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Benzoate-driven dehalogenation of chlorinated ethenes in microbial cultures from a contaminated aquifer

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Abstract Microbial dehalogenation of tetrachloroethene (PCE) and *cis*-dichloroethene (*cis*-DCE) was studied in cultures from a continuous stirred tank reactor initially inoculated with aquifer material from a PCE-contaminated site. Cultures amended with hydrogen and acetate readily dechlorinated PCE and *cis*-DCE; however, this transformation was incomplete and resulted in the accumulation of chlorinated intermediates and only small amounts of ethene within 60 days of incubation. Conversely, microbial PCE and *cis*-DCE dechlorination in cultures with benzoate and acetate resulted in the complete transformation to ethene within 30 days. Community fingerprinting by denaturing gradient gel electrophoresis (DGGE) revealed the predominance of phylotypes closely affiliated with *Desulfitobacterium*, *Dehalococcoides*, and *Syntrophus* species. The *Dehalococcoides* culture VZ, obtained from small whitish colonies in *cis*-DCE dechlorinating agarose cultures, revealed an irregular cell diameter between 200 and 500 nm, and a spherical or biconcave disk-shaped morphology. These organisms were identified as responsible for the dechlorination of *cis*-DCE to ethene in the PCE-dechlorinating consortia, operating together with the *Desulfitobacterium* as PCE-to-*cis*-DCE dehalogenating bacterium and with a *Syntrophus* species as potential hydrogen-producing partner in cultures with benzoate.

Keywords Reductive dechlorination · PCE · Benzoate · *Dehalococcoides* · *Desulfitobacterium* · *Syntrophus*

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Introduction

The lower chlorinated ethenes, *cis*-dichloroethene (*cis*-DCE) and vinyl chloride (VC), can be aerobically degraded as the sole carbon source (Coleman et al. 2002a, b), whereas the more highly chlorinated ethenes, tetrachloroethene (PCE) and trichloroethene (TCE), are resistant to breakdown under aerobic conditions. In anaerobic aquifers, reductive dechlorination is the key biological reaction for the in situ transformation of chlorinated compounds, leading to sequential removal of the halogen substituents. In a process known as dehalorespiration, bacteria are able to couple energy conservation and growth to the reduction of halogenated compounds (e.g., chlorinated ethenes) that serve as electron acceptors for anaerobic respiration. Reductive PCE dechlorination has been documented at contaminated sites, in laboratory cultures, and at engineered field sites where stimulated natural attenuation by micro-

organisms has been used for in situ remediation (Major et al. 2002; Lendvay et al. 2003; Löffler et al. 2003).

An ideal remediation strategy for groundwater contaminated with chlorinated ethenes would convert the pollutants to an environmentally harmless compound, such as ethene, and would also prevent the accumulation of partially degraded chloroethenes, which are often more toxic than the parent compounds. Most identified organisms, however, are only able to perform partial PCE dechlorination (Löffler et al. 2003) challenging the use of microbial reductive dechlorination as a meaningful remediation mechanism. Only one group of organisms that is phylogenetically related to the genus *Dehalococcoides* has been shown to completely dehalogenate chloroethenes to ethene (Maymo-Gatell et al. 1997; Cupples et al. 2003; He et al. 2003; He et al. 2005; Sung et al. 2006). Hence, recent research has focused on reductive dechlorination by *Dehalococcoides* and relatives, also attracted by the versatility of these bacteria for other organohalogen compounds (Adrian et al. 2000; Bunge et al. 2003; Fennell et al. 2004). Moreover, results of a comprehensive 16S rDNA-based survey suggested that the presence of *Dehalococcoides* is a prerequisite for the complete dechlorination of chloroethenes to ethene at contaminated sites (Hendrickson et al. 2002).

Past reports ascribe the dehalogenation of chlorinated ethenes to the activity of individual dechlorinating populations. On the other hand, the potential interactions between dechlorinators, either mutualistic or competing, are not sufficiently understood. So far, only one strain, *Dehalococcoides ethenogenes* strain 195, is known to perform complete dechlorination of PCE to ethene (Maymo-Gatell et al. 1997), all other isolates are only capable of completely reducing TCE, *cis*-DCE, and vinyl chloride (Cupples et al. 2003; He et al. 2003; He et al. 2005; Sung et al. 2006). Therefore, it is unclear to which extent these bacteria are solely responsible for the full sequential PCE dehalogenation to ethene or whether they rather interact with other dechlorinating organisms that perform the initial steps of the reaction.

