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Phylogenetic Analysis of TCE-Dechlorinating Consortia Enriched on a Variety of Electron Donors

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Two rapidly fermented electron donors, lactate and methanol, and two slowly fermented electron donors, propionate and butyrate, were selected for enrichment studies to evaluate the characteristics of anaerobic microbial consortia that reductively dechlorinate TCE to ethene. Each electron donor enrichment subculture demonstrated the ability to dechlorinate TCE to ethene through several serial transfers. Microbial community analyses based upon 16S rDNA, including terminal restriction fragment length polymorphism (T-RFLP) and clone library/sequencing, were performed to assess major changes in microbial community structure associated with electron donors capable of stimulating reductive dechlorination. Results demonstrated that five phylogenic subgroups or genera of bacteria were present in all consortia, including *Dehalococcoides* sp., low G+C Gram-positives (mostly *Clostridium* and *Eubacterium* sp.), *Bacteroides* sp., *Citrobacter* sp., and δ Proteobacteria (mostly *Desulfovibrio* sp.). Phylogenetic association indicates that only minor shifts in the microbial community structure occurred between the four alternate electron donor enrichments and the parent consortium. Inconsistent detection of *Dehalococcoides* spp. in clone libraries and T-RFLP of enrichment subcultures was resolved using quantitative polymerase chain reaction (Q-PCR). Q-PCR with primers specific to *Dehalococcoides* 16S rDNA resulted in positive detection of this species in all enrichments. Our results suggest that TCE-dechlorinating consortia can be stably maintained on a variety of electron donors and that quantities of *Dehalococcoides* cells detected with *Dehalococcoides* specific 16S rDNA primer/probe sets do not necessarily correlate well with solvent degradation rates.

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Introduction

Chlorinated ethenes are among the most common groundwater contaminants in the United States due to extensive use as cleaning solvents and improper disposal techniques. Trichloroethene (TCE) has been identified as the most common contaminant at U.S. Superfund sites (*1*). The suspected and known carcinogenic nature of chlorinated ethenes and close proximity of residential neighborhoods to contaminated sites has led to large cleanup efforts throughout the United States. Recently developed knowledge of the mechanisms for biological destruction of chlorinated ethenes has promoted the implementation of in-situ bioremediation strategies for aquifer restoration. Although in-situ bioremediation can be a cost-effective strategy for chlorinated ethenes, a more thorough understanding of the required growth substrates and community structure of microorganisms capable of degrading these compounds is necessary for optimization of these processes.

Biotransformation of chlorinated ethenes can occur via reductive dechlorination, a series of reactions that convert tetrachloroethene (PCE) to TCE, followed sequentially by dichloroethene (DCE), vinyl chloride (VC), and finally ethene (*2*) or ethane (*3*). A wide variety of methanogenic and nonmethanogenic consortia (e.g., *2*−*10*) have been previously described that are capable of sequential replacement of one or more chlorine atoms with hydrogen atoms through metabolic and cometabolic processes. In addition, numerous pure cultures of halorespiring bacteria have been identified that are capable of generating energy using PCE or TCE as a terminal electron acceptor to produce chlorinated ethene end products (e.g., *11*−*14*). To date, the only known pure cultures capable of dechlorinating all of the way to ethene are strains of the genus *Dehalococcoides*, bacteria that utilize H_2 as sole electron donor, grow slowly in pure culture, and require extremely complex medium for growth (*12*, *15*). The recent detection of *Dehalococcoides* spp. at numerous PCE and TCE contaminated sites throughout the United States and Europe (*16*) and in numerous dechlorinating enrichments (*8*, *16*−*21*) suggests that these species grow much more effectively in consortia, are fairly widely distributed in the environment, and play a critical role in communities capable of complete dechlorination to ethene.

The importance of assessing hydrological and geochemical parameters and the availability of potential electron donors to optimize enhanced reductive dechlorination strategies is well understood (*22*). Numerous laboratory and field studies have been conducted to examine the potential for stimulating reductive dechlorination of chlorinated ethenes using a wide variety of complex electron donors including molasses, flour, nonfat milk, whey, vegetable oil, municipal waste sludge, etc. However, the most commonly studied electron donors for reductive dechlorination have been defined rather than complex compounds: H_2, lactate, propionate, methanol, butyrate, and ethanol (*4*−*6*, *9*, *10*, *23*−*26*). If suitable electron donors and corresponding fermenting and dechlorinating organisms are present within a consortium, reductive dechlorination through halorespiration processes can occur at rates that are orders of magnitude faster than cometabolic processes (*27*). When organic acids and alcohols are provided as electron donors, secondary-fermenting or syntrophic bacteria are responsible for production of acetate, CO_2, and H_2 (*28*). In some consortia, H_2 is provided directly as the electron donor (*24*, *26*, *29*). The importance of delivering low H_2 partial pressures to provide a selective advantage to dechlorinators over methanogens has been well documented (*4*, *5*, *10*, *23*, *30*, *31*). Electron

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donors that are slowly fermented, such as propionate and butyrate, typically provide a steady source of electrons with corresponding low H_2 partial pressures, whereas rapidly fermented substrates such as lactate typically provide quicker release of electrons with corresponding high H_2 partial pressures. Although it is quickly fermented, methanol does not directly generate H_2; instead electrons are shuttled to the generation of methane or acetate with indirect H_2 generation (*28*).

Recently, researchers have begun to assess microbial community structure and search for known dechlorinators within PCE and TCE dechlorinating communities to better understand these complex interactions (*7*, *8*, *16*, *18*−*20*, *32*). Currently, little is known about the correlations between the microbial community structure within TCE-degrading consortia and the supplied electron donor. A better understanding of the relationship will lead to the development of more efficient strategies for optimizing enhanced bioremediation of sites contaminated with chlorinated ethenes.

This study evaluates whether enrichment of subcultures with alternate electron donors will have significant effects on the community structure and activity of a lactate-enriched consortium, ANAS, that reductively dechlorinates TCE to ethene (*8*). The objectives of this study were to (1) assess the capability of subcultures of ANAS to reductively dechlorinate TCE when enriched on alternate electron donors and to (2) evaluate changes in microbial community structure associated with the various electron donors. The four electron donors chosen for this study include lactate and methanol, two compounds that are rapidly fermented, the former with relatively high H_2 production and the latter with only indirect H_2 production, and butyrate and propionate, two compounds that are fermented relatively slowly with low H_2 generation (*4*, *5*, *10*, *23*, *30*, *31*). Enrichment culture performance and microbial community structure were evaluated by monitoring dechlorination products and by culture-independent genetic analyses such as terminal restriction fragment length polymorphism (T-RFLP), clone library construction coupled with sequencing, and quantitative polymerase chain reaction (Q-PCR).

Materials and Methods

Microbial Consortia. The source inoculum for all enrichment cultures was a dechlorinating consortium (ANAS) derived from soil taken from a solvent and waste oil contaminated site at Alameda Naval Air Station in California. ANAS was enriched with lactate as electron donor and TCE as electron acceptor (*8*), and has been functionally stable (generating ethene from TCE) for over 6 years. ANAS has been maintained in semi-batch fashion in a 1.5 L stainless steel vessel with a mean hydraulic residence time of 4 ± 1 weeks and approximately weekly culture amendments of 200−400 μM TCE and 10−20 mM sodium lactate.

Lactate, methanol, butyrate, and propionate enrichment subcultures were prepared in 160-mL serum bottles with 110 mL headspace volume containing a gas mixture of 80% N_2 and 20% CO_2 at 5 psi (Matheson Gas Products, Inc. Newark, CA) and a 50 mL liquid volume. Subcultures were made by 1:10 dilution of ANAS consortium in basal salts medium containing 10 mM electron donor and 10−110 μM TCE. All electron donors were added in excess of the stoichiometric amount required for reduction of TCE to ethene. The basal medium was prepared as previously described (*8*) and contained the following components (g L^{-1}): NaCl, 4.0; NH_4Cl, 1.0; KCl, 0.1; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.04; $NaHCO_3$, 3.5; resazurin, 0.0005; cysteine hydrochloride, 0.5; $Na_2S \cdot 9H_2O$, 0.5; yeast extract, 0.5, 5 mL per L trace metal solution (*33*), and 10 mL per L vitamin solution (*33*). Reduced, fresh basal medium was dispensed into 160 mL serum bottles, sealed with blue, butyl rubber stoppers and aluminum crimp tops, and autoclaved prior to inoculation and addition of culture amendments. 1 M anoxic stock solutions of sodium lactate (Sigma Chemical Co, 98%), methanol (Mallinckrodt, Inc., Paris, KY, 99.99%), butyric acid (Sigma Chemical Co., St. Louis, Mo, 99%), and propionic acid (Sigma Chemical Co., 99%) were prepared for electron donor amendments, and TCE (Fisher Chemical, Fair Lawn, NJ, 99.99%) saturated deionized water was used for electron acceptor amendment.

