APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2003, p. 2879–2883 Vol. 69, No. 5
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.5.2879–2883.2003

Specific Detection of *Dehalococcoides* Species by Fluorescence In Situ Hybridization with 16S rRNA-Targeted Oligonucleotide Probes

Yanru Yang and Josef Zeyer*

Soil Biology, Institute of Terrestrial Ecology, ETH Zurich, CH-8952 Schlieren, Switzerland

Received 17 September 2002/Accepted 31 January 2003

Dehalococcoides ethenogenes **is the only known cultivated organism capable of complete dehalogenation of tetrachloroethene (PCE) to ethene. The prevalence of *Dehalococcoides* species in the environment and their association with complete dehalogenation of chloroethenes suggest that they play an important role in natural attenuation of chloroethenes and are promising candidates for engineered bioremediation of these contaminants. Both natural attenuation and bioremediation require reliable and sensitive methods to monitor the presence, distribution, and fate of the organisms of interest. Here we report the development of 16S rRNA-targeted oligonucleotide probes for *Dehalococcoides* species. The two designed probes together encompass 28 sequences of 16S rRNA genes retrieved from the public database. Except *D. ethenogenes* and CBDB1, all the others are environmental clones obtained from sites contaminated with chlorinated ethenes. They are all closely related and form a unique cluster of *Dehalococcoides* species. In situ hybridization of probe Dhe1259t with *D. ethenogenes* strain 195 and two enrichment cultures demonstrated the applicability of the probe to monitoring the abundance of active *Dehalococcoides* species in these enrichment samples.**

Tetrachloroethene (PCE) and trichloroethene (TCE) are common groundwater contaminants. Under anaerobic conditions, these compounds can be reductively dehalogenated to less-chlorinated ethenes or innocuous ethene by microorganisms through dehalorespiration, which provides a promising approach for the bioremediation of contaminated sites. A number of bacteria have been identified that are capable of reductive dehalogenation of PCE and TCE (summarized in reference 13). Of these, *Dehalococcoides ethenogenes* is the only known cultivated organism capable of complete dehalogenation of PCE to ethene. Since its identification, numerous *Dehalococcoides*-like species have been found in enriched laboratory cultures and contaminated aquifers where complete anaerobic dehalogenation of PCE and TCE occurs (12, 15, 18). This suggests that *Dehalococcoides* species play an important role in natural attenuation of chloroethenes and that they are promising candidates to be used in engineered bioremediation of these contaminants. To monitor the progress of bioremediation, it is necessary to develop reliable and sensitive detection techniques for *Dehalococcoides* species.

To date, 16S ribosomal DNA-based PCR assays are the major tools available for the detection of *Dehalococcoides* species. Loeffler et al. (15) reported a nested PCR assay which allowed the detection of *Dehalococcoides* strain FL2. One to ten copies of strain FL2's 16S rRNA gene was found sufficient to yield the expected PCR product. Another study showed that as few as 10^3 cells of *D. ethenogenes* per 0.5 g of soil were sufficient to allow detection through a direct PCR assay (9). A combination of molecular assay and microcosm and site data was used to assess the indigenous reductive dechlorinating potential of a TCE-contaminated aquifer (9). More recently, Hendrickson et al. (12) designed seven sets of primers for *Dehalococcoides* species and used them to investigate the wide distribution of these bacteria in the environment. However, despite the sensitivity and applicability of these PCR assays, it is still very difficult to correlate PCR results with the actual number and activity of *Dehalococcoides* species with respect to the total microbial community due to the qualitative nature of PCR techniques (19).

During the last decade, rRNA-targeted nucleic acid probes have been increasingly used in the field of microbial ecology and demonstrated to be a readily applicable technique for detection of microbes and analysis of microbial community structures (3, 4). Compared with the 16S ribosomal DNA-based PCR assays, nucleic acid probes and probe technologies such as fluorescence in situ hybridization (FISH) allow for quantitative measurement of active microbes. However, a recent attempt to use FISH to detect *Dehalococcoides* species failed to give detectable signals (18). Here we report novel oligonucleotide probes targeting 16S rRNA of *Dehalococcoides* species and use of FISH for detection and quantification of these bacteria in two enrichment cultures.