Because molecular hydrogen is the only known electron donor directly used by *Dehalococcoides* strains, complete transformation of chlorinated ethenes relies on hydrogen delivery. Dehalorespiring organisms have a high affinity for the hydrogen produced by organic matter degradation in anaerobic environments and at low hydrogen concentrations they may outcompete bacteria that use other electron acceptors including methanogens and acetogenic bacteria (Fennell and Gossett 1998; Yang and McCarty 1998; Löffler et al. 1999). Accordingly, hydrogenotrophic dehalorespiring bacteria may belong to the preferred syntrophic partners of microorganisms that anaerobically oxidize organic compounds, which require a low hydrogen partial pressure for energy conservation (Schink 1997). Benzoate is an important intermediate in the anaerobic degradation of aromatic compounds and its anaerobic oxidation is endergonic under standard conditions. Hence, this reaction must be coupled to another process to decrease the hydrogen concentration and make it thermodynamically feasible. Although the effectiveness of benzoate as a bulk electron donor for the conversion of chloroethenes has been demonstrated at field sites and in laboratory cultures (Scholz-Muramatsu et al. 1990; Beeman et al. 1994; Yang and McCarty 1998; Yang and McCarty 2000), the microbes involved in benzoate oxidation have not yet been identified.

We study the conversion of PCE in enrichment cultures from a continuous stirred tank reactor initially inoculated with aquifer material from a PCE-contaminated groundwater site in Victoria, TX (Yang et al. 2005). In the Victoria aquifer and in laboratory cultures with material from this site, complete reductive dechlorination of PCE to ethene could be achieved by the addition of benzoate (Beeman et al. 1994; Yang and McCarty 1998; Yang et al. 2005). Contrary to the idea that a single dechlorinating organism mediates the entire dechlorination process, recent results have indicated that bacteria similar to *Desulfitobacterium* and *Dehalococcoides* species have been implicated in the overall conversion of PCE (Yang et al. 2005). We currently do not know whether the two organisms compete for the same chlorinated electron acceptors (e.g., PCE) or if the complete conversion of PCE to ethene rather depends on the sequential activity of both bacteria. The objective of our current study is to assess potential interactions of dechlorinating bacteria and to better understand their association with nondechlorinating organisms in the consortium.

Materials and methods

Cultivation of PCE- and *cis*-DCE dechlorinating communities

A set of 60-ml serum bottles sealed with butyl rubber stoppers (Bellco Glass, Vineland, NJ) and aluminum crimps was purged with N_2/CO_2, autoclaved, and filled with anaerobic medium that was prepared as previously described (Ballerstedt et al. 2004). For the first transfer after cultivation in a continuous stirred tank reactor (Yang et al. 2005), material was transferred into batch cultures (10% *v*/*v*), which were used for the investigation of the dechlorination kinetics of PCE and *cis*-DCE over a time of 62 days. The bottles (30 ml culture volume) were amended with 2.5 mM acetate and either hydrogen (10% in the headspace), acetate (2.5 mM) or benzoate (2.5 mM). Finally, PCE (>99.5% purity, Fluka, Buchs, Switzerland) or *cis*-DCE (approximately 97% purity, Fluka) was added from undiluted solutions. For each experiment, duplicate cultures were established

and incubated without shaking in the dark at 25°C during which they were periodically analyzed for dehalogenation of PCE. The dehalogenating cultures were maintained through consecutive transfers (10% *v*/*v*) into sterile mineral salts medium and routinely fed with 250 to 500 μM PCE or *cis*-DCE, 2.5 mM benzoate, and 2.5 mM acetate. In agarose shake dilutions, 1% (*w*/*v*) agar (Difco) was added to the medium. For the preparation of agarose shakes from *cis*-DCE dechlorinating enrichment cultures, the mineral medium was amended with 0.33% (*w*/*v*) low-gelling point agarose (*SeaPlaque* agarose, Biozym). The transfer of single colonies was done in an anaerobic glove box (MBraun, Garching, Germany) using 1-ml single-use syringes and needles (0.6×80 mm, Braun, Melsungen, Germany).

Analysis of chlorinated ethenes and ethene

For analytical standard preparation, liquid PCE (>99.5%, Fluka), TCE (>99.5%, Fluka), and *cis*-DCE (approximately 97%, Fluka) were used. Vinyl chloride gas mix (1,000 ppm in N_2) was obtained from Alltech Associates (Deerfield, IL), and ethene (>99.7%) was from PanGas (Dagmersellen, Switzerland). PCE, TCE, *cis*-DCE, vinyl chloride, and ethene were analyzed in 250- μl samples using a Fisons HRGC Mega 2 Series gas chromatograph (Fisons, Milan, Italy) equipped with a flame ionization detector connected to a GS-Q fused silica capillary column (length, 30 m; inside diameter, 0.53 mm; Agilent Technologies, Basel, Switzerland). The injector and detector temperature was 250°C. The oven temperature program was as follows: 45°C for 2 min, increased to 180°C at a rate of 20 K/min, and maintained at 180°C for 7 min. The compounds were identified by matching the retention times with those of the external standards. The amounts of the compounds in liquid were calculated by using published Henry's law constants (Gossett 1987).

Nested PCR detection of dehalogenating bacteria and syntrophic species

DNA was extracted from the enrichment cultures using a protocol adopted from Kuske et al. (1998). The initial PCR was performed with bacterial 16S rDNA primers fD1 and rP2 (Weisburg et al. 1991). The purified amplicons (QIAquick PCR purification kit, Qiagen) were used as the templates (1 μl) for the second PCR reaction with each of the species- or genus-specific primer sets listed in Table S1 of the Electronic supplementary material.

