FINAL REPORT

Factors Affecting Cis-Dichloroethene and Vinyl Chloride Biological Transformation Under Anaerobic Conditions

SERDP Project ER-1169

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FINAL REPORT

ER-1169
Factors affecting cis-dichloroethene and vinyl chloride biological transformation under anaerobic conditions

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Scope of this study

The chlorinated solvents, trichloroethene (TCE), tetrachloroethene (PCE), and carbon tetrachloride (CT) have been widely used by industry, the Department of Defense, and the Department of Energy as solvents for cleaning. Through leakage and poor disposal practices, these solvents have become the most frequent groundwater contaminants throughout the country, causing one of the most difficult and costly contamination problems for remediation. The biological anaerobic reductive dehalogenation of chlorinated aliphatic hydrocarbons (CAHs) such as PCE and TCE to *cis*-dichloroethene (cDCE) and vinyl chloride (VC) in groundwater was reported in the early 1980s. Further reduction of PCE and its intermediates to the harmless end product ethene was reported in 1989. Several pure cultures of anaerobic bacteria have been found to reductively dehalogenate PCE to cDCE. Rates of reduction of PCE and TCE to cDCE are high and the need for electron donor addition for the reactions is small.

However, the subsequent reduction of cDCE to VC, and then of VC to ethene is much slower and only one pure culture, at the time of the beginning of this grant, has been reported that is capable of reducing cDCE to VC or VC to ethene.
Therefore, overall objectives of this project have been:

- To develop a better understanding of the microorganisms, enzymes, and mechanisms involved in anaerobic reduction of cDCE and VC, and to develop molecular probes for monitoring these processes,
- To determine the kinetics involved in both processes,
- To evaluate potential chemical factors that may affect these processes, and
- To develop a field procedure for estimating the presence and rate of availability of electron donors for reductive dehalogenation.

Major findings

Molecular analysis of the microorganisms and enzymes involved in anaerobic reduction of cDCE and VC, and development of molecular probes

Reductive dehalogenation of vinyl chloride (VC) to ethene is the key step in complete anaerobic degradation of chlorinated ethenes. VC reductive dehalogenase was partially purified from a highly enriched culture of the VC-respiring *Dehalococcoides* sp. strain VS. The enzyme reduced VC and all dichloroethene (DCE) isomers, but not

tetrachloroethene (PCE) or trichloroethene (TCE), with comparable high rates. Using reversed genetics, the corresponding gene (*vcrA*) was isolated and characterized. Based on the predicted amino acid sequence, VC reductase is a novel member of the family of corrinoid/iron-sulfur cluster containing reductive dehalogenases. The *vcrA* gene was found to be co-transcribed with *vcrB*, encoding for a small hydrophobic protein presumably acting as membrane anchor for VC reductase, and *vcrC,* encoding for a protein with similarity to transcriptional regulators of the NosR/NirI family. The *vcrAB* genes were subsequently found to be present and expressed in other cultures containing VC-respiring *Dehaloccoides* organisms, and could be detected in water samples from a field site contaminated with chlorinated ethenes. Therefore, the *vcrA* gene identified here may be a useful molecular target for evaluating, predicting, and monitoring *in situ* reductive VC dehalogenation, which was subsequently confirmed by our experiments as well as the successful use of these molecular tools by GEOSYNTEC. The attached reprint provides more detailed information.

Vinyl Chloride and *cis*-Dichloroethene Dechlorination Kinetics and Microorganism Growth under Substrate Limiting Conditions

The reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) at contaminated sites often results in the accumulation of *cis*-1, 2-dichloroethene (DCE) and vinyl chloride (VC), rather than the non-hazardous end product ethene. This accumulation may be caused by the absence of appropriate microorganisms, insufficient supply of donor substrate or reaction kinetic limitations. Here, we address the issue of reaction kinetic limitations by investigating the effect of limiting substrate concentrations (electron donor and acceptor) on DCE and VC dechlorination kinetics and microorganism growth. For this, a model based on Monod kinetics, but also accounting for competitive inhibition between DCE and VC and the effect of low electron donor and acceptor (dual-substrate kinetics), was examined.

Model equations previously developed by us for DCE, VC and microorganism growth were used in this study. This model was previously found to describe DCE and VC dechlorination well with electron donor concentrations at non-limiting levels. However, the model was not evaluated with experimental data for the common field scenario of both electron acceptor and donor being rate limiting, nor were competitive inhibition kinetics between DCE and VC fully investigated.

<u>Competitive Inhibition</u>**.** DCE and VC were each found to be competitive to the utilization of the other. K_{ID} and K_{IV} were determined to be 3.6 ± 1.1 μM and 7.8 ± 1.5 μM, respectively, indicating that DCE (with a lower competitive inhibition value) has a more inhibitory effect on VC dechlorination than vice versa. In comparing the competitive inhibition model used here with the non-competitive inhibition model, competitive inhibition kinetics resulted in a lower sum of squares (SOS) value in a least-squares analyses, compared to non-competitive inhibition kinetics. For inhibition of VC with DCE the SOS values were 3.7 and 16.1 for competitive and non-competitive inhibition, respectively. Similarly, for inhibition of DCE with VC the SOS values were 3.0 and 15.2 for competitive and non-competitive inhibition, respectively. Thus, the competitive inhibition model provided a much better fit of the data. Inverse plots were

also evaluated and show similar near intersections of regression lines on the y-axis, which confirms competitive inhibition kinetics.

Hydrogen Half-Velocity Coefficient (K_H). An important indicator of a microorganism's ability to compete for hydrogen is the hydrogen half-velocity coefficient (K_H), however, conflicting values have previously been reported for dechlorinating microorganisms. Smatlak et al reported a relatively high value (100 ± 50 nM), indicating extremely slow growth under conditions of low electron donor. However, Ballagaprada et al. concluded that K_H was lower (9-21 nM), suggesting that dechlorinating microorganisms compete better than previously thought when hydrogen is limiting. To more accurately determine this key coefficient we investigated dechlorination at different hydrogen and DCE concentrations. The models were fit to the experimental data from both experiments using non-linear least squares analyses to determine the hydrogen half-velocity coefficient, resulting in a value of 7 ± 2 nM. This K_H value is similar to that found by Ballagaprada et al. and is significantly lower than that found by Smatlak et al, confirming that growth and dechlorination under lower hydrogen concentrations can be significant.

Substrate Limitations for Growth At a field site near a source of PCE or TCE contamination, the concentrations of DCE and VC are often very high such that supplementation with or presence of an electron donor will result in positive growth of dehalogenators and good dehalogenation, providing a seed DCE and VC dehalogenation population is present. However, as the contaminated plume moves away from the source and dehalogenation continues, PCE and TCE will disappear and the DCE plus VC concentration will drop. Model simulations suggest that as the combined concentration of DCE and VC then decreases below 100 μM, organism growth rate will decrease below the maximum rate to an extent that depends upon the hydrogen concentration present at that point. At some downgradient location in the plume then, the combined DCE, VC, and hydrogen concentrations will decrease to a level where organism growth rate will become negative. When that occurs, the dehalogenating population either cannot become established, or if bioaugmented, it cannot be sustained.

For example, the model indicates that growth will become negative at a DCE + VC concentration of 4 μM together with a hydrogen concentration of 4 nM (a typical concentration found in the field). Regardless of how high the hydrogen concentration, when the DCE plus VC concentration decreases below 0.7 μM, organism growth rate will be negative, and dehalogenation will not be sustainable (dehalogenation past this threshold is observed in batch experiments only because of a large added dechlorinating population, a scenario not typical at field sites). It is significant that 0.7 μM VC represents a concentration of 44 μg/L, well above the U.S. Environmental Protection Agency Maximum Contaminant Level for drinking water of 2 μg/L. Thus, while microbial reductive dehalogenation can have significant impact in reducing the concentrations of chlorinated ethenes in an aquifer, kinetic limitations of the microorganisms involved make it unlikely that concentrations can be reduced sufficiently to meet drinking water standards. The extent to which the VC concentration can be reduced is dependent significantly upon the hydrogen concentration that can be maintained. Thus, kinetic limitations need to be understood when considering biological dehalogenation through natural attenuation or engineered bioremediation at a field site. The simulation model presented and evaluated here should be a useful tool in helping to assess both the rate and extent of microbial dehalogenation of PCE and

TCE and the chlorinated daughter products in a contaminated aquifer, as well as the important boundary conditions that limit the extent of their removal.

Comparative Evaluation of Chloroethene Dechlorination to Ethene by *Dehalococcoides*-like Microorganisms

Reductive dehalogenation of tetrachloroethene (PCE), trichloroethene (TCE), *cis*-1,2-dichloroethene (DCE), and vinyl chloride (VC) was examined in four cultures containing *Dehalococcoides*-like microorganisms. Dechlorination and growth kinetics were compared using a Monod growth-rate model for multiple electron acceptor usage with competition. Included were the Victoria mixed culture containing *Dehalococcoides* species strain VS (from Victoria, TX), the mixed culture KB-1/VC (from Southern Ontario), the Pinellas mixed culture (from Pinellas, FL) and *Dehalococcoides ethenogenes* strain 195. All cultures, with the exception of *D. ethenogenes* strain 195 grew with VC as catabolic electron acceptor. A dilution method was developed that allows a valid comparison to be made of dehalogenating kinetics between different mixed cultures. Using this procedure maximum growth rates on VC were found to be similar for strain VS and KB-1/VC (0.42 to 0.49 ± 0.02 d^{-1}), but slower for the Pinellas culture (0.28 ± 0.01 d^{-1}). The 16S rDNA gene sequences were determined to ensure no cross contamination between cultures had occurred. Following enrichment of the VC dechlorinating microorganisms on VC, the cultures were amended with DCE, TCE or PCE. The three mixed cultures failed to dechlorinate PCE or did so very slowly. However, the dilution technique indicated all experienced growth on TCE and DCE, as well as on VC. Maximum growth rates on DCE alone were quite similar (0.43 to 0.46 d^{-1}), while the Pinellas culture grew faster on TCE alone (0.49 d^{-1}) than did the other two mixed cultures (0.33 to 0.35 d^{-1}). Half velocity and inhibition constants for growth on TCE were also determined for the three mixed cultures; both constants were found to be essentially equal and the same for the different cultures, varying between only 8.6 and 10.5 μM. The ability of the strain VS, KB-1/VC and Pinellas cultures to utilize TCE rapidly with conversion to ethene is quite different from that of any other reported microorganism. It was separately confirmed with more traditional cell-counting techniques that strain VS coupled TCE, as well as DCE and VC, utilization with growth. This is the first report of an organism obtaining energy for growth through every step in the reduction of TCE to ethene. Also, as suggested by the dilution technique, the dehalogenating organisms in the KB-1/VC and Pinellas cultures appear to obtain growth from TCE utilization as well. Such ability to grow while dehalogenating TCE to ethene will be an important advantage for their use in bioaugmentation

Chemical factors affecting the rate of cDEC and VC transformation

Previous experiments have shown that growth and VC dehalogenation rates of fresh culture can be enhanced by addition of a filter-sterilized supernatant from an actively growing culture. The supernatant was originated from mixed culture growing in bicarbonate buffer as the fresh one was grown in MOPS buffer which supports growth of dehalogenators' population. Attempts were made to characterize and quantify this phenomenon.

Several concentrations of filter-sterilized supernatant as well as 50kDa and 10kDa ultra-filtered supernatants were tested. Dehalogenation rates were measured and compared

with cultures that were grown either with supplemental reduced MOPS buffer or without additional supernatant. The highest ethylene formation rate of about 20 μmols ethylene/l*d (per bottle) was obtained in cultures supplemented with 20% supernatant, roughly double than the rate achieved with 10% supernatant. Very low rate was typically achieved when no supernatant was added. Similar rate of about 17 μmols ethylene/l*d (per bottle) was found with cultures supplemented with the filtrate of 50kDa supernatant, almost double than cultures with 50kDa retentate. However, no difference was observed between cultures supplemented with 10kDa filtrate or 10kDa retentate. Surprisingly, similar rate of about 17 μmols ethylene/l*d was found within cultures supplemented with 10% MOPS buffer instead of supernatant.

These results suggest that growth rate may be affected by several factors that exist in the supernatant, some of them might be molecule(s) smaller than 50kDa. Those factors may be produced by the dehalogenators or by other members in the consortium. However, it seems that with adequate growth conditions and redox potential, growth rate will be at the same order of magnitude as without supernatant presence

Major publications resulting from this research

Cupples AM, Spormann AM, McCarty PL 2003: Growth of a *Dehalococcoides*-like Microorganism on Vinyl Chloride and *cis*-Dichloroethene as Electron Acceptors as Determined by Competitive PCR. Appl. Environ. Microbiol. 69: 953-9.

Cupples AM, Spormann AM, McCarty PL.2004 : Vinyl chloride and *cis*-dichloroethene dechlorination kinetics and microorganism growth under substrate limiting conditions. Environ Sci Technol. 2004, 38(4):1102-7.

Cupples AM, Spormann AM, McCarty PL, 2004 "Comparative Evaluation of Chloroethene Dechlorination to Ethene by *Dehalococcoides*-like Microorganisms", Environ Sci Technol 38(18)4768-74.

Müller JA, Rosner BM, von Abendroth G., Meshulam-Simon G, McCarty PL, Spormann AM 2004: Molecular Identification of the Catabolic Vinyl Chloride Reductase from *Dehalococcoides* sp. strain VS and its Environmental Distribution, Appl. Environ.. Microbiol. 70(8):4880-8

Behrens S., Semprini L. Spormann AM 2005: Use of molecular methods to determine chloroethene transformation in soil column (manuscript in preparation)

Patents

We have obtained a patent for the use of the molecular tools we developed, entitled “Microbial Reductive Dehalogenation of Vinyl Chloride” and licensed this to GEOSYNTEC CONSULTANTS

PROJECT 1

Growth of a *Dehalococcoides*-like microorganism on Vinyl Chloride and *cis*-Dichloroethene as Electron Acceptors using Competitive PCR

Perfromer
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Summary

A competitive PCR (cPCR) assay targeting 16S rDNA was developed to enumerate a *Dehalococcoides*-like microorganism, bacterium VS, from a mixed culture derived from Victoria, Texas. The specificity, sensitivity and accuracy of the assay were investigated. The concentration of bacterium VS in the mixed culture, maintained in a continuously stirred tank reactor (CSTR), was determined with cPCR and found to be relatively constant, at 1.2 ± 0.4 X 10^7 copies/mL, over the ten-month analysis period. The constancy in CSTR cell concentration provides confidence in both the consistency of the DNA extraction method and the assay itself. Growth from the reductive dehalogenation of vinyl chloride (VC) and *cis*-1,2-dichloroethene (cDCE) was demonstrated, suggesting unique metabolic capabilities. The growth yield was 3.9 ± 1.9 X 10^8 copies/μmol Cl^- (n=19) for either chlorinated ethene as the electron acceptor and hydrogen as the electron donor (20 ± 2^0C). The maximum VC utilization rate ($\dot{q}$) ranged from 0.98-1.03 X 10^{-10} μmol Cl^-/(copy.day), indicating a maximum growth rate of 0.38-0.4 d^{-1}. These Y and $\dot{q}$ values agree well with values found by others for dechlorinating cultures. In summary, the cPCR assay enabled the investigation of kinetic parameters for one particular *Dehalococcoides*-like bacterium in a mixed culture and, more importantly, revealed the distinctive capabilities of bacterium VS to obtain energy for growth from VC dehalogenation.

INTRODUCTION

The widespread use and disposal of tetrachloroethene (PCE) and trichloroethene (TCE) has resulted in significant groundwater contamination (27). Remediation of such contaminated sites is often required because of the toxic nature of these chlorinated ethenes. A potential remediation strategy is the biological anaerobic reductive dechlorination of PCE and TCE to ethene, whereby the chlorinated ethene serves as the electron acceptor and hydrogen as an electron donor for the dehalogenating microorganism's energy metabolism. However, complete reduction to ethene is a problem due to the persistence of the reduction intermediates *cis*-1,2-dichloroethene (cDCE) and vinyl chloride (VC). Therefore, removal of cDCE to ethene is often the limiting process in utilizing reductive dechlorination as an approach for remediation. Understanding factors affecting transformation of these lesser chlorinated ethenes is hence critical for evaluating natural attenuation or for establishing engineering procedures for chlorinated solvent remediation. The only pure culture isolated to date that can dechlorinate PCE completely to ethene is *Dehalococcoides ethenogenes* strain 195 (18). This microorganism is able to grow via reductive dehalogenation using PCE, TCE or cDCE as catabolic electron acceptors, but dechlorination of VC is slow and possibly a cometabolic process. Due to this

limitation, investigating the final reduction of VC to ethene has become especially important, particularly as VC is a known human carcinogen.

Previous research on an enriched anaerobic culture seeded with aquifer material from a PCE-contaminated site in Victoria, TX, indicated a high transformation rate for VC and cDCE, with low rates of TCE and no PCE transformation (22). This research suggested that reductive dehalogenation of VC and cDCE by this mixed culture was different from previously reported reductive dehalogenations of PCE and TCE, with the high rates perhaps being symptomatic of growth. Analysis of 16S rDNA from this contaminated site (GenBank accession numbers AF388550, 11) and our culture (E. R. Hendrickson, personal communication) revealed the presence of a *Dehalococcoides* like microorganism. We suggest naming this microorganism bacterium Victoria Stanford (bacterium VS). Because of the difficulty associated with isolating the microorganism responsible for these high transformation rates, a competitive PCR (cPCR) assay was developed to quantify bacterium VS cell numbers during VC and cDCE reduction in mixed culture.

Competitive PCR was chosen for these investigations due to the many merits reported by others. The technique has been highly related to viable cell counts (16) and selective agar plate counts (13), demonstrating good accuracy. Also, good reproducibility was found (19) with low coefficients of variations between replicate samples (averaging 2.5%) (21). In addition, the method has been applied to a range of environmental samples, including soil (3, 13, 14, 20), estuarine sediments (20), rumen (21), skim milk (16) and microcosms (15).

Competitive PCR quantifies copy numbers of specific DNA templates, for example 16S rDNA or functional genes (3, 13, 19, 20, 23). Potential inconsistency due to PCR is reduced by coamplifying the target fragment with an internal standard (competitor DNA) (15, 21). The competitor DNA acts or competes in a similar manner as the target DNA in the PCR reaction (7), with the primers amplifying each with near equal efficiency. The technique involves PCR reactions for a range of known competitor DNA concentrations with a fixed amount of sample containing the target DNA. The amplified DNAs are then distinguished (and quantified) by agarose gel electrophoresis due to their difference in size. As the PCR amplifies both the sample template and the competitor DNA with the same efficiency, the ratios of the final amplification products (target: competitor DNA) reflect the ratios between the initial amounts of the two templates, allowing the amount of initial target DNA to be determined (7). We have developed a competitive PCR assay to determine cell numbers of the *Dehalococcoides*-like microorganism, bacterium VS, and applied the assay for the first documentation of growth of an organism through reductive dehalogenation of vinyl chloride.

MATERIALS AND METHODS

Chemicals. Liquid *cis*-1,2-dichloroethene (Aldrich Chem. Co., Milwaukee, WI) and VC (99+%, Scott Speciality Gases, Alltech Associates, Inc., Deerfield, IL) were used to prepare stock solutions and analytical standards. In addition, ethene (99+%, Scott Speciality Gases, Alltech Associates) was used as an analytical standard, benzoate (sodium salt, 99%, Aldrich Chem. Co.) as a substrate and sodium sulfide (Aldrich Chem. Co.) as a reducing agent.

Culture and growth conditions. A dehalogenating source culture was maintained in a closed continuously stirred tank reactor (CSTR) initially seeded with aquifer material from a PCE-contaminated site in Victoria, Texas. The reactor was anaerobically maintained, as indicated by a redox indicator (resazurin), at 20 (± 2) ^{0}C. A continuous anaerobic feed consisting of 5.9 mg L^{-1} Na_2S as a reductant, 1.7 mM sodium benzoate, 20 mg L^{-1} yeast extract, 0.5 mM TCE and trace nutrients in a basal medium (26) containing was delivered by a syringe pump at 67 mL/d, resulting in a 57 day retention time. Every three days 200 mL was removed to bring the reactor to liquid volume of 3.8 L. Complete dehalogenation of TCE to ethene occurred with all chloroethenes in the reactor remaining below 1 μM. The CSTR culture was periodically subjected to cPCR analysis.

Yield determination. Samples of CSTR culture were taken anaerobically from the reactor and purged with a mixture of 80% N_2 and 20% CO_2 to displace ethene. Batch studies were conducted in 120 mL bottles with dilutions of the CSTR culture (0, 0.5, 1, 3, 9 mL for samples with VC added and 0, 1, 9 mL for samples with cDCE added). Sufficient anaerobic media (as above without sodium benzoate or TCE) was added to bring the final volume to 60 mL with the bottle gas space being filled with a gas mixture (10%H_2, 10% CO_2, 80% N_2). Two septum liners (TFE/silicone, part number 95302, Alltech Associates) were crimped on top of the bottles, and allowed diffusion of hydrogen from an anaerobic glovebox (20 ± 2^{0}C) into the bottles (with minimal diffusion of chloroethenes or ethene out of bottles) resulting in a continuous hydrogen feed. Vinyl chloride (125μL of 99.5 % VC) was added using a gas tight syringe and cDCE (60 μl of cDCE solution) was added using a Hamilton syringe and the bottles were kept in the glovebox on a shaker. Abiotic controls with only media and chlorinated ethenes were used to demonstrate the culture was required for dechlorination. Controls that contained media and culture only, without chlorinated ethenes, were also included to illustrate that the presence of cDCE or VC was required for growth. Competitive PCR was performed on the original seed culture and within 1-2 days after dechlorination was complete.

Following complete dechlorination, three of the batch bottles were filled with a gas mixture (10% H_2, 10% CO_2, 80% N_2) and a 2 mL vial (containing the same gas mixture) with a septum allowing the outward diffusion of hydrogen from the vial, was also added to each bottle. The 2 mL vial was added to supply an extra source of hydrogen to the culture to promote maximum dechlorination rates. In addition, more VC was added (day 40) and competitive PCR was again performed within 1-2 days after dechlorination was complete.

VC growth curve. Two batch bottles were set up and maintained as described above with 1 mL inoculum of a rapidly dechlorinating culture (grown with high electron donor and acceptor) and media (59 mL). Vinyl chloride (99.5 %) was added with a gas tight syringe on day 0 (130 µL) and day 15 (250 µL). An abiotic control consisting of media (as above, 60 mL) and VC (130 uL) only, and a no VC control containing culture (1 mL) and media (59 mL), were also included. Competitive PCR was carried out on the inoculum (triplicate 1 mL culture samples), and on both the sample cultures and no VC control on day 12, 15, 17, 18 and 19 (single 1mL culture samples).

Maximum VC utilization rate ($\hat{q}$) and decay (b) determination. Monod kinetics (equation 1 and 2) were fitted, using non-linear least squares analysis (25), to the VC dechlorination data to determine values for $\hat{q}$ and b.

$$-\frac{dC}{dt} = \frac{\hat{q}XC}{K + C} \tag{1}$$

$$\frac{dX}{dt} = \frac{\hat{q}YXC}{K + C} - bX \tag{2}$$

Where C is the solution concentration (µM) of VC, X is bacterium VS concentration (copy/L), $\hat{q}$ is the maximum VC utilization rate (µmol/ copy.d) and K is the VC half-velocity coefficient, 2.6 µM (10). In addition, Y is yield (copy/µmol) and decay is represented by b (d^{-1}). Properties of the non-linear least squares estimates of $\hat{q}$ and b were analyzed following the method of Smith et al., (1998).

Decay (b) measurements. For a more direct measurement of the decay coefficient, a batch study was set up to measure dechlorination rates in cultures lacking electron acceptor (no growth conditions) for specified periods of time. Eight bottles were filled with 60 mL of mixed culture and four abiotic controls consisted of media (60mL). At day 0, 8, 14 and 21 the gas space of duplicate culture bottles and one control was filled with a gas mixture (10% H_2, 10% CO_2, 80% N_2) and VC (70 µL of 99.5 %) was added to each with a gas tight syringe. Following VC addition, VC removal and ethene formation were monitored. Hydrogen and VC levels remained high (> 3% H_2 and [VC]>>K) throughout this time (1.25 days). Competitive PCR was carried out on the culture samples at day 0 and at day 21.

Analytical methods. Analyses of ethene, vinyl chloride and *cis*-1,2-dichloroethene (250 µL of headspace) were performed with a temperature gradient (40 to 220°C) using a Hewlett-Packard model 5890 Series II gas chromatograph equipped with a flame ionization detector (Hewlett-Packard) and a GS-Q fused-silica capillary column (length, 30 m; inside diameter, 0.53 mm; J&W Scientific). Total mass values were determined with Henry's law constants (8).

DNA isolation. Genomic DNA was extracted from 1 mL of culture samples using a DNeasy® tissue system (Qiagen Inc, Valencia, CA), following the manufacture's instructions (final volume 200 µL). Replicate samples were taken from each culture to ensure consistency of

extraction. DNA was extracted (Qiagen Inc, Valencia, CA) from the CSTR culture periodically and subjected to cPCR to determine variations in culture concentration over time.

Amplification and cloning of *D. ethenogenes* 16S rRNA sequence. PCR was carried out on DNA extracted from the culture with forward primer DeF and reverse primer DeR (Table 1). Amplification reactions were performed in a total volume of 20 μL with PTC-200 Peltier

TABLE 1. Primers used in this study

Primers	Sequences (5' to 3')	Targeta
DeR	ACTTCGTCCCAATTACC	1406-1422, *D. ethenogenes* 16S rDNA
DeF	GCAAT TAAGATAGTGGC	49-65, *D. ethenogenes* 16S rDNA
VicF	TCACAGGGAAGAATAATGAC	402-422, *D. ethenogenes* 16S rDNA
CDeR	ACTTCGTCCCAATTACCGTCTCGCTAGAAAATTTAAC	1406-1422 and 1067-1086, *D. ethenogenes* 16S rDNA

Thermal Cycler (MJ Research). The reaction mixtures contained 0.5 μM of each primer (Operon) 200 μM of each deoxynucleoside triphosphate (Qiagen Inc), 1.5 units of *Taq* polymerase (Promega), PCR buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, $MgCl_2$) (Qiagen Inc) and Q solution (Qiagen Inc), 1μL of extracted DNA from the culture. The following PCR program was used: 95°C for 5 min; 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min (30 cycles); 72°C for 5 min. The PCR products were resolved by electrophoresis in 2% (wt/vol) agarose gel (Sigma) in TBE buffer, stained in ethidium bromide solution (5 μg/mL) for 20 min, followed by destaining in water for 40 min. The band, visualized upon UV excitation, was found to be of the appropriate size with a 1-kb DNA ladder (Gibco). The remaining PCR product was purified from contaminants (Qiagen Inc) and cloned into *E. coli* with a TOPO TA cloning® kit (Invitrogen Corporation, Carlsbad, CA). Plasmids were extracted from the cloned cells using a QIAprep® miniprep system (Qiagen Inc) and the insert was sequenced (PAN facility, Stanford, CA).

The resulting 16S rRNA gene sequences (1373 base pairs) obtained from the mixed culture match (100%) a sequence previously submitted to GenBank (GenBank accession AF388536, 11) and is similar to the sequence found at Victoria, TX, (GenBank accession AF388550, 11). These two sequences, differing only in 2 base pairs, have been placed into the Victoria sequence subgroup (11) and contain 14-16 base pair differences to *Dehalococcoides ethenogenes* (GenBank accession numbers AF004928).

Construction of competitor and target standards. The competitor was constructed via the approach of Celi et al. (1993) with primers VicF and CDeR (Table 1). The resulting amplicon contained the same priming sites as the target with a deletion (318 base pairs) at the 3' end. The reverse primer CDeR contained a sequence from the 3'end (positions 1406-1422), followed by a sequence from within the target (positions 1067-1086), allowing the amplification product to contain a deletion of 318 base pairs. The forward primer, VicF, was used to make the competitor and in all following competitive PCR analysis. The reverse primer for all competitive PCR analysis was DeR. Amplification with VicF and DeR resulted in 703 and 1021 base pair bands for the competitor and target, respectively.