MATERIALS AND METHODS

Organisms and culture conditions. The applicability of the probe for in situ hybridization of *Dehalococcoides* species was tested with (i) a pure culture of *D. ethenogenes* strain 195 as a positive control (17), (ii) a dehalogenating enrichment culture from Cornell University, Ithaca, N.Y., from which strain 195 was originally isolated (6, 7, 17), and (iii) a dehalogenating enrichment culture originally from a PCE-contaminated site (11, 20). The cultures were grown as described in the corresponding references.

Cell fixation. Samples were fixed in 4% paraformaldehyde–phosphate-buffered saline (PBS) (containing 0.13 M NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4 [pH 7.2]) at 0°C for 12 h, subsequently washed twice in PBS, and stored in 50% ethanol in PBS at −20°C (21, 22).

Oligonucleotide probes and stains. Sequences and target sites of the probes used in this study are given in Table 1. Oligonucleotides synthesized with 5′ Cy3 label were purchased from MWG-Biotech (Ebersberg, Germany).

The DNA-intercalating dye 4′,6′-diamidino-2-phenylindole (DAPI; Sigma, Buchs, Switzerland) was stored as a solution of 200 ng/μl and used to stain

* Corresponding author. Mailing address: ETH Zurich, Institute of Terrestrial Ecology, Soil Biology, Grabenstrasse 3, CH-8952 Schlieren, Switzerland. Phone: 41 01 633 60 44. Fax: 41 01 633 11 22. E-mail: zeyer@ito.umnw.ethz.ch.

TABLE 1. Probes used in this study

Probe	Target organism(s)	Sequence (5′–3′)[a]	*E. coli* positions	Formamide concn (%)	Reference
Eub338	Bacteria	GCTGCCTCCCGTAGGAGT	338–355	30	4
Dhe1259t	Some *Dehalococcoides* spp.	AGCTCCAGTTC**A**CACTGTTG	1,259–1,278	0–30	This study
Dhe1259c	Some *Dehalococcoides* spp.	AGCTCCAGTTC**G**CACTGTTG	1,259–1,278	0–30	This study
Non338	Negative control	ACTCCTACGGGAGGCAGC	338–355	30	4

[a] Boldface indicates a difference in nucleotides between Dhe1259t and Dhe1259c.

bacterial cells nonspecifically. Staining was performed by the addition of DAPI to the hybridization reaction mixture (final concentration, 20 ng/μl) and was always included as a control stain to detect all bacteria present in the preparation.

Hybridizations. Whole-cell hybridization was performed based on the protocol of Zarda et al. (21, 22). Eight-well, Teflon-coated slides (Erie Scientific Co., Portsmouth, N.H.) were used in the study. Just before hybridization, each well was coated with a thin layer of gelatin [0.1% gelatin, 0.01% $KCr(SO_4)_2$]. The fixed samples were spotted on single wells, air dried, and dehydrated in an ethanol series (50, 80, and 96%) for 3 min each. After applying a mixture of 1 μl of probe (25 to 30 ng/μl), 1 μl of DAPI stock solution, 8 μl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.01% sodium dodecyl sulfate [pH