Cloning and sequencing

Amplicons were purified as described above and cloned into the pGEM-T Easy Vector (Promega, Madison, WI) according to the manufacturer's instructions. Plasmids containing cloned fragments were isolated with the Wizard Plus SV Minipreps DNA Purification system (Promega), and sequencing was performed by Microsynth (Balgach, Switzerland) using vector-specific and the respective primers listed in Table S1 of the Electronic supplementary material. Closest relatives were identified using Fasta at EMBL-EBI http://www.ebi.ac.uk/fasta33/nucleotide.html) and the RDPII Sequence Match tool http://rdp.cme.msu.edu/).

DNA fingerprinting

For denaturing gradient gel electrophoresis (DGGE), 16S rRNA gene fragments were amplified from genomic DNA using the primers F-968-GC and R-1401 (Nübel et al. 1996). Electrophoresis and gel documentation followed the methods as described previously (Bürgmann et al. 2005). Reamplified PCR products from DNA of selected bands were purified, cloned, and sequenced as indicated above.

Fluorescence in situ hybridization

Culture aliquots (1 ml) were centrifuged at 10,000×*g* for 30 min, washed with 500 μl of 1x PBS, and fixed in 2% (*w*/*v*) paraformaldehyde freshly prepared from 16% (*w*/*v*) stock solutions (Electron Microscopy Sciences, Hatfield, PA) in 1x PBS at 4°C for 14 h. Fluorescently labeled oligonucleotide probes (Microsynth, Balgach, Switzerland) were applied on each well at a final concentration of 5 ng μl^{-1}. For the detection of *Desulfitobacterium*, the probes and conditions as reported by Yang et al. (2005) were used. Hybridization and washing steps were performed as described by Zarda et al. (1997). Analysis was conducted with an Axioplan fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Hamamatsu C5810 CCD camera (Hamamatsu Photonics, Solothurn, Switzerland).

CARD FISH

Catalyzed reporter deposition (CARD) fluorescence in situ hybridization (FISH) was essentially performed as described by Pernthaler et al. (2004). Cells that were permeabilized with lysozyme (10 mg ml^{-1}) and achromopeptidase (80 U ml^{-1}) revealed highest signal intensities for target cells of the HRP-labeled probes EUB338 and Dhe1259t (biomers.net, Ulm, Germany), and, compared to total DAPI-stained bacteria, maximum detection rates in mixed cultures containing *Dehalococcoides*. Optimal hybridization stringency was evaluated using variable formamide concentrations with a pure culture of *Dehalo-*

coccoides ethenogenes 195 (Maymo-Gatell et al. 1997), a culture with *Dehalococcoides* sp. strain VS (Cupples et al. 2003), and a mixed culture containing *Dehalococcoides* (this study) that were hybridized with HRP-labeled probes specific for *Dehalococcoides* species, Dhe1259t and Dhe1259c (Yang and Zeyer 2003), and the general bacterial probe EUB338 (Amann et al. 1990). A concentration of 40% (*v*/*v*) formamide in the hybridization buffer was optimal for achieving strong signals and discriminating between probes Dhe1259t and Dhe1259c.

Electron microscopy

Formvar-coated copper grids were incubated on a drop (5 μl) of a bacterial suspension for 1 min. The grids were washed three times with a drop of deionized water and negatively stained for 1 min using 4% (*w*/*v*) phosphotungstic acid. Electron micrographs were taken at 80 kV on a Zeiss EM 900 electron microscope equipped with the Variospeed SSCCD camera SM-1k-120 (TRS, Dünzelbach, Germany).

Results

Microbial transformation of PCE and *cis*-DCE

Two culture sets prepared with either PCE or *cis*-DCE were grown in the presence of different electron donors and carbon sources: (1) hydrogen and acetate, (2) benzoate and acetate or (3) acetate only (Figs. 1a–c and 2a–c). In cultures amended with hydrogen and acetate, PCE dechlorination readily occurred (Fig. 1a). However, the rapid initial PCE conversion to TCE was followed by an accumulation of *cis*-DCE and vinyl chloride, and only about 15% of the PCE was completely dechlorinated to ethene within 62 days. In contrast, an approximate lag time of 10 days was noted before the first PCE dechlorination product, TCE, was detected in the culture set containing benzoate and acetate (Fig. 1b). The dechlorination process then started immediately with the transient accumulation of the intermediates *cis*-DCE and vinyl chloride. PCE transformation was complete after 32 days.

The dechlorination time course was similar in the culture set containing *cis*-DCE. In the cultures grown with benzoate, *cis*-DCE was converted to ethene with a transient accumulation of vinyl chloride between days 8 and 24. This vinyl chloride was subsequently completely converted to ethene within 8 days (Fig. 2b). In hydrogen-amended cultures, *cis*-DCE transformation occurred without a time-lag; however, the process thereafter proceeded more slowly than in the benzoate-containing cultures (Fig. 2a). After 62 days, vinyl chloride accumulated, and only small amounts of ethene were detected.

