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Phylogenetic Characterization of Microbial Communities That Reductively Dechlorinate TCE Based upon a Combination of Molecular Techniques

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An anaerobic microbial consortium (referred to as ANAS) that reductively dechlorinates trichloroethene (TCE) completely to ethene with the transient production of *cis*-dichloroethene (cDCE) and vinyl chloride was enriched from contaminated soil obtained from Alameda Naval Air Station. ANAS uses lactate as its electron donor and has been functionally stable for over 2 years. Following a brief exposure to oxygen, a subculture (designated VCC) derived from ANAS could dechlorinate TCE only to vinyl chloride with lactate as its electron donor. Three molecular methods were used concurrently to characterize the community structure of ANAS and VCC: clone library construction/clone sequencing, terminal restriction fragment length polymorphism (T-RFLP) analysis, and fluorescent in situ hybridization (FISH) with rRNA probes. The community structure of ANAS did not change significantly over the course of a single feeding/dechlorination cycle, and only minor fluctuations occurred over many feeding cycles spanning the course of 1 year. Clone libraries and T-RFLP analyses suggested that ANAS was dominated by populations belonging to three phylogenetic groups: *Dehalococcoides* species, *Desulfovibrio* species, and members of the Clostridiaceae (within the low G + C Gram-positives). FISH results suggest that members of the Cytophaga/Flavobacterium/Bacteroides (CFB) cluster and high G + C Gram-positives (HGCs) were numerically important in ANAS despite their under-representation in the clone libraries. Parallel analyses of VCC samples suggested that *Dehalococcoides* species and Clostridiaceae were only minor populations in this community. Instead, VCC had increased populations of organisms in the β and γ subclasses of the Proteobacteria as well as significant populations of organisms in the CFB cluster. It is possible that symbiotic interactions are occurring between some of ANAS's phylogenetic groups under the enrichment conditions, including interspecies hydrogen transfer from *Desulfovibrio* species to *Dehalococcoides* species. However, the nucleic acid-based analyses performed here would need to be supplemented with chemical species data in order to test any hypotheses about functional roles of various community members. Additionally, these results suggest that an organism outside the *Dehalococcoides* genus may be capable of dechlorinating cDCE to vinyl chloride.

Introduction

Chlorinated ethenes are a major source of soil and groundwater contamination and, hence, represent a significant potential threat to human and ecological health. In situ bioremediation, the use of naturally occurring microorganisms to destroy groundwater contaminants or to convert them to harmless forms, is one of the most promising methods available for mitigating these compounds. However, our ability to understand, monitor, and manipulate the complex microbial communities performing bioremediation has been limited partially by reliance upon traditional culture-based techniques. Because nucleic acid-based techniques allow for the investigation of complex communities without relying on selective isolation of individual species, they are promising methods for the task of monitoring communities performing in situ bioremediation.

Microbial degradation of chlorinated ethenes is known to occur under both aerobic and anaerobic conditions. Under anaerobic conditions, chlorinated ethenes are transformed by reductive dechlorination catalyzed by different groups of anaerobic microorganisms (*1*−*3*). For example, a number of pure cultures have been described in recent years that can specifically use reductive dechlorination to gain biologically useful energy by using perchloroethene (PCE) or trichloroethene (TCE) as terminal electron acceptors in anaerobic respiration (*4*−*17*). Of these, only *Dehalococcoides ethenogenes* (*5*) dechlorinates past *cis*-dichloroethene (cDCE). *D. ethenogenes* uses H_2 as its sole electron donor, grows slowly in pure culture, and is fastidious in its growth conditions (*5*). It requires the extract of an anaerobic sludge community for sustained growth, suggesting that it relies on biochemical collaboration with other microorganisms and that it benefits significantly by growth in a mixed community. In general, the body of knowledge obtained from pure cultures capable of reductive dechlorination of chlorinated ethenes does not yet explain the full range of degradation patterns observed at field sites and in laboratory enrichment cultures.

In addition to the pure cultures of dehalorespirers, reductive dechlorination of chlorinated solvents has also been observed by a wide variety of methanogenic and nonmethanogenic consortia (*2*, *18*−*33*). Reductive dechlorinations catalyzed by these groups of microorganisms produce end products that vary depending on the physiological groups of bacteria involved and may occur via a combination of metabolic and cometabolic processes. Despite the considerable body of research dealing with the complete reductive dechlorination of chlorinated ethenes by mixed communities, relatively little is known about the diversity of microbial communities capable of catalyzing these reactions. In addition, in many instances, dechlorination of PCE and TCE results in the accumulation of chlorinated daughter products, including vinyl chloride (VC), a known human carcinogen. Fundamental information on the ecology, physiology, and

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biochemistry of microbial communities involved in complete reductive dechlorination, and of perturbed communities capable of only partial dechlorination, is needed in order to develop efficient anaerobic treatment strategies for remediating sites contaminated with chlorinated ethenes.

Several culture-independent molecular approaches are currently available as a means of exploring microbial communities (*34*−*39*). This paper discusses the characterization of highly enriched mixed cultures that reductively dechlorinate TCE with lactate as an electron donor/energy source using a combination of three such techniques. The community structure and activity effects of perturbation on the original consortium by a brief exposure to oxygen were also studied. 16S rDNA clone library construction and sequencing techniques were used in conjunction with terminal restriction length polymorphism (T-RFLP) analyses in order to identify individual organisms in the mixed cultures and to track population dynamics over the course of TCE conversion. Additionally, fluorescent in situ hybridization (FISH) with general and group-specific probes targeting rRNA was performed to quantify specific populations without the potential biases associated with methods based on PCR amplification of extracted DNA. When used in combination, this suite of molecular techniques can prove to be a powerful tool for not only characterizing complex communities but also tracking and monitoring specific microbial species in environmental systems. While these techniques have been applied to evaluate microbial communities for some time, they have only recently been applied for evaluating reductively dechlorinating microbial communities (*9*, *33*, *40*−*43*). To our knowledge, this is the first reported study where both PCR-based and FISH-based 16SrRNA methods have been applied in combination to characterize mixed communities performing reductive dechlorination.

Materials and Methods

Source of Inoculum and Growth Conditions. ANAS was enriched from soil obtained from a site contaminated with chlorinated solvents (mostly TCE) and waste oil at the Alameda Naval Air Station in California. The consortium was enriched using lactate as the electron donor and TCE as the electron acceptor. The consortium was grown in a basal medium and maintained at room temperature under constant stirring in a 1.5 L stainless steel vessel with side ports (containing Teflon-line stoppers) for liquid and headspace sampling. Liquid volume was maintained at approximately 500 mL, with a 1000 mL headspace. The basal medium 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; and 5 mL/L trace metal solution (*44*), 10 mL/L vitamin solution (*44*), and a gas phase of nitrogen and carbon dioxide (80%/20% by volume). Methods for the preparation and use of anaerobic media were those of Balch and Wolfe (*45*).

ANAS was maintained in semibatch fashion with a mean cell residence time of 4 ± 1 weeks. TCE (200−400 μM) and sodium lactate (10−20 mM) were supplied by injecting set volumes of neat TCE and lactate stock solution into the liquid phase through a side port. After complete conversion of chloroethenes, the vessel headspace was purged, and the culture was resupplied with fresh substrates. Subcultures were prepared by diluting a sample of ANAS 4:1 with fresh basal medium in 160 mL serum bottles (50 mL liquid volume) sealed with a butyl rubber stopper and a aluminum crimp top. Cultures were incubated at 25 or 30 °C on a shaker at 150 rpm. One subculture was exposed to oxygen overnight just after the addition of lactate and TCE. After oxygen exposure, reducing conditions were reestablished by purging the headspace with 0.2 μm filtered N_2/CO_2 (80%/20%) and re-feeding. This consortium lost the ability to dechlorinate TCE beyond VC and will be referred to as VCC.