Subculture Enrichment Protocol. Subcultures were initially amended with 100−200 μL TCE saturated deionized water (10−21 μM) and 0.5 mL of 1 M electron donor (10 mM). At the end of a feeding cycle, 2−10 mL of culture was removed for DNA extraction. Duplicate samples for DNA extraction were pelleted by centrifugation at 13 000*g* for 20 min. The cell pellets were stored at −20 °C until DNA extraction was performed as described below. Following the end of complete reductive dechlorination, 5 mL of culture was removed for 10% (v/v) culture transfers and the headspace was purged for 20−30 min with ultrahigh purity N_2 gas (Puritan-Bennett, Overland Park, KS, 99.999%). Culture transfers were subjected to the same initial enrichment protocols as above. Additionally, the butyl rubber stoppers were changed to minimize gas losses from the serum bottles. Following N_2 purging, the headspace was exchanged with an 80% N_2:20% CO_2 gas mixture pressurized to 5 psi for 2−5 min. The 50 mL culture volume was reestablished by adding 24−120 μM TCE (in the form of TCE saturated deionized water), 10 mM electron donor, and fresh basal salts medium. The enrichment subcultures were subjected to increasing TCE concentrations with each successful dechlorination time course. The electron donor was always added in excess with a 50:1 to 400:1 ratio of electron donor to electron acceptor maintained over the course of the experiments. Enrichments were incubated at 25 °C on a shaker table at 150 rpm. Enrichment subculture preparation, maintenance, and transfers were performed either by using benchtop transfer techniques outlined by Balch and Wolfe (*34*) or inside an anaerobic glovebox (Coy Laboratory Products, Inc., Grass Lake, MI) containing a 90% N_2:10% H_2 gas mixture (Matheson Gas Products, 99.999%).

Chemical Analyses. Methane, TCE, cDCE, VC, and ethene were monitored approximately every 2−7 days by taking 50 μL headspace samples. Samples were withdrawn with a gastight syringe and analyzed using a HP5890 gas chromatograph (GC) (Hewlett-Packard, Wilmington, DE) equipped with a flame ionization detector (FID) and a 30 m, 0.32 mm ID GS GasPro Capillary Column (J&W, Folsom, CA). Detector and injector temperatures were held at 250 and 220 °C, respectively. A gradient temperature program ramped the temperature from 50 to 180 °C at the rate of 15 °C/min, and held it at 180 °C for 3 min. Quantification of peak areas was based upon external calibration standards. Individual calibration standards for TCE, cDCE, VC, methane, and ethene were created by adding known amounts of each reagent grade compound to 160 mL serum bottles containing 50 mL of autoclaved deionized water. Standard curves and Henry's constants (*35*) were used to calculate total micromoles (μmol) of each compound in each 160 mL serum bottle. Daughter product generation rates were calculated from the sum of cDCE, VC, and ethene measured after 5−6 days for each enrichment. Daughter product generation rates were normalized to estimated dry cell mass calculated from extracted DNA mass quantified using a TD700 Turner Design fluorometer (Sunnyvale, CA) and the assumption that 3% of dry cell mass consists of DNA (*36*).

H_2 was measured by withdrawing 300 μL headspace samples with a gastight syringe and analyzing using a GC fitted with a Reductive Gas Detector (RGD) (Trace Analytical, Menlo Park, CA). The linear range for the instrument was up to a concentration of 7.7 μM. Calibration bottles with known

amounts of H_2 were used for standard curves, and samples that exceeded the upper linear limit were diluted in N_2-purged tubes prior to analysis.

Organic acids were analyzed using a Gynkotech high-pressure liquid chromatography (HPLC) (Gynkotech, Germering, Germany) instrument with a Bio-Rad (Hercules, California) Aminex HPX-87H ion-exclusion column and using 0.005 M H_2SO_4 as the eluent. Samples were centrifuged at maximum speed for 5 min to separate the cells and media precipitates, and the supernatants were acidified to 0.25 N H_2SO_4 by adding 45 μL of 2.5 N H_2SO_4 to 405 μL of sample. Eluent was pumped at 0.6 mL/min, and detection of organic acids was performed at 210 nm with a UV detector.

DNA Extraction and Quantification. Total community DNA from culture samples (1−5 mL) was extracted as previously described (*8*). Briefly, the protocol involves a two-step enzymatic treatment with lysozyme and proteinase K, bead mill homogenization, phenol/chloroform/isoamyl alcohol extraction, 2-propanol precipitation, and ethanol washing. Following extraction, the DNA was resuspended in 80 μL of sterile, nuclease-free water. Extracted DNA was quantified using a fluorometer. To remove low molecular weight DNA (<1 kb in size), the sample was passed through a ChromaSpin1000 DEPC-H_2O column (Clonetech, Palo Alto, CA). Samples were run on a 1% agarose gel to confirm the presence of high molecular weight DNA.

16S rDNA Amplification, Clone Library Construction, and Clone Sequencing. All 16S rDNA amplifications were performed using an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Westbury, NY). 16S rDNA amplification reactions for clone library construction were performed in accordance with the TOPO TA Cloning Kit manufacturer's instructions (Invitrogen, Carlsbad, CA). The 50 μL reaction mixtures contained 5 μL of 10x PCR Buffer (Applied Biosystems, Foster City, CA), 1.25 mM of each deoxynucleoside triphosphate (dNTP) (Applied Biosystems), 300 nM of bacterial primers 8F (5′- AGAGTTTGATCCTGGCTCAG-3′) (Operon Technologies, Alameda, CA), and 1492R (5′-GC(C/T)TACCTTGTTACGACTT-3′) (Operon Technologies), 1 U AmpliTaq Gold (Applied Biosystems), and 1 μL of community DNA or 1 μL of a 1:10 diluted sample of community DNA. The thermocycling programs outline by Dojka et al. (*32*) were used for 16S rDNA amplification. 4 μL of PCR product was visualized on a 2% agarose gel stained with ethidium bromide. A 1-kb ladder (Gibco BRL, Carlsbad, CA) was used as a molecular size standard.

The 16S rDNA PCR products for clone library construction were ligated into vector PCR2.1-TOPO using the TOPO TA Cloning Kit in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA). Selected clones were grown in either 1.5 or 5 mL of Luria Bertani (LB) medium (Becton Dickinson Microbiology Systems, Sparks, MD) amended with 50 μg/mL of ampicillin. Following harvesting, plasmid DNA was purified from cell pellets using a modified 96-well alkaline lysis procedure (*37*, *38*).

16S rDNA reamplification, digestion with restriction endonuclease *Msp*I (New England Biolabs, Beverly, MA), screening of restriction fragment length polymorphism (RFLP) patterns, and sequencing of clones were performed as described by Dojka et al. (*32*), with minor modifications. Briefly, the 30 μL PCR reamplification reaction mixtures contained 3 μL of 10x PCR Buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 300 nM of each 8F and 1492R primers, 1 U of AmpliTaq Gold, 1 μL of plasmid DNA, or 1 μL of a 1:25 diluted sample of plasmid DNA. The 20 μL restriction digestions were performed at 37 °C for 6 h and contained 12 μL of reamplified rDNA PCR products, 2 μL of 10x NEB Buffer 2 (New England Biolabs), 0.2 μL of *Msp*I, and 0.2 μL of 100x purified BSA (New England Biolabs). RFLP analysis was performed on an Agilent 2100 Bioanalyzer using DNA 7500 Assay in accordance with the manufacturer's instructions (www.agilent.com/chem/labonachip). Fragment lengths were determined using the bioanalyzer software in accordance with manufacturer's instructions. Clones were selected for sequencing based upon unique RFLP patterns, that is, observable bands in different locations along the lanes or on the Bioanalyzer chromatogram. In cases with many replicates of similar RFLP patterns, multiple clones were sequenced to confirm that they represented similar or identical species. Of the 256 clones screened by RFLP analysis, 74 were subsequently sequenced.

Clones were sequenced with an ABI 377 Sequencer (Applied Biosystems) in accordance with manufacturer's instructions. Briefly, 10 μL sequencing reaction mixtures contained 4 μL of Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems), 3 μL of plasmid DNA or 3 μL of a 1:25 diluted sample of plasmid DNA, and 900 nM of each primer. The thermocycling program was the same for the sequencing reaction except that 24 cycles were used. Partial or full sequences were obtained using T7 (5′-AATACGACTCACTATAG-3′) (Operon Technologies), 515F (5′-GTGCCAGC(A/C)GCCGCGGTAA-3′), and M13Reverse (5′AACAGCTATGACCATG-3′) plasmid primers. Plasmid DNA sequences were aligned using the AutoAssembler software package version 2.1 (Applied Biosystems). After alignment and visual checking of each sequence, the BLAST software on the Genbank website (Genbank, National Center for Biotechnology Information: www.ncbi.nlm.nih.gov/) was used to determine its putative phylogenetic association from its nearest cultured neighbor. Chimera Check analysis software (www.rdp.cme.msu.edu) was used to determine whether sequences were likely of chimeric origin.