Separation of dechlorination activities in serial dilutions

To ascertain whether the complete conversion to ethene was mediated by the successive activity of different dechlorinating populations, we initially investigated the cultures by conventional microbiological techniques. Complete PCE dechlorination to ethene occurred in serial dilutions up to 10^{-3}, whereas PCE dechlorination to *cis*-DCE was also found in higher dilutions, up to 10^{-6} (see Table S2 of the Electronic supplementary material). In all cultures where vinyl chloride accumulated transiently, this product was later converted to ethene. Likewise, TCE was always a temporary product and eventually converted to either *cis*-DCE or ethene.

Cultures diluted from 1:10 to $1:10^{-3}$ completely dehalogenated *cis*-DCE to ethene, reflecting a number of *cis*-DCE dechlorinating bacteria similar to serial dilutions of cultures spiked with PCE (Table S2 of the Electronic supplementary material). Like in the PCE-dechlorinating cultures, vinyl chloride was only detected as a transient dehalogenation product in actively dechlorinating cultures.

Stimulation of reductive dechlorination by filtered supernatant

To investigate the effect of filtered supernatant on the different dechlorinating populations, all cultures of serial dilutions that had not converted PCE or *cis*-DCE to ethene after 160 days received a second inoculation with 0.45 μm filtrate from the original undiluted culture. The addition of filtered material failed to stimulate PCE dechlorination. In contrast, in 12 of the 13 cultures that had formed *cis*-DCE before the second inoculation, complete dechlorination to ethene was stimulated by the addition of the filtered material (Table S2 of the Electronic supplementary material). This dechlorination activity was observed regardless whether the *cis*-DCE originated from the initial spike or was biogenically formed during partial PCE dechlorination.

PCR detection of bacteria potentially involved in the dechlorination process

To identify potential dechlorinators in the enrichment cultures, specific *nested* PCR primers were designed to target bacteria that dehalogenate a variety of compounds (see Table S1 of the Electronic supplementary material). Amplicons of the anticipated size were obtained from all batch cultures and positive controls in the *Dehalococcoides*-specific *nested* PCR. Sequencing of the amplified products revealed sequences identical to each other and to