The competitor was cloned into *E. coli* with a TOPO TA cloning® kit (Invitrogen Corporation, Carlsbad, CA). Plasmids were extracted from the cloned cells using a QIAprep®

miniprep system (Qiagen Inc) and were PCR amplified with the primers VicF and DeR. The PCR product was then extracted from agarose gel with a QIAquick® gel extraction kit (Qiagen Inc), stored at –20°C and kept as a stock solution for competitive PCR reactions. Target DNA was amplified from the dehalogenating culture DNA and similar to the competitor standard, the PCR products were cloned, extracted from plasmids, PCR amplified, extracted from gel, and stored at –20°C. Both stock solutions of competitor and target were quantified using a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA) calibrated with calf thymus and coumarin 311.

Primer design. The specificity of the selected primer set was examined with the PROBE-MATCH program of the Ribosomal Database Project (RDP) (17) and BLAST in GenBank (2). VicF and DeR were used for selective amplification of the 16S rDNA of bacterium VS from samples. Maximum annealing temperature to optimally amplify the target was empirically concluded to be 55°C (data not shown).

Competitive PCR. Amplification reactions were performed as described above with primers VicF and DeR, 1μL of extracted DNA from the sample and 1μL of competitor DNA. The PCR products were resolved in 2% (wt/vol) agarose gel (Sigma) in TBE buffer, stained in ethidium bromide solution (5 μg/mL) for 20 min, followed by destaining in water for 40 min. The bands were visualized by UV excitation, digital pictures were taken (Kodak DC290 zoom digital camera) and images were analyzed with Kodak ID image analysis software.

Sequencing of competitive PCR primer amplicons. Amplicons from the mixed culture with VicF and DR primers were separately cloned into *E. coli* with the TOPO TA cloning® kit (Invitrogen Corporation, Carlsbad, CA). Plasmids were extracted from the cloned cells using QIAprep® miniprep system (Qiagen Inc) and sequenced (PAN facility, Stanford, CA). The sequences were analyzed with DNAstar megaalign software.

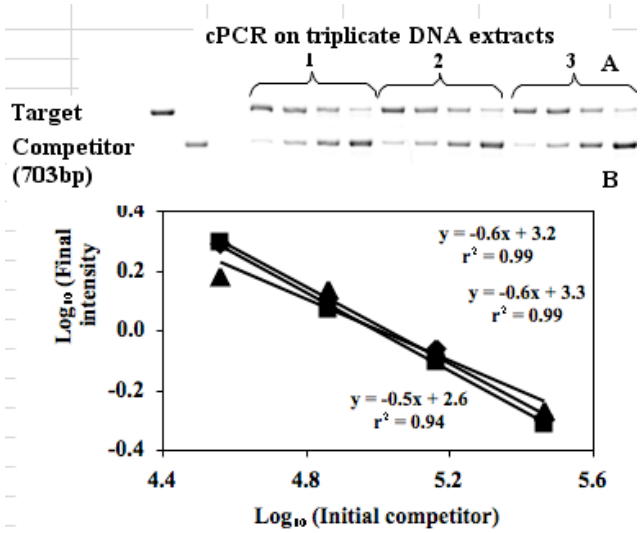

FIG. 1. cPCR on DNA extracted from three 1 mL samples of culture. Extracted DNA was coamplified with serially diluted competitor (2.9 X 10^5, 1.4 X 10^5, 0.7 X 10^5, and 0.4 X 10^5 copies/μL) and subject to gel electrophoresis. The first three lanes represent PCR on the sample only, followed by the competitor only, the blank, then cPCR on the three samples (triplicate DNA samples) (A). The ratio of the intensities of the target to the competitor for the triplicate samples (triangles, circles and squares each representing one triplicate sample) are plotted against the concentration of the competitor on a log scale (B).

RESULTS AND DISCUSSION

Quantification of *Dehalococcoides*-like bacterium VS. DNA was typically extracted from three 1mL samples of culture and competitive PCR was carried out on all three samples. The coefficient of variation (n = 15 assays) for a single measurement was 13% and for triplicate samples was 7%,

illustrating consistency in both DNA extraction and the competitive PCR assay itself. Figure 1 provides an example with three analyses, final copy number here was estimated at 2.6 ± 0.1 X 10^7 copy/mL mixed culture.

Competitive PCR is dependent on the equal amplification efficiency of the target and competitor sequences. The amplification efficiency was investigated for a range of PCR cycles (25-30) by starting with equal copy numbers of target and competitor (both from stock solutions), then determining the final ratio at cycle numbers 25, 26, 27, 28, 29 and 30. At all cycles, with equal initial concentration of competitor and target, the competitor was amplified more efficiently than the target (data not shown). Preferential amplification of a competitor or target can occur due to different lengths or because of differential denaturation (5). Competitive PCR was carried out on a range of target copy numbers to quantify and correct for the bias in amplification efficiency between the target and competitor. When the expected copy number based on the concentration of the cloned 16S rDNA gene was plotted versus the copy number calculated by the cPCR assay, the copy number was underestimated, but the relationship was linear with a slope of 0.76 (Figure 2) (standard error ± 0.037). This value was utilized as a correction factor for all cPCR assays. Such correction factors have been successfully used in other competitive PCR systems (6, 14).

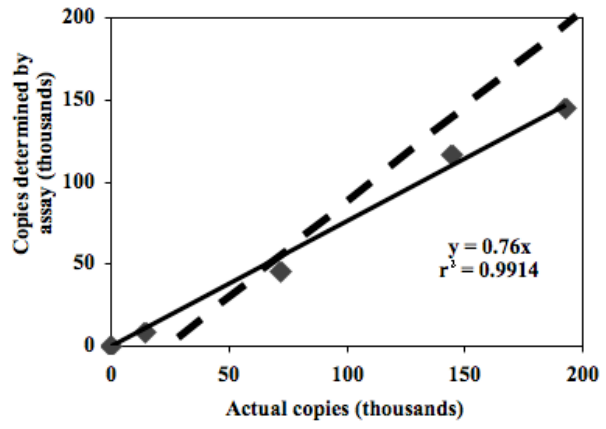

FIG. 2. Quantification of copies of 16S rDNA gene. Numbers of determined copies were determined via cPCR and compared to actual copy numbers (previously quantified using a fluorometer - see methods section). The dashed line represents a ratio of actual number of copies to determined number of copies 1:1.

To evaluate primer pair specificity, PCR (with primers VicF and DR) was carried out with the mixed culture DNA, the PCR products were cloned and ten were randomly selected for sequencing (PAN facility, Stanford, CA). Nine sequences illustrated 100% identity with the 16S rDNA previously obtained from the culture, indicating that the primers are specific to the target organism. The remaining sequence contained one base pair change, which was probably a result of PCR error. These results indicate that the cPCR primers VicF and DR are selective for the 16S rDNA of bacterium VS from the CSTR culture. It is important to note that these primers may not be selective for DNA taken from other environments containing microorganisms not present in the CSTR culture. Although the primers were checked for specificity against the current NCBI database, microorganisms perhaps exist that may hybridize to these primers but are not yet documented in the NCBI database. Notably, the primers may amplify other *Dehalococcoides* strains. Therefore, before applying this assay to samples from different environments, amplicons should be initially sequenced to ensure specificity, as was done here. The detection limit of the assay using 30 cycles was determined to be 2000 copies, equivalent to 4 X 10^5 copy/mL of mixed culture.

Competitive PCR was carried out with six either duplicate or triplicate 1 mL samples of CSTR culture over ten months. The resulting concentrations of bacterium VS remained relatively

constant over this time at 1.2 ± 0.4 X 10^7 copy/mL. The constancy in CSTR cell concentration provides confidence in both the consistency of the DNA extraction method and the assay itself.

Growth of bacterium VS through reductive dehalogenation. *D. ethenogenes* strain 195 is the only cDCE and VC dehalogenating pure culture isolated so far, however, it is difficult to grow and maintain, and hence to study. The ability to enumerate cDCE and VC dehalogenating organisms in mixed culture with competitive PCR therefore provides an opportunity to study the growth and kinetics of such microorganisms with greater ease and in the natural environment of a mixed culture.

Growth curves along with the corresponding change in mass of VC and ethene were obtained for duplicate cultures (Figure 3). The lines in Figure 3 represent numerical simulations of data discussed later. Mass balances ranged from 77-99%, with loss being attributed to VC leakage, as the abiotic control also exhibited VC loss. For all studies no dechlorination occurred in the abiotic controls and no increase in copy numbers above detection limits (4 X 10^5 copies/mL) was found in control cultures without chlorinated ethene addition, but was greater than 8 X 10^6 copies/mL in all bottles containing VC or cDCE. As expected from growth via VC dehalorespiration, a clear correlation occurs between increase in cell numbers and VC dechlorination (Figure 3). This is the first documentation, to our knowledge, of growth of a microorganism through reductive dehalogenation of VC.

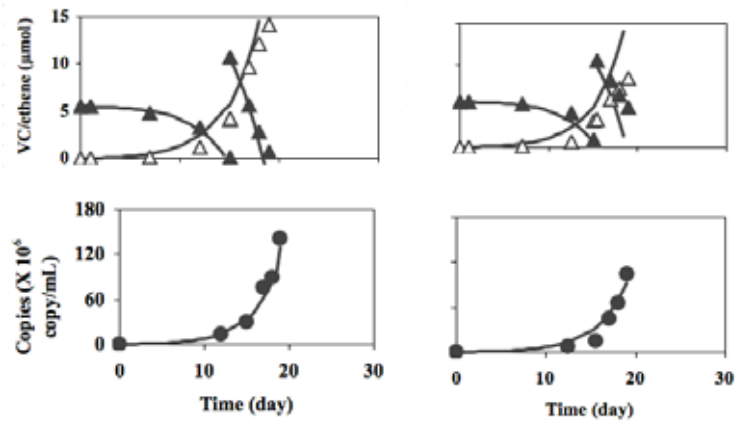

FIG 3. Vinyl chloride (closed triangles) removal and ethene (open triangles) formation (A and B) with the resulting increase in copy number (closed cirles) (C and D, respectively) in duplicate samples. Additional vinyl chloride (10.5 µmol) was added on day 15. Points are measured values and lines are those predicted by a non-linear least squares fit to the model (equation 1 and

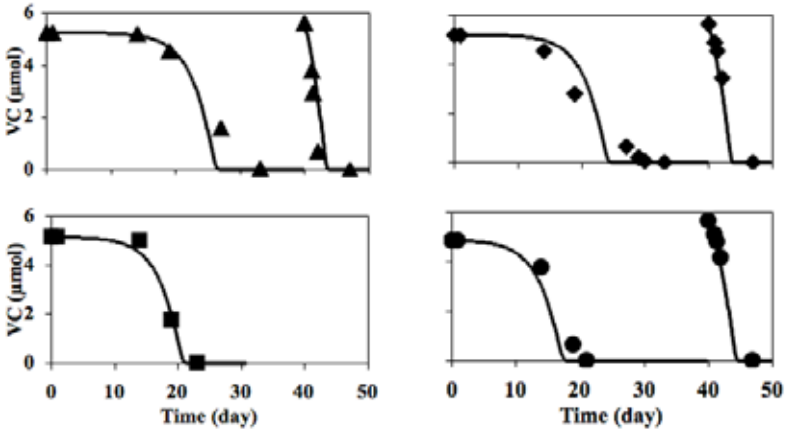

FIG 4. Non-linear least squares analysis fit of data to model (equation 1 and 2) to determine q and b. The thick lines illustrate the best fit of the model for each sample; 0.5 mL (A), 1 mL (B), 3 mL (C) and 9 mL of initial inoculum (8 X 10^6 copies/mL) (D), and the thin lines represent the best fit for all four samples combined. The measured data points for each sample are represented by triangles (A), diamonds (B), squares (C) and circles (D). Additional VC (5.6 µmol) was added on day 40 to 0.5 mL (A), 1 mL (B) and 9 mL (C) samples

In batch bottles containing a range of initial inoculum, time for complete dechlorination was dependent on initial

inoculum volume, hence the number of cells, in both VC (Figure 4) and cDCE (data not shown) amended bottles, indicating growth occurred through reductive dehalogenation. The lines in Figure 4 represent numerical simulations of data discussed later. Ethene mass balance indicated some loss occurred over the course of the incubation. Mass balances ranged from 73-86% in VC amended bottles. Initially, cDCE leakage was a major problem, however, further loss was essentially eliminated when bottles were turned upside down (Day 6). When the cultures were re-fed with VC (day 40) a faster dechlorination rate was observed compared to initial rates (Figure 4), indicating the presence of more dechlorinating cells. Copy numbers were obtained both after the initial VC and the additional VC were dechlorinated and on both occasions an increase in cells was found (data not shown), again providing evidence of growth. Growth yields were obtained from the increase in copy numbers and mass of ethene formed. Growth with either VC or cDCE as electron acceptor and hydrogen as the electron donor occurred with an average growth yield and standard deviation of $3.9 \pm 1.9 \times 10^{8}$ copies/μmol Cl^{-}, with a 95% confidence interval of 0.9×10^{8} copies/μmol Cl^{-} ($n = 19$).

Dechlorination kinetics. Using the determined growth yield, experimental data (VC, ethene and copy numbers, Figure 3) were fit to equation 1 and 2 with non-linear least squares analysis, to obtain a maximum utilization coefficient (9.8 and 1.03×10^{-10} μmol VC/copy·day for A/B and C/D) and decay coefficient (both 0.04 d^{-1}) for each culture. To apply this model (using 1.01×10^{-10} μmol VC/copy·day and 0.04 d^{-1}) to the experimental data in Figure 4, the initial inoculum was reduced to 1/10 of original to obtain the best fit. A possible explanation is the original inoculum as determined via competitive PCR was too high. The CSTR (source of the original inoculum) was maintained under low hydrogen levels resulting in an environment where the growth rate is low and decay is probably important. As the competitive PCR does not distinguish between live and dead cells (see below), dead cells may have artificially increased the original inoculum concentration. In contrast, the inoculum for the VC growth curves was an actively dechlorinating culture, growing under optimal conditions (high electron donor and acceptor), therefore the cell concentration determined via cPCR and the resulting maximum utilization and decay coefficients are considered accurate.

To determine the relationship between dechlorination activity and copy numbers and to more directly investigate decay, dechlorination activity was monitored in cultures lacking electron acceptor for specified periods of time. While there was a decrease in dechlorination activity over three weeks (Figure 5) there was no significant change in cell copy numbers ($3.4 \pm 0.4 \times 10^{10}$ and $3.7 \pm 0.4 \times 10^{10}$ copies/L at day 0 at day 21), suggesting the assay is targeting dead or unactive cells. Li and Drake (2001)

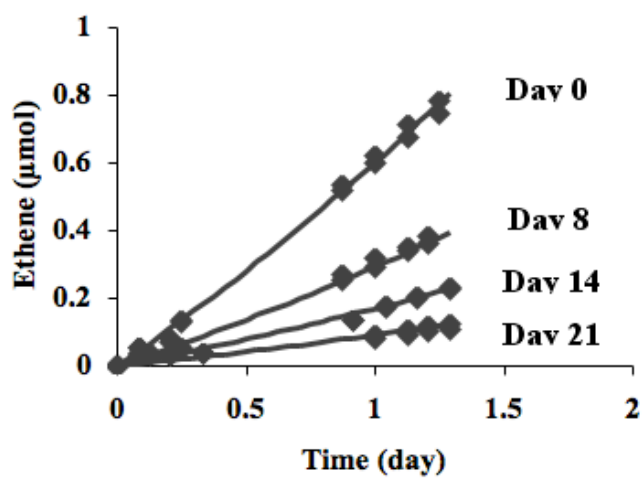

FIG. 5. Ethene formation following VC addition to duplicate cultures lacking of electron acceptor for 0, 8, 14, 21 days. Points (diamonds) are measured values and lines are those predicted by a non-linear least squares fit to the model (equation 1 and 2) to determine b.

also encountered the problem of DNA from dead cells possibly being responsible for higher than predicted bacterial numbers. These results suggest that copy number is not the best indicator of live cells under conditions were the growth rate is low and decay is important.

Equations 1 and 2 were fit to the data using non-linear least squares analysis to give a decay coefficient of 0.09 d^{-1}. The cell concentration from cPCR ($3.4 \pm 0.4 \times 10^{10}$ copies/L) was not used in the model, but was estimated (1.9×10^{10} copies/L) from the initial dechlorination rate (19.9 µmol VC/L·day) and the maximum specific dechlorination rate found previously (1.01×10^{-10} µmol VC/copy·day), as again, cPCR probably overestimated the number of active cells and dechlorination rates appear to be a more accurate description of active cells. Although it is not clear why the decay coefficient determined is higher than that obtained while the organisms were activity growing (0.04 d^{-1}), it demonstrates decay is an important for bacterium VS.

Yield and $\hat{q}$ values were converted to the more usual cell mass units assuming a conversion factor of 1.6×10^{-14} g cell/copy, based on estimates of 0.2 g cell dry weight/g cell wet weight, cell diameter of 0.5 µm (from electron micrograph of *D. ethenogenes*, 18) and one 16S rRNA gene copy/cell (from the *D. ethenogenes* sequence in GenBank). In addition, it was assumed that bacterium VS cell shape was spherical based on microscopic observation of the mixed and enriched culture containing bacterium VS and on literature describing *Dehalococcoides*-like microorganisms as irregular coccoid bacteria (1, 18). The resulting Y and $\hat{q}$ values (in cell mass units) are comparable to those found for other dechlorinating microorganisms (Table 2). It is of interest to note that the cell yield per mole of chloride released for bacterium VS is similar to that of *D. ethenogenes*. A maximum growth rate with VC (0.38-0.40 d^{-1}) was calculated from the product of $\hat{q}$ and Y, and is similar to that previously estimated by Haston (1999) for this culture (0.21-0.45 d^{-1}) from dechlorination curves from a range of initial culture inoculums.

TABLE 2. Comparison of growth yield and chloroethene utilization rate of dechlorinating cultures

	Dehalobacter restrictus	*Dehalospirillum multivorans*	*Dehalococcoides ethenogenes*	VS Strain[a]
Yield (g protein/mol Cl^-)	2.1	1.4	4.8	3.8
Utilization rate (nmol/min.mg protein)		50	69	71-75
Reference	Holliger et al., 1998	Scholz-Muramatsu et al., 1995	Maymo-Gatell et al., 1997	This study
Electron donor	Hydrogen	Hydrogen	Hydrogen	Hydrogen
Electron acceptor	PCE, TCE	PCE, TCE	PCE, TCE, cDCE	cDCE, VC

In previous studies (22), we found a strong correlation between increase in cell mass and VC dechlorination rate of this mixed culture, suggesting that growth of microbes in this culture is coupled to VC reduction. Here, we quantified growth of a *Dehalococcoides*-like microorganism, which is likely to be the dechlorinating microorganism, under VC-reducing conditions. The observed growth yield of 3.8 g protein/mol Cl^- released is consistent with growth rates of other

organo-halide respiring cultures and strongly suggests that this microbe uses VC-reduction with hydrogen as an electron donor as its catabolic reaction. Reductive dechlorination of VC is a thermodynamically favorable process with hydrogen as the electron donor (_$G^{0`}$ = -149 kJ/mol of VC) (22), it is therefore likely that VC reduction is coupled to electron transport phosphorylation.

An important finding through the use of this assay was the ability of bacterium VS to grow on vinyl chloride, in contrast to the cometabolic vinyl chloride dehalogenation exhibited by *D. ethenogenes* strain 195 (18). It appears that the different cDCE and VC dehalogenating strains with 16S rRNA similar to *D. ethenogenes* strain 195 have varying abilities for dechlorination and growth: bacterium VS exhibits high rates of cDCE and VC dechlorination, with little TCE dechlorination and no PCE dechlorination (22), whereas *D. ethenogenes* strain 195 dechlorinates cDCE, TCE and PCE well but with slow, only cometabolic, VC dechlorination (18). Such varying dechlorinating ability has been previously noted for another strain called CBDB1 sharing 98.3% similarity with *D. ethenogenes* strain 195 (1). However, in this case, the presence of chlorobenzene as an electron acceptor and hydrogen as the electron donor was essential for growth (1).

Microorganisms with 16S rRNA similar to that of *D. ethenogenes* are frequently found at contaminated sites and in mixed cultures undergoing complete dechlorination (11). Thus far, different strains can grow on the more chlorinated ethenes (*D. ethenogenes* strain 195), the lesser chlorinated ethenes (bacterium VS), or chlorobenzenes (1). We could therefore speculate that there are many different strains of *Dehalococcoides* with the ability to grow on a variety of different chlorinated compounds. The difficulty in isolating these microorganisms has hindered researchers in understanding their distinctive characteristics. The ability to enumerate these similar strains from mixed culture by cPCR should enable researchers to identify and quantify these unique growth abilities better.

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PROJECT 2

Vinyl Chloride and *cis*-Dichloroethene Dechlorination Kinetics and Microorganism Growth under Substrate Limiting Conditions

Performer
Alison M. cupples, Alfred M. Spormannn, and Perry L. McCarty

Summary

The reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE) at contaminated sites often results in the accumulation of *cis*-1, 2-dichloroethene (DCE) and vinyl chloride (VC), rather than the non-hazardous end product ethene. This accumulation may be caused by the absence of appropriate microorganisms, insufficient supply of donor substrate or reaction kinetic limitations. Here, we address the issue of reaction kinetic limitations by investigating the effect of limiting substrate concentrations (electron donor and acceptor) on DCE and VC dechlorination kinetics and microorganism growth by bacterium VS. For this, a model based on Monod kinetics, but also accounting for competition between electron acceptors for electrons from the donor and the effect of low electron donor and acceptor (dual-substrate kinetics), was examined. Competitive coefficients for VC (7.8 ± 1.5 μM) and DCE (3.6 ± 1.1 μM) were obtained and included in the model. The half velocity coefficient for hydrogen, the electron donor, was experimentally determined (7 ± 2 nM) through investigating dechlorination over different substrate concentrations. This complete model was then used, along with experimental data, to determine substrate concentrations at which the dechlorinating microorganisms would be in net decay. Notably, the model indicates net decay will result if the total electron acceptor concentration (DCE plus VC) is below 0.7 μM, regardless of electron donor levels. The ability to achieve sustainable bioremediation to acceptable levels can be greatly influenced by this threshold level.

Introduction

Biological reductive dechlorination is a potential remediation strategy for sites contaminated with the common groundwater pollutants, tetrachloroethene (PCE) and trichloroethene (TCE). One limitation of this approach is the slow removal of the reductive dechlorination intermediates *cis*-1, 2-dichloroethene (DCE) and vinyl chloride (VC), the latter a known human carcinogen. In attempting to obtain complete site remediation, it is therefore crucial to identify and understand the factors resulting in this slow removal. Key factors affecting the degradation of these contaminants are the growth characteristics of the dechlorinating microorganisms and their associated dechlorination kinetics under typical field conditions. To address this, we investigated and subsequently modeled DCE and VC dechlorination kinetics and microorganism growth under the common field scenario of limiting electron acceptor (DCE and VC) and electron donor (hydrogen).

A variety of dechlorination models exist, generally based upon Monod kinetics (*1-4*). A model developed by Haston (*1*), consisting of a combination of these models, was considered most suitable for our overall objective and culture characteristics. This model was previously found to describe DCE and VC dechlorination well with electron

donor concentrations at non-limiting levels. However, the model was not evaluated with experimental data for the common field scenario of both electron acceptor and donor being rate limiting, nor was the competition between DCE and VC for electrons from the donor fully investigated (*1*).

The model developed by Haston (*1*) has several benefits over other dechlorination models. One advantage is the inclusion of competition between DCE and VC for electrons, important because the dechlorinator under investigation (bacterium VS) uses both VC and DCE as electron acceptors in energy metabolism (*5, 6*), and co-contamination with them both is common. Another advantage is that the model also predicts growth to be dependent on both VC and DCE concentrations in an additive manner. To our knowledge, this is the first model to consider these important concepts together. Tonnaer et al. (*2*) used competitive kinetics and additive growth for PCE and TCE dechlorination, but not for DCE and VC dechlorination, as it was assumed that different microorganisms reduced these compounds. Fennel and Gossett (*3*) indicated that the same biomass was responsible for PCE, TCE and DCE dechlorination but that VC dechlorination was cometabolic, so again competitive kinetics between DCE and VC were not used. Similar to our mixed culture, Bagley (*4*) suggested that the same group of microorganisms was responsible for DCE and VC dechlorination, however he only considered competition to be important for VC dechlorination. Another benefit of the model used in the current study is the inclusion of dual-substrate kinetics with a hydrogen threshold (*3*), which enabled us to study the effect of both electron donor and acceptor concentrations and, furthermore, determine the concentrations for which growth would be limiting.

In the current study, DCE and VC competitive coefficients and the hydrogen half-velocity coefficient were determined. The complete model was then used to evaluate substrate concentrations that would be required for effective and continued DCE and VC removal. This was further investigated experimentally. The model should be useful for predicting dechlorination under substrate limiting conditions when the microorganisms involved exhibit competitive kinetics between DCE and VC. With this approach the limitations to chlorinated ethene remediation at contaminated sites under such conditions can be evaluated.

Materials and Methods

Chemicals. Liquid *cis*-1,2-dichloroethene (97% Aldrich Chem. Co., Milwaukee, WI), and gaseous VC (99.5%, Fluka, Switzerland) were used to prepare stock solutions and analytical standards. In addition, ethene (1000 PPM and 100 PPM, Scott Speciality Gases, Alltech Associates) and VC (1000 PPM and 10 PPM, Scott Speciality Gases, Alltech Associates) were used as analytical standards, benzoate (sodium salt, 99%, Aldrich Chem. Co.) as a substrate, and sodium sulfide (Aldrich Chem. Co.) as a reducing agent.

Analytical Methods. Analyses of ethene, VC and DCE were performed with a temperature program (40 to 220°C) using a Hewlett-Packard model 5890 Series II gas chromatograph equipped with a flame ionization detector (Hewlett-Packard) and a GS-Q fused-silica capillary column (length, 30 m; inside diameter, 0.53 mm; J&W Scientific). A reduction gas analyzer (Trace Analytical, Inc., Menlo Park, CA) was used to measure

hydrogen. Solution concentrations and total mass were calculated using Henry's Law Constants (*7*).

Culture. A dehalogenating source culture, initially seeded with a culture derived from aquifer material from a PCE-contaminated site in Victoria, Texas, was maintained in a closed continuously stirred tank reactor (CSTR). The reactor was anaerobically maintained, as previously described (*5*) and was either used with no dilution, or provided the seed for batch studies. Based on extensive 16S rDNA analyses made over several years, the culture has contained only a single *Dehalococcoides s* strain called bacterium VS (*5*). Previous research indicated that bacterium VS can use both DCE and VC as electron acceptors for growth (*5,6*).

Competition for electrons. Two experiments were conducted to investigate competition between DCE and VC for electrons from the donor. To ascertain the effect of DCE on VC dechlorination, batch bottles (120 mL) were filled with 40 mL of anaerobic media (as previously described without sodium benzoate or TCE, *5*) in an anaerobic chamber (10%H_2, 10% CO_2, 80% N_2). The gas phase of the batch bottles were filled with the anaerobic chamber atmosphere so that hydrogen (10%) could serve as the electron donor. Each bottle was inoculated with 20 mL of a rapidly dechlorinating culture grown from a small inoculum (1 mL CSTR culture) under optimal conditions (>3% H_2, and >40 μM VC).Gaseous VC (99.5 %) was added with a gas tight syringe (9 to 79 μL) to two sets of batch bottles to provide each set with a range of initial VC concentrations. To investigate the effect of DCE on VC dechlorination an aqueous solution of DCE was added by syringe (20 μL) to one set of batch bottles.

To ascertain the influence of VC on DCE dechlorination, the above cultures (following completion of the above experiment) were purged (20% CO_2, 80% N_2) to displace ethenes and mixed together in an anaerobic chamber where they were redistributed to clean batch bottles. An aqueous solution of DCE was added by syringe (3.4 to 30.5 μL) to two sets of batch bottles to provide each set with a range of initial DCE concentrations. To investigate the effect of VC on DCE dechlorination gaseous VC (99.5 %) was added (115 μL) to one set of batch bottles.