Pure Cultures. *Dehalospirillum multivorans* (=DSM 12446), *Desulfuromonas chloroethenica* (=DSM 12431), and *Desulfitobacterium* strain PCE1 (=DSM 10344) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. (Braunschweig, Germany). *Enterobacter* strain MS-1 was a gift from Dr. Pramod Sharma and Dr. Perry McCarty at Stanford University. *D. ethenogenes* and its specific growth medium were provided by Dr. Stephen Zinder and Ivonne Neijenhuis at Cornell University. *D. chloroethenica* was grown in a basal medium (*13*, *14*) with acetate (10 mM) as an electron donor and fumarate (10 mM) as an electron acceptor. *D. multivorans* and *Desulfitobacterium* strain PCE1 were grown as previously described (*8*, *16*).

Chemical Analyses. Ethene and chlorinated ethenes were measured by taking headspace samples (50 μL) and analyzing them on an HP5890 gas chromatograph equipped with a flame ionization detector (FID). Detector and injector temperatures were held at 250 and 220 °C, respectively. The column was a GC-GasPro (J&W Scientific, Folsom, CA). A gradient temperature program ramped the column temperature from 50 to 180 °C at the rate of 15 °C/min, and held it at 180 °C for 3 min. Standard curves and Henry's constants were used to calculate total micromoles (μmol) of each compound in vessels. Standard curves were generated from individual standards for TCE, cDCE, VC, and ethene that were created by adding known amounts of each compound to 160 mL serum bottles containing 50 mL of sterile medium.

Time Course Experiments. Liquid culture samples (replicates of 1.5 mL each) were withdrawn from ANAS at nine time points during the conversion of TCE to ethene. Samples were pelleted by centrifugation at 13000*g* for 20 min, and the supernatant was discarded. Pellets from two of the replicates were resuspended in 95% ethanol/5% water for use in microscopy analyses. The remaining pellets were stored at −20 °C until DNA extraction was performed as described in the next section.

DNA Extraction. Total community DNA from culture samples (0.5−10 mL) was extracted using the protocol of Dojka et al. (*37*) which involved two enzymatic treatment steps (with Lysozyme at 37 °C and Proteinase K at 50 °C) followed by Bead mill homogenization step in a 4% SDS solution containing 0.1 mm zirconium beads (BioSpec Product, Bartlesville, OK). Phenol/chloroform/isoamyl alcohol extraction of the DNA from the raw extraction fluid was followed by 2-propanol precipitation, washing with 70% ethanol, and final resuspension in 80 μL of sterile, nuclease-free water. To remove low molecular weight DNA (<1 kb in size), the solution was passed through a ChromaSpin1000 column (Clontech, Palo Alto, CA). Samples were electrophoresed on a 1% agarose gel to check for high molecular weight DNA.

16S rDNA Amplification, Clone Library Construction, and Clone Sequencing. For clone library construction and plasmid purification, the bacterial primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GC(C/T)TACCTTGTTACGACTT-3′) and methods of Dojka et al. (*37*) were used except that AmpliTaq rather than Pfu was used as the DNA polymerase. Following digestion with restriction enzymes *Msp*1 and *HinP*1 (Gibco, Carlsbad, CA), RFLP screening and sequencing of clones were performed as described by Dojka et al. (*37*) except that sequencing was performed on an ABI 377 sequencer (PE Biosystems, Foster City, CA). For clones that were partially sequenced, primer 515F (5′-GTGCCAGC(A/C)GCCGCGGTAA-3′) was used. For full gene sequencing, a triple pass with 515F and the plasmid primers M13Reverse (5′-AACAGCTATGACCATG-3′) and T7 (5′-AATACGACTCACTATAG-3′) was performed, and the three partial sequences were aligned using the AutoAssembler

TABLE 1. Oligonucleotide Probes Used in This Study

probe name	probe sequence, 5′−3′	% formamide in hybridization reaction	target organisms	ref
EUB338	GCTGCCTCCCGTAGGAGT	0	Bacteria[a]	*48*
EUB338II	GCAGCCTCCCGTAGGTGT	0	Bacteria[a]	*49*
EUB338III	GCTGCCTCCCGTAGGAGT	0	Bacteria[a]	*49*
Arc344	TCGCGCCTGCTGCICCCCGT	0	Archaea	*50*
Alpha1b	CGTTCGYTCTGAGCCAG	0	α-Proteobacteria	*51*
Beta	GCCTTCCCACTTCGTTT	20	β-Proteobacteria	*51*
Gamma	GCCTTCCCACATCGTTT	20	γ-Proteobacteria	*51*
HGC	TATAGTTACCACCGCCGT	20	high G + C Gram-positives	*52*
LGC	TCACGCGGCGTTGCTC	20	low G + C Gram-positives	*53*
CF319	TGGTCCGTGTCTCAGTAC	35	Cytophaga/Flavobacteria/Bacteroides cluster	*34*
SRB385	CGGCGTCGCTGCGTCAGG	35	δ-Proteobacterial sulfate reducers	*34*
DhEth	ACCTATTGTTCTGTCCATT	0	*D. ethenogenes* and relatives	this study
EubLim	CCGGAGTTTTCATGATCT	0	*Eubacterium limosum* and relatives	this study

[a] Together, EUB338, EUB338II, and EUB338III have been shown to cover most described Bacterial sequences (*49*).

software package, version 2.1 (PE Biosystems). After alignment and visual checking of each sequence, the BLAST software at the GenBank website (GenBank, National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) was used to determine its phylogenetic placement and its nearest cultured neighbor. The Chimera Check function (www.rdp.cme.msu.edu; *46*) was used to determine whether sequences were likely of chimeric origin.

T-RFLP Analysis. For T-RFLP, 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 on its 5′ end as described by Liu et al. (*35*). The mixed bacterial PCR products were restricted with the enzyme *Msp*1 (Gibco) at 37 °C for 3 h and the resulting fragments were separated and detected by running the digest on an ABI 377 sequencer. Profiles were analyzed with the GeneScan software package (PE Biosystems). Other restriction enzymes (*Hha*1 and *Taq*1) generated less complex profiles than *Msp*1, so only *Msp*1 data is presented here. In cases where archaeal T-RFLP profiles were generated, FAM-labeled 109F (5′-AC(G/T)GCTCAGTAACACGT-3′) was used as the forward primer, and three separate restrictions were performed with *Msp*1, *Hha*1, and *Taq*1 according to the manufacturers instructions (Gibco). For T-RFLP of cloned DNA, the harvested plasmid was diluted 1:25 in sterile nuclease-free water, and 1 μL of this dilution was used as template in the T-RFLP PCR reaction.

Microscopy and FISH Analyses. Hybridizations with fluorescent probes were performed in a suspension so that cells could later be dispersed and captured on 0.2 μm filters of known surface area, to facilitate microscopic counts. Specifically, 20−40 μL of culture sample suspended in 95% ethanol was spotted on the bottom of a 2 mL microfuge tube and the ethanol evaporated to dryness in a laminar flow hood or vacuum chamber. To hybridize with fluorescently labeled probes (one probe per microfuge tube), 100 μL of filter-sterilized hybridization buffer (*34*, *47*), 0−35 μL of formamide (specific amounts for each probe are listed in Table 1), and 2 μL of probe stock (50 ng/μL in sterile water) were placed into each tube. Tubes were vortexed briefly and then incubated in a water bath at 44 °C for 2−4 h. To wash and counterstain with DAPI, 1.9 mL of the appropriate filter-sterilized wash buffer (see the following discussion) and 40 μL of DAPI stock solution (0.2 mg/mL in sterile water) were added to the tubes which were then incubated at 46 °C for 20 min. Tubes were subsequently sonicated for 0.5 s (setting of 5 on a FisherBrand sonic dismembrator), and the contents of each tube were then transferred into a filter apparatus containing 15 mL of 46 °C wash buffer. Vacuum was drawn to collect the cells on 0.2 μm black polycarbonate filters, and the filters were rinsed by drawing through an additional 20 mL of warmed (46 °C) wash buffer. Each filter was placed on a slide and one drop Citifluor antiquenching agent (Citifluor, U.K.) was added before applying a cover slip. Samples were viewed using an Olympus BH2 epifluorescent microscope with the filter set G (with excitation filter BP545) for viewing Cy3 and filter set U (with excitation filter UG1) for viewing DAPI fluorescence. For each sample at least 400 DAPI-stained cells were counted. For photomicrogaphs, the fluorescent cell wall stain 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF) was used as described in Bhupathiraju et al. (*54*) to obtain images with more morphological detail than DAPI provided.