T-RFLP Analysis. T-RFLP analysis was performed as described previously (*8*) using the procedures outlined by Liu et al. (*39*). Briefly, whole community DNA was amplified with the same procedure and bacterial primers (8F and 1492R) as for clone library construction, except that the 8F primer was labeled with 6-FAM (6-carboxyfluorescein) on its 5′ end. The mixed bacterial PCR products were digested with the enzyme *Msp*I at 37 °C for 3 h, and the resulting fragments were separated and detected by running the digest on an ABI 377 Sequencer and analyzed with the GeneScan software package (PE Biosystems, Foster City, CA).

Quantitative PCR (Q-PCR). Q-PCR was performed with primers targeting the 16S rDNA for *Dehalococcoides* spp. using extracted DNA samples for each electron donor enrichment. TaqMan primers and probes for Q-PCR were designed using Primer Express software version 2.0 (Applied Biosystems, Foster City, CA). The sequences of the probes were designed to target the 16S rDNA of all known *Dehalococcoides* strains in Genbank. The nucleotide sequences of the forward and reverse hybridization primers were 5′GGTAATACGTAGGAAGCAAGCG-3′ and 5′- CCGGTTAAGCCGGGAAATT-3′, respectively. The fluorogenic probe (5′-ACATCCAACTTGAAAGACCACCTACGCTCACT-3′) was labeled with 6-FAM (6-carboxyfluorescein) on its 5′ end as the reporter and with TAMRA (6-carboxytetramethylrhodamine) at the 3′ serving as the quencher dye.

Q-PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) in accordance with manufacturer's instructions. The 25 μL reaction mixtures contained PCR 2x master mix (12.5 μL based on AmpliTaq Gold DNA polymerase), 1 μL of community DNA, 100 μM of each primer, 100 μM of the fluorogenic probe, and sterile water. The following thermocycling program was used for 16S rDNA amplification: 2 min at 50 °C, followed by 10 min at 95 °C to activate AmpliTaq Gold DNA polymerase. This program was repeated for 40 cycles with 10 s denaturing steps at 95 °C, 1 min annealing steps at 60 °C, and a final annealing and extension step at 55 °C for 1 min.

For construction of the *Dehalococcoides* 16S rDNA standard curve, a fragment spanning positions 8−1417 of the gene (TIGR or Genbank AF004928) was cloned out of *D. ethenogenes* 195 (courtesy of Professor Stephen Zinder at Cornell University) using the TOPO TA Cloning Kit according to the manufacturer's instructions. Following cloning, plasmid DNA was purified and quantified fluorometrically. The plasmid preparation was used to create a dilution series spanning 7 orders of magnitude. Plasmid DNA was chosen as standard curve template due to its increased resistance to enzymatic attack as compared to linear DNA. Finally, DNA concentrations were converted to *Dehalococcoides* 16S rDNA copy numbers using the known size of the plasmid and insert.

Clone Library Designations and Genbank Accession Numbers. Individual clones selected for DNA sequencing were identified based on enrichment type (i.e., 10^{-3} dilution of TCE dechlorinating enrichment culture using lactate as electron donor or LTCE-T2A), 96-well column and row designation (i.e., 2C), and RFLP pattern designation (i.e., ED-ANAS-1). Sequences determined in this study have been deposited into the Genbank database under accession numbers AY217379−AY217449.

Results

Evaluation of Dechlorination Ability with Alternate Electron Donors. Two rapidly fermenting electron donors, lactate and methanol, and two slowly fermenting electron donors, propionate and butyrate, were selected to evaluate the effects of alternate electron donors on a lactate-enriched consortium capable of complete TCE dechlorination. A lactate-enriched subculture was included in this study to control for the effects of successive culture transfers and new subculture conditions. Prior to microbial community analysis, electron donor enrichments were subcultured three times by 10% v/v serial transfers to fresh basal medium with TCE and 10 mM electron donor.

The initial generation of subcultures was subjected to increasing TCE concentrations (10−110 μM) over a period of several months. Biotransformation of TCE to ethene with transient DCE and VC production occurred with each of the alternate electron donors with similar temporal patterns and no observable lag time for acclimation (Figure 1a). Subsequent second and third generation subcultures also demonstrated complete biotransformation of TCE to ethene. The rate of TCE utilization for all electron donor enrichments increased with each successive TCE amendment.

Patterns of hydrogen and methane generated by the various electron donors are shown in Figure 1b and c. Lactate resulted in a sharp increase in hydrogen during the first 5 days, followed by a rapid decrease to concentrations consistent with the other three electron donors. In addition, methanol and butyrate reproducibly generated a relatively small hydrogen peak at approximately 4 days, which subsequently leveled off. Hydrogen production was consistent with observations that each of the electron donors was converted to equivalent amounts of acetate and propionate within the first 5 days, after which concentrations remained stable. Methane generation was significantly higher in the lactate culture than in the other cultures and was most active during the decline of hydrogen (days 3−6), suggesting that hydrogenotrophic methanogens were active during this time.

T-RFLP Profiles for Electron Donor Enrichments. Following observations of some functional similarities among alternate electron donor enrichments, the microbial community structures were analyzed to determine if alternate electron donors stimulated significant population shifts. T-RFLP was chosen as an initial assessment tool to evaluate major changes in the diversity of the enrichments due to its ease of use and potential for rapid analysis (*7*, *8*, *39*). Samples for T-RFLP were collected at the onset of ethene production for the third subculture of each enrichment. Figure 2a−d shows electropherograms of the 5′ T-RFLP patterns from *Msp*I digests of amplified community rDNA. Additionally, Figure 2e shows the overlaid T-RFLP plots of relevant individual clones from the original ANAS consortium (*8*). To account for differences in extracted DNA masses between samples, dominant peaks were identified after individual T-RFLP peak heights were normalized to total fluorescence intensity (FI) using procedures similar to Dunbar et al. (*40*), with a normalized FI cutoff value of 200. A total of 15 dominant peaks were identified among the four enrichment cultures as shown in Table 1. Corresponding T-RFs from clones identified from ANAS, along with predicted T-RFs from T-RFLP simulation software (TAP-TRFLP; www.rdp.cme.msu.edu), were used to predict phylogenetic associations of dominant peaks. A tolerance limit of ±2 base pairs (bp) was used for phylogenetic predictions based on previous conclusions from Liu et al. (*39*) on the sizing accuracy of T-RFs ranging from 37 to 600 bp.

T-RFLP results revealed interesting shifts in the community structure between lactate and the other enrichments. The lactate enrichment contained nine dominant peaks (Figure 2a) and appeared to exhibit the most diverse community structure of the enrichments. No peak in this enrichment represented more than 15% of the total normalized FI (Table 1). Methanol (Figure 2b), propionate (Figure 2c), and butyrate enrichments (Figure 2d) contained seven, eight, and seven dominant peaks, respectively. Three dominant peaks were identified in all four enrichments; given with their corresponding presumptive phylogenetic associations, they were peaks 3 (95 bp; *Bacteroides* sp.), 5 (161 bp; *Desulfovibrio* sp.), and 7 (498 bp; *Citrobacter* sp.). Seven of the nine dominant peaks in the lactate enrichment were conserved in either the methanol, the propionate, or the butyrate enrichments. Peaks 8 (529 bp) and 9 (545 bp) were only observed in the lactate enrichment, presumptively associated with *Clostridium* sp. and an unknown species, respectively. T-RFLP results for the methanol enrichment revealed the absence of peaks 1, 4, 8, 9, and 12−15 and the appearance of peaks 10 (221 bp) and 11 (296 bp) with a possible phylogenetic association to *Eubacterium* sp. and *Clostridium* sp., respectively. Peak 7, presumptively associated with *Citrobacter* sp., along with peak 10 dominated the profile, representing over 49% of the total normalized FI (Table 1). The propionate enrichment revealed an absence of peaks 1, 4, 8−11, and 15 but demonstrated an appearance of peaks 12 (492 bp), 13 (513 bp), and 14 (520 bp), presumptively belonging to *Dechlorosoma* sp., *Dehalococcoides* sp., and *Clostridium* sp., respectively. Finally, the butyrate enrichment revealed the absence of peaks 2, 6, and 8−13, with the appearance of peak 15 (103 bp), corresponding to an unknown species. Peaks presumptively associated with *Mycobacterium* sp. (71 bp), *Bacteroides* sp. (95 bp), and *Citrobacter* sp. (498 bp) represented over 45% of the total normalized FI for this enrichment. Although *Dehalococcoides* sp. was only detected as a dominant peak in the propionate enrichment, it was detected below the dominant peak threshold (<200 normalized FI) in all third generation subcultures.

Previous T-RF length identification associated with sequenced ANAS consortium clones (20), along with predicted T-RF lengths from TAP-TRFLP, allowed for the presumptive identification of four phylogenic genera of bacteria, *Mycobacterium*, *Flavobacterium*, *Bacillus*, and *Lactobacillus*, which were not previously identified within the original ANAS consortium. Additionally, *Serratia* sp. (156 bp) and *Propionibacterium* sp., which were previously identified in the ANAS consortium clones (494 bp), were not detected in the enrichments.