In both experiments, hydrogen was non-limiting (> 3%) and dechlorination rates (formation of dechlorination product) were measured over 8 hours. Each dechlorination rate was determined using a regressions line ($r^2 > 0.98$) through three data points. In all experiments, an abiotic control with media, VC, and DCE was included to demonstrate the culture was required for dechlorination. All bottles were sealed with a butyl rubber stopper and aluminum seal (Bellco Glass, Inc., Vineland, NJ). For each study, following the addition of the chlorinated ethenes, the cultures were moved to a shaker (60-80 RPM) where they remained throughout the experiment (20 ± 2°C). In all batch studies, headspace samples were periodically removed (250 μL) for chlorinated ethene and hydrogen analyses.

The Effect of Hydrogen on Dechlorination. The influence of the concentration of hydrogen, the electron donor, was investigated for both VC dechlorination and DCE dechlorination. These studies were conducted in bottles (120 mL) with 60 mL (DCE batch study) or 30 mL (VC batch study) of the CSTR culture taken anaerobically from the reactor. The cultures were then purged (80% N_2, 20% CO_2) to displace ethene. Different hydrogen feed rates and thus, concentrations, were obtained by adding 1 to 4 small vials (2 mL) (Part number 98380, Alltech Associates), each containing a gas

mixture (10% CO_2, 10% H_2, 80% N_2) to the bottles. Hydrogen diffused through the vial septum (TFE/Butyl liners, Part number 98380, Alltech Associates) to provide a continuous hydrogen supply to the culture. When needed, an aqueous solution of DCE (70 µL) was added by syringe and gaseous vinyl chloride (99.5 %) was added with a gas tight syringe (55 µL).

Hydrogen Half-Velocity Coefficient (K_H). Dechlorination over a range of electron donor and acceptor concentrations was investigated to determine K_H. Experiment 1 contained five different, duplicated treatments, consisting of two concentrations of DCE and three of hydrogen. DCE was added in an aqueous solution by syringe (7.6 or 34 µL) and different hydrogen levels were obtained by adding different vial numbers (as above) to the cultures. Experiment 2 consisted of six different, duplicated, treatments with three different concentrations of DCE and two concentrations of hydrogen. DCE was added in an aqueous solution by syringe (3.8, 7.6 or 30 µL) and different hydrogen levels were obtained as above.

The initial active dechlorinating cell concentrations for these experiments were determined by subjecting duplicate cultures to non-limiting substrate levels, and measuring the rate of product formation. From these data, the active cell concentration was obtained by dividing the product formation rate (µmol L^{-1} d^{-1}) by the maximum utilization coefficient (µmol $cell^{-1}$ d^{-1}) ($\hat{q}$) previously determined for bacterium VS (*5*). The mean determined cell concentration was then the starting cell concentration for use in the model to estimate K_H.

Dechlorinating Biomass. To confirm the model's prediction of dechlorinating biomass, the active cell concentration of bacterium VS was experimentally determined at the end of the above two experiments (day 8 for experiment 1 or day 8 or 14 for experiment 2). At this time, the cultures were purged (20% CO_2, 80% N_2), and then supplied with non-limiting concentrations of electron donor and acceptor. Initial product formation rates were determined, and from this active cell concentrations were calculated as above. Resulting cell concentrations where then compared to cell concentrations predicted by the model.

Dechlorination Model. The model equations for DCE, VC and microorganism growth used in this study are, respectively;

$$-\frac{dDCE}{dt} = \left(\frac{\hat{q}\ X\ DCE}{DCE + K_D\left(1 + \frac{VC}{K_{IV}}\right)}\right)\left(\frac{(H - H^*)}{(H - H^*) + K_H}\right) \quad (1)$$

$$-\frac{dVC}{dt} = \left[\left(\frac{\hat{q}\ X\ VC}{VC + K_V\left(1 + \frac{DCE}{K_{ID}}\right)}\right) - \left(\frac{\hat{q}\ X\ DCE}{DCE + K_D\left(1 + \frac{VC}{K_{IV}}\right)}\right)\right]\left(\frac{(H - H^*)}{(H - H^*) + K_H}\right)$$

(2) $$\frac{dX}{dt} = \mu X\left(\frac{VC}{VC + K_V\left(1 + \frac{DCE}{K_{ID}}\right)} + \frac{DCE}{DCE + K_D\left(1 + \frac{VC}{K_{IV}}\right)}\right)\left(\frac{(H - H^*)}{(H - H^*) + K_H}\right) - bX$$

(3)

Where $\hat{q}$ is the maximum utilization coefficient (μmol cell $^{-1}$ d^{-1}), X is the concentration (cell L^{-1}) of bacterium VS (therefore, $\hat{q}$ X is the maximum utilization rate on a liquid basis, μmol L^{-1} d^{-1}), b is the decay rate (d^{-1}), μ is the maximum growth rate (d^{-1}). K_V and K_D are the half-velocity coefficients for VC and DCE (μM), values for which were determined previously for bacterium VS (10) as listed in Table 1.

TABLE 1. Growth, decay and substrate utilization kinetic coefficients for the reductive dechlorination of DCE and VC by bacterium VS. 95% confidence intervals for some coefficients are also displayed.

Coefficients	Units	DCE	VC	Reference
Half-velocity (K_D, K_V)	μM	3.3 ± 2.2	2.6 ± 1.9	*(10)*
Max. utilization * Bacterium VS concentration ($\hat{q}$ X)	μmol Cl^- $(L\cdot d)^{-1}$	14.3 ± 1.6	10.8 ± 1.1	Fig 1
Competition (K_{ID}, K_{IV})	μM	3.6 ± 1.1	7.8 ± 1.5	Fig 1
Max. utilization ($\hat{q}$)	μmol Cl^- $(cell\cdot d)^{-1}$	7.8 X 10^{-10}	7.8 X 10^{-10}	*(5)*
Max. growth rate (μ)	d^{-1}	0.4	0.4	*(5)*
Decay rate (b)	d^{-1}	0.09	0.09	*(5)*
H_2 half-velocity (K_H)	nM	7 ± 2	7 ± 2	Fig 4
Initial Bacterium VS concentration (X)	cell L^{-1}	5 X 10^9 6 X 10^9	5 X 10^9 6 X 10^9	Fig 4A-E Fig 4F-G

For competition between DCE and VC for electrons from the electron donor, the model was formulated similar to that for enzyme competitive inhibition, although it is

recognized here that competition for electrons from the electron donor is mechanistically different from that of competition for a given enzyme. How well this model fits the case in point was investigated. K_{ID} and K_{IV} are the competitive coefficients for DCE and VC (μM) and K_H is the hydrogen half-velocity coefficient (nM) (*5, 10,* Table 1). DCE and VC are the solution concentrations of VC and DCE (μM) and H is the solution hydrogen concentration (nM) Mass additions of DCE and VC and hydrogen head space concentrations were converted to solution concentrations through knowledge of head space and liquid volumes and use of dimensionless Henry's coefficients, as previously discussed (5). H* is the hydrogen threshold concentration for dehalogenation (nM). Several researchers have reported H* values: Fennel and Gossett (*3*) suggested it was at least as low as 1.5 nM; Yang and McCarty (*11*) observed a threshold of 2.2 ± 0.9 nM; and Smatlak et al. (*12*) indicated a value of less than 2 nM. Based on the lowest hydrogen concentration measured during dechlorination in the current study we set H* equal to 0.9 nM. Hydrogen concentration was measured every 1-4 days and not for every time step (0.5 day) used in the model.. Hydrogen concentrations for use in the model at intermediate time steps were taken to be an average of values measured before and after that time step.

Modeling Approach. Kinetic coefficients were determined using non-linear least squares analysis with an excel spreadsheet method previously developed for the evaluation of biochemical reaction rate coefficients (9). The method allows calculation of 95% confidence intervals for the determined coefficients. Using this approach, the VC and DCE dechlorination data (without competitor present) from the competitive for electrons experiments were fit to Monod kinetics (equation 1 and/or 2 without competitive kinetics or dual-substrate kinetics) to determine the maximum utilization coefficient on a liquid volume basis ($\hat{q}X$) for each. Then, (using the appropriate $\hat{q}X$ value), the competitive model (equation 1 and/or 2 without dual-substrate kinetics) was fit to the experimental data for VC and DCE with the competitive acceptor present to obtain the appropriate competitive coefficients (K_{IV} and K_{ID}) along with their 95% confidence intervals. Therefore, for each experiment the non-linear least squares analysis contained only one unknown (either $\hat{q}X$ or K_I) reducing the uncertainty associated with the results. Growth (equation 3) was not included in these calculations as the experiments lasted less than 8 hours during which little growth would have occurred.

Using these competitive coefficients, the hydrogen half-velocity (K_H) coefficient was then determined by fitting the entire model (equations 1, 2 and 3) to the dechlorination data over a range of electron acceptor and donor concentrations. The initial bacterium VS cell concentration (X) for this modeling phase was determined from dechlorination activity assays (as described above).

Results and Discussion

Evaluation of Competitive Kinetics. Research with our culture indicated that DCE and VC were dechlorinated at similar rates (*13*), and that growth of bacterium VS occurred with either of these compounds (*5*). Additionally, a partially purified VC-reductase from a culture, highly enriched (>99%) with bacterium VS, reduced DCE and VC at similar rates (*6*).

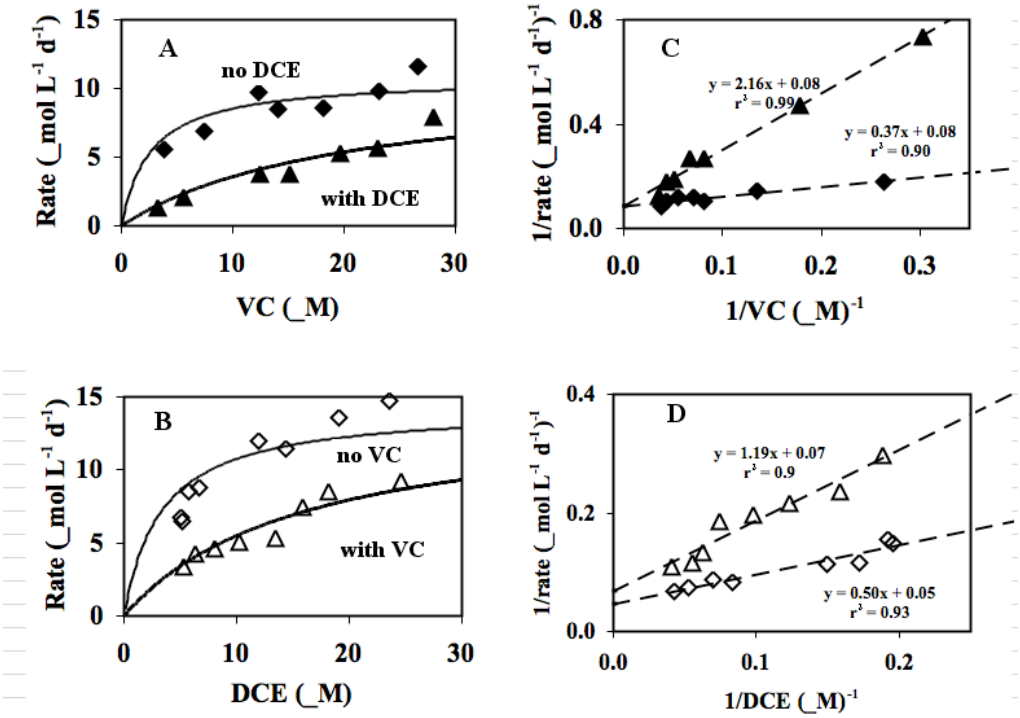

FIGURE 1. (A) VC dechlorination over a range of initial VC concentrations, with DCE (24 _M) (solid triangles), and without (solid diamonds). (B) DCE dechlorination over a range of initial DCE concentrations with VC (30 _M) (open triangles), and without (open diamonds). For each experiment, the lighter and the heavier lines (A and B) represent the best fit of the model (equations 1 and 2) to determine the maximum utilization coefficient (_mol Cl^- $(L\cdot d)^{-1}$) and the competitive coefficient (_M), respectively. Lineweaver-Burk plots for these data; (C) VC dechlorination and (D) DCE dechlorination (D).

DCE and VC were each found to be competitive to the utilization of the other. K_{ID} and K_{IV} were determined to be 3.6 ± 1.1 μM and 7.8 ± 1.5 μM, respectively (Figure 1A and B), indicating that DCE (with a lower competitive coefficient) has a more inhibitory effect on VC dechlorination than vice versa. In comparing the competitive model used here with one similar to the non-competitive inhibition enzyme model, a lower sum of squares (SOS) value was obtained in a least-squares analyses for the competitive coefficient compared to the non-competitive coefficient. For the effect of DCE on VC utilization, the SOS values were found to be 3.7 16.1 for competitive and non-competitive kinetics, respectively. Similarly, for the effect of VC on DCE utilization the SOS values were 3.0 and 15.2 for competitive and non-competitive kinetics, respectively. Thus, the competitive model provided a much better fit of the data. Inverse plots (Figure 1C and D) were also evaluated and show similar near intersections of regression lines on the y-axis, which suggests a good fit with the competitive kinetics model.

The Effect of Hydrogen on Dechlorination. Hydrogen levels affected both VC (Figure 2) and DCE (Figure 3) dechlorination trends. Even over the narrow hydrogen ranges investigated (5 - 13 and 5 - 11 nM) a noticeable difference in dechlorination rate is apparent, indicating that available hydrogen concentration at contaminated sites can have a large impact on dechlorination rates. Model predictions illustrated with experimental results in Figures 2 and 3 are discussed below.

Hydrogen Half-Velocity Coefficient (K_H). The ability to compete for electron donor greatly influences the survival and growth of microorganisms at contaminated sites. An important indicator of a microorganism's ability to compete for hydrogen is the hydrogen half-velocity coefficient (K_H), however, conflicting values have previously been reported for dechlorinating microorganisms. Smatlak et al (*12*) reported a relatively high value (100 ± 50 nM), indicating extremely slow growth under conditions of low electron donor. However, Ballagaprada et al. (*14*) concluded that K_H was lower (9-21 nM), suggesting that dechlorinating microorganisms compete better than previously thought when hydrogen is limiting. To more accurately determine this key coefficient we investigated dechlorination at different hydrogen and DCE concentrations. Using the determined initial active cell concentration for the culture used in these experiments (5 X 10^9 and 6 X 10^9 cells L^{-1} for experiments 1 and 2), the models (equations 1, 2 and 3) were fit to the experimental data from both experiments using non-linear least squares analyses to determine the hydrogen half-velocity coefficient, resulting in a value of 7 ± 2 nM (Figure 4). This K_H value is similar to that found

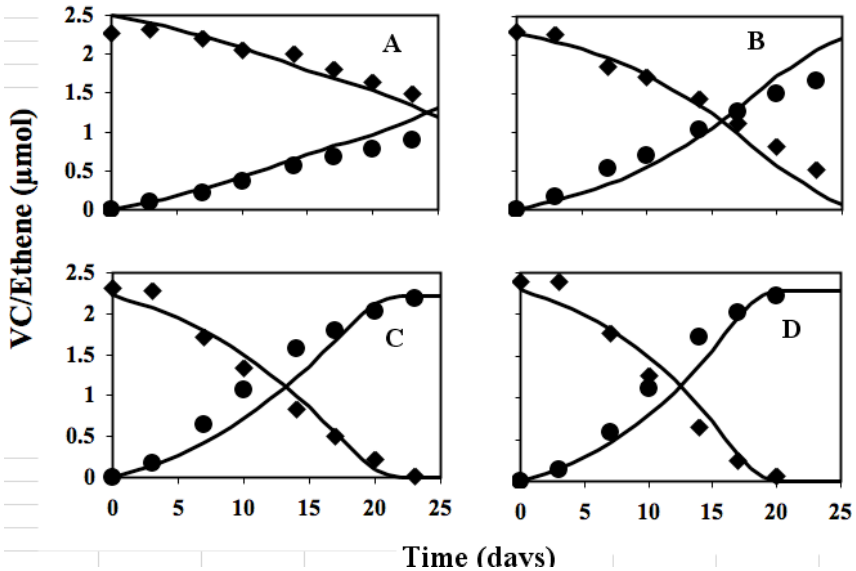

FIGURE 2. Dechlorination of VC over a range of hydrogen concentrations as follows: (A) 5 ± 1 nM, (B) 8 ± 2 nM, (C) 11 ± 2 nM and (D) 13 ± 2 nM. Points represent measured masses of VC (diamonds) and ethene (circles), and lines represent best fits of the data to the model (see discussion).

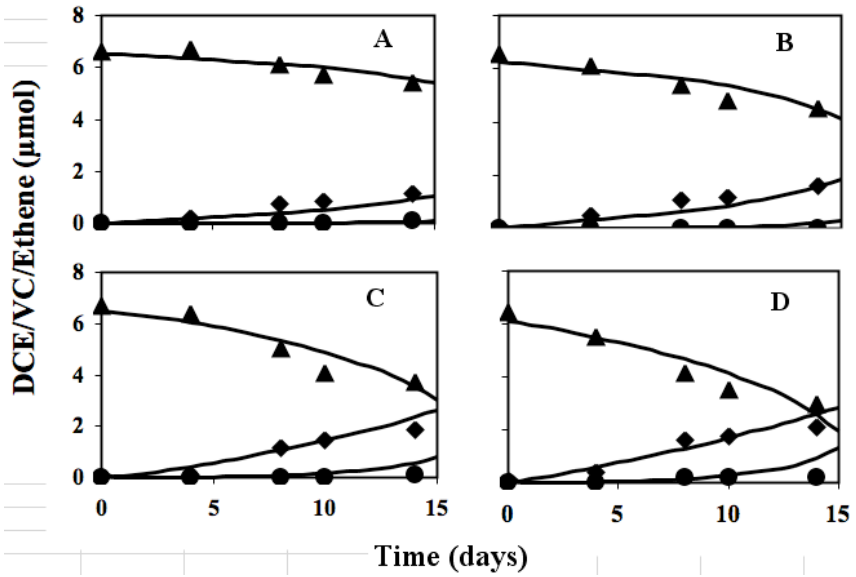

FIGURE 3. Dechlorination of DCE over a range of hydrogen concentrations as follows: (A) 5 ± 2 nM, (B) 8 ± 4 nM, (C) 9 ± 3 nM and (D) 11 ± 6 nM. Points represent measured masses of DCE (triangles), VC (diamonds) and ethene (circles), and lines represent best fits of the data to model (see discussion).

by Ballagaprada et al. (*14*) and is significantly lower than that found by Smatlak et al (*12*), confirming that growth and dechlorination even under very low hydrogen concentrations can be significant.

The determined K_H value was used to predict DCE and VC dechlorination rate in the previously discussed experiments (Figures 2 and 3). However, because the initial cell concentrations were not measured, the initial cell concentrations were the fitting parameters, resulting in values of 4 X 10^9 and 3 X 10^9 cells L^{-1} (Figures 2 and 3, respectively).

Dechlorinating Biomass The dechlorinating cell concentrations at the end of the above two experiments, were experimentally investigated using the dechlorination activity assay and compared to those concentrations predicted by the model (Figure 5). For each experiment, the initial cell concentration is depicted by a square. Final cell concentrations below this value represent cultures that experienced a net decline in population and those above represent those that experienced a net increase over the study period. Most measured concentrations (perhaps except at the higher concentrations) fall close to the predicted values, providing reasonable confidence in model predictions.

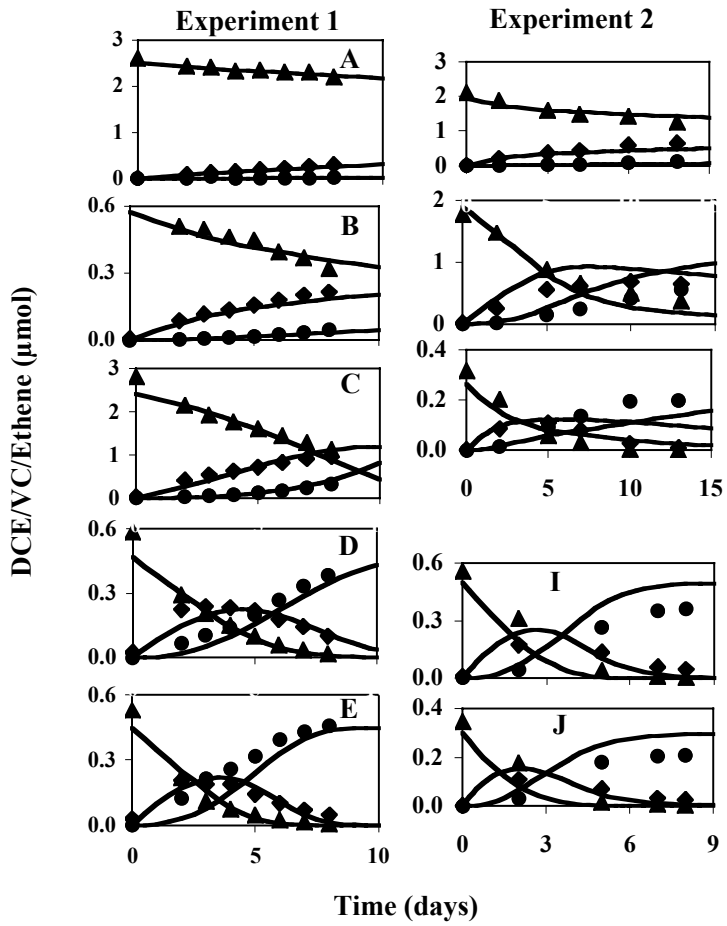

FIGURE 4. Dechlorination over a range of DCE and hydrogen concentrations, respectively, as follows (with DCE masses converted using Henry's law to respective concentration units): Experiment 1, (A) 37 µM and 2 ± 0.4 nM; (B) 9 µM and 2 ± 0.6 nM; (C) 36 µM and 13 ± 7 nM; (D) 7 µM and 11 ± 3 nM; (E) 7 µM and 25 ± 6 nM; Experiment 2, (F) 30 µM and 4 ± 2; (G) 28 µM and 18 ± 8 nM; (H) 5 µM and 3 ± 2 nM; (I) 7 µM and 20 ± 11 nM; (J) 4 µM and 10 ± 9 nM. Points represent measured means from duplicates of DCE (triangles), VC (diamonds) and ethene (circles). Lines represent best fits of the data to model to determine K_H. Note differences in axis scales.

An important parameter for the model's estimation of biomass, thus dechlorination rates, is the decay coefficient. For these investigations the decay coefficient used was based on previous work with this culture (5). A higher decay coefficient would result in a slower overall growth rate, lower dechlorination rate, and hence higher substrate concentrations required for positive growth, whereas a lower decay coefficient would have the opposite effect. Thus, good predictions of substrate

limitations require that the decay coefficient be known with reasonable confidence.

Substrate Limitations for Growth At a field site near a source of PCE or TCE contamination, the concentrations of DCE and VC are often very high such that supplementation with or presence of an electron donor will result in positive growth of dehalogenators and good dehalogenation, provided a seed DCE and VC dehalogenation population is present. However, as the contaminated plume moves away from the source and dehalogenation continues, PCE and TCE concentrations will decrease to zero and the DCE plus VC concentration will drop. The model simulation results in Figure 6 suggest that as the combined concentration of DCE and VC then decreases below 100 µM, organism growth rate will decrease below the maximum rate to an extent that depends upon the hydrogen concentration present at that point. At some downgradient location in the plume then, the combined DCE, VC, and hydrogen concentrations will decrease to a level where organism growth rate will become negative. When that occurs, the dehalogenating population either cannot become established, or if bioaugmented, it cannot be sustained. Consequently, unless some other degradation process such as aerobic degradation occurs or an engineered approach that overcomes this limitation is invoked, complete site remediation will fail due to the persistence of DCE and VC. One engineered approach that has proven successful is recycling of the downgradient plume, with below population sustaining concentrations, to a location up gradient where high population-sustaining donor and acceptor concentrations are present (*15*).

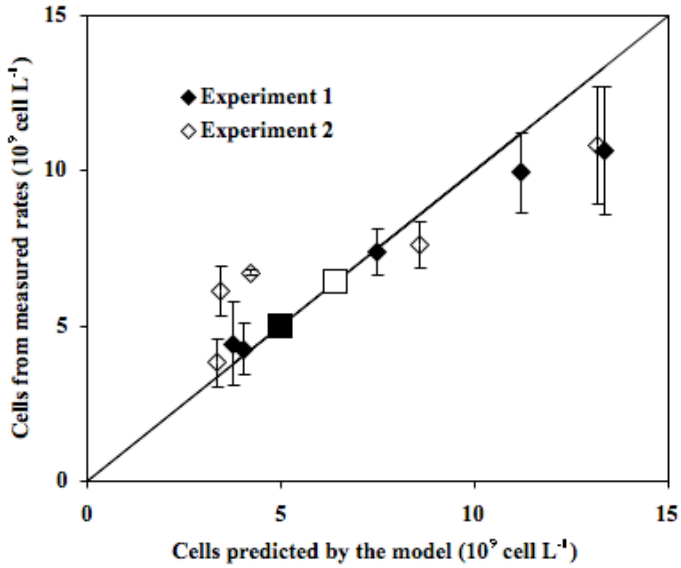

FIGURE 5. A comparison between cell concentrations (filled diamonds for experiment 1 and open diamonds for experiment 2) determined by dechlorination rates and predicted by the model (after 8 days for experiment 1 and after 8 or 14 days for experiment 2), with the line representing perfect correspondence. The filled and open squares represent the initial cell concentration for experiment 1 (5 X 10^9 cell L^{-1}) and 2 (6 X 10^9 cell L^{-1}), respectively. Cell concentrations greater than the corresponding square represent a net increase in cells, and concentrations smaller than the corresponding square represent a net decrease over the dechlorination period. Error bars represent the maximum and minimum values (*n*=2).

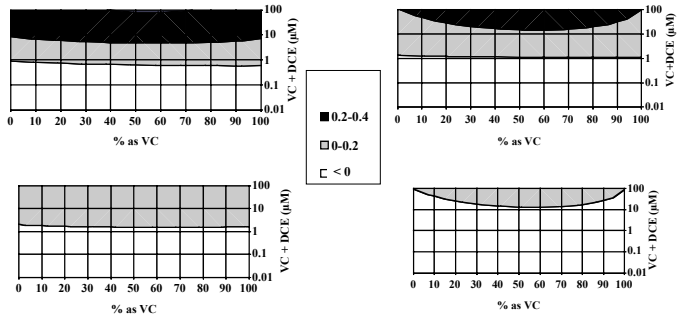

FIGURE 6. Predicted specific growth rates at four different hydrogen concentrations (A-D), with a range of DCE and VC concentrations.

For example, the model indicates that growth will become negative at a DCE + VC concentration of 4 μM together with a hydrogen concentration of 4 nM (a typical concentration found in the field). Regardless of how high the hydrogen concentration, when the DCE plus VC concentration decreases below 0.7 μM, organism growth rate will be negative, and dehalogenation will not be sustainable (dehalogenation past this threshold is observed in batch experiments only because of a large added dechlorinating population, a scenario not typical at field sites). It is significant that 0.7 μM VC represents a concentration of 44 μg/L, well above the U.S. Environmental Protection Agency Maximum Contaminant Level for drinking water of 2 μg/L. Thus, while microbial reductive dehalogenation can have significant impact in reducing the concentrations of chlorinated ethenes in an aquifer, kinetic limitations of the microorganisms involved make it unlikely with natural attenuation alone that concentrations can be reduced sufficiently by reductive dehalogenation to meet drinking water standards. Other processes such as aerobic degradation or dilution may be required. The extent to which the VC concentration can be reduced is dependent significantly upon the hydrogen concentration that can be maintained. Thus, kinetic limitations need to be understood when considering biological dehalogenation through natural attenuation or engineered bioremediation at a field site. The simulation model presented and evaluated here should be a useful tool in helping to assess both the rate and extent of microbial dehalogenation of PCE and TCE and the chlorinated daughter products in a contaminated aquifer, as well as the important boundary conditions that may limit the extent of their removal.