The oligonucleotide probes used in this study are presented in Table 1 (*34*, *48*−*53*). Additionally, NON338 (the complement of EUB338) was used as a negative control. Each probe was labeled on the 5′ end with the fluorochrome Cy3 (Amersham Pharmacia, Cleveland, OH). Positive and negative pure culture controls were analyzed for each probe. Hybridizations with formamide were performed with the hybridization buffer described in Snaidr et al. (*47*). Formamide concentrations in the hybridization buffers are also listed in Table 1. Formamide-free hybridizations were performed in a buffer containing 0.9 M NaCl, 100 mM TrisHCl, and 0.1% sodium dodecyl sulfate (SDS). Wash buffers contained 100 mM TrisHCl, 5 mM NaEDTA, 0.1% SDS, and NaCl concentrations that depended on the percent formamide used during hybridization: 0.9, 0.308, and 0.102 M for 0%, 20%, and 35% formamide, respectively.

In addition to previously described probes, probes were designed that were specific for the 16S rRNA of (1) *D. ethenogenes* (GenBank accession number AF004928) and (2) *Eubacterium limosum* (=ATCC number 8486; GenBank accession number EUBRR16SA). Sequences of dominant clone types were closely related (97−99% identical) to these organisms and none of the available group-level probes would target these particular cloned sequences. Both primary and secondary structure of the 16S rRNA molecule were taken into consideration when designing these probes. Available secondary structures of related organisms—*Eubacterium brachy* and *Thermomicrobium roseum* for *E. limosum* and *D. ethenogenes*, respectively—were used as secondary structure templates (available at www.rna.icmb.utexas.edu; *55*). Probe DhEth targets 16S rRNA nucleotides 1166−1184 of *D. ethenogenes* (GenBank accession number AF004928) and is also complimentary to Bacterium CBDB1 and *Dehalococcoides* sp. FL2 (GenBank accession numbers AF230641 and AF357918, respectively). For DhEth, no other known organism's 16S rRNA gene was complementary to the probe. Using the ProbeCheck software available from the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu; *46*), it was determined that there were at least two mismatches between

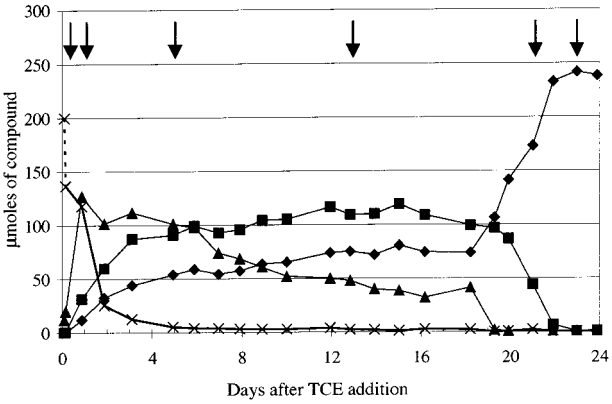

FIGURE 1. Conversion of TCE (×) to ethene (◆) with the transient build-up of cDCE (▲) and vinyl chloride (■) by the ANAS consortium. At times indicated by arrows, samples were taken for DNA extraction and subsequent T-RFLP analysis. TCE was added as neat liquid at time zero.

the DhEth probe sequence and nontarget organisms. Probe EubLim targets 16S rRNA nucleotides 214−231 of *Eubacterium limosum* (EUBRR16SA) and is also complementary to other *E. limosum* strains (GenBank accession numbers AF064242, AF064241, and ELU67159) as well as their close relatives *E. callanderi* (=DSM number 3662; GenBank accession number ECRNA16S) and *Butyribacterium methylotrophicum* (GenBank accession number AF064241). Nearest nontarget matches in RDP had at least three mismatches with EubLim. No formamide was required in hybridizations with these probes. Negative control pure cultures were *Methanobacterium thermoautotrophicum* (four nucleotide mismatches) and *Bacillus subtilis* (four nucleotide mismatches) for DhEth and EubLim, respectively.

GenBank Accession Numbers. Sequences determined in this study have been deposited into the GenBank database under accession numbers AF427907 through AF427957.

Results

Successive Dechlorination of TCE to Ethene. Figure 1 presents a plot of TCE transformation to ethene over time in the functionally stable Alameda Naval Air Station (ANAS) consortium. Conversion of TCE began immediately, and for much of the time course, the daughter products of dechlorination (cDCE, VC, and ethene) coexisted until the final conversion to ethene. Initial TCE concentration was estimated from the amount spiked into the liquid phase of the reactor at day zero since headspace/liquid equilibrium was not achieved prior to TCE consumption (possibly due to sorption to the side-port septum). While complete conversion to ethene always occurred, there was some variation in the length of time that the conversion took. In fact, dechlorination often proceeded at a faster rate than shown in Figure 1 (complete conversion of TCE to ethene in 10 days was not uncommon). This particular conversion cycle is shown because it corresponded with the intensive liquid sampling performed to collect the T-RFLP data shown in Figure 2. At each time point indicated with an arrow in Figure 1, samples were taken for T-RFLP analyses.