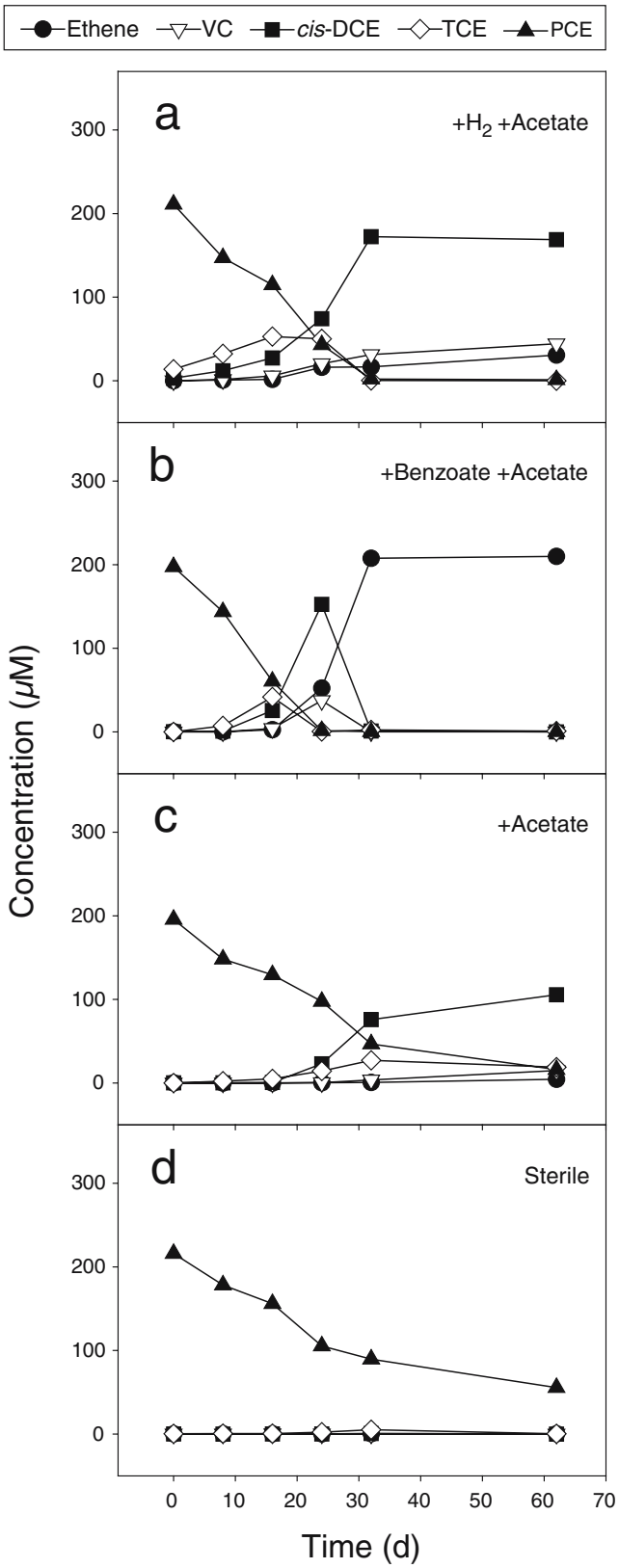

Fig. 1 Dechlorination products of PCE in cultures amended with hydrogen and acetate (**a**), benzoate and acetate (**b**) or acetate (**c**). The inoculum originated from a PCE-dechlorinating chemostat culture (Yang et al. 2005). No dechlorination products were detected in sterile controls (**d**). Values shown are means for two parallel cultures

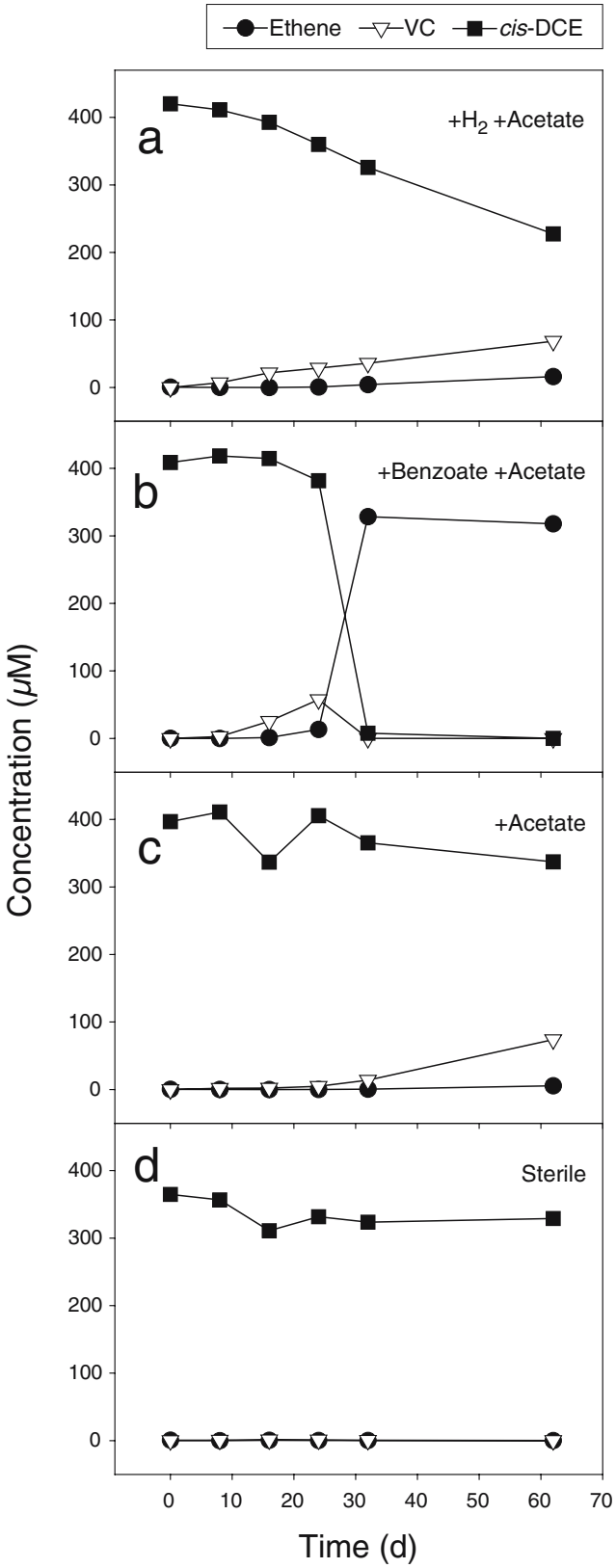

Fig. 2 Time course of the reductive dechlorination of *cis*-DCE in cultures grown with hydrogen and acetate (**a**), benzoate and acetate (**b**) or acetate (**c**). **d** Presents the time course in cultures incubated with autoclaved inoculum. Values are means for duplicate cultures

Dehalococcoides sp. strain VS. Samples from all cultures, except the sterile inoculum and negative controls, also yielded PCR products with the *Desulfitobacterium*-specific primers. The sequences exhibited 100% identity with *Desulfitobacterium hafniense* 16S rDNA sequences. PCR for all other investigated dechlorinating species was negative. In all enrichment cultures, PCR conducted with *Syntrophus*-specific primers yielded amplicons of the expected size. The recovered sequences exhibited 99% identity with *Syntrophus gentianae* (Wallrabenstein et al. 1995).