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PROJECT 3

Comparative Evaluation of Chloroethene Dechlorination to Ethene by *Dehalococcoides*-like Microorganisms

Performers

Alison M. Cupples, Alfred M. Spormann and Perry L. McCarty

Abstract

Reductive dehalogenation of tetrachloroethene (PCE), trichloroethene (TCE), *cis*-1,2-dichloroethene (DCE), and vinyl chloride (VC) was examined in four cultures containing *Dehalococcoides*-like microorganisms. Dechlorination and growth kinetics were compared using a Monod growth-rate model for multiple electron acceptor usage with competition. Included were the Victoria mixed culture containing *Dehalococcoides* species strain VS (from Victoria, TX), the mixed culture KB-1/VC (from Southern Ontario), the Pinellas mixed culture (from Pinellas, FL) and *Dehalococcoides ethenogenes* strain 195. All cultures, with the exception of *D. ethenogenes* strain 195 grew with VC as catabolic electron acceptor. A dilution method was developed that allows a valid comparison to be made of dehalogenating kinetics between different mixed cultures. Using this procedure maximum growth rates on VC were found to be similar for strain VS and KB-1/VC (0.42 to 0.49 ± 0.02 d^{-1}), but slower for the Pinellas culture (0.28 ± 0.01 d^{-1}). The 16S rRNA gene sequences were determined to ensure no cross contamination between cultures had occurred. Following enrichment of the VC dechlorinating microorganisms on VC, the cultures were amended with DCE, TCE or PCE. The three mixed cultures failed to dechlorinate PCE or did so very slowly. However, the dilution technique indicated all experienced growth on TCE and DCE, as well as on VC. Maximum growth rates on DCE alone were quite similar (0.43 to 0.46 d^{-1}), while the Pinellas culture grew faster on TCE alone (0.49 d^{-1}) than did the other two mixed cultures (0.33 to 0.35 d^{-1}). Half velocity and inhibition constants for growth on TCE were also determined for the three mixed cultures; both constants were found to be essentially equal and the same for the different cultures, varying between only 8.6 and 10.5 μM. The ability of the strain VS, KB-1/VC and Pinellas cultures to utilize TCE rapidly with conversion to ethene is quite different from that of any other reported

microorganism. It was separately confirmed with more traditional cell-counting techniques that strain VS coupled TCE, as well as DCE and VC, utilization with growth. This is the first report of an organism obtaining energy for growth through every step in the reduction of TCE to ethene. Also, as suggested by the dilution technique, the dehalogenating organisms in the KB-1/VC and Pinellas cultures appear to obtain growth from TCE utilization as well. Such ability to grow while dehalogenating TCE to ethene will be an important advantage for their use in bioaugmentation.

Introduction

Tetrachloroethene (PCE) and trichloroethene (TCE) are among the most common organic groundwater contaminants. Under anaerobic conditions, they may be converted biologically through reductive dehalogenation to *cis*-1, 2-dichloroethene (DCE) (*trans*-dichloroethene or 1,1-dichloroethene may also be formed, *1*), vinyl chloride (VC), and then ethene. As yet, no microorganism has been reported that can grow through every step in the reductive dechlorination of PCE or TCE to ethene. Several phylogenetically different bacteria have been identified with the ability to convert PCE and TCE to DCE, but the reduction of DCE to VC and ethene appears to be carried out for the most part by organisms of the genus *Dehalococcoides*. The first such organism identified, *Dehalococcoides ethenogenes* strain 195 (*2*), obtains energy for growth from reduction of PCE, TCE, and DCE to VC. It can convert VC to ethene, but does so only cometabolically. Two recently reported *Dehalococcoides* microorganisms, *Dehalococcoides* species strain VS (*3, 4*), and *Dehalococcoides* isolate BAV1 (*5*) can derive energy from both DCE and VC reduction to ethene. *Dehalococcoides* isolate BAV1 uses TCE and PCE only cometabolically *(5)*. The possible ability of strain VS to use TCE or PCE has not previously been indicated.

In the field, complete transformation to ethene, the desired endpoint, often does not occur naturally because of insufficiency in required electron donor, reaction kinetics, the absence of necessary dehalogenating bacteria, or other environmental factors. Engineering efforts to enhance complete microbial reductive dehalogenation at contaminated sites generally involve adding electron donor. Mixed cultures, such as Pinellas *(6)* and KB-1 *(7,8),* containing *Dehalococcoides*-like strains, have been successfully used in field bioaugmentation

demonstrations to bring about complete dehalogenation to ethene. Other cultures with the ability to reductively dechlorinate VC to ethene rapidly may be found. The question of which culture is best for use arises when considering bioaugmentation. We present here a method that can be used to make such a judgment together with a comparative study of the utilization of VC, DCE, TCE, and PCE by four of the above mentioned cultures or strains.

Materials and Methods

Chemicals. Liquid *cis*-1,2-dichloroethene (97% Aldrich Chem. Co., Milwaukee, WI), trichloroethene (99+% Aldrich Chem. Co.), tetrachloroethene (99.9+% Sigma-Aldrich) and gaseous vinyl chloride (99.5%, Fluka, Switzerland) were used neat, to prepare stock solutions and analytical standards. In addition, ethene (1000 PPM and 100 PPM, Scott Speciality Gases, Alltech Associates) and vinyl chloride (1000 PPM and 10 PPM, Scott Speciality Gases, Alltech Associates) were used as analytical standards, benzoate (sodium salt, 99%, Aldrich Chem. Co.) as a substrate, and sodium sulfide (Aldrich Chem. Co.) as a reducing agent.

Analytical methods. Analyses of ethene, VC, DCE, TCE and PCE were performed with a temperature program (40 to 220°C) using a Hewlett-Packard model 5890 Series II gas chromatograph equipped with a flame ionization detector (Hewlett-Packard) and a GS-Q fused-silica capillary column (length, 30 m; inside diameter, 0.53 mm; J&W Scientific). A reduction gas analyzer (Trace Analytical, Inc., Menlo Park, CA) was used to measure hydrogen. Solution concentrations and total mass were calculated using Henry's Law Constants (*9*).

Stock cultures. The Victoria mixed culture containing strain VS was maintained in a closed continuously stirred tank reactor (CSTR) as previously described (*3*). A VC enrichment of this culture, maintained over 5 years, was also used in these investigations. Liquid cultures of the three other cultures evaluated (Pinellas, KB-1/VC, and *Dehalococcoides ethenogenes* strain 195) were kept in anaerobic bottles, stored in an anaerobic chamber (10%H_2, 10% CO_2, 80% N_2). The bottles were amended with TCE (1-3µL neat liquid), VC (250 µL gas), and PCE (1-3µL neat liquid) being added to each, respectively, approximately every two weeks.

Batch studies. For the experimental portion of this research, two studies were undertaken, one a comparative VC growth study, and the other a comparative growth and substrate utilization study with different chlorinated ethenes. These studies compared the dechlorination abilities of *Dehalococcoides ethenogenes* strain 195 and the *Dehalococcoides*-like microorganisms in the three mixed cultures (Victoria, KB-1/VC, and Pinellas). For the first study, a VC enriched inoculum was first developed for each of the three mixed cultures. Here, bottles (120 mL) were filled with 60 mL of anaerobic media in an anaerobic chamber as previously described (*3*), and inoculated with 200 µL of each stock culture. Gaseous VC (99.5 %) was added with a gas tight syringe (200 µL). The cultures were then moved to a shaker (60-80 rpm) in an anaerobic chamber (20 ± 2°C). Immediately after 5 µmol of ethene was formed in a bottle, 200 µL of the culture was transferred to fresh media (60 mL), in triplicate, and VC was again added (200 µL). The inoculum for the growth study was that formed after exactly 4 µmol of ethene was produced following this second transfer. To compare growth rates on VC, a range in concentration of each inocula (0.1, 0.3, 1, 3, 9 mL) was transferred to fresh media (as above) to result in a liquid volume of 60 mL, and gaseous VC (200 µL) was added to each. An abiotic control with media and VC was included in all batch studies to demonstrate the cultures were required for dechlorination. Hydrogen served as the electron donor and was non-limiting (> 3%) in all studies. Headspace samples were periodically removed by syringe (250 µL) for chlorinated ethene analyses.

The purpose of the second study was to compare dechlorination rates of PCE, TCE and DCE between the three different cultures that grew on VC (Victoria, KB-1/VC and Pinellas). The development of inocula here was performed with VC, just as in the first study. For the growth and substrate utilization experiments, triplicate bottles were set up for each culture containing 1 mL of inoculum and either neat PCE (0.4 µL), neat TCE (0.3 µL), saturated DCE solution (34 µL) or gaseous VC (200 µL).

To further investigate TCE removal, bottles (120 mL) were filled with 20 mL of a highly enriched culture of strain VS made from the Victoria culture. Duplicate bottles were supplied with gaseous VC (50 µL on day 0, then 150 µL on day 3) or TCE (0.4 µL neat liquid). Dechlorination was followed until all VC or TCE was converted to ethene (day 12). The change in strain VS concentration during VC and TCE utilization was then determined both by competitive PCR and by an activity procedure evaluated previously for strain VS (*3, 10*). In the

activity procedure a non-growth limiting concentration of VC (150 μL) along with excess hydrogen is added to a culture and the initial dechlorination rate is measured (over < 1 day) A good estimate of strain VS concentration can then be obtained using the previously determined (*3*) strain VS maximum VC utilization rate (7.8 X 10^{-10} μmol Cl^- (cell • d)$^{-1}$). The increase in strain VS concentration during TCE or VC utilization was determined with these two procedures on the culture before and after TCE and VC utilization.

Comparative dechlorination and growth kinetics. With mixed cultures, the concentration of organisms carrying out a particular reaction is generally unknown and difficult to determine. Thus, reaction rates with mixed cultures are generally reported in units that cannot be used for comparative purposes. For example, dechlorination rates have been reported in mass removal rate per microcosm or bottle (*11*), per unit liquid volume (*7*), per unit of total biomass (*12*) or per unit of total protein (*13*). However, the approach described here allows us to make direct comparisons of basic dechlorination and growth kinetics between cultures. The main parameter used in this comparison is organism maximum growth rate, which is directly related to dechlorination kinetics. The maximum growth rate for each culture was determined from a least-squares model fit to experimental data (*14*). For this purpose, the dechlorination of TCE, DCE, and VC was modeled using Monod kinetics with competitive kinetics between the multiple electron acceptors involved in dechlorination (for the case when electron donor is non-rate-limiting):

$$-\frac{dVC}{dt} = \left(\frac{\left(\frac{\mu_V}{Y}\right) X\, VC}{VC + K_V\left(1 + \frac{DCE}{K_{ID}} + \frac{TCE}{K_{IT}}\right)} \right) - \left(\frac{\left(\frac{\mu_D}{Y}\right) X\, DCE}{DCE + K_D\left(1 + \frac{VC}{K_{IV}} + \frac{TCE}{K_{IT}}\right)} \right) \qquad (1)$$

$$-\frac{dDCE}{dt} = \left(\frac{\left(\frac{\mu_D}{Y}\right) X\, DCE}{DCE + K_D\left(1 + \frac{VC}{K_{IV}} + \frac{TCE}{K_{IT}}\right)} \right) - \left(\frac{\left(\frac{\mu_T}{Y}\right) X\, TCE}{TCE + K_T\left(1 + \frac{VC}{K_{IV}} + \frac{DCE}{K_{ID}}\right)} \right) \quad (2)$$

$$-\frac{dTCE}{dt} = \left(\frac{\left(\frac{m_T}{Y}\right) X\, TCE}{TCE + K_T\left(1 + \frac{VC}{K_{IV}} + \frac{DCE}{K_{ID}}\right)} \right) \quad (3)$$

$$\mu = \left(\frac{\mu_V VC}{VC + K_V\left(1 + \frac{DCE}{K_{ID}} + \frac{TCE}{K_{IT}}\right)} + \frac{\mu_D DCE}{DCE + K_D\left(1 + \frac{VC}{K_{IV}} + \frac{TCE}{K_{IT}}\right)} + \frac{\mu_T TCE}{TCE + K_T\left(1 + \frac{VC}{K_{IV}} + \frac{DCE}{K_{ID}}\right)} \right) - b$$

(4)

Here, μ_V, μ_D and μ_T are the maximum growth rates (d^{-1}) on VC, DCE and TCE respectively, b is the cell decay rate (d^{-1}), X is the concentration (cells L^{-1}) of dechlorinating cells and Y is the yield (cells $(\mu mol)^{-1}$). *VC, DCE* and *TCE* are the solution concentrations (μM) of VC, DCE and TCE respectively. K_V, K_D and K_T are the half-velocity coefficients (μM) for VC, DCE and TCE. K_{IV}, K_{ID} and K_{IT} are the competitive coefficients (μM) for VC, DCE and TCE respectively. As summarized in Table 1 these coefficients for VC and DCE were determined previously (*10, 12*), while the coefficients for TCE were determined as part of this study.

In order to determine the maximum growth rates from these equations from measured concentrations of TCE, DCE, and VC, values for Y, X, b, K_i, and K_{ij} are needed, where i and j are designators for v, d, t and D, T, respectively. The values Y and X, in particular, are difficult to measure with mixed cultures. However, this difficulty can be surmounted with the batch technique used. The inoculum approach used resulted from growth of a very small seed concentration on a known amount of VC. The concentration of VC dehalogenating organisms in the inocula is thus equal to YS_m/V_i, where S_m is the mass (μmol) of VC consumed and V_i is the culture volume of the seed vessel. When x ml of this inocula is transferred to the 60 ml of media in the batch bottles, the concentration is reduced by the dilution factor, D, which here equals

$x/60$. This gives an initial organism concentration, X^o, in the batch culture, which is related to the dilution used,

$$X^o = DYS_m / V_i \quad \text{or} \quad \frac{X^o}{Y} = DS_m / V_i \tag{5}$$

Organism growth rate is related to the change in organism concentration as follows:

$$\mu_i = \frac{dX/dt}{X}, \quad \text{or} \quad \frac{dX}{Y} = \mu \frac{X}{Y} dt \tag{6}$$

In the numerical approach used here, the value X/Y in Eqs. 1 to 3 from one time step in the numerical model is increased by dX/Y as indicated in Eq. 6 for the next time step. In other words,

$$\frac{X_k}{Y} = \frac{DS_m}{V_i}\left[(1+\mu_1\Delta t)(1+\mu_2\Delta t)...(1+\mu_k\Delta t)\right] \tag{7}$$

where X_k is the microorganism concentration after k time steps. With the dilution procedure, the value on the right side of Eq. 7 is substituted into Eqs. 1 to 3, resulting in the cancellation of X and Y, so that neither need be known to solve the system of equations.

While the values of K_i is also an unknown, except for strain VS, this was relatively unimportant in the determination of maximum growth rates since the initial substrate concentrations used were well above this value so that growth rates were highly insensitive to K_i.

Modeling approach. The above procedure was utilized to compare VC, DCE and TCE maximum growth rates between the *Dehalococcoides* like-microorganisms in the three cultures (strain VS, KB-1/VC and Pinellas). Previously determined VC and DCE half-velocity and competitive coefficients were included in this modeling approach (Table 1). Also, a previously determined (*3*) decay coefficient (0.05 d^{-1}) for cells growing under optimal conditions (non-limiting electron acceptor and donor) was included. This leaves the maximum growth rates on

individual chlorinated ethenes (μ_V, μ_D, or μ_T) as the only fitting parameters, which were determined, together with the 95% confidence interval) using non-linear least-squares fit (*14*) to the experimental data.

DNA isolation and competitive PCR. For the Victoria, Pinellas, and KB-1/VC genomic DNA was extracted (1 mL) from the inoculum used in the VC growth comparison study and also from cultures in the comparative substrate utilization study following complete TCE dechlorination. DNA was extracted using a DNeasy® tissue system (Qiagen Inc, Valencia, CA), following the manufacture's instructions (final volume 200 μL). DNA was extracted from the *D. ethenogenes* strain 195 culture following dechlorination of DCE. Competitive PCR was carried out, as previously described (*3*), on DNA extracted from the VC enrichment culture of strain VS following TCE and VC dechlorination (day 12), in order to determine cell concentration.

Amplification and cloning of 16S rRNA genes. PCR with forward primer DeF and reverse primer DeR, as previously described (*3*), was carried out on DNA extracted from all cultures. Aliquots of the PCR products were resolved by electrophoresis in 2% (wt/vol) agarose gel (Sigma) in TBE buffer, stained in ethidium bromide solution (5 μg/mL) for 20 min, followed by destaining in water for 40 min. The bands, visualized upon UV excitation, were found to be of the appropriate size with a 1-kb DNA ladder (Gibco). The remaining PCR products were cloned into *E. coli* with a TOPO TA cloning® kit (Invitrogen Corporation, Carlsbad, CA) following the manufacturers instructions. Plasmids were extracted from the cloned cells using a QIAprep® miniprep system (Qiagen Inc) and the inserts were sequenced (Stanford University's Protein and Nucleic acid facility, PAN).

For DNA extracted from the KB-1/VC and Pinellas cultures (used for inoculum in the VC growth comparison study) partial sequences (1091-1320 base pairs) from nine clones each were obtained. For DNA extracted from *D. ethenogenes* strain 195 and for DNA extracted from the Victoria inoculum (used in the VC growth comparison study) partial sequences (ranging from 421-930 base pairs) from five clones each were obtained. For DNA extracted from cultures following TCE dechlorination, partial sequences (960-1140 base pairs) from four (Pinellas) or five (Victoria and KB-1/VC) clones each were obtained. The sequences were compared to known sequences using BLAST in GenBank (*15*) and were analyzed with DNASTAR MegAlign

software.

Results and Discussion

VC dechlorination. Dechlorinating microorganisms in the Victoria, KB-1/VC, and Pinellas cultures dechlorinated VC to ethene, forming 5 μmol of ethene in 18 days (Victoria and KB-1/VC) or 21 days (Pinellas) (Figure 1). This conclusion is corroborated by recent research demonstrating the expression of the same VC-reductase gene in all three cultures (*4*). As expected of cells growing on VC, the initial dechlorination rate was low following inoculation and increased exponentially with time. Standard deviations from the second transfer (triplicate cultures), illustrate the level of reproducibility typical of these experiments (Figure 1). *D. ethenogenes* strain 195 did not dechlorinate VC, indicating no growth on VC, but did dechlorinate DCE to VC (with no further reduction to ethene), as expected (Figure 2).

The results of the VC-fed serial dilution study for each of the three mixed cultures are illustrated in Figure 3. The μ_v values and 95 % confidence intervals obtained by least-squares fitting of Eq. 4 to 7 to these results of the Figure 3 results are summarized in Table 2. The growth rates of the dehalogenators in the Victoria and KB-1/VC cultures are similar (0.49 and 0.42 d^{-1}, respectively), while that of the Pinellas culture is lower (0.28 d^{-1}). The value of 0.49 d^{-1} compares with that of 0.4 d^{-1} determined previously using the more traditional method of cell counting (3). The dilution method used here is believed to be more precise because of the normal errors in cell counting methods, especially with mixed cultures. In summary, evidence for growth of dehalogenating bacateria in these three cultures on VC provided by the current study includes the repeated transfer of VC dehalogenating microorganisms, the increased rate of VC consumption following each transfer, increased VC dechlorination in cultures with greater inocula, and their similarity in VC dechlorination kinetics. Since several *Dehalococcoides* strains have now been shown to grow on VC, this capability may be more typical than was once thought.

DCE, TCE and PCE Dechlorination. In the second study, triplicates of each individual mixed culture grown on VC was supplied with DCE, TCE or PCE. Using the dilution procedure, the dehalogenation results from cultures fed DCE were used to determine the maximum growth

rate on DCE alone (μ_D) using least-squares analysis and the values for μ_V from Table 2. The maximum growth rates found for growth on DCE alone (μ_D) are similar for the three mixed cultures (0.43 to 0.46 d^{-1}), and are also similar to that on VC by the Victoria and KB-1/VC cultures (0.42 to 0.49 d^{-1}) (Figure 4). In agreement with this, recent research indicates that the purified VC-reductase from strain VS from the Victoria culture dehalogenates DCE and VC at similar rates (*4*).

Figure 5 illustrates a representative model fit for TCE removal (Pinellas culture). An unexpected finding was that all three mixed cultures dehalogenated TCE. Since the half velocity (K_T), inhibition coefficient (K_{IT}), and maximum growth rate (μ_T) for TCE were unknown, the three coefficients were used as fitting parameters along with previously determined values for μV and μD from Table 2 in a least-squares fit to the TCE utilization data. The maximum growth rate found for TCE utilization alone was again the same for the Victoria and KB-1/VC cultures (0.23 d^{-1}), but lower than that for the Pinellas culture (0.49 d^{-1}). Also of interest is that the half velocity and inhibition coefficients for TCE utilization by the three cultures (KT and KIT) are equal to each other and about the same for the three cultures varying between 8.6 and 10.5 (Table 3). The near equality between the half velocity and inhibition coefficients was found previously for DCE. With VC on the other hand, the inhibition coefficient (found from an earlier study, *10*) is significantly higher than the half-velocity coefficient, the higher value meaning the presence of VC is less inhibitory to the dehalogenation of DCE and TCE.

The rapid utilization of TCE by the cultures came somewhat as a surprise because the recently isolated, VC-respiring *Dehalococcoides* isolate BAV1 (*5*) uses TCE only co-metabolically. This rapid utilization of TCE and the strong suggestion from the model that this utilization is coupled with growth (μ_T values of 0.35 to 0.49 d^{-1}) was further investigated using the VC enrichment of the Victoria culture. Here in a separate study using a highly enriched (>95% purity) strain VS culture developed from the Victoria culture, the separate utilization of TCE and VC was investigated. Figures 6 and 7 illustrate a comparison between experimental data and modeled results using Table 1 to 3 coefficients, except for μ_T, K_T, and K_{IT}, which were determined using the dilution technique and the data in Figure 6. The value for μ_T was found to match that found with the mixed Victoria culture (0.35 d^{-1}). The values for K_T, and K_{IT} were similar (Table 3). Growth yields on both TCE and VC were determined using competitive PCR, resulting in values of $4.7 \pm 0.3 \times 10^8$ cell/μmol Cl^- when grown on TCE and $5.2 \pm 0.4 \times 10^8$

cell/μmol Cl^- when grown on VC. A third method to confirm growth occurred on TCE was obtained from a comparison of the estimated increase in cell numbers while growing on TCE and VC obtained by measuring maximum VC utilization rates initially and after utilization of TCE and VC was complete (see Batch Studies under Materials and Methods). The cultures dechlorinated the same chlorine mass during either TCE or VC dechlorination, and at the same rate (Figure 8). Thus, if growth occurred on TCE, then the increase in cell numbers while growing on TCE should be the same as that while growing on VC. This was found to be the case (Table 4). Thus three separate lines of evidence, one a modeling fit to the data and two others where increase in cell numbers was determined, confirm that growth of strain VS occurred from TCE dehalogenation alone.

The three methods used above all indicate that growth yields with TCE dehalogenation to ethene are similar to that for VC dehalogenation to ethene. If the TCE degradation was co-metabolic, as has been found for the recently isolated, VC-respiring, *Dehalococcoides* isolate BAV1 (*5*), then the ethene formation rate and the growth yield for the TCE fed cells would be two thirds of that found for the VC fed cells. While such detailed analysis of growth yield through cell counting was not conducted for the KB-1/VC and Pinellas cultures, the maximum growth rates similar determined by the dilution procedure, and their close similarities in TCE utilization to strain VS suggests that they too obtain energy from TCE dehalogenation.

The Pinellas and KB-1/VC cultures failed to dechlorinate PCE. PCE dechlorination did occur after a lag period of 6-12 days with the Victoria mixed culture, suggesting that the presence of an additional dechlorinating microorganism in the culture might be responsible for the eventual PCE dechlorination.

16S rRNA gene sequences. Since the Victoria, KB-1/VC, and Pinellas cultures all exhibited such similar dehalogenation kinetics, a question arises as to whether they all contain the same strain of *Dehalococcoides*. The Victoria culture contains a *Dehalococcodies* strain (Strain VS) which is distinctly different from that in the other two cultures (*3, 16*). However, the similarity in 16S rRNA sequence between the Pinellas and KB-1/VC cultures makes it difficult to ascertain if these two cultures contain the same or a different *Dehalococcoides* sp. To ensure no cross contamination occurred during this study, the 16S rRNA gene sequences for the

three cultures were obtained, and the numbers of differences in base pairs from that of *Dehalococcoides ethenogenes* strain 195 (GenBank Accession no. AF004928) were determined. These sequences were 99.4-100% identical with those previously reported for these cultures *(16)* The 16S rRNA gene sequences were also determined from cultures taken at the end of the growth experiments to investigate whether contamination between cultures might have occurred during these experiments. All sequences so obtained agreed with those of the starting cultures, thus we conclude that no cross-contamination occurred. It is of interest to note that while the nucleotide sequence for strain VS is quite different from that of the other mixed cultures, their activity towards PCE, TCE, DCE, and VC was very similar. This indicates that 16S rRNA-based methods alone cannot be used for characterizing a culture's potential for dechlorination.

In summary, a dilution procedure was developed that can be used for comparing maximum growth and dehalogenation rates in mixed cultures. This procedure was used to compare three mixed cultures and one pure culture containing *Dehalococcoides*-like microorganisms. *Dehalococcoides*-like bacteria within the three mixed cultures, Victoria, KB-1/VC, and Pinellas, could dehalogenate TCE, DCE, and VC at similar rates, but could not grow on PCE, suggesting they have similar mechanisms for dechlorination. Maximum growth rates for dehalogenating bacteria in the Victoria and KB-1/VC cultures were very similar, and about the same on DCE and VC (0.42 to 0.49 d^{-1}), but lower on TCE (0.35 d^{-1}). The Pinellas culture had about the same growth rate on DCE (0.43 d^{-1}), but lower on VC (0.28 d^{-1}) and higher on TCE (0.49 d^{-1}). However, the three mixed cultures performed quite differently from *D. ethenogenes* strain 195, which does dechlorinate PCE with growth, but dechlorinates VC only co-metabolically. They also differ from the isolate *Dehalococcoides* isolate BAV1, which grows with VC, but uses TCE only co-metabolically. The most significant finding of this research is the confirmed ability of the strain VS to obtain energy from each step in the dehalogenation of TCE to ethene. Similar dehalogenation characteristics of *Dehalococcoides*-like strains in the KB1/VC and Pinellas cultures cultures suggests they too have this ability. This should be an important advantage when using these cultures for bioaugmentation of TCE contaminated sites.

This study also provided additionally evidence for the ability of the model represented by Eq. 4, or its equivalent given elsewhere for the case when electron donor is also limiting (10), to correctly simulate dehalogenation rates, providing information on the parameters of importance. This equation indicates that the overall growth rate (μ) is equal to the sum of growth rates

individually on TCE, DCE, and VC. This may suggest that the maximum growth rate would be greater if growing on all three electron donors rather than on one alone. However, this need not be true because the growth rate is modified by the competitive coefficients, which reduces the growth rate on one electron acceptor when others are present. Thus, in order to obtain correct growth and dehalogenation rates, the complete model with competitive coefficients as provided here is needed. Although we have not addressed here the biological basis for this model of competitive inhibition, possible explanations include a competition of several chloroethenes for one enzyme or the competition of different, chloroethene-specific reductive dehalogenases for a common pool of reducing equivalents, or other mechanism(s).

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TABLES AND FIGURES

TABLE 1. Kinetic coefficients and growth rates, along with 95 % confidence intervals, for the reductive dechlorination of DCE and VC developed previously with the Victoria culture and used in this study.

TABLE 2. Maximum growth rates and 95% confidence intervals for reductive dehalogenation of TCE, DCE, and VC by three different mixed cultures and one highly enriched culture.

TABLE 3. Half velocity and inhibition coefficients for reductive dehalogenation of TCE by three different mixed cultures and one highly-enriched culture.

FIGURE 1. VC (squares) and ethene (triangles) by Victoria (A), KB-1 (B) and Pinellas (C) cultures. Following the formation of 5 μmol of ethene (day 18 for A and B, day 21 for C), culture (200 μL) was transferred to triplicate media bottles and VC was added (200 μL), with the results shown. Error bars, following these transfers, represent standard deviations for triplicate samples. Lines do not represent model simulations.

FIGURE 2. DCE (circles), VC (squares) and ethene (triangles) mass change with time in *D. ethenogenes* strain 195 cultures. Cultures were supplied with either VC (A) or DCE (B). Lines do not represent model simulations.

FIGURE 3. VC (squares) and ethene (diamonds) by Victoria , KB-1/VC and Pinellas cultures inoculated with 9 , 3 , 1 , 0.3 or 0.1 mL of culture. Lines represent model simulations as discussed in the text.

FIGURE 4. Reductive dehalogenation of DCE to ethene by (A) Victoria culture, (B) KB-1/VC culture, and (C) Pinellas culture. Symbols represent DCE (diamonds), VC (triangles), and ethene (circles). Lines represent model simulations as discussed in the text.