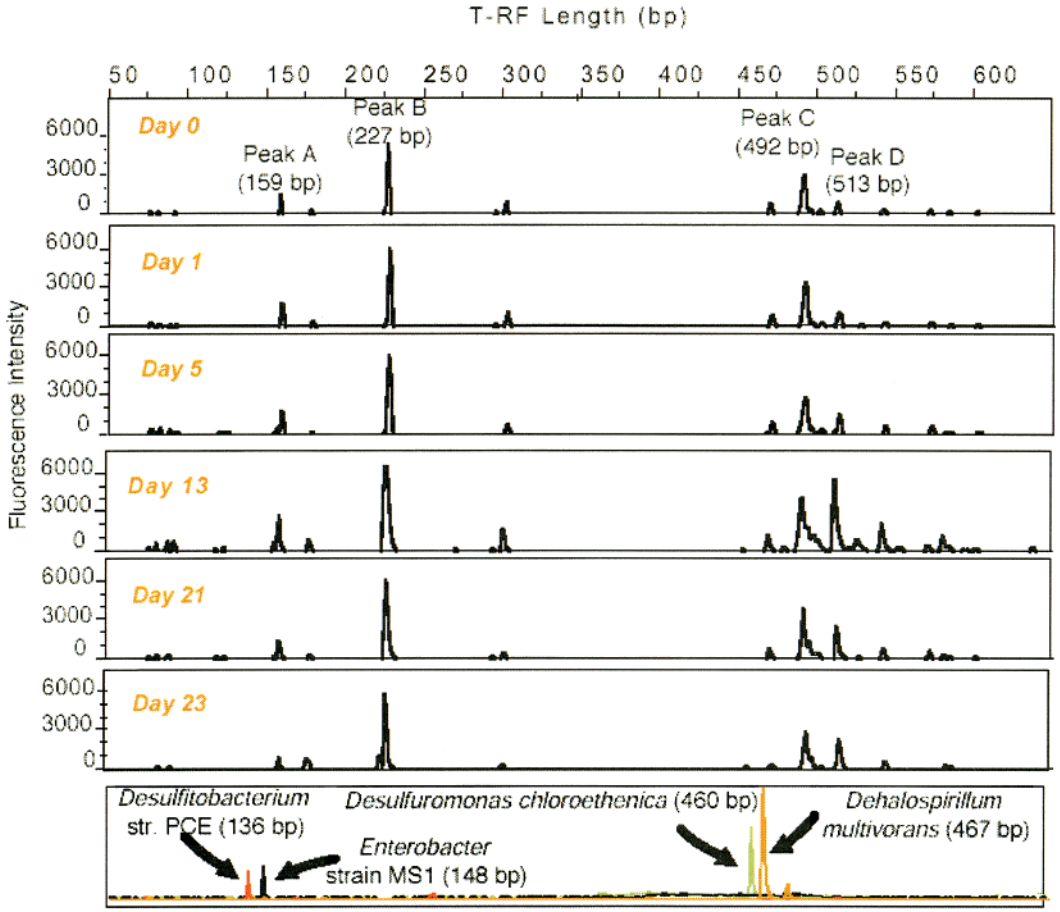

FIGURE 2. T-RFLP profiles generated from ANAS community DNA with samples taken at times denoted by arrows in Figure 1. Bacterial primers (8F-FAM and 1492R) were used and restrictions were performed with *Msp*1 (recognition sequence, CCGG). Also shown (bottom panel) are the overlaid T-RFLP profiles generated with DNA extracted from pure cultures of known dechlorinating organisms.

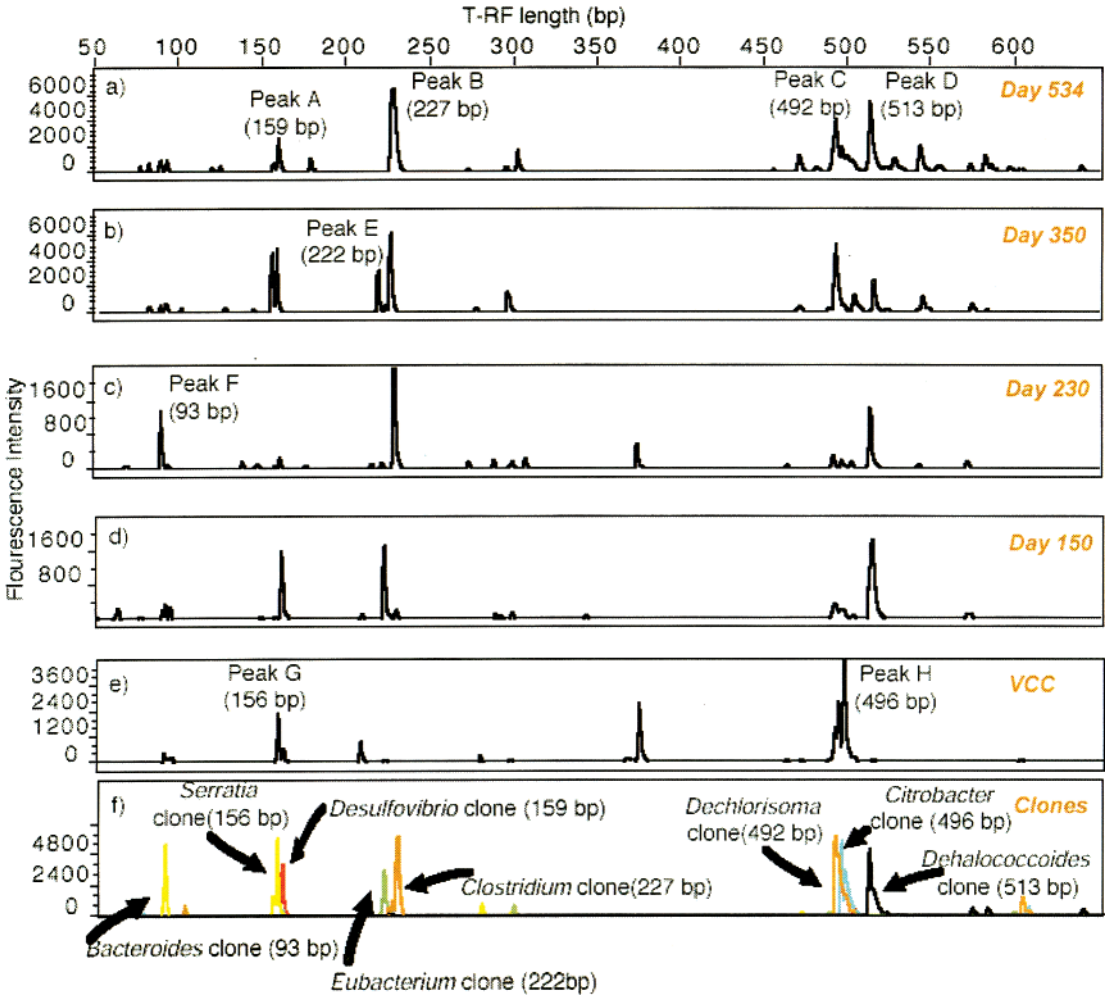

FIGURE 3. T-RFLP profiles generated from ANAS community DNA sampled over the course of 1 year (a−d). A T-RFLP profile of DNA from consortium VCC which dechlorinates TCE only to vinyl chloride is shown in panel (e). Panel (f) contains the overlaid T-RFLP profiles generated by individual clones from the clone libraries.

Community T-RFLP Profiles during a Single Feeding Cycle. In Figure 2, the T-RFLP profiles of amplified community rDNA using bacteria-specific primers are presented for sampling times indicated with arrows in Figure 1. Although there were some variations in minor peaks as shown in Figure 2, bacterial T-RFLP profiles were largely unchanged over the course of the feeding cycle despite the fact that total microbial concentrations doubled during this cycle as determined by DAPI staining. Major peaks at 159 (peak A), 227 (peak B), 492 (peak C), and 513 (peak D) base pairs (bp) were present throughout the time course, and each of these peaks accounted for at least 4% of the total area of all detected peaks in a given T-RFLP profile. T-RFLP profiles from days 3, 8, and 18 also showed similar patterns (data not shown).

To investigate whether known chloroethene−dechlorinating bacteria were present in the consortium, community ANAS T-RFLP profiles were compared to the T-RFLPs obtained from pure cultures of some known dechlorinating bacteria and the predicted T-RF lengths (see the following discussion) of other documented dechlorinating bacteria. The pure culture T-RFLP plots are overlaid in the last panel of Figure 2, with peaks observed at 136, 148, 460, and 467 bp for *Desulfitobacterium* strain PCE, *Enterobacter* strain MS-1, *D. chloroethenica*, and *D. multivorans*, respectively. While *D. ethenogenes* was available in pure culture, T-RFLP profiles produced from this culture contained more than one significant peak (including one peak at 513 bp), possibly due to the presence of remnant DNA fragments from the sludge supernatant used as a growth factor for this organism. With the possible exception of a peak corresponding to *D. ethenogenes*, no major peaks were found in the ANAS T-RFLP profiles at the lengths that corresponded to the tested dechlorinating bacteria.

For a number of other dechlorinating isolates, theoretical calculations of the T-RF length for each were made using the published 16S rDNA sequence (GenBank). Using this technique, it was determined that peaks for *D. restrictus* strain TEA and *D. restrictus* PER-K23 should both fall at 145 bp. No significant peaks are seen at this location in the plots in Figure 2. Using data from the TIGR Microbial Genomes database (www.tigr.org), the predicted T-RF length for *D. ethenogenes* is 513 bp, which corresponds to a significant peak in Figure 2 plots.

T-RFLP profiles of archaeal PCR products from ANAS samples produced using three different restriction enzymes each contained only one significant peak (at 170, 244, and 704 bp for *Msp*1, *Hha*1, and *Taq*1, respectively). Because some methane is produced by the ANAS culture, these peaks most likely correspond to a methanogen. The T-RFLP data is consistent with only one described species of methanogen, *Methanobacterium congolense*.