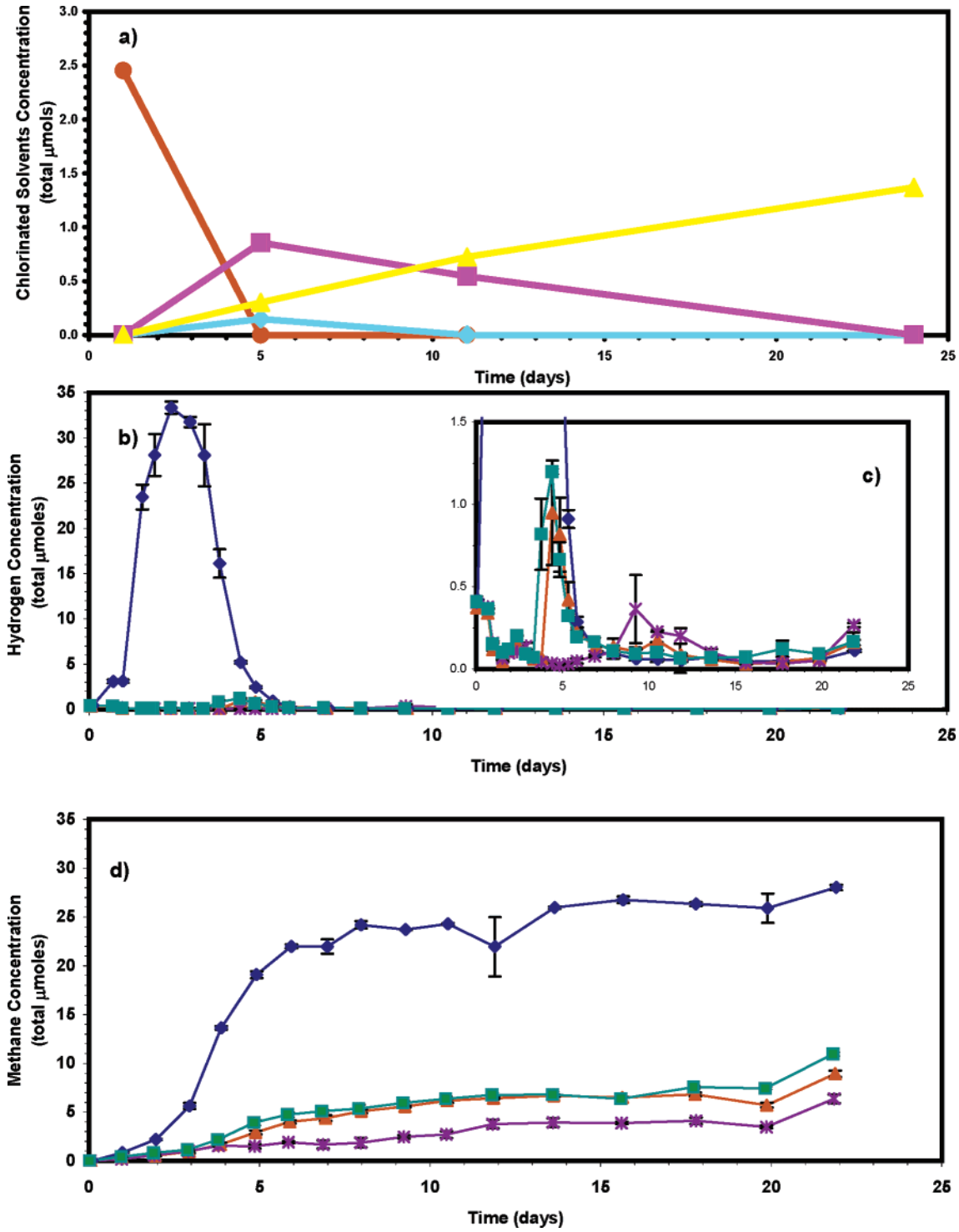

FIGURE 1. (a) Typical dechlorination pattern (conversion of TCE (red ●), to ethene (yellow ▲) with the transient build-up of cDCE (aqua ◆) and vinyl chloride (pink ■)) observed for each enrichment subculture. Part (a) represents the first TCE amendment for the first generation (10^{-1} dilution) lactate subculture. Hydrogen (b and c) and methane (d) concentration data for the first generation subcultures (lactate (blue ◆), methanol (green ■), propionate (purple *), and buytrate (orange ▲)).

Clone Libraries of Electron Donor Enrichments. Because T-RFLP results indicated that fundamental changes in the microbial community had occurred as a result of enrichment on alternate electron donors, the phylogenetic associations of the enrichments were evaluated by constructing clone libraries from rDNA extracted from the third generation of each enrichment. Table 2 is a summary of the bacterial clones identified in the four enrichments and their corresponding putative phylogenetic association. Also included in Table 2 is the clone breakdown for the original ANAS culture (*20*). A total of 256 clones containing rDNA inserts were screened for unique RFLP patterns, and 74 partial and complete 16S rDNA sequences were subsequently submitted to Genbank (AY217379−AY217449). Of these 74 sequences, 54 had ≥95% similarity to an identified and/or cultured neighbor. Each of the four enrichments contained similar numbers of distinct RFLP patterns, with lactate, methanol, propionate, and butyrate containing 32, 32, 20, and 33, respectively

The majority of the clones identified in all of the enrichments showed sequence similarity to low G+C Gram-

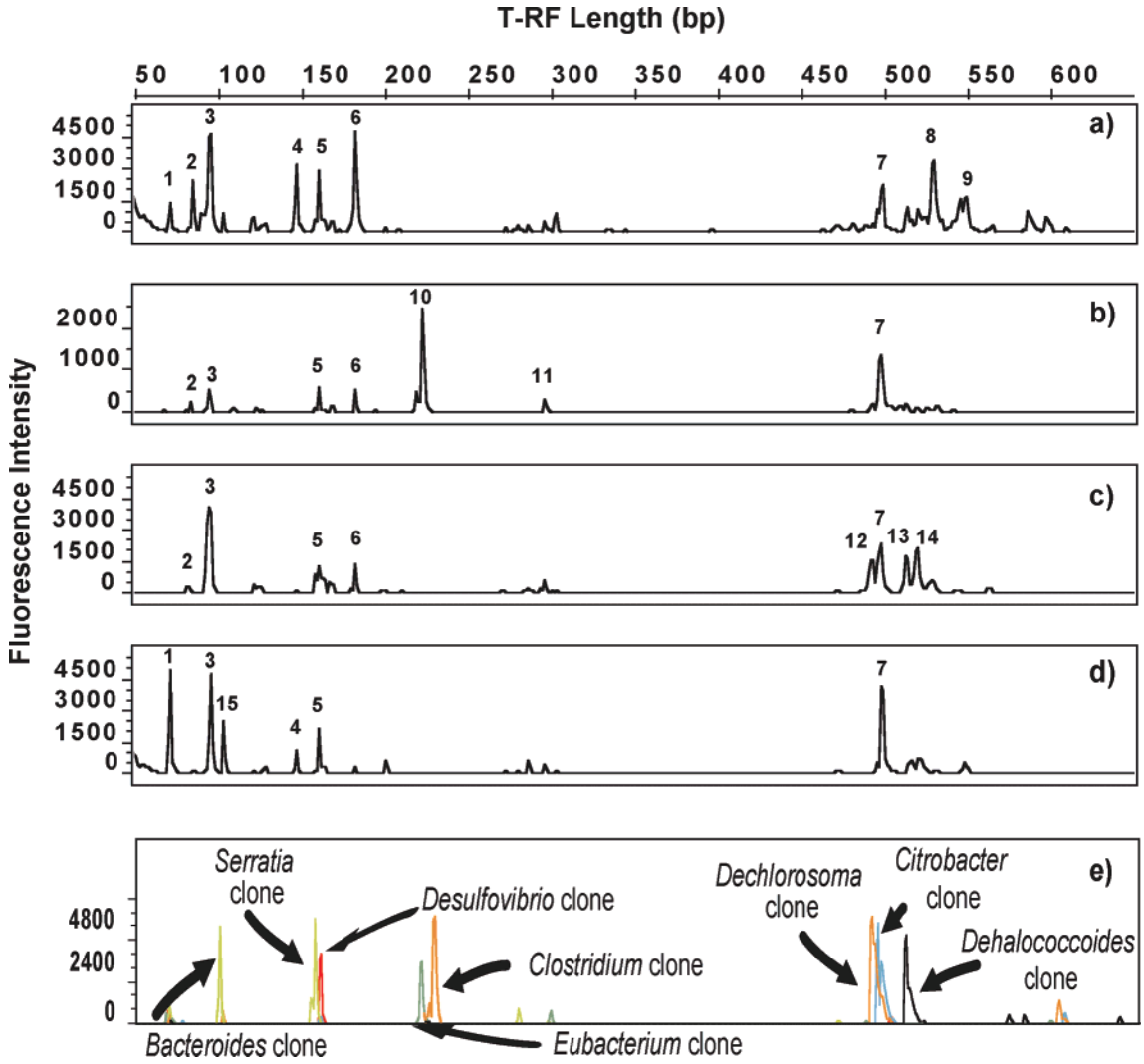

FIGURE 2. T-RFLP profiles generated from community rDNA extracted from electron donor enrichment subcultures. Samples were taken from 10^{-3} dilution subcultures following either the beginning of complete dechlorination (start of ethene production) or the end of complete dechlorination. Bacterial primers (8F-FAM and 1492R) were used, and restrictions were performed with *Msp*I (recognition sequence, CCGG). (a) Lactate, (b) methanol, (c) propionate, (d) butyrate, (e) T-RFLP profiles from sequenced clones of the ANAS consortium previously described by Richardson et al., 2002 (*8*).