FIGURE 5. Reductive dehalogenation of TCE to ethene by the Pinellas culture. Symbols represent TCE (squares), DCE (diamonds), VC (triangles), and ethene (circles). Lines represent model simulations as discussed in the text.

FIGURE 6. TCE (A), DCE (B), VC (C) dechlorination and ethene formation (D) in duplicates of the VC enrichment of strain VS from the Victoria culture. Lines represent model simulations, as discussed in the text.

FIGURE 7. VC dechlorination (A) and ethene formation (B) in duplicates of the VC enrichment of strain VS. Lines represent model simulations, as discussed in the text.

FIGURE 8. Comparison of mass dechlorinated (μmol chloride produced) with time by the VC enrichment of strain VS supplied with either TCE (triangles and crosses) or VC (squares and diamonds).

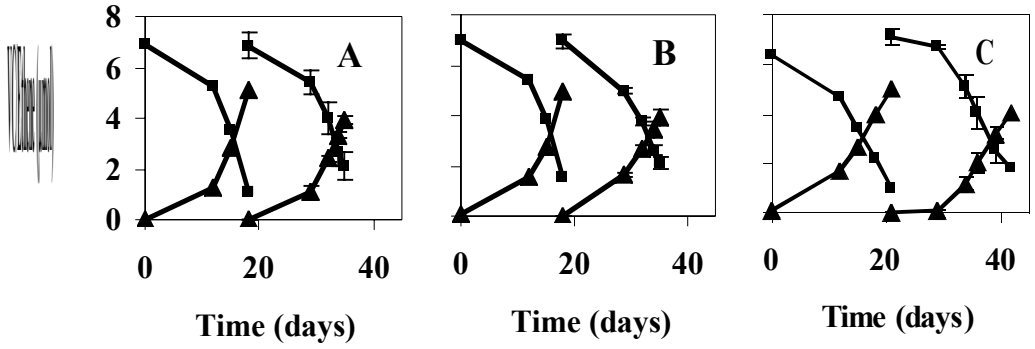

FIGURE 1. VC (squares) and ethene (triangles) by Victoria (A), KB-1/VC (B) and Pinellas (C) cultures. Following the formation of 5 µmol of ethene (day 18 for A and B, day 21 for C), culture (200 µL) was transferred to triplicate media bottles and VC was added (200 µL), with the results shown. Error bars, following these transfers, represent standard deviations for triplicate samples. Lines do not represent model simulations.

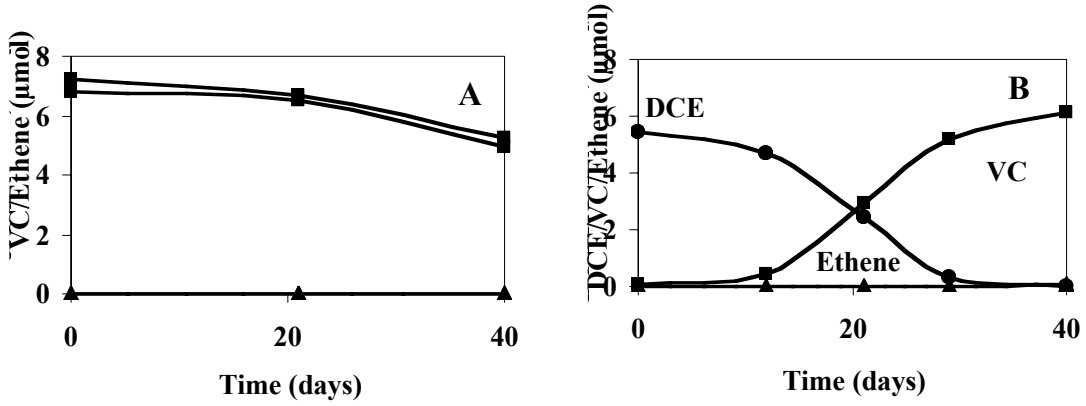

FIGURE 2. DCE (circles), VC (squares) and ethene (triangles) mass change with time in *D. ethenogenes* strain 195 cultures. Cultures were supplied with either VC (A) or DCE (B). Lines do not represent model simulations.

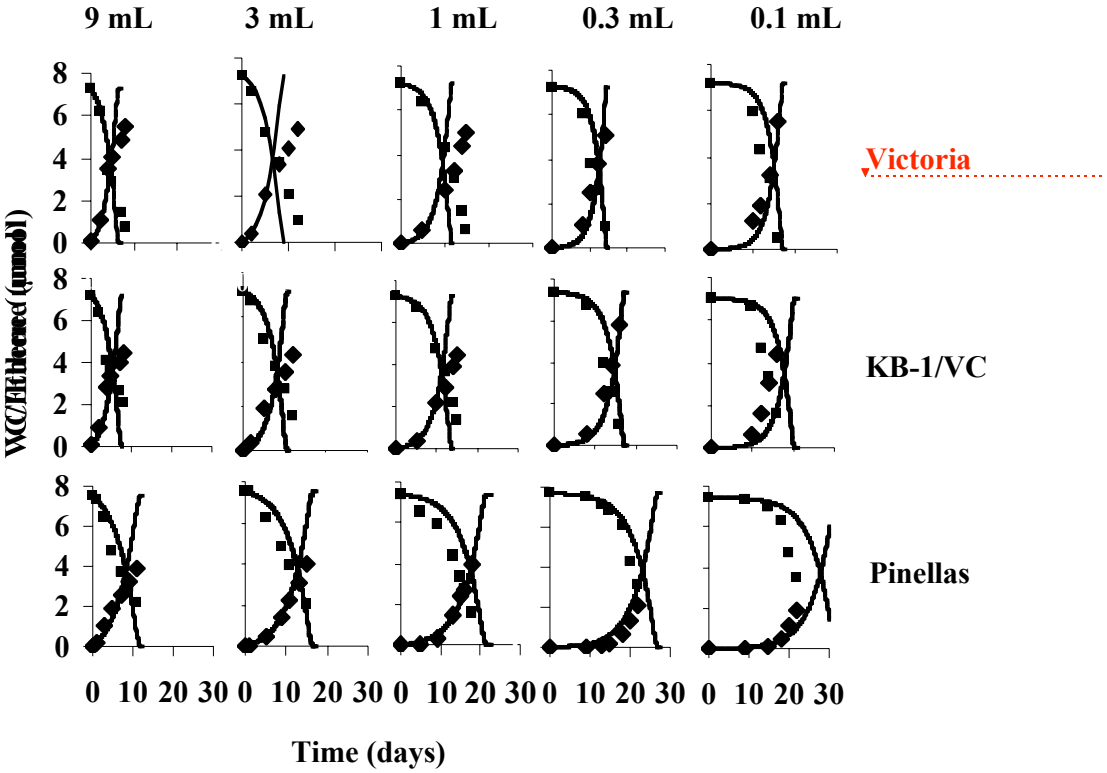

FIGURE 3. VC (squares) and ethene (diamonds) by Victoria , KB-1/VC and Pinellas cultures inoculated with 9 , 3 , 1 , 0.3 or 0.1 mL of culture. Lines represent model simulations as discussed in the text.

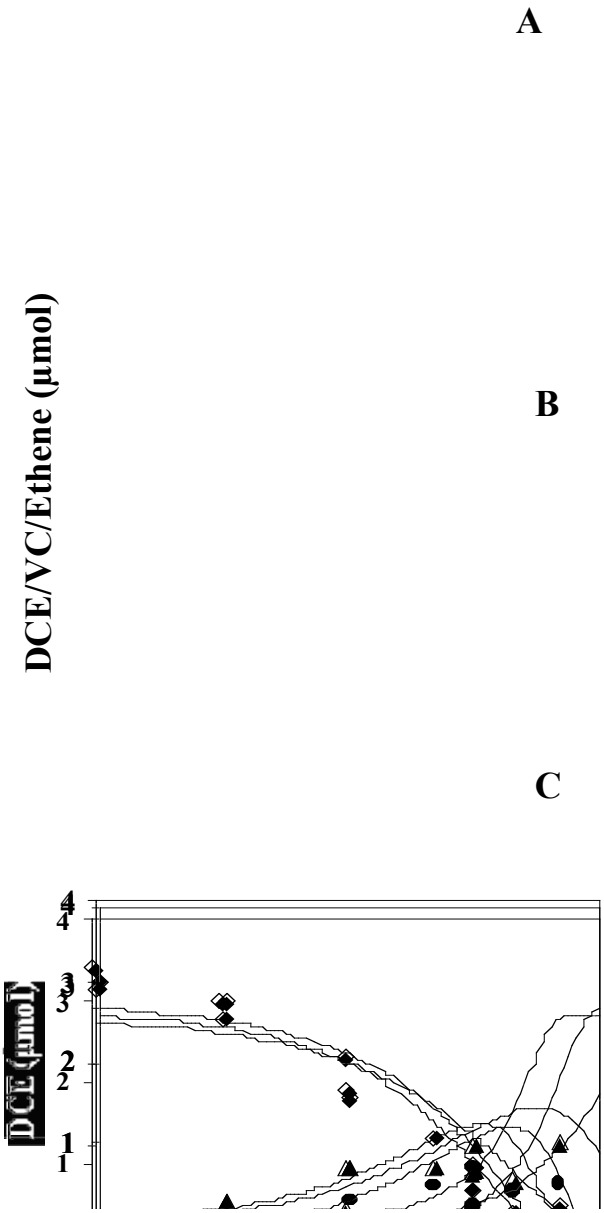
DCE/VC/Ethene (µmol)
A
B
C
DCE (µmol)
4
4
4
3
3
3
2
2
1
1

FIGURE 4. Reductive dehalogenation of DCE to ethene by (A) Victoria culture, (B) KB-1/VC culture, and (C) Pinellas culture. Symbols represent DCE (diamonds), VC (triangles), and ethene (circles). Lines represent model simulations as discussed in the text.

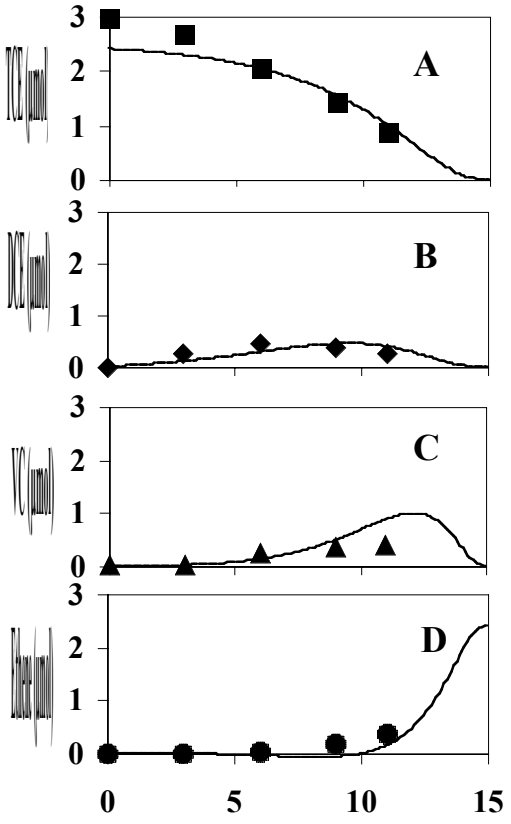

FIGURE 5. Reductive dehalogenation of TCE to ethene by the Pinellas culture. Symbols represent TCE (squares), DCE (diamonds), VC (triangles), and ethene (circles). Lines represent model simulations as discussed in the text.

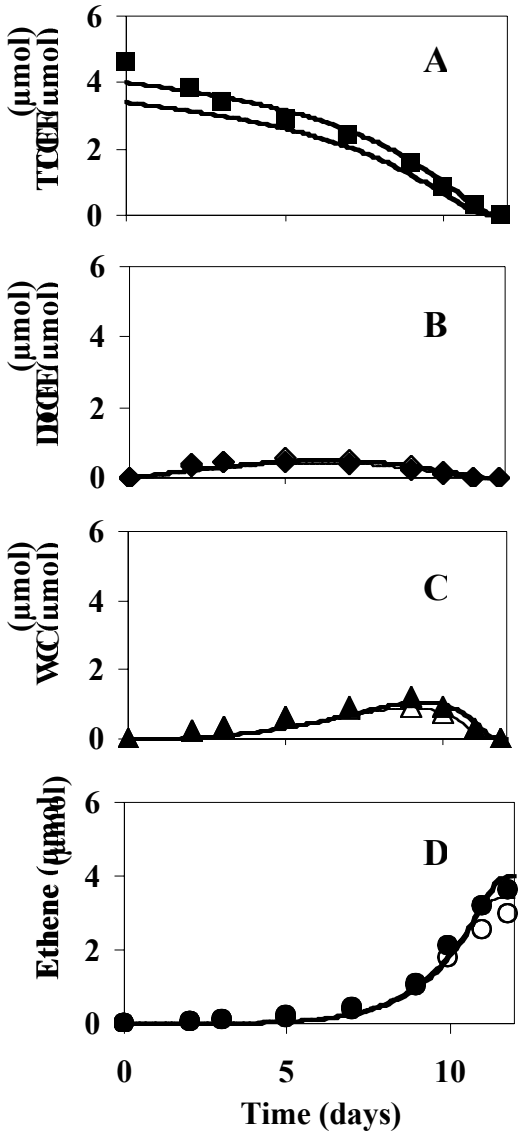

FIGURE 6. TCE (A), DCE (B), VC (C) dechlorination and ethene formation (D) in duplicates of the VC enrichment of strain VS from the Victoria culture. Lines represent model simulations, as discussed in the text.

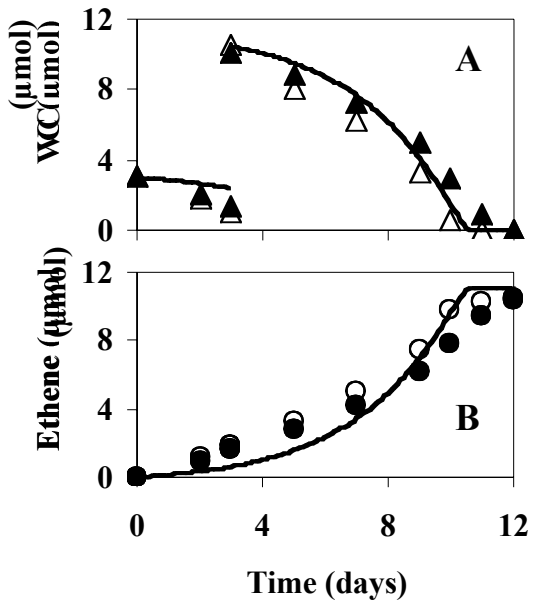

FIGURE 7. VC dechlorination (A) and ethene formation (B) in duplicates of the VC enrichment of strain VS. Lines represent model simulations, as discussed in the text.

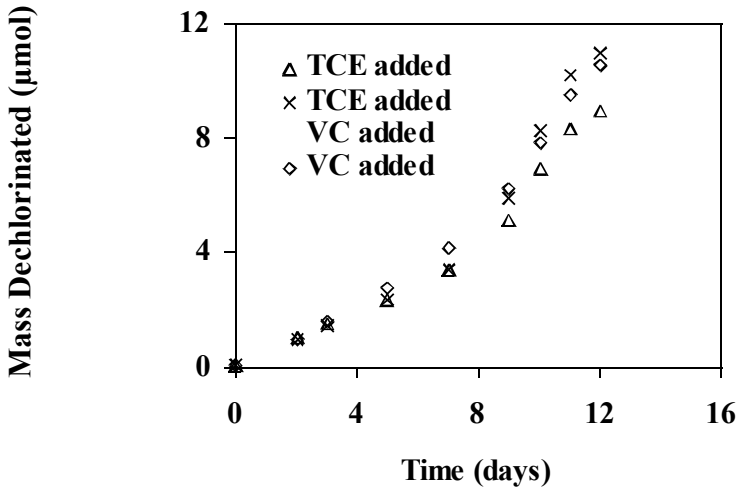

FIGURE 8. Comparison of mass dechlorinated (µmol chloride produced) with time by the VC enrichment of strain VS supplied with either TCE (triangles and crosses) or VC (squares and diamonds).

TABLE 1. Kinetic coefficients and growth rates, along with 95 % confidence intervals, for the reductive dechlorination of DCE and VC developed previously with the Victoria culture and used in this study.

Coefficients	Units	DCE	VC
Half-velocity (K_D, K_V)	μM	3.3 ± 2.2[a]	2.6 ± 1.9[a]
Competitive (K_{ID}, K_{IV})	μM	3.6 ± 1.1[b]	7.8 ± 1.5[b]

[a] Reference 12, [b] Reference 10.

TABLE 2. Maximum growth rates and 95% confidence intervals for reductive dehalogenation of TCE, DCE, and VC by three different mixed cultures and one highly enriched culture.

	Maximum Growth Rate (d^{-1})		
Culture	μ_T	μ_D	μ_V
VS Mixed	0.35 ± 0.07	0.46 ± 0.04	0.49 ± 0.02
Highly enriched	0.35 ± 0.13		
KB-1/VC	0.33 ± 0.06	0.44 ± 0.02	0.42 ± 0.03
Pinellas	0.49 ± 0.03	0.43 ± 0.00	0.28 ± 0.01

TABLE 3. Half velocity and inhibition coefficients for reductive dehalogenation of TCE by three different mixed cultures and one highly-enriched culture.

	Coefficient (μM)	
Culture	K_T	K_{IT}
VS Mixed	9.0 ± 0.4	8.6 ± 0.4
Highly enriched	12.4 ± 0.6	6.8 ± 1.4
KB-1/VC	10.0 ± 2.0	10.0 ± 0.6
Pinellas	10.5 ± 0.7	10.5 ± 0.7

TABLE 4. *Dehalococcoides* species strain VS growth yields on TCE and VC as determined from VC utilization rate measurements, the computed increase in cell biomass, and the mass dechlorinated.

	VC	**TCE**
Initial VC rate (μmol $(d{\cdot}L)^{-1}$)	28 ± 2^{a}	28 ± 2^{a}
Initial cells (cell L^{-1})[b]	3.6 ± 0.3^{a} X 10^{10}	3.6 ± 0.3^{a} X 10^{10}
Final VC rate (μmol $(d{\cdot}L)^{-1}$)	228, 204	312, 204
Final cells (cell L^{-1})[b]	2.9 X 10^{11}, 2.6 X 10^{11}	4.0 X 10^{11}, 2.6 X 10^{11}
Mass dechlorinated (μmol)	10.39, 10.52	10.94, 8.97
Yield (cell μmol^{-1})[c]	**4.6 ± 0.3^{a} X 10^{8}**	**5.8 ± 0.8^{a} X 10^{8}**

[a]Average and range of replicates.
[b]Determined by dividing VC utilization rate by standardized rate of 7.8 x 10^{-10} μmol ethene/cell · d
[c]Yield = (20mL sample/1000mL)(final cells - initial cells)/mass dechlorinated

PROJECT 4

Molecular Identification of the Catabolic Vinyl Chloride Reductase from *Dehalococcoides* sp. strain VS and its Environmental Distribution

Performer

Jochen A. Müller, Bettina M. Rosner, Gregory von Abendroth, Galit Meshulam-Simon, Perry L. McCarty, and Alfred M. Spormann

Summary

Reductive dehalogenation of vinyl chloride (VC) to ethene is the key step in complete anaerobic degradation of chlorinated ethenes. VC reductive dehalogenase was partially purified from a highly enriched culture of the VC-respiring *Dehalococcoides* sp. strain VS. The enzyme reduced VC and all dichloroethene (DCE) isomers, but not tetrachloroethene (PCE) or trichloroethene (TCE), with comparable high rates. Using reversed genetics, the corresponding gene (*vcrA*) was isolated and characterized. Based on the predicted amino acid sequence, VC reductase is a novel member of the family of corrinoid/iron-sulfur cluster containing reductive dehalogenases. The *vcrA* gene was found to be co-transcribed with *vcrB*, encoding for a small hydrophobic protein presumably acting as membrane anchor for VC reductase, and *vcrC,* encoding for a protein with similarity to transcriptional regulators of the NosR/NirI family. The *vcrAB* genes were subsequently found to be present and expressed in other cultures containing VC-respiring *Dehaloccoides* organisms, and could be detected in water samples from a field site contaminated with chlorinated ethenes. Therefore, the *vcrA* gene identified here may be a useful molecular target for evaluating, predicting, and monitoring *in situ* reductive VC dehalogenation.

INTRODUCTION

Contamination of groundwater with the chlorinated solvents tetrachloroethene (PCE) and trichloroethene (TCE) threatens numerous drinking water supplies (6, 36). Conventional approaches for groundwater remediation have placed a multi billion dollar burden on society and consequently stimulated research in alternative clean-up strategies (20). One such strategy, the removal of these contaminants by naturally occurring, chloroethene-degrading microorganisms (bioremediation) appears to be a viable and cost-effective alternative. The microbial degradation of PCE and TCE has been observed most frequently under anaerobic conditions where the chlorinated ethenes can be reductively dehalogenated via the lesser chlorinated ethenes *cis*-1,2-dichloroethene (cDCE) and vinyl chloride (VC) to harmless ethene. However, at many chloroethene-contaminated sites, reductive dehalogenation ceases, or is significantly slowed down, at the level of VC resulting in its accumulation. Because VC is a known human carcinogen and the most toxic compound of all chloroethenes, reduction of VC to ethene is the key step in the complete anaerobic degradation of these compounds.

Reductive dehalogenation of VC has been linked to the genus *Dehalococcoides* (3, 7-9, 18). *Dehalococcoides ethenogenes* strain 195, the first microorganism isolated in pure culture that dehalogenates VC to ethene (18), catalyzes this reduction only in a slow, co-metabolic reaction (14, 15, 19). Recently, enrichment cultures containing *Dehalococcoides*-like organisms which couple VC reduction with energy conservation have been reported (3, 7). The isolation of an axenic culture of one of those organisms, strain BAV1, was described subsequently (8).

While reductive dehalogenation of higher chlorinated ethenes and of some chlorinated aromatic compounds has been studied on a biochemical, chemical, and genetic level (14-16, 21-

24, 26, 31-35), little is known about molecular features of reductive dehalogenation of VC. In *D. ethenogenes* 195, VC-reduction is mediated by the TCE-reductive dehalogenase (TceA) (14, 15). The VC-reduction rate of TceA is, however, less than 1 % of its activity of TCE- and cDCE-reduction. In a previous study with the VC-degrading enrichment culture maintained in our laboratory, we showed the presence of a membrane-bound activity that reduced VC and cDCE, but not TCE or PCE, with high rates (29).

Using the highly enriched *Dehalococcoides* sp. strain VS, we provide here, for the first time, insights into the molecular composition of a VC-reductase as well as the encoding gene. This gene was subsequently found by molecular probing to be present both in VC-dehalogenating mixed cultures used in bioremediation approaches and in groundwater samples from a contaminated field site undergoing bioremediation. These observations suggest that the here described enzyme is an environmentally relevant VC-reductase, and that molecular probing methods, based on the *vcrA* gene sequence, can be useful tools to assess the capacity of contaminated sites for VC reduction.

MATERIALS AND METHODS

Materials. Titanium(III)NTA stock solutions contained 100 mM Ti^{3+} chelated by 150 mM nitrilotriacetate, and were prepared as described elsewhere (25). FeS was prepared according to Ehrenreich and Widdel (4). All other chemicals and gases used were of reagent grade or better and from standard commercial sources. Fast-protein liquid chromatography (FPLC) columns were obtained from Pharmacia.

Bacterial culture and growth conditions. The VC-dehalogenating culture (Victoria-culture) was cultivated under strictly anoxic conditions in a MOPS-buffered (20 mM, pH 7.2) mineral salt water medium essentially as described (29) with the alteration of the buffering system. The culture was grown under an N_2/H_2 [90:10 (vol/vol)] atmosphere in 5 l carboy bottles containing 4 l medium with FeS (~ 0.5 mmol/l) and 0.2 mM titanium(III)NTA as reducing agents. Addition of 10% of sterile-filtered supernatant of a mixed culture containing *Dehalococcoides* sp. strain VS grown with bicarbonate-buffer was needed to obtain sufficient growth. The electron acceptors VC or 1,1-DCE were added discontinuously over time in 200 μmol/l increments up to a total of 5-10 mmol/l; the electron donor H_2 was replenished when needed. The cultures were stirred in the dark at 30°C for several weeks with frequent exchange of the headspace of the bottles in an anoxic chamber (Coy Laboratory Products, Ann Arbor, MI). The mixed cultures KB-1, Pinellas, and WS were grown in reduced mineral medium with VC as electron acceptor essentially as described previously (37).

Enzyme assays. Cells were harvested by centrifugation (20,000 × *g*, 20 min, 4°C) under strictly anoxic conditions using the anoxic chamber, washed and resuspended in degassed MOPS buffer (20 mM, pH 7.2) supplemented with 2 mM DTE and 0.2 mM titanium(III)NTA (buffer A). The buffer was incubated overnight in the anoxic chamber before addition of reducing agents. Cell-free extracts were obtained by anoxic disruption of cells by French Press treatment (138 MPa; two passages), followed by two centrifugation steps (30,000 x *g*, 15 min, 4°C).

Assays of VC-reduction were conducted as described previously in 2-ml glass vials under an N_2/H_2 atmosphere with Ti(III)-reduced methyl viologen as artificial electron donor (29). The protein concentration in the assay varied between 10 and 100 μg/ml. The test was started by addition of gaseous VC to the assay mix (total aqueous volume 0.3 ml), and change in VC and ethene concentrations were followed with time by gas chromatography. Experiments testing for reduction of liquid chlorinated ethenes (PCE, TCE, DCE-isomers)

were carried out essentially as described above with the appropriate substitution of the chlorinated ethene. The chlorinated compounds were added from aqueous stock solutions. Oxygen sensitivity of VC-reducing activity in cell-free extract was investigated in buffer amended with resazurin as redox indicator. Prior to addition of VC, the assay was exposed to air until the resazurin underwent a color-change from colorless to pink. Subsequently, the assay was rendered anoxic again by exchanging the gas phase inside the anoxic chamber followed by addition of Ti(III)NTA (5 mM final concentration). All enzyme assays were carried out at ambient temperature, and given activities are means of at least three independent measurements.

Partial purification of VC reductase. Protein purification was performed at 4^0C in the anoxic chamber. The membrane fraction was obtained by centrifugation of cell-free extract at 100,000 x *g* for 90 min at 4°C. The pellet was resuspended in 1 ml buffer A supplemented with 2 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), and incubated for 1 h on ice. After subsequent centrifugation (100,000 x *g*, 90 min, 4°C) the solubilized membrane fraction was diluted with an equal volume of buffer B (50 mM 1,3-Bis[tris(hydroxymethyl)methylamino]propane (Bis-Tris Propane), pH 9.6, 2 mM dithiothreitol, 0.2 mM titanium(III)NTA, 20 mM CHAPS), and loaded on a 1-ml HighTrap Q column (Amersham Pharmacia) equilibrated with buffer C (same as buffer B but with 2 mM CHAPS). VC reductive dehalogenase activity was eluted as a single peak (at 550 to 580 mM NaCl) with 10 ml buffer C followed by a 40-ml linear gradient from 0-700 mM NaCl in buffer C at a flow of 1 ml/min.

Fractions containing the highest activity were pooled and mixed with an equal volume of buffer A containing 20 mM CHAPS, and applied to a Superose-6 column equilibrated with the same buffer. Enzyme activity was eluted with buffer A at a flow rate of 0.2 ml/min. The protein concentration of the samples was determined according to Bradford with bovine serum albumin as a standard.

Amino acid sequencing and tryptic digest. Peptides present in active enzyme fractions were separated by 12 % SDS-polyacrylamide gel electrophoresis and adsorbed onto polyvinylidene difluoride membrane by electroblotting. The transferred peptides were excised and their N-termini sequenced at the Stanford Protein and Nucleic Acid (PAN) Facility. To obtain internal peptides, active enzyme fractions were separated using SDS gel electrophoresis, bands were excised, digested with trypsin, separated by High-Performance Liquid Chromotagraphy (HPLC), and sequenced.