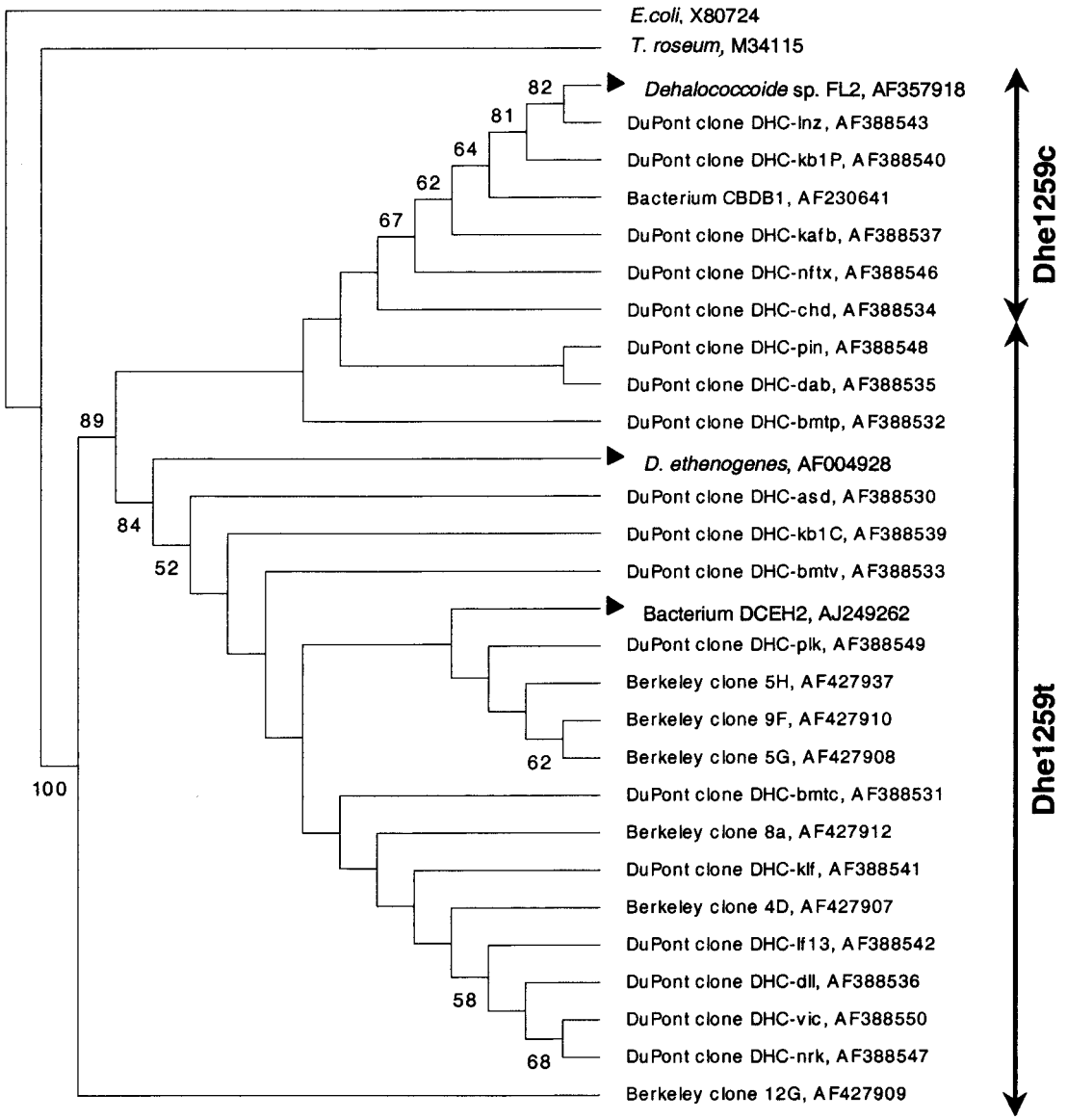

FIG. 1. Phylogenetic relationship of *Dehalococcoides* species and the coverage of designed probes. The tree was inferred by the maximum-likelihood method from approximately 1,400 nucleotide bases by using PHYLIP. Bootstrap values were determined from 100 iterations of the maximum-likelihood calculation. All sequences were labeled with their original names (name of research group and submission name in case of environmental clones) followed by GenBank accession numbers. *E. coli* and *T. roseum* were used as references. The black arrows in front of some sequences indicate the organisms and/or clones whose 16S rRNA sequences were used for the probe design. Probe Dhe1259c is fully complementary to the first 7 sequences, and probe Dhe1259t is fully complementary to the other 21 sequences.

7.2], and a specific amount of formamide according to the probe used) (Table 1) on each well, the slides were incubated for 2 h in a humid chamber at 42°C. Subsequently the slides were washed in prewarmed buffer for 15 min at 48°C, rinsed with deionized water, and air dried. The washing buffer contained 20 mM Tris-HCl (pH 7.2), 10 mM EDTA, 0.01% sodium dodecyl sulfate, and 102 mM NaCl (when 30% formamide was used during hybridization).

Microscopy. Slides were mounted with Citifluor (London, United Kingdom) solution and examined at ×400 magnification (Zeiss Plan-Neofluar 40×, 1.30 numerical aperture, in oil; Oberkochen, Germany) and ×1,000 magnification (Zeiss Plan-Neofluar 100×, 1.30 numerical aperture, in oil) with a Zeiss Axiophot microscope equipped with the filter sets 02 (Zeiss) for DAPI-conferred fluorescence and HQ-Cy3 (Cy3) (AHF Analysen Technik, Tuebingen, Germany) for Cy3-conferred fluorescence.