positive bacteria (LGCs; most belonging to *Clostridium* and *Eubacterium* genera), *Bacteroides* sp., and *Citrobacter* sp. Unsurprisingly, the data indicate that fermenting bacteria dominated each of the libraries. The percent of clones for *Clostridium* spp., *Bacteroides* spp., and *Citrobacter* spp. ranged from 78% to 88% for the four enrichments (Table 2). In contrast, these three genera represent only 38% of the clones in the parent ANAS culture. In fact, although *Bacteroides* spp. were well represented in each of the enrichment libraries (29−68% of clones), this enteric obligate anaerobe that is capable of carbohydrate, polysaccharide, and simple sugar fermentation was poorly represented in the ANAS library (3% of clones in ANAS-I and 0% of clones in ANAS-II libraries (*8*)). In addition, the enrichments produced more clones of *Citrobacter* sp., a facultative aerobe that ferments simple sugars and is capable of hydrogen production. Although *Desulfovibrio spp.*, sulfate reducers that can also grow by fermentation, was well represented in the ANAS consortium (13%), clones of this genus were found only in low numbers in the methanol (2.2%) and butyrate enrichments (2.6%). Another sulfate reducer and fermentor, *Desulfobulbus elongatus*, was detected in the lactate enrichment. Interestingly, although *Dehalococcoides* spp. was a dominant representative of the clones from the original ANAS consortium (33%), they were not found in the lactate, propionate, and butyrate clone libraries, and they only represented 6.7% of the clones from the methanol enrichment. In fact, three different *Dehalococcoides* clones were identified in this enrichment, each with only 97−98% similarity to the cultured *D. ethenogenes* 195.

Application of Q-PCR for Detection of *Dehalococcoides* sp. Although all four electron donor enrichments demonstrated functional similarities in their ability to perform the complete biotransformation of TCE to ethene, *Dehalococcoides* spp. was only detected in the propionate enrichment during T-RFLP analysis, and only in the methanol enrichment during clone library construction. Previous investigators have reported difficulties detecting *Dehalococcoides* spp. using universal bacterial primers, and subsequently have used nested PCR with *Dehalococcoides* specific primers to improve detection (*16*, *18*, *19*, *21*). In this study, Q-PCR was applied to quantify copy numbers of specific 16S rDNA sequences associated with *Dehalococcoides* in the first and third generations of each enrichment culture (Table 3). Because *Dehalococcoides* contains only one copy of the 16S rDNA gene on its chromosome (determined from the genome sequence at www.tigr.org (*41*)), the quantification of *Dehalococcoides* 16S rDNA genes in each sample is expected to be

TABLE 1. Summary of Dominant T-RF[a] Lengths Present in Electron Donor Enrichment Subcultures

peak number	actual T-RF length (bp)	percentage of total normalized fluorescence intensity[b]				predicted genus association[c]
		lactate	methanol	propionate	butyrate	
1	71	2.2			15.0	*Mycobacterium* sp.
2	85	3.9	2.6	3.0		*Flavobacterium* sp.
3	95	12.2	5.5	22.4	17.7	*Bacteroides* sp.
4	147	5.0			3.5	*Bacillus* sp.
5	161	4.6	5.9	11.1	6.8	*Desulfovibrio* sp.
6	182	7.3	5.4	4.3		*Lactobacillus* sp.
7	498	6.5	16.5	11.1	12.5	*Citrobacter* sp.
8	529	5.3				*Clostridium* sp.
9	545	5.0				unknown
10	221		32.1			*Eubacterium* sp.
11	296		3.0			*Clostridium* sp.
12	492			7.7		*Dechlorisoma* sp.
13	513			5.3		*Dehalococcoides* sp.
14	520			9.3	2.3	*Clostridium* sp.
15	103				8.0	unknown

[a] T-RF = terminal restriction fragment. [b] Dominant T-RFs are based on peak heights > 200 normalized total fluorescence intensity. Total normalized fluorescence intensity is calculated using procedures similar to Dunbar et al. (2001) (*40*). [c] Predicted genus is based on observed T-RFs lengths, clone library data, TAP T-RFLP analysis (www.rdp.cme.msu.edu), and previous identification of clones from Richardson et al. (*20*) (Figure 2e).

representative of the actual number of *Dehalococcoides* cells extracted from each sample. In contrast to the T-RFLP and clone library results, *Dehalococcoides* was detected in all four enrichments by Q-PCR. Because the cell density and extraction efficiency were expected to vary considerably across enrichments, copy numbers were normalized to mass of DNA extracted, to facilitate specific comparisons. Detected numbers of *Dehalococcoides* cells varied widely among the enrichments. Both the bulk and the mass-normalized numbers indicate that the first and third generation butyrate enrichments and the third generation propionate enrichment contained the largest amount of *Dehalococcoides* 16S rDNA, with numbers slightly larger than those of the parent ANAS culture. The first generation methanol culture and the third generation lactate and methanol cultures all contained the same order of magnitude of copies of *Dehalococcoides* 16S rDNA per mass of DNA.

Although a detailed kinetic study was not performed as part of this study, the initial daughter product generation was measured for the ANAS consortium and each of the first and third generation enrichments (Table 3). Complete conversion of TCE to ethene generally occurred for all of the enrichments within 24 days. Measured daughter product generation rates were similar across the first generation subcultures, with the exception of the butyrate subculture that demonstrated a cell mass-normalized rate that was 5−7 times slower than the other enrichments. Conversely, the third generation methanol and butyrate subcultures exhibited cell mass-normalized rates that were more than 2−9 times faster than the other subcultures. The normalized dechlorination rate for ANAS was 10−65 times faster than that for the first generation subcultures and 10−100 times faster than that for the third generation subcultures. Interestingly, although functional similarity in TCE degradation abilities was observed among all cultures, there was not a good correlation between the quantity of *Dehalococcoides* cells detected by Q-PCR and the measured degradation rates.

Discussion

The objective of this study was to assess the effects of enriching TCE-dechlorinating communities with alternate electron donors and to evaluate microbial community changes associated with the enrichments. Although PCE or TCE-dechlorinating enrichment cultures have been studied previously, there is still an incomplete understanding of the relationship between known dechlorinating bacteria, such as *Dehalococcoides* spp., and associated fermenting species that may be required to provide H_2 and essential vitamins and nutrients. This paper presents the first study of the use of molecular tools to assess microbial community changes resulting from enrichment of a TCE-dechlorinating culture with alternate electron donors.

Results of this study indicate that lactate, methanol, propionate, and butyrate were all suitable electron donors to support TCE dechlorination to ethene by the studied consortia. Although different electron donors resulted in different hydrogen patterns during the first week following feeding, hydrogen concentrations and methane generation rates were equivalent for all cultures from the second week onward. The rapid H_2 production observed during the fermentation of lactate resulted in increased methanogenic activity during the first week, suggesting that the majority of electrons produced from lactate fermentation were being shuttled to methane production rather than supporting enhanced dechlorination, as reported previously (*23*). The lower methane production observed in the butyrate and propionate enrichments was likely due to diminished selection for methanogens caused by lower hydrogen production as shown previously by Fennel et al. (*23*).

Five phylogenic genera or subgroups of bacteria including *Dehalococcoides*, low G+C Gram-positives (mostly *Clostridium* and *Eubacterium*), *Bacteroides*, *Citrobacter*, and δ *Proteobacteria* (mostly *Desulfovibrio*) were present in all donor enrichments. Daughter product dechlorination rates were similar for the various enrichments with complete conversion to ethene within 24 days, but generally decreased in subsequent generations.

The use of T-RFLP to provide initial screening of the microbial consortia qualitatively illustrated shifts in the community structure as a result of the enrichment on alternate electron donors. Further, the putative presence of *Bacteroides* spp., *Desulfovibrio* spp., and *Citrobacter* spp. in the T-RFLP profiles of all enrichments suggests that these genera likely play a key role in the syntrophic relationship with *Dehalococcoides*. However, T-RFLP profiles did not demonstrate the presence of *Dehalococcoides* as a dominant peak in three of the four enrichments, consistent with its use as a qualitative tool for detecting major shifts in microbial communities rather than for tracking individual organisms.