Community T-RFLP Profiles over the Course of 1 Year. In Figure 3 parts a−d, a reverse chronology of T-RFLP profiles are presented for samples collected from ANAS over the course of a year: day 534 (Figure 3a), day 350 (Figure 3b), day 230 (Figure 3c), and day 150 (Figure 3d). Day 534 in this long-term series corresponds to day 13 of the individual feeding cycle (Figures 1 and 2). While there is more observable

TABLE 2. Phylogenetic Summary of TCE-Dechlorinating Communities Based on Clone Libraries/Sequence Analyses[a]

putative phylogenetic affiliation	distinct RFLP patterns (% identity of clone sequence to nearest cultured neighbor in GenBank)	% of clones from ANAS I[b]	% of clones from ANAS II[c]	% of clones from VCC[d]
green non-sulfur bacteria				
Dehalococcoides ethenogenes	I-03 (99); I-04 (98); I-32 (97); I-33 (99); II-01 (99); II-02 (97); II-20 (97); II-22 (99); I-21 (96); I-23 (96)	36.6	33.3	
low G + C Gram-positive bacteria				
Eubacterium sp.	I-05 (99); I-22 (99); I-29 (98); II-05	20.4	2.6	
other Clostridiaceae	I-25 (98); I-18 (95); I-24 (91); I-15 (99); II-03 (93−97);[e] II-14 (98); II-16 (98); II-17 (95); II-19 (97)	5.4	34.6	
Ruminococcus sp.	I-17 (94)	2.2		
Aminobacterium sp.	I-06 (94); I-30 (95); II-07 (94); II-15 (92); II-18 (94)	3.2	5.1	
Bacillus sp.	I-28 (96)	1.1		
Dehalobacter sp.	I-27 (93)	1.1		
δ-Proteobacteria				
Desulfovibrio sp.	I-01 (99); I-02 (99); I-19 (99); I-13 (96) I-7 (93); I-12 (90); II-04 (99); VCC-13 (95)	20.4	12.8	2.5
Pelobacter sp.	I-20 (97)	1.1		
Bacteroidaceae	I-08 (91); I-14 (96); I-16 (97); VCC-03 (90); VCC-10 (89)	3.2		7.5
γ-Proteobacteria				
Citrobacter sp.	II-08 (99); VCC-01 (99)		3.8	50
Pseudomonas sp.	VCC-07 (98)			2.5
Serratia sp.	VCC-02 (95)			7.5
β-Proteobacteria				
Dechlorisoma sp.	VCC-05 (96); VCC-06 (99); VCC-08 (97); VCC-09 (92)			17.5
high G + C Gram-positive bacteria				
Propionibacterium sp.	VCC-04 (94)			5
Spirochaeta	I-26 (90); II-12 (89); VCC-14 (92)	1.1	1.3	2.5
chimeras[f]	II-21		1.3	
not sequenced	I-09; I-10; I-11; I-31; II-06; II-09; II-11; II-13; VCC-11; VCC-12	4.3	5.1	5.0
number of clones analyzed		93	78	40

[a] Clone libraries were constructed using bacterial primers 8F and 1492R. [b] Samples were taken from the reactor at day 150 (after complete conversion of TCE to ethene). [c] Samples were taken from the reactor at day 230 (2 days after TCE addition). [d] Samples taken from the reactor after complete conversion of TCE to VC. [e] For RFLP type II-03, different clones had slightly different sequences. [f] Chimeras determined using the Chimera Check analysis software at the RDP-II website: http://rdp.cme.msu.edu (Maidak et al.).

variation throughout the year than during the individual feeding cycle shown in Figure 2, each of the major peaks A−D identified during the individual feeding cycle were present over the course of the year. In addition to those persistent peaks, other peaks emerge when the longer timescale is considered, including peaks E, F, and G (at 222, 93, and 156, respectively) which are present and significant in at least one ANAS T-RFLP profile. Clone libraries of the ANAS culture were used to assist in identification of individual T-RFLP peaks (see the following section).

Comparison of T-RFLP Profiles of ANAS and VCC. A subculture of ANAS that was exposed to oxygen overnight lost the ability to dechlorinate TCE beyond vinyl chloride. This subculture, designated VCC, grew for several feeding cycles using lactate as the electron donor and TCE as the electron acceptor and consistently accumulated vinyl chloride. This characteristic was observed repeatedly for a number of ANAS subcultures upon brief oxygen exposure. Figure 3 shows a comparison of the ANAS T-RFLP profiles (Figure 3a−d) with the T-RFLP profile of VCC (Figure 3e). A clone library of the VCC culture was used to assist in identification of individual T-RFLP peaks. It is important to note that VCC was incubated at a higher temperature (30 °C) than ANAS (25 °C). However, other ANAS subcultures that were grown at 30 °C but were not exposed to oxygen were able to consistently dechlorinate TCE completely to ethene. While the molecular characterization of these cultures was not performed as part of this study, DTAF staining was performed and showed that major morphologies observed in the original ANAS—most notably, flattened cocci fitting the description of *D. ethenogenes* strain 195—were also observed in the 30 °C ANAS subcultures but not in VCC.

Clone Libraries from ANAS and VCC. The phylogenetic association of bacterial clones from three constructed libraries are summarized in Table 2. Archaeal clone libraries were not constructed, as only a faint PCR product was obtained with archaeal primers and T-RFLP plots generated using those primers showed only one significant peak corresponding to *M. congolense*. The sequence of the archaeal PCR product appeared clean and was most similar to the 16S rDNA of *M. congolense* (with >98% identity). Additionally, treatment of ANAS subcultures with BES (an inhibitor of methanogenesis) did not affect its ability to dechlorinate TCE completely, suggesting that Bacteria were more functionally important than methanogenic Archaea.

Data presented in Table 2 are the percentages of clones that were determined to have sequences belonging to a particular phylogenetic group as evaluated by sequence analyses. Additionally, the total number of clones analyzed for each library is presented. For ANAS, two separate clone libraries were constructed from samples collected months apart and at different times in the dechlorination cycle. ANAS I was collected after complete conversion of TCE to ethene (at day 150 of the stable consortium) while ANAS II was collected during a later feeding cycle 2 days after the addition of TCE and lactate (at day 230 of the stable consortium). The third clone library was constructed using DNA from VCC

TABLE 3. T-RF Lengths from Important Clones and Comparisons with T-RF Lengths Predicted from Nearest-Neighbor Sequences

clone RFLP type	nearest cultured neighbor	accession number[a]	% identity to nearest neighbor	T-RF length (bp) predicted by nearest-neighbor's sequence[b]	actual T-RF length (bp) from clones[c]	corresponding peak as labeled in Figures 2 and 3
I-03; II-01	*D. ethenogenes*	AF004928	99	513[d]	513	D
I-01; I-02; II-04	*Desulfovibrio desulfuricans*	AF192153	99	162	159	A
I-05	*E. limosum*	AF064242	99	221	222	E
I-08	*Bacteroides* sp.	AB003389	91	105	93	F
II-03	*Clostridium propionicum*	CP16SRRN	93−97[e]	489	227	B
VCC-01	*Citrobacter freundii*	AF025365	99	496	496	H
VCC-06	*Dechlorisoma* sp. SDGM	AF170349	99	492	492	C
VCC-02	*Serratia fonticola*	SF0233429	95	N/A[f]	156	G
VCC-04	*Propionibacterium lymphophilum*	J003056	94	161	494	

[a] GenBank accession number of nearest neighbor (www.ncbi.nlm.nih.gov). [b] Predicted T-RFs were estimated from the 16S rDNA sequence of the nearest neighbor when full sequence was available. [c] Major peak in T-RFLP profile generated from individual clones. [d] For prediction of the T-RF for *Dehalococcoides*, the genome data provided by the Institute for Genomic Research (www.tigr.org) was used. [e] For RFLP type II-03, different clones had slightly different sequences. [f] N/A: not available (published sequence begins after the location of the primer (8F) used in the T-RFLP PCR reactions).

taken after the third spike of TCE was completely converted to vinyl chloride (4 weeks following oxygen exposure).