Microbial community composition

Characterization of the PCE-dechlorinating community grown in the presence of benzoate and acetate by denaturing gradient gel electrophoresis (DGGE) showed only a few bands per lane indicating cultures with minimal complexity (Fig. 3). DNA sequences from the most intense bands were closely affiliated (>99% identity) with phylotypes representing bacteria of the genera *Desulfitobacterium* (band 3), *Syntrophus* (band 4), and *Dehalococcoides* (band 5). Fingerprinting of 16S rDNA fragments was similar in the other PCE-dechlorinating cultures but some additional bands were detected. The sequences representing bands 1, 2, and 6 were similar to those of known isolates with the highest similarities to *Clostridium glycolicum* RD-1 (AJ291746), *Sedimentibacter* sp., clone C7 (AY766466), and a *Desulfovibrio* species (AY548774) (Fig. 3).

Enrichment of dechlorinating bacteria

As shown by FISH, up to 28% of the bacteria stained by DAPI in PCE-dechlorinating cultures represented *Desulfitobacterium* cells, which were subsequently obtained from single colonies in agar shake dilution series, and identified by FISH. In experiments with spiked PCE, the cultures produced *cis*-DCE as end product indicating that this bacterium was responsible for the initial attack on tetrachloroethene. Cultures spiked with *cis*-DCE did not show any dechlorination within 12 weeks of incubation.

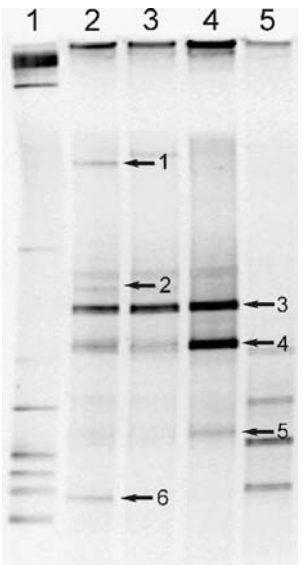

Fig. 3 DGGE analysis of 16S rDNA fragments from a culture set with PCE and grown with either hydrogen and acetate (*lane 2*), acetate (*lane 3*) or benzoate and acetate (*lane 4*). *Lane 1*, marker 1 Kb Plus DNA Ladder (Invitrogen); *lane 5*, control incubation with autoclaved inoculum. The *numbers* within the gel refer to bands mentioned in the text

Conventional FISH provided insufficient signals for the detection and quantification of *Dehalococcoides* in cultures older than 3 weeks, probably due to the low ribosomal content after extended incubation time and the small cell size of these bacteria (Zinder and Dworkin 2006). To overcome difficulties in detecting and quantifying such small bacteria under the microscope, we adapted and applied a sensitive CARD FISH protocol for the analysis of *Dehalococcoides* (Pernthaler et al. 2004). In cultures older than 3 months, CARD FISH permitted detection rates up to five times higher than the conventional FISH technique.

Using the horseradish peroxidase-labeled probe Dhe1259t for CARD FISH, samples of each transfer were monitored for the abundance of *Dehalococcoides* cells. Continued cultivation with *cis*-DCE, benzoate, and acetate enriched the culture for *Dehalococcoides* cells and resulted in a relative cell number of about 54% in the fifth transfer. Thereafter, cultures were grown in a dilution series prepared with solid medium (0.3% (*w*/*v*) *Seaplaque* low-gelling point agarose) containing *cis*-DCE, hydrogen, and acetate. Dechlorination products of *cis*-DCE in the culture headspace were first detected after 2 months of incubation. PCE was not anymore dechlorinated by these cultures (test period=24 weeks). In the following transfers of single colonies in liquid cultures, *Dehalococcoides* cells were detected in all active cultures that converted *cis*-DCE to ethene; however, microscopic examination revealed the presence of several other morphotypes including short vibroid cells and long rod-shaped bacteria in most of these cultures. Monitored by CARD FISH analysis, we were able to obtain a microscopically homogenous culture from following *cis*-DCE dechlorinating liquid cultures that were generated from small whitish colonies in preceding agarose shake dilution series. The identified *Dehalococcoides* culture was designated strain VZ (stands for Victoria-Zurich). Under our cultivation conditions, the bacteria mainly grew in aggregates of cells with irregular size with a diameter between 200 and 500 nm (Fig. 4a). Electron microscopy indicated a spherical or biconcave disk-shaped morphology of the cells (Fig. 4b).

Discussion

Serial dilutions of the cultures amended separately with either PCE or *cis*-DCE allowed us to show that the