DAPI- and Cy3-labeled images were captured separately from the same examining field (exposure time, 0.64 and 1.20 s, respectively) with a cooled digital video camera C5810 (Hamamatsu Photonics KK, Hamamatsu-city, Japan).

Phylogenetic analysis. Twenty-eight 16S rRNA gene sequences of *Dehalococcoides* species and two control sequences (*Escherichia coli* and *Thermomicrobium roseum*) (Fig. 1) were retrieved from the public domain databases RDP-II (http://rdp.cme.msu.edu) (16) and GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) (2) and were aligned with ClustalW (http://www.bork.embl-heidelberg.de/Alignment/). Phylogenetic analyses were performed by the maximum-likelihood method by using PHYLIP software package modules (8). Inferred trees (Fig. 1) were viewed and edited with Tree Explorer, version 2.12, from the MEGA2 software package (14).

RESULTS AND DISCUSSION

Design of 16S rRNA-targeted probes. Currently *D. ethenogenes* is the only cultivated organism in the group of *Dehalococcoides* capable of PCE dehalogenation. In spite of the numerous environmental clones extracted from either laboratory cultures or contaminated sites, direct evidence of association of these clones with complete PCE dehalogenation only existed for two of these clones, *Dehalococcoides* strain FL2 (15) and bacterium DCEH2. Therefore, the initial design of the probe was based on an alignment of 16S rRNA gene sequences of these three *Dehalococcoides* species (*D. ethenogenes*, *Dehalococcoides* strain FL2, and bacterium DCEH2). Probe design was carried out by using the PROBE_DESIGN program of the ARB software package (http://arb-home.de) (3).

The resulting probe candidates were screened first by assessing sequence specificity with PROBE_MATCH from RDP (16) and BLAST from GenBank (2), since both databases are updated frequently.

In the second screening step, the accessibility of the probes was evaluated by comparing the 16S rRNA targeting position of the probes with the *E. coli* probe accessibility table developed by Fuchs et al. (10). For that purpose, the secondary structure of the 16S rRNA of *T. roseum* (5), which is also a green nonsulfur bacterium, was used as a template for *Dehalococcoides* due to the lack of that for *Dehalococcoides* species. Several target regions appeared to be very specific and diagnostic for *Dehalococcoides* species, which were mainly around helices 10 (183 to 192 [*E. coli* 16S rRNA positions]), 20 (537 to 555), 23 (658 to 675), and 46 (1,259 to 1,278) (*E. coli* positions are given in parentheses). Three probe candidates in helices 10, 20, and 46 were experimentally evaluated, and only the probe in helix 46, Dhe1259, resulted in an ideal signal. The failure of the other two probes might be caused by poor accessibility, especially for the probe in helix 10, of which the secondary structure is very different from that of *E. coli*. The probe candidate in helix 23 was not tested due to technical problems encountered when synthesizing the probe. Although this site has relatively low accessibility in *E. coli* 16S rRNA, reasonably good probes for bacteria other than *E. coli* were designed based on this region (10); therefore, it would be worthwhile to further test the usefulness of this probe.

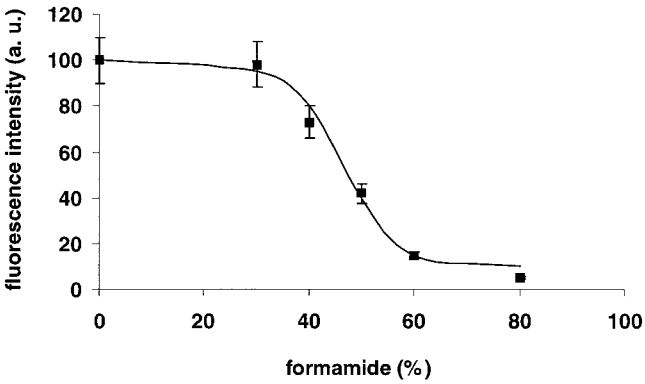

FIG. 2. Probe dissociation curve of Dhe1259t under increasingly stringent hybridization and washing conditions. Each data point represents the mean value of fluorescence intensity of 100 cells. Sigmoidal fitting of the melting curve was carried out with Prism 3 (GraphPad software). Error bars indicate the standard deviations. a.u., arbitrary units.