Clone libraries provided species identification associated with shifts in microbial community structure due to the enrichments. Phylogenetic associations indicated only minor shifts at the group and genus level with more prevalent shifts at the species level. The predominance of *Bacteroides* spp.

TABLE 2. Phylogenetic Summary of TCE-Dechlorinating, Electron Donor Enrichment Subcultures Based on Clone Library Construction and Sequence Analysis[a]

putative phylogenetic association	% of clones from each enrichment				
	lactate[b]	methanol[b]	propionate[b]	butyrate[b]	ANAS-II[c]
***Dehaloccoccoides* group**					
Dehalococcoides sp.		6.7			33.3
total *Dehalococcoides* sp.		**6.7**			**33.3**
low G+C Gram-positive bacteria					
Clostridium akagii	18.0	6.7	7.2		
Clostridium cellulovorans		2.2			
Clostridium hastiforme		2.2			
Clostridium hydroxybenzoicum	11.5	6.7	4.3	7.9	
Clostridium orbiscindens		2.2			
Clostridium peptidovorans			5.8		
Clostridium pascui		2.2			
Clostridium propionicum		20.0	7.2		
Clostridium thiosulforeducens		2.2	10.1	2.6	
total *Clostridium* sp.	**29.5**	**44.4**	**34.6**	**10.5**	**34.6**
Eubacterium limosum		2.2			2.6
eubacterium clone vadinCA02		2.2			
eubacterium clone vadinHA73		2.2			
uncultured eubacterium WCHB1-29	4.9		2.9		
total *Eubacterium* sp.	**4.9**	**6.7**	**2.9**		**2.6**
Acidaminococcus fermentans	1.6		1.4		
Ruminococcus albus	1.6				
Sporomusa malonica	1.6		4.3		
Syntrophomonas sp. *MGB-C1*		2.2			
Tissierella praeacuta			1.4		
Aminobacterium sp.					5.1
total other low G+C Gram-positives	**4.9**	**2.2**	**7.2**		**5.1**
Bacteroidaceae					
Bacteroides sp. *ASF519*	26.2	17.8	40.6	31.6	
Bacteroides sp. *253c*		2.2			
Bacteroides caccae			2.9		
Bacteroides distasonis		6.7			
Bacteroides forsythus	1.6	2.2		2.6	
Bacteroides merdae	1.6			34.2	
total *Bacteroides* sp.	**29.4**	**28.9**	**43.5**	**68.4**	
γ Proteobacteria					
Citrobacter braakii			1.4	5.3	
Citrobacter freundii	1.6	2.2	1.4	2.6	
Citrobacter werkmanii	24.6	2.2	7.2		
total *Citrobacter* sp.	**26.2**	**4.4**	**10.0**	**7.9**	**3.8**
δ Proteobacteria					
Desulfovibrio desulfuricans		2.2		2.6	
Desulfobulbus elongatus	1.6				
total ∂ *Proteobacteria*	**1.6**	**2.2**		**2.6**	**12.8**
Spirochaeta					
total *spirochaeta* sp.					**1.3**
closest to unidentified bacterium					
bacterium DCE29			1.4		
uncultured bacterium clone p-4322-4Wa3	1.6				
uncultured bacterium ckncm229-4FM.2F5	1.6				
uncultured bacterium SHA-7				7.9	
total unidentified bacterium	**3.3**		**1.4**	**7.9**	
chimeras[d]		**4.4**		**2.6**	**1.3**
total number of clones analyzed	**80**	**46**	**70**	**60**	**78**

[a] Clone libraries were constructed using Bacterial primers 8F and 1492R. [b] DNA was extracted from third generation enrichments after the onset of ethene production. [c] Clone library results for the ANAS consortium were previously described in Richardson et al., 2002 (*20*). [d] Screening for chimeric sequences was performed using the Chimera Check analysis software located at the RDP-II website: http://rdp.cme.msu.edu (Maidak et al., 2001).

and *Clostridium* spp. in the clone libraries suggests that these versatile fermentors play an important role in H_2 transfer to *Dehalococcoides*. The dominant *Bacteroides* sequence detected in the enrichments was most closely related to *Bacteroides distasonis* (*42*) and represented approximately 26%, 18%, 41%, and 32% of the clones from the lactate, methanol, propionate, and butyrate enrichments, respectively. The dominant *Clostridium* sequences detected in the enrichments were closely related to *Clostridium akagii* and *Clostridium hydroxybenzoicum*. *Clostridium akagii*, a bacteria capable of fermenting glucose to acetate, lactate, butyrate, H_2, and CO_2 (*43*), represented approximately 7−18% of clones from the lactate, methanol, and propionate enrichments, but none in the butyrate enrichment. Although Kuhner et al. (*43*) did not directly test lactate, methanol, and propionate as suitable carbon sources during the characterization of *Clostridium akagii*, it is likely that these carbon sources are also fermented to H_2 by this bacteria. *Clostridium hydroxybenzoicum*, an amino acid-utilizing, anaerobic rod that requires yeast extract or acetate for growth in pure culture, is the closest sequence to 4−12% of clones from the four enrichments. *Clostridium* spp. likely plays an indirect role

TABLE 3. Q-PCR Results Using *Dehalococcoides* 16S rDNA Primers

electron donor enrichment	total extracted community DNA (μg/mL)	copies[c]/mL of sample	copies/mg of DNA	daughter product generation rate (μmol L^{-1} day^{-1})[e]	daughter product generation rate (μmol mg cell $mass^{-1}$ day^{-1})[e,f]
ANAS	2.0	$(1.0 \times 10^{+07}) \pm (2.9 \times 10^{+06})$[d]	$(1.0 \times 10^{+08}) \pm (2.9 \times 10^{+07})$	32	0.477
first subculture[a]					
lactate	3.2	$(7.3 \times 10^{+05}) \pm (3.7 \times 10^{+04})$	$(4.5 \times 10^{+06}) \pm (2.3 \times 10^{+05})$	4.2	0.040
methanol	4.1	$(1.4 \times 10^{+06}) \pm (3.2 \times 10^{+05})$	$(7.0 \times 10^{+06}) \pm (1.6 \times 10^{+06})$	6.1	0.044
propionate	3.5	$(6.9 \times 10^{+06}) \pm (1.0 \times 10^{+06})$	$(3.9 \times 10^{+07}) \pm (5.7 \times 10^{+06})$	5.6	0.048
butyrate	6.0	$(4.7 \times 10^{+07}) \pm (3.4 \times 10^{+05})$	$(1.6 \times 10^{+08}) \pm (1.1 \times 10^{+06})$	1.4	0.0071
third subculture[b]					
lactate	13	$(5.8 \times 10^{+06}) \pm (2.1 \times 10^{+05})$	$(4.4 \times 10^{+07}) \pm (6.1 \times 10^{+06})$	2.1	0.0047
methanol	2.6	$(3.5 \times 10^{+05}) \pm (1.4 \times 10^{+05})$	$(2.7 \times 10^{+07}) \pm (1.1 \times 10^{+07})$	3.6	0.041
propionate	8.7	$(1.2 \times 10^{+07}) \pm (5.7 \times 10^{+06})$	$(1.3 \times 10^{+08}) \pm (6.5 \times 10^{+07})$	2.5	0.0087
butyrate	1.9	$(1.3 \times 10^{+07}) \pm (1.8 \times 10^{+07})$	$(1.4 \times 10^{+08}) \pm (4.8 \times 10^{+07})$	3.8	0.018

[a] First generation subculture is 10^{-1} dilution from ANAS consortium. [b] Third generation subculture is 10^{-3} dilution from ANAS consortium. [c] Copies of *Dehalococcoides* 16S rDNA/mL of sample. [d] Standard deviation. [e] Daughter product generation rates were calculated from the sum of cDCE, VC, and ethene measured after 5−6 days for each enrichment. Complete conversion of TCE to ethene occurred for the ANAS consortium and each of the first and third generation enrichments within 24 days. [f] Rates were normalized to total cell mass based on the total mass of extracted community DNA and the assumption of DNA representing 3% dry cell mass (*36*).

in the TCE degradation by either H_2 transfer or supply of important nutrients to *Dehalococcoides* as indicated by previous investigators (*8*, *11*). Clone library results showed that 2−25% of sequenced clones from the lactate, methanol, and propionate enrichments were most closely related to *Citrobacter werkmanii*, a known fermentor of lactate and other simple organics. The presence of clones related to *Syntrophomonas* spp. and *Eubacteriaum limosum* in the methanol enrichment suggests that methanol may stimulate inter-relationships with the syntroph providing hydrogen, while the acetogenic methylotroph generates acetate and vitamin B12 for *Dehalococcoides* (*29*, *44*).

T-RFLP results (Table 1) were in general agreement with clone library results (Table 2) for *Bacteroides* spp., *Citrobacter* spp., and *Clostridium* spp., but some inconsistencies were observed for *Desulfovibrio* spp., *Eubacterium* spp., and *Dehalococcoides* spp. detection. However, T-RFLP results indicated the presence of *Desulfovibrio* spp. in all enrichments, while clone library results indicated its presence only in the methanol and butyrate enrichments. Also, *Eubacterium* spp. was detected by T-RFLP only in the methanol enrichment, whereas it appeared in clone library results of all enrichments except for butyrate.