While there are some differences in the breakdown of clones from ANAS I and ANAS II, most of the changes occur within a larger phylogenetic group. The three main phylogenetic groupings represented in both of these libraries were *Dehalococcoides* species, low G + C Gram-positives (LGCs; most belonging to the Clostridiaceae), and *Desulfovibrio* species (within the δ-Proteobacteria). Between 33% and 37% of the clones had sequences that were similar to *Dehalococcoides* (> 96% identical). LGCs were also significant (33−42% of clones) as were δ-Proteobacteria (13−21% of clones). With a single exception (a *Desulfovibrio*-like clone), no clones from the VCC library were associated with these groups. Instead, dominant sequences were associated with γ- and β-Proteobacteria (60% and 17% of clones, respectively) especially *Citrobacter* and *Dechlorisoma* species. In addition to listing the nearest cultured neighbor to each clone type, Table 2 includes the percentage identity between that neighbor's sequence and the clone's sequence. Notable in Table 2 are the 10 distinct *D. ethenogenes*-related sequences. There is also evidence for the presence of organisms in ANAS and VCC that represent putative new genera (specifically, the clones related with 89−94% identity to described species) within the low G + C Gram-positives, Spirochaeta, and Bacteroidaceae.

Table 3 shows predicted and actual T-RF lengths for selected clones. The correspondence of these T-RF lengths with major peaks identified in Figures 2 and 3 is also shown. Measured T-RF lengths were determined by performing T-RFLP on individual clones. The predicted T-RF lengths were calculated using the published 16S rDNA sequences of the nearest-neighbor. This was done by searching for the *Msp*1 restriction site (CCGG) closest to the 8F primer recognition site and counting the number of nucleotides before the cut site. When the nearest-neighbor's sequence was partial (i.e., did not include the 8F recognition site), it was not possible to predict an exact length of the T-RF that the sequence would produce. This was the case for *Serratia fonticola*.

The overlaid T-RFLP plots of eight individual clones from the clone libraries of ANAS and VCC are shown in Figure 3f. These are clones with sequences related to species of *Bacteroides* (at 93 bp), *Dehalococcoides* (513 bp), *Eubacterium* (222 bp), *Clostridium* (227 bp), *Desulfovibrio* (159 bp), *Citrobacter* (496 bp), *Serratia* (156 bp), and *Dechlorisoma* (492 bp).

Given the presumptive T-RFLP peak identifications inferred by Table 3 data, a comparison of T-RFLP patterns from ANAS and VCC (Figure 3, parts a−d and e) with those of the identified clones (Figure 3f) shows some interesting results. For example, although *Dechlorisoma* sp. was not detected in the ANAS clone libraries, a T-RFLP peak at 492 corresponding to *Dechlorisoma* was significant in all ANAS T-RFLP profiles (although it is less dominant in plots corresponding to the clone libraries; days 150 and 230). Because a large diversity of organisms can generate T-RFs at 492 bp, this peak in ANAS profiles may correspond to a number of other genera besides *Dechlorisoma*.

Fluorescent in Situ Hybridization (FISH) of ANAS and VCC. Samples of ANAS I (day 150) and VCC were analyzed using FISH with 16S rRNA probes. Domain-level probes were applied first followed by more specific Group and Genus-level probes. Figure 4 presents these results in percent of total cells as determined by DAPI staining. For comparison, also shown in Figure 4 is the clone breakdown as percentages of total clones from the libraries constructed for ANAS I and VCC.

Of the organisms that did hybridize with one of the two domain-level probes, most were bacteria (65% ± 2% and 69% ± 6% of total cells for ANAS and VCC, respectively). Only 2% ± 0.2% of cells hybridized with the archaeal probe in ANAS. In VCC, archaeal populations were below the detection limit set by NON338 (1% of total cells). Domain-level probing was also performed on time course samples, and results were similar (data not shown).

Figure 4 also shows data obtained with the more resolved set of probes including the newly designed EubLim probe. In ANAS, most of the hybridizing Bacteria also hybridized with probes targeting the sulfate-reducing δ-Proteobacteria (15% ± 1% of total cells), the Cytophaga/Flavobacteria/Bacteroides (CFB) cluster (12% ± 1%), the high G + C Gram-positives (7% ± 2%), low G + C Gram-positives (9.6%), α-Proteobacteria (7% ± 2%), and *Eubacterium limosum*/*callenderi* (6% ± 1%). While *Eubacterium* falls within the grouping of low G + C Gram-positives, the group-level probe is not complementary to this genus' 16S rRNA. For this reason, a new probe was developed to target the *E. limosum*-like clone sequence, and the results were plotted independently. Some organisms targeted by the γ- (4% ± 1%) and β- (2% ± 1%) Proteobacteria probes are also present in ANAS but in smaller proportion. Coccoid cells fitting the morphological description of *D. ethenogenes* made up approximately 20% of the total number of organisms in ANAS. These organisms did not hybridize with any of the applied probes. A species-specific probe for *D. ethenogenes* (DhEth) was designed, constructed, and applied via FISH. Only very weak signals were observed, even in actively growing and respiring pure