PCR amplification, cloning and sequencing. Standard protocols were used for DNA cloning and transformation (30). Chromosomal DNA was purified according to Owen and Borman (27). Purification of PCR-products and Plasmids were performed using Qiaprep spin colums (Qiagen). Clones were generated in pETBlue-1 (Novagen) or in pDrive (Qiagen). Sequencing was carried out on an ABI Prism 373 sequencer using ABI Big-Dye sequencing chemistry (PE Applied Biosystems) at the Stanford Protein and Nucleic Acid (PAN) Facility. Southern blot analysis was performed using dioxiginin labeled probes following the Genius kit protocol (Boehringer Mannheim).

Based on the N-terminal amino acid sequence (EANSTKDQPWYVKHREHFDP) and on one internal amino acid sequence (DALFYAVTQPF) of the 62 kDa peptide (see below), degenerate oligonucleotides were designed: Nterm13F (5'-ACV AAR GAY CAR CCD TGG TA-3') and Intern4R (5'-TTY TAY GCM GTI ACV CAR CC-3'). PCR-conditions were as follows: 100 ng of genomic DNA, 200 nM of each primer, 200 μM dNTP, 1 U *Taq* Polymerase in PCR-buffer with 1.5 mM $MgCl_2$ (Roche). PCR-parameters were as follows: 3

min at 92^0C, 30 cycles of 1 min at 94^0C, 1 min at 47 ^{0}C, 1 min at 72^0C, followed by 5 min at 72^0C. A single amplification product of 292 bp was cloned into pET-Blue and was used to generate a probe for Southern blot analysis.

Southern blot analysis with genomic DNA from the VC-degrading mixed culture identified a 3.0-kb *Hin*dIII fragment, a 3.0-kb *Acc*I fragment, and a 1.6-kb *Ssp*I fragment that hybridized to the probe. Subsequently, inverse-PCR with genomic DNA from the VC-degrading mixed culture was carried out as described below. Genomic DNA was digested with *Ssp*I, the reaction mix was purified with Qiaprep spin colums, and the digested DNA ligated (0.5 ng/μl) with T4 DNA Ligase in the presence of ATP overnight at 12^0C. The ligated DNA was purified with Qiagen-Plasmid Preparation and used in inverse-PCR amplification. Based on the previously determined 292 bp DNA sequence, primers vcrSspf (5'-CTA TTT TAC GCC GTC ACC CAA CCT-3') and vcrSspr (5'-TGT AAT CGT AGG GTC AAA ATG CTC-3') were designed. The Reaction contained 25 ng/μl circularized DNA, 150 nM of each primer, 200 μM dNTP, 1 U *Taq* Polymerase in PCR-buffer with 1.5 mM $MgCl_2$, and Q-solution (Qiagen) in a total volume of 20 μl. PCR-parameters were as follows: 3 min at 92^0C, 35 cycles of 1 min at 94^0C, 1 min at 56 ^{0}C, 2 min at 72^0C, followed by 7 min at 72^0C. A single 1.4-kb fragment was amplified, cloned, and sequenced. Based on this sequence, the primer pair vcrAcc/Hinf (5'-ACG CGA GAT GGG GTT TGT A-3') and vcrAcc/Hinr (5'-AAT TCG CTT CTT TTG CTC TTC AC-3') was designed for a second round of inverse-PCR with genomic DNA digested with *Acc*I or *Hin*dIII, respectively. The $MgCl_2$ concentration in the PCR-mixtures was raised to 2.0 mM. PCR-parameters were as follows: 3 min at 92^0C, 35 cycles of 1 min at 94^0C, 1 min at 51 ^{0}C, 3 min at 72^0C, followed by 7 min at 72^0C. A single 2.8-kb fragment from each PCR-reaction was amplified, cloned, and sequenced. To obtain further downstream sequence, a new probe for Southern blotting was generated from the 3'-end of the *Hin*dIII fragment. This probe hybridized to a 1.5 kb *Nco*I fragment. Inverse-PCR with *Nco*I-digested genomic DNA yielded a 1.1-kb fragment. Primers and PCR-parameters were as follows: vcrNcof (5'-GCA AAA CGG CAG ACA GGTA TTA TC-3') and vcrNcor (5'-GCC ACG CCC AAC TGA ATA GG-3'); 3 min at 92^0C, 35 cycles of 1 min at 94^0C, 1 min at 56 ^{0}C, 3 min at 72^0C, followed by 7 min at 72^0C. The sequences of *vcrA* and *vcrB* were verified by PCR-amplification with *Pfu* polymerase (Stratagene) and sequencing products from three independent PCR assays.

For PCR-amplification of *vcrAB* operons from other VC-degrading mixed cultures (KB-1, Pinellas-culture, WS) the following degenerate primers were designed: 5'-ACVAARGAYCARCCDTGGTA-3' and 5'-TYGGTCCYTCYTCYTTCC-3'. A single 1392 bp product was obtained from each culture, cloned, and sequenced. The flanking regions of those products were PCR-amplified using primers designed from the *vcrAB* genomic locus of strain VS. The possibility of a contamination of the three mixed cultures with strain VS was excluded by amplification of the *vcrAB* operon from genomic DNA isolated externally and by testing for the presence of the 16S rRNA gene of strain VS in those cultures.

For PCR-amplification of *vcrAB* operon from groundwater samples, total DNA from sediment material of 2 liters of groundwater samples was isolated with the UltraClean™ Soil DNA Isolation Kit (Mo Bio Laboratories, Inc.). The following primer pair was used: 5'-CTATGAAGGCCCTCCAGATGC-3' and 5'-GTAACAGCCCCAATATGCAAGTA-3'.

Reverse-Transcriptase PCR (RT-PCR). Total RNA was prepared from cells in mid-log phase by a combination of TRIzol®-extraction (Invitrogen) and RNA clean-up with the RNeasy Mini Kit (Qiagen). DNA was removed from the RNA by three treatments with RNase-free DNase I (Qiagen). cDNA was synthesized from 0.2 to 0.8 μg RNA and 2 pmol of

specific primer with SuperScript II RNAase H^- Reverse Transcriptase (Invitrogen) as described by the supplier. The PCR amplification mixtures contained 6 µl cDNA, 200 nM of each primer, 200 µM dNTP, 1 U *Taq* Polymerase in PCR-buffer with 1.5 mM $MgCl_2$.

For amplification of parts of *vcrA*, *vcrB*, *vcrC*, and intergenic regions of *vcrA* and *vcrB*, and *vcrB* and *vcrC*, five sets of primers were chosen for RT-PCR: vcrAf (5'-TGC TGG TGG CGT TGG TGC TCT-3') and vcrAr (5'-TGC CCG TCA AAA GTG GTA AAG-3'); vcrBf (5'-CTT GGC ATA TTG GGG CTG TTA C-3') and vcrBr (5'-ATT TGT CTA CCC TGC GTC TTA CTG-3'); vcrCf (5'-GTG GCC CTC TTA CGG TTG TT-3') and vcrCr (5'-CTAAGTGGCGAGAAAGAATAATG-3'); vcrABf (5'-AAA ATA GTAAA AGG TGT TGT TGC-3') and vcrABr (5'-TAT TTG TCT ACC CTG CGT CTT A-3'); vcrBCf (5'-TGC GGC AAG ATC AGT AAG ACG-3') and vcrBCr (5'-GTA AGA GGG CCA CCA TAA CCA TAG-3'). PCR-parameters were as follows: 3 min at 92^0C, 30 cycles of 0.45 min at 94^0C, 1 min at 55^0C for the primer pairs vcrAf/vcrAr, vcrBf/vcrBr, vcrCf/vcrCr, and vcrBCf/vcrBCr, and at 50 ^{0}C for primer pair vcrABf/vcrABr, 1 min at 72^0C, followed by 7 min at 72^0C.

To determine the region of the transcriptional start site of *vcrABC*, the following primer pairs were used: vcrT1f (5'-TAT CTT TGC GTA TTT TGT GC-3') and vcrT1r (5'-GCC CGC TGA TCC CCT CTC-3'); vcrT2f (5'-TTG TAC TGA GGA AAC GCT TAT GG-3') and vcrT2r (5'-GCC CGC TGA TCC CCT CTC C-3'); vcrT3f (5'-CTT ATG GAT ATT TGG CGT TCA GGA -3') and vcrT3r (5'-AAT TCG CTT CTT TTG CTC TTC ACC-3'). PCR-parameters were essentially as above with an annealing temperature of 51^0C.

Computer analysis. The nucleotide and amino acid sequences were analyzed using the DNAStar software package (DNASTAR, Madison, WI), as well as Simple Modular Architecture Research Tool SMART (http://smart.embl-heidelberg.de). Preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org and from the Joint Genome Institute (http://www.jgi.doe.gov).

Nucleotide accession number. All sequence data have been deposited in the GenBank data base under accession no. AY322364.

RESULTS

Cultivation of *Dehalococcoides* sp. strain VS. Anaerobic dehalogenation of VC was studied with a mixed bacterial culture (Victoria culture) comprised to greater than 99 % of small cocci. The culture was grown in reduced mineral medium with VC or 1,1-DCE as electron acceptor, H_2 as electron donor, and acetate as a carbon source. An axenic culture of those small cocci was obtained since then (J. A. Müller, G. Meshulam Simon, and A. M. Spormann, unpublished data), and its 16S rRNA gene sequence was found to be identical to that of our previously described *Dehalococcoides*-like bacterium VS (3). The mixed culture contained low numbers of three morphologically different types of organisms. These microorganisms were rapidly enriched for with either pyruvate or vanillate instead of VC as catabolic substrate. After four consecutive transfers (1 %, v/v) in the presence of pyruvate or vanillate, these cultures had lost the ability to reduce VC and the small cocci could not be detected microscopically.

Partial purification and characterization of VC reductase. Our previous work had shown that the VC-reductive dehalogenating activity of the mixed culture was associated with the bacterial membrane fraction (29). For purification of VC-reductase, the highly enriched mixed culture was grown for 2 months in 20 l medium containing either VC or 1,1-DCE as electron acceptor and H_2 as an electron donor. Cell-free extract was prepared

anoxically, and VC-reductive dehalogenating activity was solubilized from membranes in the presence of 2 mM CHAPS. The VC-reductase was partially purified about 4 fold to an apparent electrophoretic homogeneity of 50 % by means of anion exchange chromatography and gelfiltration (Table 1, Fig. 1). A single peak of VC-reductase activity was obtained at both

TABLE 1. Purification scheme for VC reductive dehalogenase of *Dehaloccoides* strain VS

Step	Protein (mg)	Activity[a] (mU)	Sp act (mU/mg protein)	Yield (%)	Purification factor
Cell-free extract	40.5	8910	220	100	
Membrane fraction	16.2	5612	346	63	1.5
Solubilized membrane fraction	9.9	3047	308	34	1.4
Anionic exchange chromotagraphy	0.9	607	638	7	2.9
Gelfiltration	0.5	449	990	5	4.5

[a]Activity was monitored as described in Materials and Methods by gas chromatography with reduced methyl viologen as electron donor (29), and given activities are means of at least three independent measurements. One milli unit (mU) activity is defined as the rate of reduction of one nmol VC per minute.

chromatographic steps, suggesting that only one such enzyme was present in the culture. However, the activity eluted during gelfiltration as a relatively broad peak which was likely due to aggregation and/or micell formation of the detergent. During the anion exchange chromatography step, a high percentage (up to 90 %) of the enzyme activity was irreversibly lost. Slightly better recovery of the activity was achieved with the pH of the elution buffer adjusted to pH 9.6 from 7.4, or 8.5, respectively. Addition of glycerol (20 %) to the buffer or varying the detergent concentration (0.5 to 10 mM) did not increase the recovery of activity. Attempts to further purify VC-reductase with additional chromatographic steps (sequential anion exchange chromatography at different pH-values, hydrophobic interaction chromatography, hydroxyapatite, native- and blue native gel electrophoresis) were unsuccessful. In those latter cases, recovery of activity was essentially zero. Therefore, low yields after chromatographic steps in combination with low available biomass at the onset impeded purification of the protein to homogeneity. After gelfiltration, protein fractions with VC-reductase activity displayed two major bands on SDS-PAGE gels, corresponding to an apparent molecular mass of 62 kDa and 45 kDa (Fig. 1).

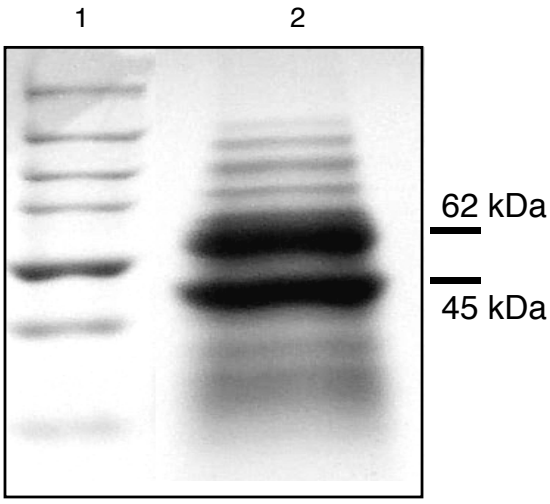

FIG. 1. SDS-polyacrylamide gel of partially purified VC-reductive dehalogenase (7 µg) of *Dehalococcoides* sp. strain VS. Lane 1, molecular weight standard (BioRad) with sizes (in kDa) of 250, 150 100, 75, 50, 37, and 25; lane 2, active fraction after gelfiltration with sizes of major peptides indicated. The gel was stained with Coomassie Brilliant Blue R-250.

These two peptides were estimated to comprise 95 % of the total protein in active fractions. Minor peptide bands of 30, 34, 76, 107, 140 and 175 kDa were also present. However, the bands at 30, 34, 76, 140 and 175 kDa were not detectable in all FPLC-fractions where VC-reductase activity was found (data not shown).

Active protein fractions obtained after gelfiltration catalyzed the reduction of VC (350 nmol min^{-1} [mg protein]$^{-1}$) and all three DCE-isomers at high rates (350 - 390 nmol min^{-1} [mg protein]$^{-1}$) with reduced methyl viologen as electron donor. The DCE isomers were first dehalogenated to VC, which was then further reduced to ethene. Reduction of TCE to cDCE was very slow and occurred at only 5 % of the reduction rate for cDCE to VC. PCE was not transformed during the course of the enzyme assay (2 h). The reduction rate of VC was not affected in the presence of saturating concentrations of PCE or TCE. Thus, the enriched enzyme has preferred substrate specificity to VC and DCEs over TCE and PCE and was therefore designated as VC-reductase. The VC reductase activity was sensitive towards exposure to air with an activity half-life 5 ± 3 min.

The N-terminal amino acid sequences of the two major peptide bands of 62 and 45 kDa as well as of the minor peptide band of 107 kDa were obtained. Furthermore, internal peptides of the 62 kDa band were obtained after a tryptic digest of the excised band. The amino acid sequence of the N-terminus of the 62 kDa peptide (EANSTKDQPWYVKHREHFDP) was found to be similar to a 20 amino acid region of the N-terminus of TceA from *D. ethenogenes* (14). The sequence run showed no indication of background protein contamination. Three internal peptide fractions obtained after tryptic digestion of the 62 kDa peptide band were sequenced. Two of those fractions appeared to be homogenous [peptide1: VYEGPPDA(P)FT(S/T) and peptide2: VGTLVQMF(L); ambiguous amino acid residues in parenthesis]. The third fraction contained two different peptides (peptide3: DALFYAVTQPFPG and peptide4: ESIXTFTLP) of roughly equal abundance. All four sequences were found to have moderate identity (25 – 33 %) with internal sequences of TceA.

The N-terminus of the 45 kDa peptide (AVREQVYGFFIPSVTLIGIG) was nearly identical to N-termini of some alcohol dehydrogenases. A BLASTP search in the deduced proteome of *D. ethenogenes* using the complete sequence of the alcohol dehydrogenase with the most similar N-terminus (accession number ZP_00128696) revealed a putative alcohol dehydrogenase gene located about 30 kb distant from *tceAB*. Interestingly, this 30 kb region contains almost exclusively phage-related genes and genes involved in DNA-recombination.

The N-terminus of the 107 kDa peptide [ANQD(W)SKISLPGSGATG(G/A)YV] was highly similar (90 % identity) to a amino acid sequence in the N-terminal region of a deduced protein of *D. ethenogenes*. This protein of a predicted molecular mass of 106 kDa showed no significant similarities to any other protein in the databases.

Based on the abundance of the 62 kDa peptide in active protein fractions, the molecular similarity of this protein with other known reductive dehalogenases, as well as the absence of any other obvious candidate protein, we concluded that the 62 kDa peptide constitutes the catalytically active subunit of the VC-reductase.

Cloning of the genes encoding VC-reductase. Degenerate oligonucleotide primers were designed from the N-terminal amino acid sequence and one internal sequence of the 62 kDa peptide (see Material and Methods). PCR-amplification with those primers yielded a single 292 bp product, which, after sequencing, was found to be comprised of a single open reading frame (*orf*). The predicted amino acid sequence of this *orf* included the amino acid sequences of the N-terminus as well as those of peptide1 and peptide3 of the 62 kDa peptide.

A probe generated from the 292 bp fragment was found in Southern blot analyzes to hybridize to a 1.6 kb *Ssp*I fragment, a 2.8 kb *Acc*I fragment and a 2.8 kb *Hin*dIII fragment of restriction enzyme-digested genomic DNA of the mixed culture. The flanking DNA regions of the 292 bp product were subsequently amplified by several inverse PCR reactions using circularized *Ssp*I-, *Acc*I-, *Hin*dIII-, and *Nco*I-digested genomic DNA as templates. The respective PCR-products were sequenced, and the four sequences were assembled into a 5.1-kb contig revealing four *orfs* (Fig. 2). The N-terminus and all 4 internal peptide sequences of the 62 kDa peptide were

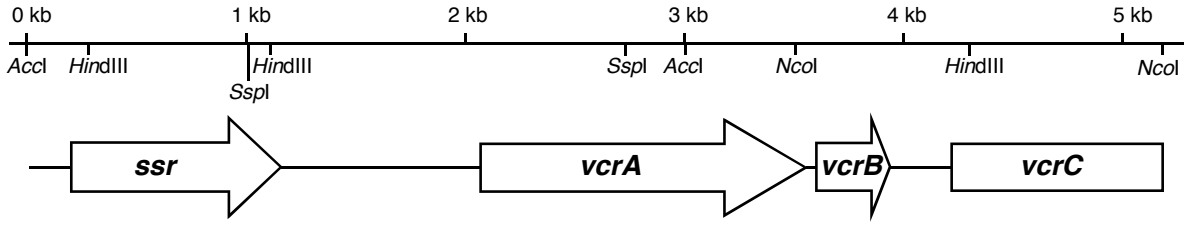

FIG. 2. Physical map of the *vcr* gene locus in *Dehalococcoides* sp. strain VS. The location and direction of open reading frames are indicated by arrows. Relevant restriction enzyme recognition sites are shown.

present in one *orf*, designated *vcrA* (for vinyl chloride reductase).The deduced VcrA sequence, however, contained an isoleucine at position 330 instead of the (ambiguous) leucine as indicated by the peptide2 sequence. A second *orf*, designated *vcrB*, was found immediately downstream of *vcrA* (Fig. 2). An *orf* that started 228 bp downstream of *vcrB* was found and designated *vcrC*. One additional *orf* upstream of *vcrA* was identified and designated *ssr* (for site-specific recombinase, see below).

Computational analysis of *vcrA* and VcrA. The *vcrA* gene is 1560 bp in length and predicted to encode a polypeptide, VcrA, of 519 amino acids with a calculated molecular mass of 57,506 Da. The GC content of *vcrA* (44.6 %) is similar to the GC content of the *D. ethenogenes* genome (48.9 %) and the average GC content of the putative dehalogenase genes (48.3 %) in this organism. A putative ribosome binding site and a sigma70-type promoter sequence (see below) were found upstream of the predicted start codon of *vcrA*. The N-terminal amino acid sequence of the partially purified vinyl chloride reductase matches amino acid residues at positions 44 to 63, respectively, of VcrA, consistent with the predicted polypeptide containing a leader sequence, which is cleaved off, leaving a mature polypeptide of 476 amino acids with a calculated molecular mass of 53,115 Da. The proposed leader sequence containing a twin-arginine motif (Tat-motif) is predicted by motif-search using SMART (http://smart.embl-heidelberg.de), and is similar to those found in other reductive dehalogenases (14, 16, 23, 33, 35). Two motifs for iron-sulfur clusters were identified at positions 400-411 and 444-456. Both motifs are similar to the ferredoxin-type 4Fe4S-cluster ($CX_2CX_2CX_3CP$); here with the variations that the first motif contains a valine after the fourth cysteine instead of the canonical proline, and the second motif displays three amino acids between the first two cysteines instead of two as in the consensus sequence. As for all other reductive dehalogenases, the described binding motif for corrinoids ($DXHX_2G$) found in various corrinoid-containing enzymes (12) is absent in VcrA.

The sequence of VcrA was aligned with those of described reductive dehalogenases (Fig. 3), as well as with putative dehalogenases identified in the genomes of *D. ethenogenes* and *Desulfitobacterium frappieri*. Closest similarity (36 % identity) was with TceA from *D. ethenogenes*. Identities with PCE dehalogenases from *Sulfurospirillum* [formerly *Dehalospirillum* (13)] *multivorans* (PceA-Sm) (23), *Dehalobacter restrictus* (PceA-Dr) (17), and *Desulfitobacterium* sp. strain Y51 (PceA-Y51) (33), and *ortho*-chlorophenol reductive

FIG. 3. Amino acid sequence alignment of VcrA from *Dehalococcoides* sp. strain VS with TceA from *D. ethenogenes* (accession number AF228507), PceA from *S. multivorans* (accession number AF022812), PceA from *Desulfitobacterium* sp. strain Y51 (accession number AB070709), and CprA from *D. dehalogenans* (accession number AF204275). Amino acid residues identical in all 5 sequences are highlighted in black. Functionally similar amino acid residues (2 distance units) and amino acid residues that are conserved in only some of the sequences are boxed. Horizontal bar, twin-arginine motif; *plus* sign, first amino acid residue, E^{44}, of the mature VcrA; asterisks, conserved cysteines.

dehalogenase from *Desulfitobacterium dehalogenans* (CprA) (35) were between 14 and 19 %. VcrA was more identical to the putative dehalogenases from *D. ethenogenes* (up to 34 % identity) than to that of *Ds. hafniense* (up to 23 % identity). In addition to the leader sequence and the C-terminal two iron-sulfur cluster motifs, several highly conserved amino acid residues, including a conserved histidine, H469, is present in VcrA and all other reductive dehalogenases. Furthermore, the sequence in VcrA ranging from amino acid 198 to 215 displays strong similarity to a region in TceA and all putative dehalogenases from *D. ethenogenes*.

Computational analysis of *vcrB* and VcrB. *vcrB*, a gene of 282 bp, is located 41 bp downstream of *vcrA* and preceded by a putative ribosome binding site. The 94, predominantly hydrophobic amino acids account for a calculated molecular mass of 10,641 Da (VcrB). Sequence analysis of VcrB with Protean (DNAStar) and SMART predicted the presence of three transmembrane spanning regions (not shown). The predicted VcrB sequence shows some identity to proposed membrane anchors (B-proteins) for described as well as putative reductive dehalogenases. The most similar sequence to VcrB was TceB with 43% identity. Canonical binding motifs for redox-active cofactors, i.e, iron-sulfur cluster, heme, NAD/FAD, or flavin were not detected in VcrB.

Computational analysis of *vcrC* and VcrC. The presumed start codon of *vcrC* is 228 bp downstream of *vcrB*. No transcriptional stop codon for *vcrC* was found in the DNA fragment analyzed. The partial *vcrC* gene translates into a protein, VcrC, of at least 305 amino acids with a calculated molecular mass of at least 33,363 Da. Four potential membrane-spanning helices can be predicted to occur in VcrC (not shown). The amino acid sequence of VcrC displays some similarity (25 % identity) to CprC from *D. dehalogenans*. CprC was postulated to function as a NosR/NirI-type transcriptional regulator in *ortho*-chlorophenol respiration (31). Four *orfs* similar to VcrC were also found in the deduced proteome of *D. ethenogenes* (38 - 47 % identity). Two of those are located at loci next to genes encoding putative dehalogenases. The other two *orfs* are located directly downstream to homologs of *tatB* and *tatC*, components of the translocation apparatus for proteins carrying the Tat-motif.

Transcriptional organization of *vcrA, vcrB,* and *vcrC*. Transcription of *vcrA*, *vcrB*, and *vcrC* was investigated in a series of reverse transcriptase experiments. Reverse Transcriptase-PCR was performed on RNA isolated from VC-grown cells. RT-PCR assays with primer pairs designed to amplify internal fragments of *vcrA*, *vcrB*, and *vcrC*, respectively, yielded products of the expected sizes (Fig. 4). PCR-products were also obtained for the intergenic regions of *vcrA* and *vcrB* as well as of *vcrB* and *vcrC*. The RNA-specific recovery of these products demonstrates that *vcrA*, *vcrB*, and *vcrC* are expressed as a polycistronic unit during growth on VC. Two DNA sequences upstream of *vcrA* were nearly identical to the -10 and -35 regions of an *E. coli* σ70-promoter (Fig. 5). RT-PCR assays were conducted to determine whether the transcriptional start site of the *vcrABC* operon is located downstream of that σ70-promoter consensus sequence. PCR products were obtained only with forward primers matching regions downstream but not upstream of the putative transcriptional start site. These results map the transcriptional start of the *vcr* operon to a region containing a σ70-type promoter sequence.

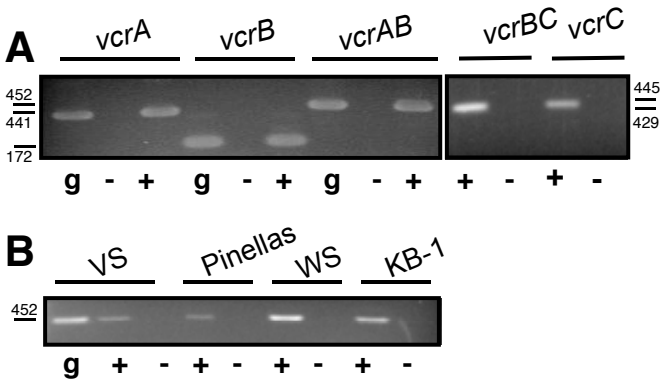

FIG. 4. Reverse Transcriptase-PCR (RT-PCR) analysis of *vcrA*, *vcrB*, and *vcrC* in *Dehalococcoides* sp. strain VS. (A) Transcriptional organization of *vcrA, vcrB* and *vcrC* as an operon in strain VS. Agarose gel electrophoresis of RT-PCR assays with primers targeting *vcrA*, *vcrB*, and *vcrC*, and intergenic regions of *vcrA* and *vcrB*, and *vcrB* and *vcrC*, respectively. (B) Transcription of *vcrA* in strain VS, and in the mixed cultures Pinellas, WS, and KB-1. "g" stands for genomic DNA as template, "+" for assays with RNA as template and conducted with Reverse Transcriptase, and "-" for assays with RNA as template conducted without Reverse Transcriptase. Sizes of products are indicated and were as predicted (for vcrA 441 bp, for vcrB 172 bp, for vcrAB 452 bp, for vcrBC 429 bp, for vcBC 445 bp). Absolute positions of used primers, vcrAf, 2691-2711; vcrAr, 3111-3131; vcrBf, 3773-3793; vcrBr, 3921-3944; vcrCf, 4346-4365; vcrCr, 4767-4790; vcrABf, 3472-3494; vcrABr, 3924-3945; vcrBCf, 3910-3930; vcrBCr, 4335-4358.

DNA region upstream of *vcr* operon. Upstream (850 bp) of the start-codon of *vcrA*, an open reading frame, *ssr*, of 972 bp was identified (Fig. 2). It translates into a predicted protein of 324 amino acid residues with a calculated mass of 36,553 Da. A BLASTP search revealed low similarity (25 – 28 % identity) to site-specific recombinases of the resolvase family containing a Pin domain (28). A putative ribosome binding site was not identified immediately upstream of the start codon.