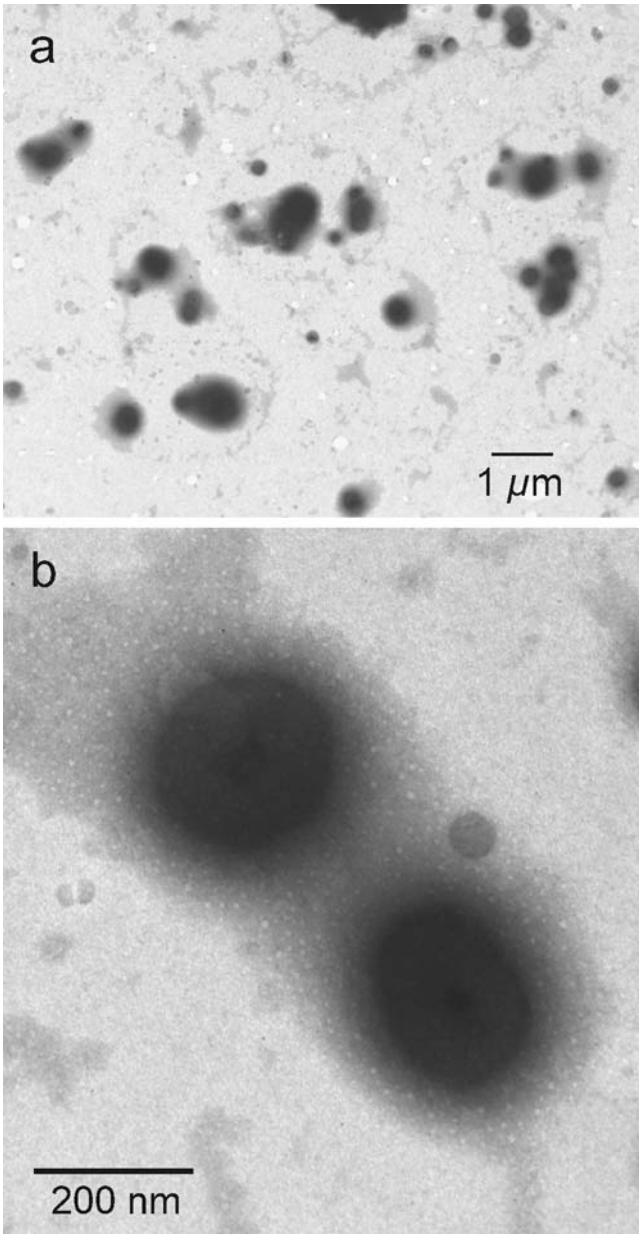

Fig. 4 Electron micrographs of *Dehalococcoides* sp. strain VZ (**a**, **b**). Image of a cell aggregate, the cell size varied between 200 and 500 nm (**a**). The central regions of higher contrast might indicate a biconcave shape of the cells (**b**)

sequential PCE dechlorination was carried out by two populations with different cell numbers and substrate specificity. Although the PCE-dehalogenating strain was present in higher dilutions, resulting in the formation of *cis*-DCE as the dechlorination end product, the activity of another population was required for complete dehalogenation to ethene. Furthermore, our results indicate that the reaction proceeds through *cis*-DCE as an interspecies intermediate and thus precludes the possibility that both populations compete for the same chlorinated substrates, i.e., PCE and *cis*-DCE.

The involvement of two dechlorinating populations is further supported by selective stimulation of the *cis*-DCE dechlorinating bacteria with filtered inoculum. Reductive dechlorination to ethene was only observed in those cultures where *cis*-DCE was present, which either originated from the initial spike (in the *cis*-DCE amended cultures) or was biogenically formed during partial PCE dechlorination. Our results may suggest selective stimulation of the *cis*-DCE dechlorinators by key trace components that are provided in the filtered supernatant in accordance with the findings of Maymo-Gatell et al. (1997). On the other hand, the cells of the known *Dehalococcoides* species are small, e.g., for strain 195 with a diameter of 400 to 500 nm and a height of 100 to 200 nm (Zinder and Dworkin 2006). Although passing of *Dehalococcoides* cells through 0.22 μm or 0.45 μm filters has not yet been reported, we observed a higher cell size variability of *Dehalococcoides* sp. strain VZ in our cultures, with a diameter of 200 to 500 nm. Therefore, it is possible that the stimulating effect of filtered supernatant for the dechlorination of *cis*-DCE to ethene in previously inactive cultures was caused by the passing of additional *Dehalococcoides* cells through 0.45 μm filters rather than by stimulation through nutrient factors in the supernatant. If a filter-assisted cultivation strategy would be also useful for other strains of *Dehalococcoides*, this method may offer a simple and easily implementable selective enrichment technique for laboratory cultures and intrinsic *Dehalococcoides* bacteria at field sites.

Of the substrates tested, we observed the highest dechlorination rates in batch cultures amended with benzoate, whereas cultivation with hydrogen, which is the ultimate electron donor for complete PCE dehalogenation, was less effective. According to the DGGE patterns, hydrogen and acetate promoted growth of a wider range of bacteria, phylogenetically similar to organisms with acetogenic and sulfate-reducing capabilities (Kuesel et al.