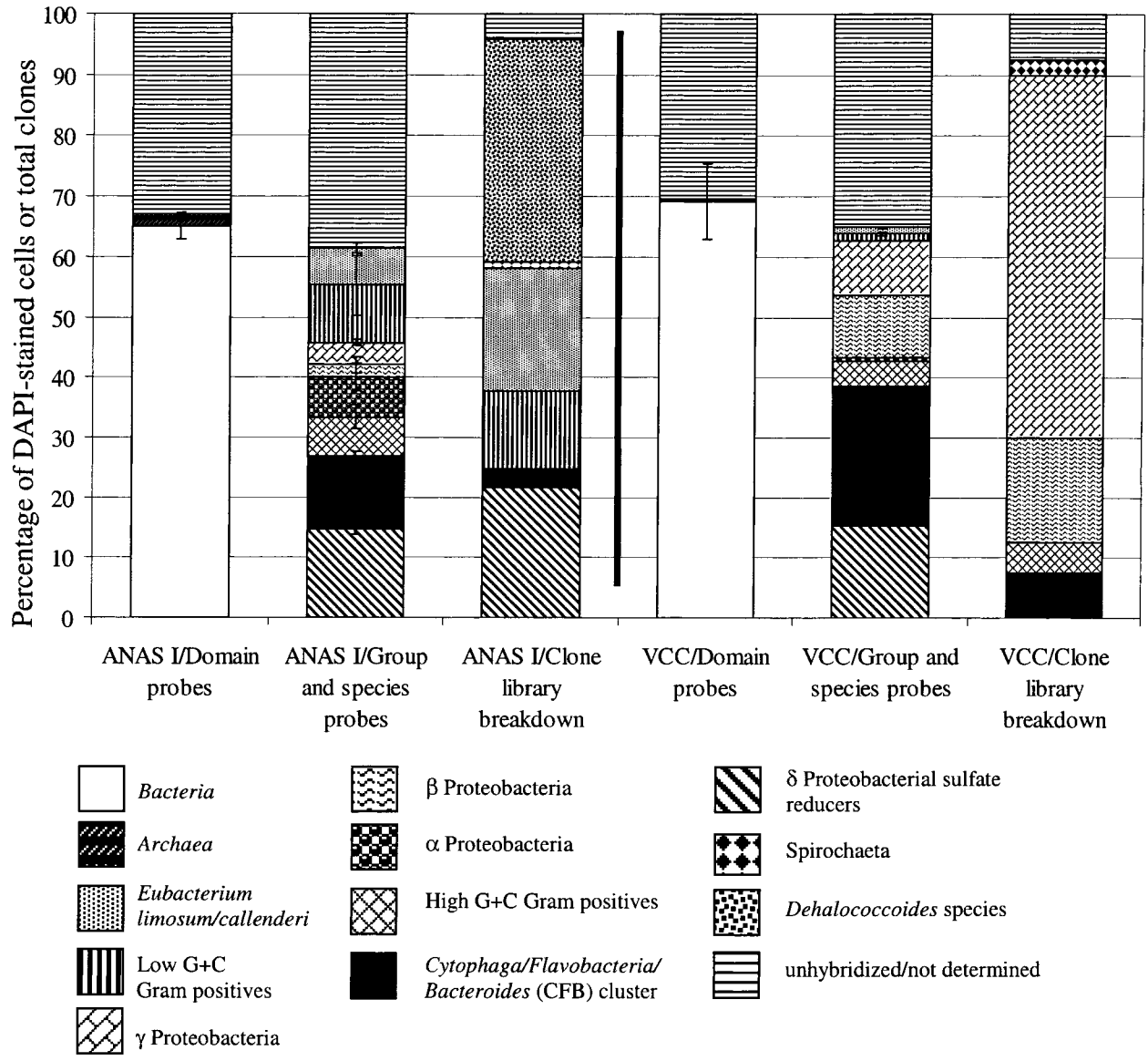

FIGURE 4. Characterization of dechlorinating consortia (ANAS and VCC) using FISH. For each consortium, results using Domain-level probes are presented in the first bar: Results using Group or Genus level probes are shown in the second bar: In the third bar, the clone breakdown of the corresponding clone library (from Table 2) are presented in terms of percentage of total clones. In addition to groups targeted in FISH studies, there were clones associated with two other groups: Spirochaeta and *Dehalococcoides* species. In each bar there is a remaining percentage of cells/clones that were unhybridized/not determined. The ANAS sample was taken after conversion of TCE to ethene (day 150) and the VCC sample was taken after conversion of TCE to vinyl chloride.

cultures of *D. ethenogenes*. Even when two fluorescent probes (EUB338 and the DhEth) were employed in concert to amplify the signal from each ribosome, these cells did not show detectable fluorescent signal. It is possible that low ribosome concentration or poor membrane permeability may explain this poor signal. The flattened morphology of *D. ethenogenes* strain 195 (only ~0.2 μm thick; *5*) may make it particularly difficult to visualize with 16S rRNA probes.

FISH data from VCC are also shown in Figure 4. Members of the δ-Proteobacteria and CFB group were the most dominant numerically (15% and 23%, respectively). Results also showed an increase in the prevalence of γ- and β-Proteobacteria (10% and 9% of total cells as compared to 4% and 2%, respectively, in ANAS) and a decrease in relative populations of LGCs, *Eubacterium limosum/callenderi*, and α-Proteobacteria (all present near the 1% detection limit). High G + C Gram-positives were also significant in VCC (4% of the total microbial count). Morphologically, very few (<2%) of the DAPI-stained organisms in VCC resembled *D. ethenogenes*.

The data used for the clone breakdown results plotted in Figure 4 are presented in Table 2. This introduces two new groups to the figure for which FISH data was not available, *Dehalococcoides* species and Spirochaeta. Some groups were detected by both FISH and clone library analyses. In ANAS I, these include δ-Proteobacteria, *E. limosum*-type organisms, other low G + C Gram-positives, and CFB-cluster organsims. Other groups detected by FISH (notably high G + C Gram-positives and α-Proteobacteria) were absent from the clone library. In VCC, β-, γ-, and δ-Proteobacteria, high G + C Gram-positives, and CFB-cluster organisms were detected by both methods.

Figure 5 displays photomicrographs of ANAS I and VCC (Figure 5, parts a and b, respectively) using the general cell wall stain 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF) as well as the parallel micrograph for a pure culture of *D.*

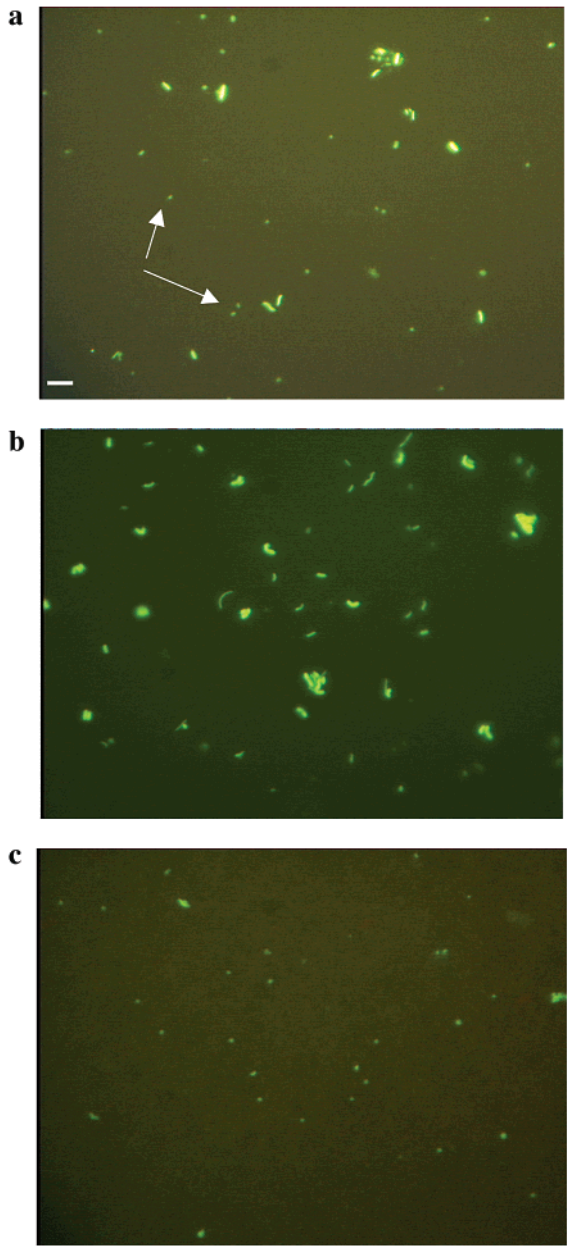

FIGURE 5. Photomicrographs of ANAS I (a) VCC (b) and a pure culture of *D. ethenogenes* (c) following staining with DTAF to highlight cell morphology. Scale bar is 5 μm.

ethenogenes (Figure 5c). Differences in cell morphologies are readily visible between VCC and ANAS. There is similarity between a significant member of the ANAS consortium (arrows) and the organism *D. ethenogenes*. Both the pure culture and the ANAS population could be visualized with DTAF and DAPI, but neither was detectable using FISH. Numerically, the irregular coccoid population accounted for $^{1}/_{2}$ to $^{2}/_{3}$ of the unhybridizable cells in ANAS.

Discussion

In this study, a combination of molecular techniques gave a composite picture of dechlorinating mixed communities. The use of clone libraries to provide peak identification for rapid T-RFLP analysis allowed for much more information to be collected with less effort than repeated clone libraries would have required. However, specific measurement of T-RF lengths for clones was important because the mere phylogenetic affiliation of a clone with cultured neighbors does not allow for the prediction of the T-RF length that it would generate. In fact, not surprisingly, it was not possible to accurately predict clones' T-RF lengths from the nearsest-neighbor's sequence when sequence identity was <97% (Table 3).

The ANAS consortium is a complex microbial community with certain persistent species that endure not only over the course of a feeding cycle but also over the course of many feeding cycles spanning months. The use of clone libraries in conjunction with T-RFLP allowed for the presumptive identification of species that correspond with particular peaks in the T-RFLP profiles. The more rapid T-RFLP method could then be used to detect specific population changes over time or when the consortium was amended or otherwise perturbed, as in the case of VCC. While results from FISH alone would have overlooked important ANAS species (e.g., *D. ethenogenes*), they did confirm the importance of some species identified via the clone library results and highlighted some organisms (such as members of the Cytophaga/Flavobacteria/Bacteroides cluster and the high G + C Gram-positives) that were underrepresented in the clone libraries.