Presence of *vcrAB* homologs in other cultures containing *Dehalococcoides spp.* Degenerate PCR primers were designed and used to probe for the presence of the *vcrA* gene in three mixed cultures KB-1 (17), Pinellas (5), and WS (W. Wu, unpublished data). These cultures contain *Dehalococcoides*-like organisms that can grow by reductive dehalogenation of VC to ethene (Cupples, A. M., A. M. Spormann, and P. L. McCarty, submitted for publication). PCR products of size of 1393 bp were obtained from genomic DNA of all three cultures. The products were cloned and sequenced, and found to be highly similar to the corresponding sequence of *vcrA* previously identified in *Dehalococcoides* sp strain VS. Additional primers were designed to amplify the complete *vcrA* and *vcrB* genes from those cultures. The thereby obtained nucleotide sequences showed high identities (> 98 %) to *vcrAB* of *Dehalococcoides* sp. strain VS, and the deduced amino acid sequences contained only a few conservative changes suggesting that they are true homologs. The expression of *vcrA* during growth of KB-1, Pinellas, and WS with VC electron acceptor was demonstrated by RT-PCR analysis (Fig. 4). *Dehalococcoides* strains 195, CBDB-1 (1) and a culture containing strain FL-2 (11) were examined for the presence of a *vcrA* homolog by PCR. None of these cultures have been reported to grow by reductive dehalogenation of VC. With the primer set used, no PCR product was obtained with genomic DNA from those cultures as template (data not shown).

Detection of a *vcrAB* homolog in contaminated groundwater samples. The strong correlation of the presence of a *vcrA* homolog with reductive VC dehalogenation, as observed in the above described dehalogenating culture, suggested that the *vcrAB* sequence could be useful as a molecular probe for testing for *in situ* VC-reduction potential at contaminated field sites. Such molecular probes could prove valuable when evaluating, monitoring or predicting complete removal of chlorinated ethenes from groundwater in field scale bioremediation projects. Therefore, we queried for the presence of the *vcrAB* genes groundwater samples from a chlorinated ethene-contaminated aquifer at Moffett Field, CA, where complete reductive dehalogenation to ethene was achieved through biostimulation of indigenous microorganism (2). DNA extracted from groundwater wells exhibiting VC reduction as well as from areas where VC dehalogenation did not occur was used as template in PCR experiments using the primers described above. PCR products were obtained only from groundwater samples undergoing VC reduction to ethene. One of the PCR-products was sequenced, and its *vcrAB* genes were found to be nearly identical (> 98 %) to those obtained from *Dehalococcoides* sp. strain VS.

DISCUSSION

Reductive dehalogenation of VC has long been considered as the most critical step for complete anaerobic removal of PCE/TCE from groundwater and contaminated soils (20), but the molecular features of reductive VC dehalogenation have been largely unknown. Here, for the first time, a VC-reductive dehalogenase was partially purified, and its encoding genes, *vcrAB*, were identified and characterized. Furthermore, a strong correlation between the presence of *vcrAB* in other cultures and catabolic reductive VC dehalogenation was found. A PCR assay testing for the *in situ* presence of *vcrAB* was successfully developed and used on groundwater samples from a site contaminated with chlorinated ethenes. The data suggest that the *vcrAB* genes identified here may be widely distributed, be of relevance for *in situ* VC-reductive dehalogenation, and be a useful target for molecular probing of samples from chloronated ethene-contaminated sites.

The VC reductive dehalogenase was partially purified from a highly enriched culture of *Dehalococcoides* sp. strain VS. The partially purified enzyme reduced VC and the three DCE isomers with similar high rates. TCE was reduced with significantly lower activity, and PCE was not converted. This is in agreement with our earlier results on chloroethene reduction rates in cell-free extract of the parent mixed culture (29). As with most other chloroethene reductive dehalogenases, VC reductive dehalogenase was associated with the membrane fraction, which is consistent with this enzyme being involved in energy conservation during VC reduction to ethene. Analysis of the identified genes encoding VC reductase revealed that this enzyme is a novel member of the family of corrinoid/iron-sulfur cluster containing reductive dehalogenases. The hydrophobic VcrB protein presumably acts as a membrane anchor for the catalytic subunit of VC reductase. The VcrC protein likely plays a role in regulation of transcription of the *vcr* operon. The precise function, however, remains to be elucidated.

Chlorinated ethenes have been assumed to be introduced into the environment essentially due to human activities within the last decades, although this has been questioned recently for VC (10). Interestingly, the *vcr* operon is downstream of a putative site-specific recombinase gene. It is noteworthy that also *tceAB* in *D. ethenogenes* 195 (The Institute for GenomicResearch, Bethesda, Md) and *pceAB* in *Desulfitobacterium* sp. strain Y51 (33) are located in close proximity to putative transposase genes. While the evolutionary origin of those reductive dehalogenases remains unknown, it is tempting to speculate that the parent genes might have encoded enzymes catalyzing the reductive dehalogenation of naturally occurring chlorinated compounds, and that through recent gene duplication/transposition, mutation and selection the substrate specificity towards the anthropogenic chlorinated ethenes was acquired.

So far, only microorganisms affiliated with the *Dehalococcoides*-group are known to be able to grow on VC. Because not all *Dehalococcoides* strains use VC as catabolic electron acceptors (8), simply demonstrating the presence of members of the *Dehalococcoides*-group (based on phylogenetic analysis) at chlorinated ethene-contaminated sites is insufficient for predicting growth-linked, hence rapid, VC degradation. The occurence and expression of homologs of *vcrAB* in cultures containing *Dehalococcoides*-like organisms growing on VC, but not in members of the *Dehalococcoides*-group which are unable to grow by reductive dehalogenation of VC, strongly suggest that the *vcrAB* gene sequence can be used to determine the presence of VC-respiring bacteria at contaminated field sites. Indeed, during this study the presence of *vcrAB* in a contaminated aquifer undergoing complete reductive dehalogenation of chlorinated ethene to ethene was demonstrated. This methodology could allow for better assessing the potential for biological degradation of chlorinated ethenes at numerous

contaminated field sites, and, in combination with bioaugmentation, may become a powerful tool for the clean-up of chlorinated solvents.

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Project 5

Spatial Distribution and Activity of *Dehalococcoides* Subpopulations Inhabiting a PCE-Dechlorinating Continuous Flow Bioreactor

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Summary

We report an integrated approach that addresses the complex interplay of structure and function in a PCE dechlorinating anaerobic microbial community. The microbiologically-mediated reductive dehalogenation of chlorinated ethenes was used as a model to investigate the causal relationships between the spatial distribution of dehalogenating bacteria, microbial dechlorinating activity, gene abundance and expression of process-specific reductive dehalogenases (RDases). Anaerobic reductive dehalogenation of tetrachloroethene (PCE) was studied in a laboratory scale flow column using soil from contaminated field sites and *Dehalococcoides* cultures which have been linked to the reductive dehalogenation of chlorinated ethenes. Using real-time PCR targeting process-specific RDase genes we could link the expression of different RDase genes to the transformation of PCE and lesser chlorinated ethenes in different column sections on a subspecies-level of resolution. We found strong support for causal relationships between *in situ* microbial dechlorinating activity and the reductive dehalogenation of chlorinated ethenes. Variability in RDase gene expression was not reflected by *Dehalococcoides* cell numbers. However, the detection and quantification of metabolic dechlorinating activity at subspecies-level resolution provides accurate means for monitoring and evaluation of bioremediation on the basis of individual activities of *Dehalococcoides* strains.

Introduction

Microbial communities are defined by a complex interrelationship between microbial geno- and phenotypes and by the interaction of each community member with the physical-chemical environment. A profound knowledge of the physiology and ecology of microbial communities is essential to manage their application in contaminant removal to protect and sustain human and environmental health.

Tetrachloroethene (PCE) and trichloroethene (TCE) are the most abundant groundwater contaminants in the United States (35). *Dehalococcoides* species are able to couple the reductive dehalogenation of PCE to growth. They can transform PCE in a four-step series of reductive dechlorination reactions via the intermediates TCE, cis-dichloroethene (DCE), and vinyl chloride (VC) to ethene. While PCE-dehalogenating bacteria of genera other than *Dehalococcoides* dechlorinate PCE to DCE, dechlorination past DCE has been linked exclusively to members of the genus *Dehalococcoides*. However, *Dehalococcoides* strains differ in their ability to catalyze different steps in the complete dechlorination of PCE. *Dehalococcoides* strain 195 and strain FL2 metabolically degrade PCE to VC while the dechlorination of VC to ethene in these strains is co-metabolic (14, 34, 47). Two other *Dehalococcoides* strains named VS and GT dechlorinate TCE to ethene but fail to dechlorinate PCE (6, 37, 49). *Dehalococcoides* strain BAV1 dechlorinates DCE isomers and VC to ethene but does not degrade higher chlorinated ethenes (16). *Dehalococcoides* strain CBDB1, initially isolated for reductive dehalogenation of tetra- and trichlorobenzenes, dechlorinates PCE no further than DCE (1, 24). In order to reveal the contribution of different *Dehalococcoides* strains to the overall dechlorinating activity of an anaerobic microbial community inhabiting a PCE dechlorinating flow column we analyzed the abundance of specific *Dehalococcoides* strains and the expression of functional genes catalyzing various

dechlorination reactions. The continuous flow column served as a model system to apply molecular tools to quantify the abundance and activity of *Dehalococcoides* subpopulations under relevant environmental conditions.

Analyzing the strain diversity of *Dehalococcoides* species is a challenging task because cell abundance is low and species are very closely related based on 16S rRNA gene sequence comparison. Recent studies have demonstrated that common phylogenetic markers, like the 16S rRNA gene, are not sufficient to distinguish between closely related *Dehalococcoides* strains exhibiting different dechlorinating activities (9, 15, 42, 43). However, molecular probing of genes that encode enzymes catalyzing different dechlorination reactions provides the resolution to distinguish between *Dehalococcoides* strains with different metabolic abilities to grow with chlorinated ethenes as electron acceptor.

Reductive dehalogenases (RDases) are the key enzymes in the respiratory chain of halorespiring microorganisms. Whole genome sequencing revealed that *Dehalococcoides* microorganisms contain multiple non-identical RDase gene copies (24, 47). Phylogenetic sequence comparison led to the identification of subclusters of highly similar reductive dehalogenase homologues (*rdh*) that are shared by different *Dehalococcoides* strains (19, 51). Nevertheless, the two complete *Dehalococcoides* genome sequences revealed that 15 out of 32 (46.9%) *rdh* genes in strain CBDB1 and 7 out of 17 (41.2%) in strain 195 are unique for the respective strain.

To date three reductive dehalogenases have been characterized biochemically. For *Dehalococcoides* strain 195 a PCE (PceA) and TCE (TceA) reductase have been shown to catalyze the reduction of PCE to TCE (32) and TCE to VC (31), respectively. Mueller et al. purified and characterized the first VC reductase (VcrA) from *Dehalococcoides* strain VS. They showed that the enzyme reduces VC and all DCE isomers at high rates and that the gene can be found in DNA extracts from field sites contaminated with chlorinated ethenes (37). Subsequently, transcription analysis of VC-grown *Dehalococcoides* strain BAV1 cultures led to the molecular identification of a putative RDase (BvcA) involved in reductive dechlorination of vinyl chloride to ethene (23). These enzymes are the only reductive dehalogenases from *Dehalococcoides* microorganisms for which either direct biochemical or indirect molecular data strongly suggests a catabolic role in the complete dechlorination of PCE to ethene. Because of lack of experimental support linking the PCE reductase of *Dehalococcoides* strain 195 to the nucleotide sequence of *rdh* gene DET0318 (40) we focused our study on the three remaining RDases for which experimental evidence on corresponding nucleotide sequence information was available (23, 31, 37).

Using newly design primers for the three well-characterized RDase genes, we monitored gene abundance and expression in DNA and RNA extracts from the soil matrix of a PCE dechlorinating continuous flow column by quantitative real-time (RT-)PCR. In addition, more general primers were developed and used to quantify absolute *Dehalococcoides* and Eubacterial cell numbers. PCE and VC dechlorination rates obtained from microcosm studies are discussed in context of total bacterial and *Dehalococcoides* cell numbers, the abundance and distribution of different *Dehalococcoides* subpopulations, and the relative expression of process-specific RDase genes on the column.

Material and Methods

Column material. The aquifer material was obtained from the Hanford Formation at a quarry near Pasco, WA and consisted of alluvial deposit of sands and gravels of mixed basaltic and granitic origin (27). The material was classified as clean sand (SW) with approximately 30% fine gravels and less than 5% silt and clay. It contained approximately 6 wt % total Fe, 0.15 wt % available Fe, less than 0.001 wt % organic matter. The particle density was 2900 kg/m^3. The sediment was initially screened to remove large particles (> 1 cm) and subsequently washed several times with tap water. The dry aquifer materials were sifted with a 60 mesh

sieve (0.175 mm openings) to remove fine particulates (Tyler Standard Screen Scale, Mentor, Ohio).

Column construction and operation. The aquifer material was packed in a glass chromatography column (30 cm L x 4.5 cm ID; Knontes CHROMAFLEX chromatography column, Vineland, NJ), sealed with Teflon endcaps. 1/8" PEEK tubing (Upchurch Scientific, Oak Harbor, WA) was used to connect the column with sampling valves at the column influent and effluent ends. PEEK tubing was chosen because of its rigidity and low O_2 permeability. Tubing and pumps were connected to establish flow from bottom to top. The column was packed by adding aquifer-material to the top of the column in small increments as water was slowly pumped upward. No attempt was made to do this anaerobically, since the sediment had been previously exposed to air. The column was packed with about 1 kg of aquifer-material yielding a porosity of 0.35 and a pore volume of 192 mL, based on weight and volume measurements.

Solutions were pumped through the column using a Dionex HPLC gradient pump (Sunnyvale, CA). The connection tubing in the pump heads was replaced with PEEK tubing to prevent penetration of atmospheric oxygen. Solutions were pumped through the column at flow rate of 0.1 mL/min from two reservoirs: one reservoir containing a PCE-saturated solution and the second reservoir containing synthetic groundwater and lactate as an electron donor (initial concentration 0.34 mM). The reservoirs consisted of two 5 L Kimble GL-45 media bottles. To maintain anaerobic conditions in the bottles they were sealed with Kimble "Economy HPLC 3-Hole" caps that were equipped with three _-28 ports. The bottles were purged with nitrogen gas and pressurized at about 10 psi.

The PCE solution was prepared from 5 L of deionized water and 2 mL pure PCE. The solution was vigorously stirred with a magnetic stir bar while purged for 3-4 h with N_2 to obtain a saturated aqueous PCE solution of 1 mM. After purging the bottle was capped and pressurized. Non-aqueous PCE remained at the bottom of the bottle indicating the solution was saturated. Throughout the experiment the PCE solution was constantly stirred at low speed (~60 rpm) to ensure the reagents in the bottle remained well mixed. The pressure on the reservoirs was checked periodically. The reservoirs were re-pressurized if the pressure dropped below 1 psi.

Bioaugmentation culture. The column was inoculated with the Evanite (EV) enrichment culture, obtained from contaminated groundwater at the Evanite site in Corvallis, OR (54). Under strictly anaerobic conditions the enrichment culture catalyzes the complete dechlorination of PCE to ethene. Hydrogen or butanol were used as sole electron donors. Yu et al. (2004 and 2005) studied the transformation rate kinetics of complete PCE dechlorination (55, 56). Maximum utilization rates (k_{max}) were reported on a protein basis and half-saturation coefficients (K_s) were determined for each dechlorination step. Prior to bioaugmentation of the continuous flow column the EV culture was maintained in batch fed reactors supplemented with PCE and butanol as described previously (55).

Experimental design. The chronology of column performance has been describes elsewhere (companion paper Azizian et al. in preparation). In brief: Bromide tracer and PCE transport tests were performed in deionized water. Aqueous solutions of 0.6 mM bromide and 0.09 mM PCE were pumped through the column at a nominal flow rate of 0.1 mL/min and breakthrough concentrations of bromide and PCE were measured in the column effluent. PCE was continuously injected for six weeks in order to achieve steady-state effluent concentrations. The column was then amended with synthetic Hanford groundwater supplemented with trace elements, vitamins, PCE, and Lactate. Synthetic groundwater was prepared to approximate the chemistry of Hanford groundwater as reported by Last et al. (25). It contained 250 _M NaCl, 110 _M KCl, 140 _M Na_2SO_4, and 1 mM $NaHCO_3$ (38). Trace elements and vitamins were added as anaerobic solutions used to grow the Evanite augmentation culture (55). The initial lactate concentration in the synthetic groundwater

reservoir was 0.34 mM. The flow column was inoculated with 20 mL of the EV culture (~ 1.5 mg protein) directly harvested from a batch fed reactor. The EV culture was injected into the column bottom inlet using a 25 ml gas tight syringe. After eight weeks of column operation (39 pore volumes) the lactate concentration in the synthetic groundwater reservoir was raised to 0.67 mM. Sixteen weeks into the experiment (84 pore volumes) the lactate concentration in the groundwater reservoir was double for a second time to reach a final concentration of 1.34 mM.

Analytical methods. The EPA 502.2 purge-and-trap method (2) was used to determine the concentrations of PCE and its transformation products, including ethene. Aqueous samples (1 mL) were injected into an HP 7695 purge-and-trap system. The CAHs and ethene were sorbed onto a Vocarb-3000 trap (Supelco, Bellefonte, PA). A 2 min desorption time at 250°C provided sharp peaks and good peak resolution. Chromatographic separations were achieved with a megabore HP-624 column (30 m x 0.53 µM I.D.) from Agilent (New Castle, DE) operated in a HP6890 series gas chromatograph (GC) connected to a photo ionization detector (PID) followed by flame ionization detection (FID).

Column sampling. After 170 days (128 pore volumes) of column operation aquifer solids were sampled for nucleic acid extraction and the construction of microcosms. In order to maintain anaerobic conditions during the sampling the column was placed in an anaerobic glove box with an atmosphere of 10% H_2 and 90% N_2. The column was split into 6 sections of 5 cm length each. For the 30 cm long column, the following intervals were collected: 0-5 cm, 5-10 cm, 10-15 cm, 15-20 cm, 20-25 cm, and 25-30 cm. The 0-5 cm section was closest to the column influent port. Section 25-30 cm at the opposite end of the column lay next to the column effluent port. Aquifer solids were sampled by scooping out solids from the center of each section with an autoclaved spatula. The column solids were placed in a separate autoclaved sample container and homogenized before sub-samples for nucleic acid extraction and microcosm construction were taken. For DNA extraction 20 g of aquifer solids were placed in 50 mL polypropylene tubes and stored at -20 ºC. For RNA extraction aquifer solids were mixed with equal volumes of RNA stabilization solution (RNAlater®, Ambion, Inc.) and stored at -80 ºC immediately. Homogenized aquifer solids of each of the six column sections were also used to inoculate fed batch microcosms.

Microcosm studies. Microcosms were constructed to evaluate the rates of PCE and VC transformation for each column section. They were constructed in an anaerobic glove box following the procedure described by Yu and Semprini (2002) (57). Approximately 20 g of homogenized solids were added to autoclaved 156 mL serum bottles. Two sets of microcosm were constructed for each section to perform kinetic tests on PCE and VC dechlorination. After distributing solids into the microcosms, 100 mL synthetic groundwater was added to each microcosm, leaving approximately 55 mL of headspace. The bottles were closed tightly with butyl rubber septa and screw caps. Electron donor and CAHs were subsequently added while the microcosms remained in the anaerobic glove box.

Lactate served as electron donor in all microcosm experiments and was added to each bottle from an anaerobic stock solution to a final concentration of 1 mM. The headspace of the PCE and VC microcosms was further amended with 3 mL of Hydrogen. 1 mL of 1 mM saturated PCE solution was added to each PCE microcosm to achieve an aqueous concentration of about 1.5 mg/L. The anaerobic stock of VC gas was made by adding 10 mL of pure VC to an N_2-sparged 156 mL Wheaton bottle. 0.9 mL of anaerobic VC stock solution was added to each microcosm to achieve an aqueous concentration of approximately 1 mg/L.

After electron donor and acceptor addition, the PCE and VC microcosms were analyzed for their initial rates of dechlorinated product formation. Headspace samples (100 _L) were analyzed by gas chromatography. The H_2 concentration in headspace of the microcosms was monitored periodically to ensure ample hydrogen was present. The microcosms were incubated at 20° C in an inverted position on a rotary shaker at 200 rpm.

DNA extraction. Total DNA from the column soil was prepared according to SDS-based DNA extraction methods (50, 59). Up to 0.5 g of soil were mixed with 0.25 mL of 1x TE buffer (100 mM Tris-HCl, 100 mM EDTA, pH 8.0). A spatula tip of acid washed glass beads (0.1 to 0.15 µm in diameter, Sigma-Aldrich, Inc.) and SDS to final concentration of 2% were added to the soil slurry. The samples were vortexed shortly and incubated in boiling water for 2 min. Following the incubation in boiling water samples were immediately frozen in liquid nitrogen and kept on ice until they were completely thawed. The thawed samples were amended with 0.05 mL of a 10% BSA solution and vortexed for 10 min. After centrifugation at 4 °C for 3 min at 12,000 xg the soil slurry (~ 0.85 mL) was combined with 0.43 mL extraction buffer (0.8 M NaCl, 500 mM Na acetate, pH 5.5). Each sample was split in half and DNA was extracted at least twice with 1.5 volumes of phenol:chloroform:isoamylalcohol (25:24:1 vol, pH 8.0, Sigma-Aldrich, Inc.) in 2 mL phase-lock tubes (heavy gel, Eppendorf). After the final centrifugation (5 min, 13,200 xg) DNA in the aqueous phase was precipitated with 2.5 volumes of absolute ethanol at –20°C for at least 2 hours. The precipitated DNA was collected by centrifugation for 30 min at 13,200 xg at 4°C. DNA pellets were washed in 75% ethanol, centrifuged and dried for 15 min at room temperature. DNA was dissolved over night at 4°C in 40 µL nuclease-free water and aliquots were stored at –20°C.

Preparation of total RNA. Isolation of total RNA was performed after a modified protocol of Oelmüller et al. (1990) (39). All solutions were treated with 0.1% (vol/vol) diethylpyrocarbonate (DEPC, Fluka) and subsequently autoclaved prior to use. Soil samples in RNAlater® (Ambion, Inc.) were removed from the –80°C freezer and thawed on ice. Samples were extensively vortexed and 0.5 mL of the RNALater soil slurry were mixed with 10 mL ice-cold AE buffer (20 mM sodium acetate, 1 mM disodium acetate, pH 5.5). Cells and soil particles were filtered onto polycarbonate membrane filters (type GTTP, pore size, 0.2 µm; diameter, 25 mm; Millipore, Eschborn, Germany) and rinsed with double-distilled water. For each sample up to 8 membrane filters were collected in 1.8 mL cold AE buffer, frozen in liquid nitrogen, and stored at –80°C until further usage. Membrane filters were thawed on ice (4 filter pieces in 0.9 mL AE buffer), mixed with SDS to a final concentration of 2%, and extracted with an equal volume of hot phenol:chloroform (60°C, 5:1 vol, pH 5.1, Ambion, Inc.) in 2 mL phase-lock tubes (heavy gel, Eppendorf). Samples were rigorously mixed by vortexing and incubated at 60°C for 10 min, inverting the tubes every 2 min. Afterwards samples were centrifuged for 5 min at 13,200 xg at 4°C. The aqueous phase was supplemented with 3 M sodium acetate, pH 5.5, to a final concentration of 0.3 M and repeatedly extracted (at least 2 times) with phenol-chloroform (5:1 vol), until no interphase was visible any more. Nucleic acids were precipitated with 2.5 volumes of absolute ethanol by incubation at –20°C for at least 16 h over night. Following precipitation nucleic acids were pelleted by centrifugation for 30 min at 13,200 xg at 4°C. RNA pellets were dissolved in 20 µL RNase-free water and additionally purified from humic substances using the RNA clean up spin columns from the Fast RNA® Pro Soil-Direct Kit (Qbiogene, Inc). Residual RNA was removed by incubation with 5 U RNase-free DNase (1U/µL, Roche Diagnostics) in DNase buffer (40 mM Tris-HCl, 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$, pH 8.0) in a total volume of 100 µL. After DNA digestion RNA was purified with the RNeasy MinElute Cleanup Kit (Qiagen, Inc.) following the manufacturers instructions. Finally, RNA was eluted in 14 µL RNase-free water and aliquots were stored at –80°C.

Reverse transcription and PCR. cDNA synthesis from total RNA was carried out following the manufacturers instructions for the SuperScriptIII Reverse Transcriptase (Invitrogen). In brief, 0.5 to 5 µg of total RNA were mixed with 250 ng of random hexanucleotides, 500 µM dNTPs and the final volume was adjusted to 13 µL with RNase-free water. Samples were heated to 65°C for 5 min and cooled on ice before 4 µL of 5x SuperScriptIII transcription buffer, 40U RNase inhibitor (Invitrogen), 5 mM DTT, and 200U SuperScriptIII reverse transcriptase were added. The total volume of the reaction was 20 µL. Samples were

incubated for 5 min at 25°C, 1 h at 50°C, and 15 min at 70°C. Target RNA was selectively digested by addition of 2U RNase H and incubation for 20 min at 37°C. Samples with no reverse transcriptase functioned as negative control.

PCR amplifications were carried out in 0.2 mL thin-walled PCR tubes (E&K Scientific, Campbell, CA), with 20- or 50 µL volumes containing a final concentration of 0.5 µM of each primer (Table 1), 200 µM of each dNTP, 0.5 U of Tag-Polymerase (QIAGEN GmbH, Germany), and 1x QIAGEN PCR buffer containing 1.5 mM $MgCl_2$ (pH 8.0). 0.5 µL of the cDNA reaction mixture was used as template. PCR amplification parameters included an initial denaturation at 94°C for 5 min, followed by 29 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72°C for 1 min. The last cycle was followed by a final extension step at 72°C for 9 min. PCR amplifications were performed in a PTC-200 Gradient Cycler (MJ Research, Inc. Watertown, MA). Amplification products were separated in a 5% polyacrylamide gel (1 mm thick) in 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.4) and stained with ethidium bromide (45).

Table 1. PCR primers used in this study

Name	Sequence (5' -> 3')	Position[a]	Application	Specificity
Eub341F	CCTACGGGAGGCAGCAG	341-357	real-time PCR	Eubacteria, 16S rRNA genes
Eub534R	ATTACCGCGGCTGCTGGC	534-517	real-time PCR	Eubacteria, 16S rRNA genes
Dehalo505F	GGCGTAAAGTGAGCGTAG	505-522	real-time PCR	*Dehalococcoides* spp., 16S rRNA genes
Dehalo686R	GACAACCTAGAAAACCGC	703-686	real-time PCR	*Dehalococcoides* spp., 16S rRNA genes
vcrA880F	CCCTCCAGATGCTCCCTTTA	880-899	real-time PCR	*Dehalococcoides* sp. strain VS
vcrA1018R	ATCCCCTCTCCCGTGTAACC	999-1018	real-time PCR	*Dehalococcoides* sp. strain VS
bvcA277F	TGGGGACCTGTACCTGAAAA	277-296	real-time PCR	*Dehalococcoides* sp. strain BVA1
bvcA523R	CAAGACGCATTGTGGACATC	504-523	real-time PCR	*Dehalococcoides* sp. strain BVA1
tceA511F	GCCACGAATGGCTCACATA	511-529	real-time PCR	*Dehalococcoides ethenogenes* strain 195 *Dehalococcoides* sp. strain FL2 Bacterium PM-VC1, RC-VC2, YK-TCE1
tceA817R	TAATCGTATACCAAGGCCCG	798-817	real-time PCR	*Dehalococcoides ethenogenes* strain 195 *Dehalococcoides* sp. strain FL2 Bacterium PM-VC1, RC-VC2, YK-TCE1

Real-Time Quantitative PCR. The relative quantification of genes and transcripts in the aquifer solids from the continuous flow bioreactor was performed using the iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA) and gene specific primers for different reductive dehalogenases (Table 1). Each sample had a 30 _L reaction volume containing, 1 x iQ SYBR Green Supermix, forward and reverse primers at a concentration of 500 nM, and 6 or 2 _L of the prepared DNA or cDNA, respectively. PCR amplification and detection were conducted in an iCycler (BioRad Laboratories, Hercules, CA). Real-Time PCR conditions were as follows: 3 min 95 ºC followed by 50 cycles of 10 sec at 95 ºC and 45 sec at 61.5 ºC. The amplification program was succeeded by a melt curve analysis starting with the incubation of the PCR product for 1 min at 95 ºC and 1 min at 55 ºC followed by 80 ten seconds cycles in which the temperature was increased by 0.5 ºC each cycle. Each reaction (primer/template combination) was performed in triplicates and triplicate measurements were repeated twice on independent nucleic acid preparations. To verify amplification and correct amplicon size, aliquots from real-time PCR were examined on ethidium bromide-stained 5% polyacrylamide gels. For real-time PCR data analysis the background subtracted raw data was exported from the iCycler system and analyzed using the 'Real-Time PCR Miner' algorithm available online at *http://www.miner.ewindup.info/*. This objective and noise-resistant algorithm calculates the efficiency and threshold cycle based on the kinetics of individual real-time PCR reactions without the need of a standard curve (58). The relative rDH gene abundance and expression was calculated using the following equation with E for the PCR reaction efficiency, C for the threshold cycle, and N representing the template concentration.

$$N_1 / N_2 = [(1+E_2)^{C2}] / [(1+E_1)^{C1}] \quad (1)$$

Results and Discussion

Column performance, sampling, and real-time PCR approach

The performance of the PCE/lacate continuous flow column was monitored over a 24 week period. Prior to bioaugmentation no PCE dechlorination was detected (data not shown). A detailed description on column setup and operation conditions is reported elsewhere (companion paper Azizian et al. in preparation). After 150 days of operation complete dechlorination of PCE to vinyl chloride and ethene was observed. The column experiment was terminated after 170 days when 70 % of the PCE pumped into the column was transformed to ethene, and only 30% left the column as vinyl chloride. No methane production was detected in the column during the entire experiment.