Conflicting T-RFLP and clone library results highlight the importance of recognizing that limited numbers of representative clones, potential PCR bias, and variations in extraction efficiencies can potentially lead to misinterpretations of community structure. Potential PCR biases include cell lysis, DNA contamination, PCR chimera formation, PCR amplification, poor cloning efficiencies, and rDNA copy number (*45*). Attempts were made to minimize the introduction of biases through the use of a physical cell lysis method utilizing bead mill homogenization as discussed previously (*8*, *32*, *46*) and by removing low molecular weight DNA. In addition, the variation in number of 16S rDNA gene copies present in some species in the enrichments may have contributed to a skewed picture of the actual microbial community by over-representing their presence (*47*). For example, *Clostridium* spp. and *Bacteroides* spp. contain up to 15 and 5 16S rDNA gene copies per cell, respectively, whereas *D. ethenogenes* 195 contains only one (www.tigr.org/tdb/mdb, www.rrndb.cme.msu.edu). Further, given the divergent approaches inherent in the two methodologies, focusing primarily on dominant structure and diversity, respectively, it is not surprising that each would yield unique results. Therefore, T-RFLP and clone library results should be recognized as providing complementary pictures of organisms contributing to microbial community structure rather than a quantitative analysis of specific species distribution.

Previous results by Hendrickson et al. (*16*) indicated that the 16S rDNA copies of *Dehalococcoides* in a mixed culture increased as TCE dechlorination proceeded toward ethene. Therefore, in this study, samples for DNA extraction were collected at the onset of ethene production for each enrichment culture. Despite this, *Dehalococcoides* clones were found only in the library from the methanol enrichment. In contrast, Q-PCR with *Dehalococcoides*-specific probes and primers detected significant numbers of these cells in all enrichments. These results are consistent with the functional similarity observed for all enrichments. Interestingly, Q-PCR detected a greater number of *Dehalococcoides* cells with the slower H_2 producing electron donor enrichments, butyrate and propionate, confirming that rapid H_2 production does not necessarily correlate with enhanced *Dehalococcoides* presence or growth (*23*). Recent studies have demonstrated that additional strains of *Dehalococcoides* exist, including some that are capable of growth on the transformation of VC to ethene (*17*, *21*). In addition, although all of the *Dehalococcoides* strains described to date would be detected by the primer/probe sets used in this study, there are many dechlorinators that catalyze only the TCE to DCE reaction that would not be detected by this primer/probe set. Therefore, it is possible that the observed discrepancy between the rate of dehalogenation and the number of detected *Dehalococcoides* cells in the enrichments is due to the presence of additional dehalogenating species that were not detected with the applied primer/probe set. Alternatively, it is possible that the dissimilar growth conditions resulting from the different electron donors caused unequal expression patterns of the dehalogenases generated by the *Dehalococcoides* cells.

The optimal electron donor to stimulate TCE reductive dechlorination in field applications will be a strong function of the hydrological and geochemical site conditions as well as the indigenous microbial communities (*19*). This study showed that lactate and three alternate electron donors all appear to be suitable electron donors to promote reductive dechlorination due to the presence of versatile fermenting bacteria such as *Bacteroides* spp., *Clostridium* spp., *Citrobacter* spp., and *Desulfovibrio* spp. in dechlorinating consortia. Further evaluation of dominant fermenting bacteria and dehalogenators in field samples using Q-PCR would increase our understanding of the inter-relationships among

these species so that biostimulation strategies can be optimized.

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Literature Cited

(1) *Sources, Emission and Exposure for Trichloroethylene (TCE) and Related Chemicals*. National Center for Environmental Assessment-Washington Office, Office of Research and Development. U.S. Environmental Protection Agency: Washington, DC, 2001.

(2) Freedman, D. L.; Gossett, J. M. Biological Reductive Dechlorination of Tetrachloroethylene and Trichloroethylene to Ethylene Under Methanogenic Conditions. *Appl. Environ. Microbiol.* **1989**, *55*, 2144−2151.

(3) de Bruin, W. P.; Kotterman, M. J.; Posthumus, M. A.; Schraa, G.; Zehnder, A. J. Complete Bological Reductive Transformation of Tetrachloroethene to Ethane. *Appl. Environ. Microbiol.* **1992**, *58*, 1996−2000.

(4) Ballapragada, B. S.; Stensel, H. D.; Puhakka, J. A.; Ferguson, J. F. Effect of Hydrogen on Reductive Dechlorination of Chlorinated Ethenes. *Environ. Sci. Technol.* **1997**, *31*, 1728−1734.

(5) Carr, C. S.; Hughes, J. B. Enrichment of High-Rate PCE Dechlorination and Comparative Study of Lactate, Methanol, and Hydrogen As Electron Donors To Sustain Activity. *Environ. Sci. Technol.* **1998**, *32*, 1817−1824.

(6) DiStefano, T. D.; Gosset, J. M.; Zinder, S. H. Reductive Dechlorination of High Concentrations of Tetrachloroethene to Ethene by an Anaerobic Enrichment Culture in the Absence of Methanogenesis. *Appl. Environ. Microbiol.* **1991**, *57*, 2287−2292.

(7) Flynn, S. J.; Loffler, F. E.; Tiedje, J. M. Microbial Community Changes Associated With a Shift From Reductive Dechlorination of PCE to Reductive Dechlorination of Cis-DCE and VC. *Environ. Sci. Technol.* **2000**, *34*, 1056−1061.

(8) Richardson, R. E.; Bhupathiraju, V. K.; Song, D. L.; Goulet, T. A.; Alvarez-Cohen, L. Phylogenetic Characterization of Microbial Communities That Reductively Dechlorinate TCE Based Upon a Combination of Molecular Techniques. *Environ. Sci. Technol.* **2002**, *36*, 2652−2662.

(9) Smatlak, C. R.; Gossett, J. M.; Zinder, S. H. Comparative Kinetics of Hydrogen Utilization for Reductive Dechlorination of Tetrachloroethene and Methanogenesis in an Anaerobic Enrichment Culture. *Environ. Sci. Technol.* **1996**, *30*, 2850−2858.

(10) Yang, Y. R.; McCarty, P. L. Competition for Hydrogen Within a Chlorinated Solvent Dehalogenating Anaerobic Mixed Culture. *Environ. Sci. Technol.* **1998**, *32*, 3591−3597.

(11) Chang, Y. C.; Hatsu, M.; Jung, K.; Yoo, Y. S.; Takamizawa, K. Isolation and Characterization of a Tetrachloroethylene Dechlorinating Bacterium, Clostridium Bifermentans DPH-1. *J. Biosci. Bioeng.* **2000**, *89*, 489−491.

(12) Maymo-Gatell, X.; Chien, Y.; Gossett, J. M.; Zinder, S. H. Isolation of a Bacterium That Reductively Dechlorinates Tetrachloroethene to Ethene. *Science* **1997**, *276*, 1568−1571.

(13) Scholtz-Muramatsu, H.; Neumann, A.; Messmer, M.; Moore, E.; Diekert, G. Isolation and Characterization of Dehalosprillium Multivorans Gen. Nov., Sp. Nov., a Tetrachlorethene-Utilizing, Strictly Anaerobic Bacterium. *Arch. Microbiol.* **1995**, *163*, 48−56.

(14) Sharma, P. K.; McCarty, P. L. Isolation and Characterization of a Facultatively Aerobic Bacterium That Reductively Dehalogenates Tetrachloroethene to Cis-1,2-Dichloroethene. *Appl. Environ. Microbiol.* **1996**, *62*, 761−765.

(15) He, J.; Ritalahti, K.; Yang, K.; Koenigsberg, S.; Loffler, F. Detoxification of Vinyl Chloride to Ethene Coupled to Growth of an Anaerobic Bacterium. *Nature* **2003**, *424*, 62−65.

(16) Hendrickson, E. R.; Payne, J. A.; Young, R. M.; Starr, M. G.; Perry, M. P.; Fahnestock, S.; Ellis, D. E.; Ebersole, R. C. Molecular Analysis of Dehalococcoides 16S Ribosomal DNA From Chloroethene-Contaminated Sites Throughout North America and Europe. *Appl. Environ. Microbiol.* **2002**, *68*, 485−495.

(17) He, J.; Ritalahti, K. M.; Aiello, M. R.; Loffer, F. E. Complete Detoxification of Vinyl Chloride by an Anaerobic Enrichment Culture and Identification of the Reductively Dechlorinating Population As a Dehalococcoides Species. *Appl. Environ. Microbiol.* **2003**, *69*, 996−1003.

(18) Loffler, F. E.; Sun, Q.; Li, J.; Tiedje, J. M. 16S RRNA Gene-Based Detection of Tetrachloroethene-Dechlorinating Desulfuromonas and Dehalococcoides Species. *Appl. Environ. Microbiol.* **2000**, *66*, 1369−1374.