Dhe1259 is a mixture of two oligomers (Dhe1259t and Dhe1259c) with 1 base difference. The degenerate site is the 12th base from the 5′ end (A or G). Besides the 16S rRNA gene sequences of the three *Dehalococcoides* species (*D. ethenogenes*, bacterium DCEH2, and *Dehalococcoides* species FL2) used for probe design, 25 other 16S rRNA gene sequences were found that were complementary to Dhe1259; only one belonged to a cultivated organism, bacterium CBDB1, a chlorobenzene-degrading bacterium (1), the other 24 belonged to environmental clones all interestingly obtained from microcosms or sites where complete anaerobic dehalogenation of chlorinated ethenes were occurring. All these sequences were then retrieved from GenBank, aligned, and phylogenetically analyzed. The results indicated a close phylogenetic relationship of these sequences (>95% similarity in 1,400 bp), forming a unique *Dehalococcoides* species cluster (Fig. 1). All other 16S rRNA gene sequences currently in the public database had at least 4 mismatches with the probe sequence.

Specific detection and quantification of *Dehalococcoides* species. Due to the unavailability of any culture that can supposedly hybridize with Dhe1259c, only Dhe1259t was tested in this study. The optimal hybridization stringency was determined with a pure culture of *D. ethenogenes* strain 195 by increasing the formamide concentration in the hybridization buffer in increments of 10% at a constant hybridization temperature of 42°C. Probe-conferred signals remained at the same level following the addition of formamide up to 30% and then decreased rapidly (Fig. 2). Therefore, 30% formamide was used in the later hybridization. The in situ hybridization results are displayed in Fig. 3. As shown in Fig. 3D, Dhe1259t allowed us to successfully visualize *D. ethenogenes* strain 195 cells as irregular cocci. The hybridized cells varied in sizes, a lot of which were extremely small, <0.5 μm in diameter.

Subsequently, probe Dhe1259t was applied to different enrichment cultures. In the mixed culture from which *D. ethenogenes* 195 was originally isolated (6, 7, 17), *Dehalococcoides*