Because genomic DNA serves as the template in these PCR-based methods, one possible bias in these methods relates to the number of gene copies for 16S rRNA an organism harbors, because results may be skewed to favor organisms with more than one copy of the 16S rDNA operon (*56*). For example, organisms such as Clostridiaceae (a subset of the low G + C Gram-positives) may have up to 15 copies of this gene on their genome, whereas *D. ethenogenes* maintains only one copy (TIGR Microbial Genomes, www.tigr.org/tdb/mdb; *57*; RDP 16S copy number database, http://rrndb.cme.msu.edu; *46*). PCR-products may then significantly misrepresent the ratio of organisms in the original sample. This particular bias would actually result in an underestimation of the population of *D. ethenogenes*-like organisms in a consortium.

Results from the two employed PCR-based methods (clone libraries and T-RFLP) generally supported each other with two exceptions. First, the absence of *Dechlorisoma* sp. from the clone libraries of ANAS samples and, second, the presence of a peak at 373 base pairs in VCC which could not be ascribed to any dominant clone type in the VCC library. Discrepancies between FISH results and PCR-based results may be explained by the biases associated with each method. For example, with Clostridiaceae (and other low G + C Gram-positives such as *Eubacterium*), FISH results quantifying their presence in ANAS (15.7%) at percentages well below those suggested by the corresponding clone library (32%) may reflect the PCR-bias in their favor due to their multiple 16S rDNA gene copies. While FISH suggests that CFB cluster organisms are significant numerically in ANAS, only 3 ANAS I clones had sequences related to these organisms (the *Bacteroides*-related sequences; clone RFLP types I-08, I-14, and I-16). To specifically identify the occurring CFB species, future work should employ primers (e.g., ref *58*) that specifically target those organisms in preparing clone libraries. Similarly, FISH suggests that δ-Proteobacteria are numerically important in VCC, despite their minor presence in the VCC clone library.

Although *D. ethenogenes*-like organisms represented no hybridizable fraction in FISH studies, they likely represent a significant fraction of the population in ANAS, as suggested by the clone libraries (34−37%) and the phenotypic features of populations in the consortium (Figure 5). Flattened cocci fitting the morphological description of *D. ethenogenes* were observed when nonspecific DAPI staining or cell wall staining with DTAF was applied to ANAS (Figure 5a), but were not a significant population in VCC. These cocci grew at the same rate as the overall ANAS community (they consistently represented 20% ± 5% of the total cells). This implies that

they are active organisms and that their failure to hybridize with probes may be an artifact of the protocol used. The inability to successfully apply FISH to *Dehalococcoides* frustrates attempts at quantifying this genus' actual presence in the consortium or in environmental samples. Additional strategies for successful hybridization are being employed that focus upon amplifying FISH signal intensity.

It is notable that while a number of clones obtained in the libraries from ANAS samples contain 16S rDNA that are almost identical (≥99% identity) to the original *D. ethenogenes* strain 195 sequence deposited in GenBank (accession number AF004928), other clones showed RFLP patterns and sequences that were unique from the original and from each other (<98% sequence identity). This suggests that distinct species of *Dehalococcoides* may exist in this consortium. Fortunately, most of the observed sequence differences did not occur in locations recently selected as targets for genus-specific *Dehalococcoides* probes (*9*, *40*). All *Dehalococcoides*-like sequences in these clone libraries would be detected by the *Dehalococcoides*-specific primers in Loeffler et al. (*9*) (although the predicted amplicon sizes range from 436 to 452 bp). Two sequences (clone RFLP types I-21 and I-23), however, show more than one mismatch to the primer set introduced by Fennell et al. (*40*). For the sequences that perfectly match these primers, amplicons are all predicted to be 816 base pairs in length.

While it seems that *Dehalococcoides* species are important elements in the ANAS community that dechlorinates completely to ethene, it is likely that other organisms are involved, either directly or indirectly, in converting TCE to ethene. When grown in pure culture, *D. ethenogenes* produces at least as much VC as ethene as an end product (*5*). In fact, the pattern observed in ANAS of complete TCE conversion to ethene has only been documented in mixed communities (e.g., ref *33*). Comparing the compositions of ANAS and VCC suggests that *Dehalococcoides* species may be directly responsible for the complete conversion of TCE to ethene, while LGCs and *Desulfovibrio* species may produce important biochemicals and substrates utililized by *Dehalococcoides* species. Considering the evidence of TCE-declorinating catalytic ability of *Clostridium bifermentans* (*4*), it is also possible that some of the Clostridiaceae may take a more direct role in the enzymatic reduction of TCE.

Considering the growth needs of *D. ethenogenes* and the physiological capabilities of LGCs and *Desulfovibrio* species, it is possible to conceive of a chemical ecology among these organisms. When grown in the absence of sulfate and in the presence of hydrogen-utilizing bacteria, *Desulfovibrio* can ferment substrates such as lactate and produce hydrogen (*59*, *60*), a requisite electron donor for *D. ethenogenes*. One ANAS subculture was consistently fed H_2 and acetate rather than lactate. Its T-RFLP profile was similar to the ANAS profile except that the peak corresponding to *D. desulfuricans* was diminished (data not shown). Defined co-culture studies reported by Drzyzga et al. (*61*) provide evidence of such syntrophic interspecies hydrogen transfer from a *Desulfovibrio* species to the dehalorespirer *Desulfitobacterium frappieri* TCE1 during the conversion of PCE to cDCE (with lactate serving as the source of electrons and in the absence of sulfate, as in ANAS). Certain LGCs such as *Eubacterium limosum* (a major clone type in the ANAS I library) have been reported to generate cofactors including Vitamin B_{12} (*62*), which is required by *D. ethenogenes* strain 195 for dechlorination. Additional studies tracking biochemicals (H_2, B_{12}, and organic acids) and employing selective physiological inhibitors will help elucidate how populations interact.

Dehalococcoides-type sequences have been detected in other dechlorinating mixed communities (*40*, *41*, *63*). In Pulliam-Holoman et al. (*41*), a mixed community dechlorinating 2,3,5,6-tetrachlorobiphenyl (grown at 30 °C rather than 25 °C) was characterized using clone libraries. Despite the difference in the electron-accepting substrate, the breakdown of clones in that report is surprisingly similar to the ANAS libraries analyzed in this study, with δ-Proteobacteria, LGCs, and *Dehalococcoides*-like ribotypes dominating the clone library. This further supports the theory that δ-Proteobacteria or LGCs interact symbiotically with *Dehalococcoides* to effect dechlorinating activity.

The data presented in this paper describe the ANAS community structure and provide clues about organisms with which *Dehalococcoides* species might have symbiotic or mutualistic relationships. However, these data also emphasize that caution must still be used when applying molecular methods to glean quantitative and functional information about mixed communities. Reliance upon application of multiple molecular methods suffering the same biases should be avoided. In fact, it seems that adding chemical species data (organic acids, H_2-levels, or cofactors) in combination with one or two nucleic acid-based methods might generate the most efficient collection of evidence pertaining to the functional roles of various species in mixed communities.

The results suggest that the organisms required to catalyze the final dechlorination step, from vinyl chloride to ethene, are extremely oxygen sensitive or can easily be overtaken by facultatively aerobic competitors. From a remediation standpoint, additional investigations into the effect of transient oxygen exposure on these communities are needed, as are studies that determine which populations in VCC are responsible for dechlorinating cDCE to vinyl chloride because no reported pure cultures terminate dechlorination at vinyl chloride. Isolation efforts employing selective inhibitors are currently underway. Recently, a correlation was reported between the presence of *Dehalococcoides*-like 16S rDNA and complete dechlorination to ethene at contaminated field sites (*63*). If even brief exposure can decimate such organisms, it may alter subsurface microbial communities such that vinyl chloride becomes the predominant end product. At such sites, efforts could be made to actively restrict oxygen from entering the aquifer or, alternatively, to encourage aerobic conditions once they are encountered, employing successive anaerobic/aerobic treatment of subsurface chloroethene-contaminated sites.

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