Quantitative real-time PCR has been described as the method of choice for the quantification of *Dehalococcoides* and other metabolic reductively dechlorinating bacteria since alternative methods like fluorescence in situ hybridization with rRNA-targeted oligonucleotide probes and immunostaining assays so far have not been successfully applied to detect and quantify *Dehalococcoides* species in soil samples (12, 14, 15, 26, 28, 42, 43, 46, 48, 49, 53). However, the application of real-time PCR for the absolute quantification of cell numbers in environmental samples has limitations that have to be considered when analyzing real-time PCR data (5, 22, 30). Absolute quantification approaches are further biased by differences in target complexity and amplification efficiencies of standards and samples. We restrained from absolute quantification based on plasmid standards, with exception of the total Eubacterial and *Dehalococcoides* cell numbers (Figure 1) that have to be interpreted carefully for the given reasons. Instead we used a relative quantification approach by comparing threshold cycles and amplification efficiencies of two primer pairs in individual reactions using the same template. Threshold cycles and amplification efficiencies were calculated for individual reactions using the MINER algorithm (58).

Several studies have shown a correlation between the presence of 16S rRNA gene sequences closely related to *Dehalococcoides* species, and reductive dehalogenation at contaminated sites (10,

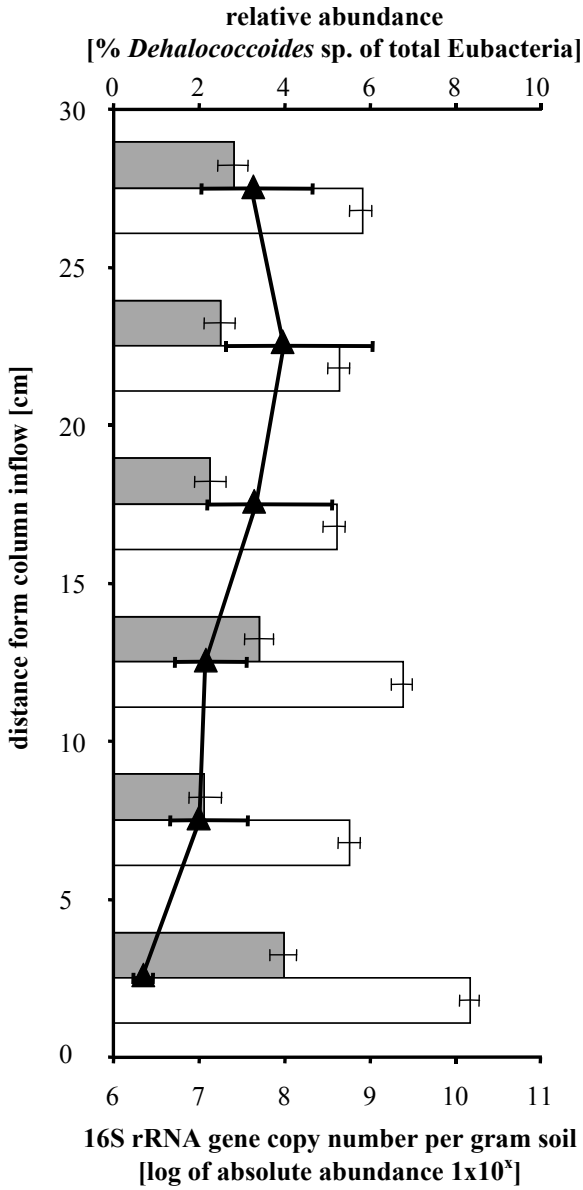

Figure 1. Absolute and relative abundance of *Dehalococcoides* sp. across the vertical profile of the flow column. White bars, total Eubacterial 16S rRNA gene copy numbers per gram column aquifer solids; gray bars 16S rRNA gene copy numbers for the genus *Dehalococcoides* sp.. Triangles represent the relative abundance of *Dehalococcoides* species expressed as percentage of total Eubacterial 16S rRNA gene counts.

11, 17, 26, 29, 42, 44). So far it has not been possible to link positive PCR results to the population abundance of different *Dehalococcoides* strains *in situ*, or to infer metabolic activities of these microorganisms in environmental samples once their presence has been confirmed by PCR (17, 29, 42).

Total *Dehalococcoides* sp. abundance

We analyzed the microbial community composition of the flow column with a focus on the genus *Dehalococcoides*. Aquifer solids from the column were sampled for molecular analysis after 170 days of column operation. The column was split in six 5 cm sections and solids of each site were used for DNA extraction. DNA of each section served as template in real-time PCR assays to quantify *Dehalococcoides* organisms on the column. 16S rRNA gene specific primers targeting the whole genus *Dehalococcoides* (Dehalo505F/686R) and general Eubacterial 16S rRNA gene primers (Eub341F/534R) were used (Table 1). Results of the absolute quantification of *Dehalococcoides* sp. and total Eubacteria for each sampled location along the column are depicted in Figure 1. Eubacterial 16S rRNA gene copies ranged from 5 _ 10^8 to 1 _ 10^{10} per gram soil with higher numbers close to the column inlet where the bioaugmentation culture and media had been injected (Figure 1). *Dehalococcoides* cell numbers decreased slightly from about 1 _ 10^8 cells per gram soil in the first 5 cm to 4.5 - 6 _ 10^7 cells per gram of column matrix throughout the rest of the column. When expressed as percentage of total Eubacteria, *Dehalococcoides* cell numbers increase from 0.5% in the first 5 cm to about 4% of all Eubacteria towards the column outflow (Figure 1).

The relative *Dehalococcoides* species abundance of the bioaugmentation culture was determined by real-time PCR to be 73.5 ± 6.1% of total Eubacteria prior to column inoculation. After the 6 month of column operation the relative abundance of the *Dehalococcoides* population in the upper column part agreed with an estimated 4-5% of reducing equivalents from lactate fermentation attainable for reductive dechlorination (companion paper Azizian et al. in preparation). This can be estimated by the occurrence of Fe(III) and sulfate reduction reactions on the column which may have limited the flow of electrons from lactate fermentation to reductive dehalogenation.

PCE and VC transformation in microcosms

We constructed microcosms from aquifer solids of each column section and measured the initial PCE and VC transformation rates in order to approximate the in situ PCE and VC dechlorination rates in the column. Aquifer solids from the six different vertical sections of the flow column served as inocula. One set of microcosms was fed PCE and another set VC. Initial reduction rates were integrated over the first 3 to 8 hours after addition of electron acceptor. VC transformation rates were calculated from measured ethene formation in VC fed microcosms. Using the *Dehalococcoides* and total Eubacterial cell numbers we calculated the ratio of dechlorination rate per cell for each of the six column sections. The average ratio of ethene formation per *Dehalococcoides* cells on the column (1.32 _ 10^{-10} _mol of Cl^- ($copy^{-1}$ day^{-1})) was in the order of previously reported maximum VC utilization rates of 7.8 _ 10^{-10} _mol of Cl^- ($copy^{-1}$ day^{-1}) (7). Individual ratios for each column section did not vary greatly (factor of ± 2), indicating that members of the genus *Dehalococcoides* were most likely the main VC dechlorinating organisms on the column (Figure 2 A). Notably, the ratios of PCE dechlorination per *Dehalococcoides* cell were about two orders of magnitude higher than the VC/*Dehalococcoides* ratios. The PCE transformation rates per cell decreased significantly from the column inlet to the effluent (Figure 2 A). The fact that PCE reduction decreased with distance while *Dehalococcoides* cell numbers were equal or less variant indicated that *Dehalococcoides* was not the main PCE transforming microorganism in the system. On the contrary the relative *Dehalococcoides* cell numbers increased with distance due to continued VC dehalogenation.

In general ratios of dechlorination rates to total Eubacterial cell numbers were lower than ratios based on the *Dehalococcoides* population size (Figure 2 B) because the total

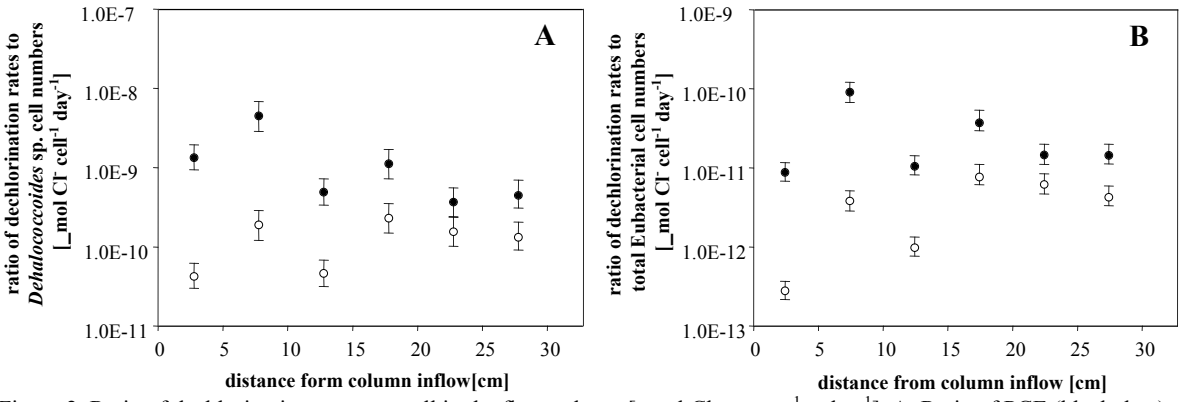

Figure 2. Ratio of dechlorination rates per cell in the flow column [_mol Cl^- _ $copy^{-1}$ _ day^{-1}]. A. Ratio of PCE (black dots) and VC (white dots) dechlorination rates to *Dehalococcoides* sp. cell numbers. B. Ratio of PCE (black dots) and VC (white dots) dechlorination rates to total Eubacterial cell numbers.

Eubacterial community included many other microorganisms besides PCE dehalogenators. The lactate fermenting fraction of the Eubacterial community is supplying the electron donor for PCE dechlorination which results in a direct correlation, between lactate utilization and PCE reduction. Both processes proceeded simultaneously leading to a fairly good correlation between PCE dehalogenation and Eubacterial cell numbers (Figure 2 B).

Yu et al. (2005) conducted kinetic studies with the Evanite culture to evaluate inhibition of reductive dechlorination by chlorinated ethenes (55). They found that higher chlorinated ethenes inhibited the reductive dechlorination of the less chlorinated ethenes. VC dechlorination to ethene in the column is therefore strongly inhibited by the presence of TCE and cis-DCE. We observed a similar effect in the PCE amended microcosms in which VC and ethene formation was two orders of magnitude lower than ethene formation in the VC amended microcosms (companion paper Azizian et al. in preparation). However the VC formation rates measured in the PCE microcosms did not correlate with *Dehalococcoides* or Eubacterial cell abundance and distribution on the column. On the other hand VC reduction rates in the VC amended microcosms were highest in the first 5 cm indicating that in the absence of inhibitory PCE, TCE, and/or cis-DCE, VC dechlorination activity is restored (data not shown).

In general, microcosm studies and continuous flow column performance are only indirectly comparable. In microcosms, chlorinated compounds, electron donor, and micronutrients are added on a periodic batch basis. These conditions are very different from those existing under continuous flow operation. In microcosms, conditions are spatially homogeneous and changing over time, while continuous flow systems mimic more environmentally relevant conditions found at contaminated field sites that tend to vary spatially.

Relative quantification of process-specific rDH genes

In order to quantify gene abundance and expression of strain-specific, functionally well characterized reductive dehalogenase genes in different sections along the column profile, we designed specific primers for three reductive dehalogenases that have been identified as key enzymes in the complete dechlorination of TCE and VC to ethene. The primers targeted the vinyl chloride reductase of *Dehalococcoides* strain VS (vcrA880F/1018R) (37), the vinyl chloride reductase of *Dehalococcoides* strain BAV1 (bvcA277F/523R) (23), and the TCE reductase (DET0079) of *Dehalococcoides* strain 195 (tceA511F/817R) (31). The tceA511F/817R primers pair also targeted the TCE reductases of *Dehalococcoides* strain FL2, and bacterium PM-VC1, RC-VC2, and YK-TCE1 (Table 1).

All primers were tested on genomic DNA from *Dehalococcoides* strain VS under real-time (RT)-PCR conditions. Only the use of the *vcrA*-specific primers resulted in product

amplification using strain VS genomic template DNA, proving the high degree of primer specificity towards the respective rDH genes (data not shown).

The primer pairs tceA511F/817R, vcrA880F/1018R *and* bvcA277F/523R were used in individual real-time PCR experiments with template DNA obtained from different flow column sections to study the relative abundance of the different reductive dehalogenases genes in the column. Results were summarized in Figure 3. The reductive dehalogenase gene abundances are displayed relative to the total *Dehalococcoides* 16S rRNA gene copies present in each section. Calculating the ratio of rDH genes to *Dehalococcoides* 16S rRNA genes is sensible because both genes appear as single copies in the genome of *Dehalococcoides* species. *vcrA*, *bvcA*, and *tceA* gene abundance showed divergent but systematic trends within the *Dehalococcoides* populations of each column section and across the columns vertical profile. *Dehalococcoides* strains possessing a *bvcA*-type vinyl chloride reductase increased from about 2% close to the inflow port to about 60-70% of all *Dehalococcoides* at the outflow of the column. *Dehalococcoides* strains possessing a *vcrA*-type vinyl chloride reductase showed a more uniform distribution along the column maintaining a relative abundance of 15 to 20% in the first 20 cm of the column before dropping to about 5% of all *Dehalococcoides* at the column end. *Dehalococcoides* strains possessing a TCE reductase of the *tceA*-type decreased in abundance from about 3.5% to less than 0.5% near the column outflow (Figure 3).

The detection frequency of *Dehalococcoides* subspecies carrying one or the other rDH gene targeted in this study increased from about 25% of the total *Dehalococcoides* population to more than 70% with increasing distance from the column inflow. The fraction of the *Dehalococcoides* population possessing a vinyl chloride reductase comprised two third of all *Dehalococcoides* species 25 to 30 cm from the column inflow. Interestingly, there seemed to be spatial heterogeneity in the distribution of *vcrA*- and *bvcA*-possessing *Dehalococcoides*. Whereas the *vcrA*-type containing *Dehalococcoides* microorganisms seemed to be present at high PCE/lactate concentration conditions near the column inflow, maintaining a stable population size of about 15% in the first 10 cm, *bvcA*-possessing *Dehalococcoides* cells increased from about 2 to 12% in transition from the 5 to the 10 cm section. *bvcA*-containing *Dehalococcoides* reached their numerical maximum near the outflow of the column, spatially separated from the high PCE/lactate concentration at the inflow end of the column. Under continuous flow conditions *Dehalococcoides* subpopulations with functionally identical rDH genes established at

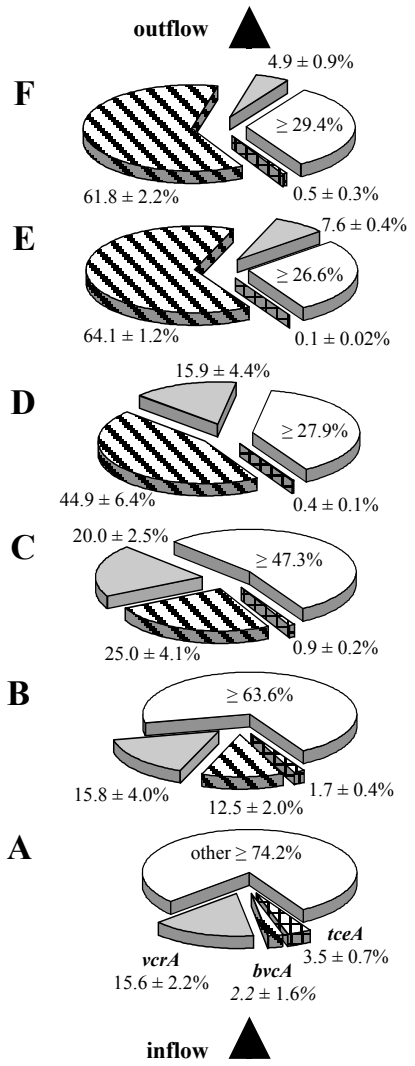

Figure 3. Relative abundance of three process-specific reductive dehalogenase genes expressed as percentage of *Dehalococcoides* sp. 16S rRNA gene copies per column section. Gray, *vcrA,* vinyl chloride reductase of *Dehalococcoides* strain VS; striped, *bvcA*, vinyl chloride reductase of *Dehalococcoides* strain BAV-1; squared, *tceA*, trichloroethene reductase of *Dehalococcoides* strain 195, strain FL2, and Bacterium PM-VC1, RC-VC2, YK-TCE1; white, other rDH genes. Panel A through F represent different column sections with increasing distance to the reactor inflow port. A 5 cm; B 10 cm; C 15 cm; D 20 cm; E 25 cm; and F 30 cm.

spatially separated locations. *Dehalococcoides* subpopulations possessing a *vcrA*- or *bvcA*-type reductive dehalogenase might have different VC dechlorinating-phenotypes that drive niche specialization. Subspecies community analysis using functional rDH gene markers revealed spatial differences in *Dehalococcoides* strain distribution indicating a level of diversity beyond the 16S rRNA gene resolution.

rDH expression profiles

Whereas functional rDH gene marker may only provide information on the genetic capacity or phenotypic potential of *Dehalococcoides* genotypes *in situ*, direct mRNA quantification provides insights into the metabolic activity of different *Dehalococcoides* subpopulations. We analyzed the relative mRNA abundance of the three described rDH genes along the column to verify whether the observed spatial distribution of *Dehalococcoides* genotypes is reflected in a spatial rDH gene expression pattern.

Total RNA was extracted from each column section and reverse transcribed into cDNA. The cDNA served as template in quantitative real-time PCR assays to investigate expression of the target rDH genes. To check for DNA contamination in the total RNA preparations we omitted the cDNA synthesis step and performed real-time PCR reactions on the purified RNA. No product amplification was observed in all 6 environmental RNA preparations as documented

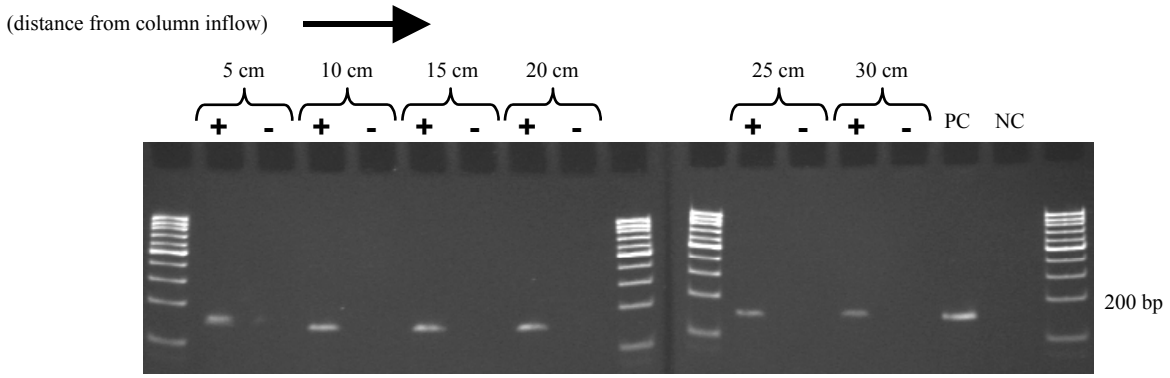

Figure 4. RT-PCR using *vcrA* gene specific primers and total RNA extracted from different regions of the flow column. + reverse transcription performed with reverse transcriptase; – reverse transcription performed without reverse transcriptase; PC, positive control using *Dehalococcoides* strain VS genomic DNA; NC, negative control with no template added.

by gel electrophoresis (Figure 4). We therefore concluded that amplification products obtained after cDNA synthesis were solely based on reversely transcribed total RNA.

We quantified rDH gene transcripts in total RNA extracts from soil relative to the number of *Dehalococcoides* 16S rRNA genes and normalized the data to the absolute *Dehalococcoides* species numbers per section (Figure 5). The change in relative gene expression of the three target rDH genes along the column did not correlate with changes in total *Dehalococcoides* cell numbers or rDH-gene defined *Dehalococcoides* subpopulations. The 5 cm section showed the highest absolute *Dehalococcoides* cell

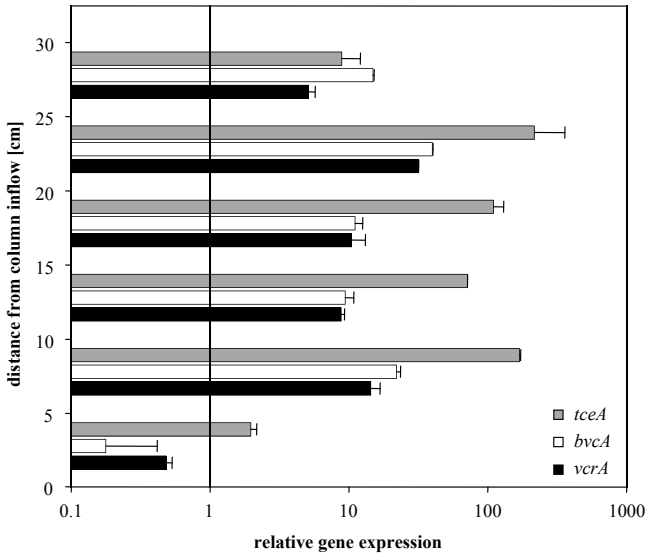

Figure 5. Relative gene expression of three reductive dehalogenases from Dehalococcoides species at different distances from the inflow of the PCE dechlorinating flow column. Black, gene vcrA of strain VS; white, gene bvcA of strain BAV-1; dark gray, gene tceA of strain 195, FL2, and bacterium PM-VC1, RC-VC2, and YK-TCE1

numbers (Figure 1) but only less than 25% of the *Dehalococcoides* organisms in this region contain one of the targeted, functionally known rDH genes (Figure 3 A). Likewise expression of all three studied rDH genes is comparably low in this section (Figure 5). This may suggest the contribution of either *Dehalococcoides* or non-*Dehalococcoides* species that possess so far functionally unknown rDH genes with PCE dechlorination activity to the overall high dechlorination rates observed in the first 5 cm (Figure 2). To fully understand the complex process of chloroethene reduction in the environment it is therefore important to further isolate and biochemically characterize the enzymes encoded by *rdh*s to provide more suitable targets for the evaluation of bioremediation using molecular tools.

Comparison of rDH gene abundance and expression in all column sections suggests differences in rDH gene regulation. Figure 5 shows variations in relative gene expression for all three reductive dehalogenases with increasing distance from the column inflow. Despite a relative *vcrA* gene abundance of 15.6 ± 2.2% in the first 5 cm almost no *vcrA* expression was detectable in this region. Column conditions in the first 5 cm seem to negatively affect *vcrA*, *bvcA*, and *tceA* expression in general because all three rDH genes showed relatively low expression in this section. Most likely PCE concentrations near the column inflow selectively affected expression of genes coding for reductive dehalogenases with substrate specificity for lesser chlorinated ethenes (55). Relative *bvcA* gene expression in the 10 cm section is more than twice that high as in the 15 cm section whereas *bvcA* possessing *Dehalococcoides* cells double in relative abundance from about 12.5 ± 2.0% to 25.0 ± 4.1% 10 to 15 cm from the column inflow (Figure 3 B,C and 5). The highest relative *bvcA* expression was measured at 25 cm from the column inflow port. Relative to the increased *bvcA* gene abundance this translates into a 2 fold induction over the observed expression at 10 cm and a 4 fold induction over the measured expression at 15 cm (Figure 5). Despite the low abundance of *tceA*-possessing *Dehalococcoides* the overall *tceA* expression is about 10 times higher than that of both vinyl chloride reductases in every column section. The only exception is the 30 cm section where *bvcA*-containing *Dehalococcoides* dominate in cell number and gene activity.

Johnson, et al. (2005) studied the expression of the *tceA* gene from *Dehalococcoides* sp. strain 195 in a *Dehalococcoides*-containing microbial enrichment cultures and reported constitutive *tceA* expression independent from chlorinated ethene concentration, hydrogen concentration, or the presence of alternate bacterial electron acceptors (20, 21). No putative regulatory genes have been found in close proximity of the described TCE reductase gene (*tceA*) in the genome of *Dehalococcoides* sp. strain 195 (47). However, our data explicitly suggests spatial differences in *tceA* gene expression on the flow column. Under continuous flow conditions gene expression of *tceA*-containing *Dehalococcoides* subpopulations differs from what has been reported based on lab culture observations of strain 195. We designed our *tceA* primers to target not only the TCE reductase of strain 195 but also highly similar *tceA*-like genes in other *Dehalococcoides* strains (Table 1) for which constitutive gene expression has not been shown in lab cultures yet. The complex interplay of biotic and abiotic factors under which reductive dehalogenation is occurring in a continuous flow system may result in more complex yet not fully understood spatial gene regulation patterns as opposed to what has been found in batch culture studies. Recently, Rahm et al. (2006) reported differences in RDase gene expression between pure and mixed cultures. They found that the expression of a putative *rdh* (DET1545) from *Dehalococcoides ethenogenes* strain 195 increased about 5 fold in mixed culture compared to its relative expression in pure culture (40). These and the results shown in this study emphasize the importance of how continuous flow systems that simulate refined conditions at contaminated field sites can contribute to a more differentiated understanding of the distribution and activity of *Dehalococcoides* subpopulation in situ.

Conclusions

Recent studies have proven that natural attenuation and bioremediation of chlorinated organic hydrocarbons can be applied at field sites but also showed that effective technology implementation depend on a comprehensive understanding of the regulatory mechanisms and cellular responses to changes in environmental conditions affecting contaminant degradation *in situ* (3, 4, 8, 11, 13, 17, 18, 33, 36, 41, 52). Functional rDH marker genes provide promising targets to complement existing 16S rRNA gene-based approaches to more comprehensively describe a chloroethene dechlorinating *Dehalococcoides* community. We identified *Dehalococcoides* subpopulations containing either a *vcrA* or *bvcA*-type vinyl chloride reductase gene and demonstrated that under continuous flow condition these subpopulations enrich at separate locations along the column. Although both subpopulations are functionally similar in terms of their ability to dechlorinate VC they occupy different ecological niches. Furthermore, quantification of gene expression of reductive dehalogenases known to catalyze the dechlorination of TCE and VC *in situ* revealed remarkable changes in the dechlorination activity along the column. The change in gene expression along the vertical profile of the flow column can not be explained by the distribution of total *Dehalococcoides* cells or individual *Dehalococcoides* subpopulations. Therefore we conclude that species abundance, while important, is not adequate by itself for the evaluation of bioremediation. However, investigating gene regulation and activity of *Dehalococcoides* subpopulations in relation to changing environmental gradients will deepen our knowledge on how these microorganisms can efficiently be used for chlorinated solvent removal in the future. A broad understanding of the complex interplay of parameters that lead to a functionally stable dechlorinating microbial community is a prerequisite for critical evaluation of bioaugmentation and remediation projects.

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