or absence of 400 μM 1,2-DCA did not influence the growth of the isolated *Acetobacterium* sp. strain on all growth substrates tested in this study. These findings indicate that 1,2-DCA conversion was not coupled to energy conservation. Furthermore, (i) the higher dechlorination rates known for dehalorespiring anaerobes (at least 25 times higher), (ii) the low percentage of overall metabolism attributed to dechlorination of 1,2-DCA, and (iii) the low dechlorination rate with autoclaved cells compared with living cells suggest that the 1,2-DCA conversion originates from cometabolic transformation by growing *Acetobacterium* cells.

DTT is able to reduce 1,2-DCA in the presence of vitamin B_{12} (Holliger et al. 1992), which was added to the media at a concentration of 0.05 mg/l. However, the dechlorination rates observed in media without *Acetobacterium* sp. cells were almost 25-fold lower than in the presence of formate growing cells. The stimulating effect of DTT in combination with vitamin B_{12} for the dechlorination of carbon tetrachloride was reported earlier (Assafanid et al. 1994). That this effect of DTT and vitamin B_{12} was significantly less pronounced with 1,2-DCA may be one of the reasons for the recalcitrance of this toxic molecule, even under low redox potential conditions.

The *Acetobacterium* sp. strain lost its dechlorination during several transfers in pure culture, possibly due to lacking nutritional requirements in the growth media. The loss of 1,2-DCA dechlorination activity has also been investigated with the dehalorespiring bacterium *Dehalococcoides ethenogenes* strain 195, which needs the addition of bacterial extracts from dechlorinating mixed cultures (Maymo-Gatell et al. 1997). Growth experiments with preserved cultures of the isolated *Acetobacterium* sp. strain under different nutritional conditions might further explore the exact requirements for dechlorination activity.

Highly reduced chlorinated ethanes and ethenes are almost not susceptible to further reductive dechlorination reactions that could totally dechlorinate and detoxify them (De Wildeman et al. 2003). Hence, especially these compounds accumulate in reductive conditions. Inducing the reductive dechlorination capacity of these toxic pollutants in mixed bacterial cultures, and selecting for acetogenic bacteria as described in this study, may be helpful to compare, elucidate and apply their dechlorination capabilities at anoxic polluted sites.

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