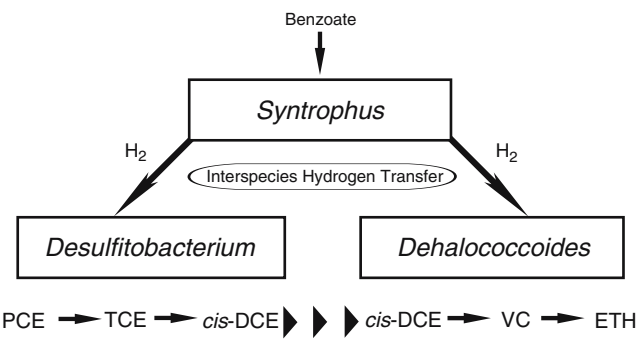

Fig. 5 Proposed interaction scheme of organisms mediating the sequential reductive dechlorination of PCE. Cultivation with benzoate and PCE favors the growth of a *Syntrophus* sp. that oxidizes benzoate and provides hydrogen to the dechlorinators. Acetate may support partial dechlorination of PCE. *cis*-DCE is the interspecies intermediate in the reductive conversion of PCE to ethene and has to be channeled to the *Dehalococcoides* organism

2001; Kaksonen et al. 2004). Thus, our findings support the assumption that catabolic intermediate substrates (such as benzoate) that generate a slow and steady release of hydrogen at a limited concentration may provide a competitive advantage to dehalorespiring bacteria resulting in sustained dechlorination and selective enrichment of dechlorinating bacteria.

The complete in situ reductive dehalogenation of chlorinated ethenes was demonstrated in the aquifer at the Victoria site using benzoate as the electron donor (Beeman et al. 1994). PCE-dechlorinating laboratory cultures from the same site, which were the basis for our current study, have been also fed with benzoate (Yang and McCarty 1998; Yang et al. 2005); however, the benzoate-oxidizing microorganisms have not been identified. The interaction of benzoate oxidizers with dehalogenating bacteria was first discovered in the classic example of the reductive 3-chlorobenzoate-dechlorinating consortium comprising *Desulfomonile tiedjei* (Dolfing and Tiedje 1986). Bacteria of the genus *Syntrophus* can obtain energy from the anaerobic oxidation of benzoate and other organic acids in syntrophic association with hydrogen-using microorganisms (Wallrabenstein et al. 1995). *Syntrophus*-like organisms were detected at organohalogen-contaminated sites where reductive dechlorination was observed (Dojka et al. 1998) and in chlorophenol-dehalogenating laboratory cultures (Becker et al. 2001). Becker et al. (2005) clearly demonstrated that *Syntrophus* is involved in the anaerobic degradation of 3-chlorobenzoate and 2-chlorophenol. In this study, we show that *Syntrophus* is part of a stable, defined PCE-dechlorinating consortium with *Desulfitobacterium* and a *Dehalococcoides* species as dehalogenating partners. Benzoate has been converted to hydrogen, acetate, and CO_2. The complete transformation of PCE and *cis*-DCE appeared to be dependent on hydrogen because acetate alone did not support the complete reduction of the spiked chlorinated ethenes.

Previous work suggested that the successive attack by different dechlorinating bacteria mediated the complete PCE reduction in the Victoria aquifer (Beeman et al. 1994). In support of this idea, in a PCE-dechlorinating chemostat derived from that aquifer, Yang et al. (2005) showed that both *Dehalococcoides* and *Desulfitobacterium* are present in the PCE to ethene dechlorinating cultures. Desulfitobacteria produce TCE or *cis*-DCE from tetrachloroethene (Löffler et al. 2003). The interaction of these versatile bacteria, which catalyze the initial steps of PCE dechlorination with *Dehalococcoides* spp. for sequential PCE dechlorination has not yet been demonstrated. Based on our data, we propose a metabolic network as illustrated in Fig. 5.

The 16S rDNA sequence of the identified *Dehalococcoides* was identical to the sequence of *Dehalococcoides* sp. strain VS that had been enriched from the same contaminated site. Recent work, however, shows that the comparison of 16S rRNA is of limited value for *Dehalococcoides* classification (Duhamel et al. 2004; Sung et al. 2006), and it remains unclear whether both strains have identical properties (substrate specificity and inventory of dehalogenase genes or other genome characteristics).

In fact, not all *Dehalococcoides* strains are able to convert chlorinated ethenes (Adrian et al. 2000). Our results indicate that also in case of *Dehalococcoides* strains that are able to transform chlorinated ethenes, the presence and activity of syntrophic organisms or other dehalogenating bacteria involved in the early dechlorination steps is equally important for the overall process. Therefore, our findings further challenge PCR detection of *Dehalococcoides*, regardless whether it is based on 16S rRNA/DNA or functional genes, as a sole tool for evaluating the potential for complete PCE dechlorination. Likewise, previous efforts have focused on the identification of the dechlorinating bacteria while syntrophic associations between dehalogenating and non-dehalogenating bacteria have been rarely studied. In particular, the role of *Syntrophus–Desulfitobacterium–Dehalococcoides* interactions for complete dehalogenation of chlorinated solvents has not been recognized so far.

It remains unknown whether *Syntrophus* is involved in the turnover of chlorinated solvents in aquifer ecosystems or in the reductive transformation of other organohalogen compounds where in situ reactions occur via benzoate or other slowly hydrogen-releasing compounds. If this is the case, the results may help to stimulate the conversion of persistent halogenated compounds in the environment and may offer guidance for the implementation and adjustment of bioremediation strategies.

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