(19) Fennell, D. E.; Carroll, A. B.; Gossett, J. M.; Zinder, S. H. Assessment of Indigenous Reductive Dechlorinating Potential at a TCE-Contaminated Site Using Microcosms, Polymerase Chain Reaction Analysis, and Site Data. *Environ. Sci. Technol.* **2001**, *35*, 1830−1839.

(20) Hohnstock-Ashe, A. M.; Plummer, S. M.; Yager, R. M.; Baveye, P.; Madsen, E. L. Further Biogeochemical Characterization of a Trichloroethene-Contaminated Fractured Dolomite Aquifer: Electron Source and Microbial Communities Involved in Reductive Dechlorination. *Environ. Sci. Technol.* **2001**, *35*, 4449−4456.

(21) Cupples, A. M.; Spormann, A. M.; McCarty, P. L. Growth of a Dehalococcoides-Like Microorganism on Vinyl Chloride and Cis-Dichloroethene As Electron Acceptors As Determined by Competitive PCR. *Appl. Environ. Microbiol.* **2003**, *69* , 953−959.

(22) Morse, J. J.; Alleman, B. C.; Gossett, J. M.; Zinder, S. H.; Fennell, D. E.; Sewell, G. W.; Vogel, T. M. *Draft Technical Report-A Treatability Test for Evaluating the Potential Applicability of the Reductive Anaerobic Bilogical In Situ Treatment Technology (RABITT) to Remediate chloroethens*; DoD Environmental Security Technology Certification Program, 1998.

(23) Fennel, D. E.; Gossett, J. M.; Zinder, S. H. Comparison of Butyric Acid, Ethanol, Lactic Acid, and Propionic Acid As Hydrogen Donors for the Reductive Dechlorination of Tetrachloroethene. *Environ. Sci. Technol.* **1997**, *31*, 918−926.

(24) Gibson, S. A.; Sewell, G. W. Stimulation of Reductive Dechlorination of Tetrachloroethene in Anaerobic Aquifer Microcosms by Addition of Short-Chain Organic Acids or Alcohols. *Applied Environ. Microbiol.* **1992**, *58*, 1392−1393.

(25) Gibson, S. A.; Roberson, S. D.; Russell, H. H.; Sewell, G. W. Effects of Three Concentrations of Mixed Fatty Acids on Dechlorination of Tetrachloroethene in Aquifer Microcosms. *Environ. Toxicol. Chem*. **1994**, *13*, 453−460.

(26) DiStefano, T. D.; Gossett, J. M.; Zinder, S. H. Hydrogen As an Electron Donor for Dechlorination of Tetrachloroethene by an Anaerobic Mixed Culture. *Appl. Environ. Microbiol.* **1992**, *58*, 3622−3629.

(27) McCarty, P. L. Breathing With Chlorinated Solvents. *Science* **1997**, *276*, 1521−1522.

(28) Schink, B. Energetics of Syntrophic Cooperation in Methanogenic Degradation. *Microbiol. Mol. Biol. Rev*. **1997**, *61*, 262−280.

(29) Maymo-Gatell, X.; Tandoi, V.; Gossett, J. M.; Zinder, S. H. Characterization of an H2-Utilizing Enrichment Culture That Reductively Dechlorinates Tetrachloroethene to Vinyl Chloride and Ethene in the Absence of Methanogenesis and Acetogenesis. *Appl. Environ. Microbiol.* **1995**, *61*, 3928−3933.

(30) Loffler, F. E.; Tiedje, J. M.; Sanford, R. A. Fraction of Electrons Consumed in Electron Acceptor Reduction and Hydrogen Thresholds As Indicators of Halorespiratory Physiology. *Appl. Environ. Microbiol*. **1999**, *65*, 4049−4056.

(31) Mazur, C. S.; Jones, J. W. Hydrogen Concentrations in Sulfate-Reducing Estuarine Sediments During PCE Dehalogenation. *Environ. Sci. Technol.* **2001**, *35*, 4783−4788.

(32) Dojka, M. A.; Hugenholtz, P.; Haack, S. K.; Pace, N. R. Microbial Diversity in a Hydrocarbon- and Chlorinated-Solvent-Contaminated Aquifer Undergoing Intrinsic Bioremediation. *Appl. Environ. Microbiol*. **1998**, *64*, 3869−3877.

(33) Tanner, R. S. In *Manual of Environmental Microbiology*; Hurst, C. J., Knudsen, A. R., McInerney, M. J., Stetzenbach, L. D., Waller, M. V., Eds.; American Society for Microbiology: Washington, DC, 1997; pp 52−62.

(34) Balch, W. E.; Wolfe, R. S. *Appl. Environ. Microbiol.* **1976**, *32*, 781−791.

(35) Gossett, J. M. Measurement of Henry's Law Constants for C_1 and C_2 Chlorinated Hydrocarbons. *Environ. Sci. Technol.* **1987**, *21*, 202−208.

(36) Madigan, M.; Martinko, J.; Parker, J. *Brock: Biology of Microorganisms*, 8th ed.; Prentice-Hall: Upper Saddle River, NJ, 1997.

(37) Hugenholtz, P.; Pitulle, C.; Hershberger, K. L.; Pace, N. R. Novel Division Level Bacterial Diversity in a Yellowstone Hot Spring. *J. Bacteriol.* **1988**, *180*, 366−376.

(38) Ng, W.-L.; Schummer, M.; Cirisano, F. D.; Baldwin, R. L.; Karlan, B. Y.; Hood, L. High-Throughput Plasmid Mini Preparations Facilitated by Micro-Mixing. *Nucleic Acids Res.* **1996**, *24*, 5045−5047.
(39) Liu, W. T.; Marsh, T. L.; Cheng, H.; Forney, L. J. Characterization of Microbial Diversity by Determining Terminal Restriction Fragment Length Polymorphisms of Genes Encoding 16S RRNA. *Appl. Environ. Microbiol.* **1997**, *63*, 4516−4522.
(40) Dunbar, J.; Ticknor, L. O.; Kuske, C. R. Phylogenetic Specificity and Reproducibility and New Method for Analysis of Terminal Restriction Fragment Profiles of 16S RRNA Genes From Bacterial Communities. *Appl. Environ. Microbiol.* **2001**, *67*, 190−197.
(41) Seshadri, R.; Adrian, L.; Fouts, D. E.; Eisen, J. A.; Phillippy, A. M.; Methe, B. A.; Ward, N. L.; Nelson, W. C.; Deboy, R. T.; Khouri, H. M.; Kolonay, J. F.; Dodson, R. J.; Daugherty, S. C.; Brinkac, L. M.; Sullivan, S. A.; Madupu, R.; Nelson, K. E.; Kang, K. H.; Impraim, M.; Tran, K.; Robinson, J. M.; Forberger, H. A.; Fraser, C. M.; Zinder, S. H.; Heidelberg, J. F. Genome Sequence of the PCE-Dechlorinating Bacterium *Dehalococcoides Ethenogenes*. *Science* **2005**, *307*, 105−108.
(42) Dewhirst, F. E.; Chien, C. C.; Paster, B. J.; Ericson, R. L.; Orcutt, R. P.; Schauer, D. B.; Fox, J. G. Phylogeny of the Defined Murine Microbiota: Altered Schaedler Flora. *Appl. Environ. Microbiol.* **1999**, *65*, 3287−3292.
(43) Kuhner, C. H.; Matthies, C.; Acker, G.; Schmittroth, M.; Gossner, A. S.; Drake, H. L. Clostridium Akagii Sp. Nov. and Clostridium Acidisoli Sp. Nov.: Acid-Tolerant, N(2)-Fixing Clostridia Isolated From Acidic Forest Soil and Litter. *Int. J. Syst. Bacteriol.* **2000**, *50*, 873−881.
(44) Lebloas, P.; Loubiere, P.; Lindley, N. D. Use of Unicarbon Substrate Mixtures to Modify Carbon Flux Iimproves Vitamin B-12 Production With the Acetogenic Methylotroph Eubacterium-Limosum. *Biotechnol. Lett.* **1994**, *16*, 129−132.
(45) Amann, R. I.; Ludwig, W.; Schleifer, K. H. Phylogenetic Identification and in Situ Detection of Individual Microbial Cells Without Cultivation. *Microbiol. Rev.* **1995**, *59*, 143−169.
(46) Pulliam-Holoman, T. R.; Elberson, M. A.; Cutter, L. A.; May, H. D.; Sowers, K. R. Characterization of a Defined 2,3,5,6-Tetrachlorobiphenyl-Ortho-Dechlorinating Microbial Community by Comparative Sequence Analysis of Genes Coding for 16S RRNA. *Appl. Environ. Microbiol.* **1998**, *64*, 3359−3367.
(47) Farrelly, V.; Rainey, F. A.; Stackebrandt, E. Effect of Genome Size and Rrn Gene Copy Number on PCR Amplification of 16S RRNA Genes From a Mixture of Bacterial Species. *Appl. Environ. Microbiol.* **1995**, *61*, 2798−2801.

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