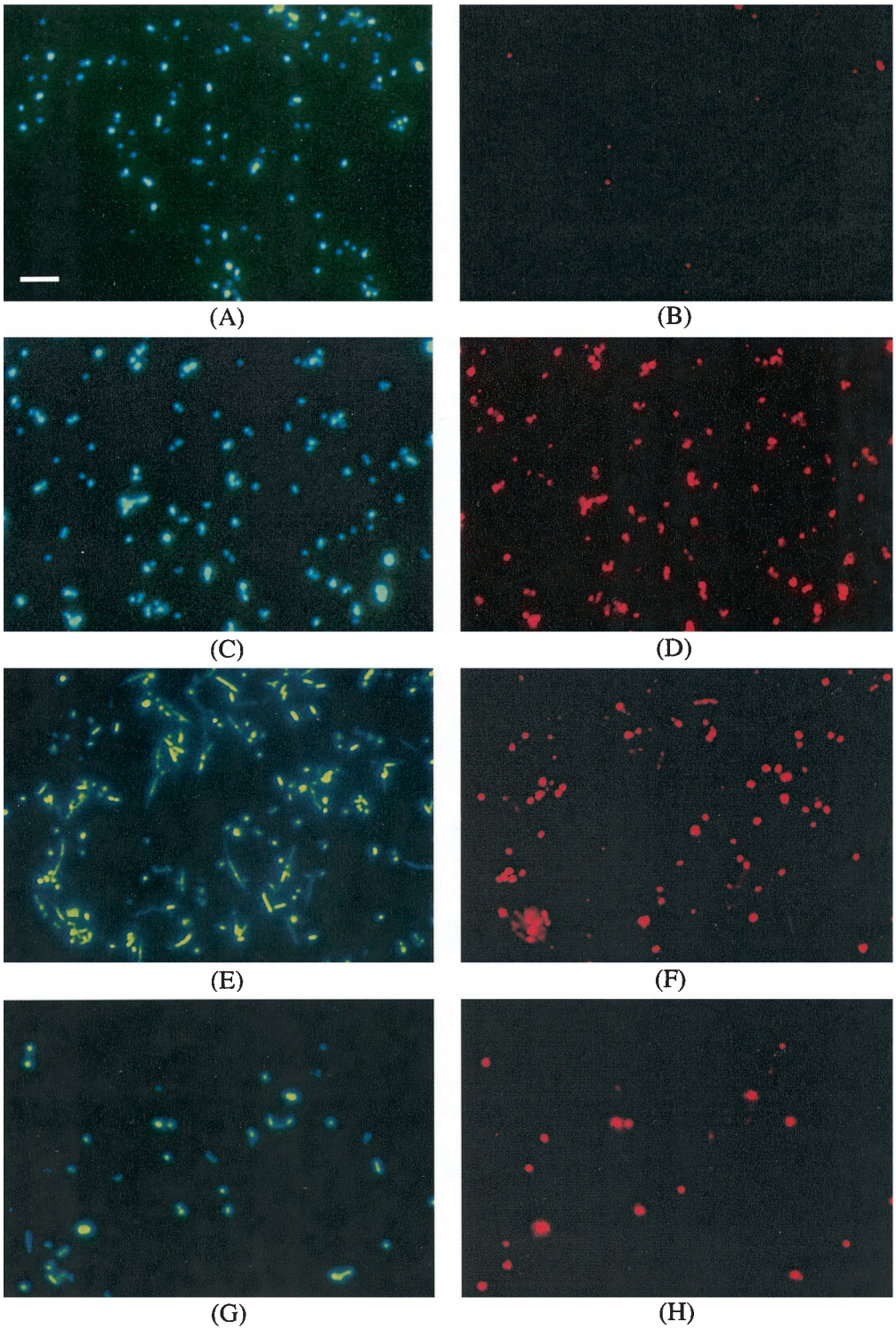

FIG. 3. FISH microscopic images of *D. ethenogenes* strain 195 (rows 1 and 2), the enrichment where strain 195 was isolated (row 3), and the enrichment originally from a PCE-contaminated groundwater site (row 4). Panels A, C, E, and G are micrographs of DAPI staining; panel B is a micrograph of the same field shown in panel A when hybridized with probe Non338; panels D, F, and H are micrographs of the same fields shown in panels C, E, and G, respectively, when hybridized with newly designed probe Dhe1259t. Scale bar, 5 μm.

species accounted for 32% ± 7% of the total DAPI-stained cells (Fig. 3F). *Dehalococcoides* species were also detected in a culture which was originally retrieved from a groundwater site contaminated with PCE and later maintained in the laboratory in a closed continuous stirred-tank reactor with continuous feed of sodium benzoate and PCE (11, 20). About 5% ± 3% of the total DAPI-stained cells hybridized with probe Dhe1259t in the closed continuous stirred-tank reactor culture

(data not shown). Serial transfers were performed to enrich the culture by using PCE and hydrogen as the energy source. During the enrichment process, the percentage of *Dehalococcoides* species with respect to the total DAPI-stained cells steadily increased and eventually reached 38% ± 7% in 3 months after three transfers (Fig. 3H). The presence of *Dehalococcoides* species in this culture was consistent with the fact that it was capable of complete dehalogenation of PCE to ethene, although it cannot be excluded that other species may also play a role in the process. In contrast, when tested with a few cultures incapable of dehalogenation, the signals obtained were no better than those of the background controls (data not shown).

Interestingly, the recently developed probe DhEth for *D. ethenogenes* and relatives (18) is only 2 bp upstream of Dhe1259. While Dhe1259t could successfully detect *Dehalococcoides* species, DhEth only gave very weak signals (data not shown). Besides the small size of the bacteria, this might also be a result of the poorer accessibility of DhEth (about 30% relative accessibility according to the *E. coli* probe accessibility table) (10) or the difference in microscopic setup.

ACKNOWLEDGMENTS

We thank P. L. McCarty, Steve Zinder, Ivonne Nijenhuis, and Jochen Muller for providing us with the different cultures used in this study. We also thank W. Ludwig and colleagues for providing free ARB software.

REFERENCES

1. **Adrian, L., U. Szewzyk, J. Wecke, and H. Goerisch.** 2000. Bacterial dehalorespiration with chlorinated benzenes. Nature **408:**580–583.
2. **Altschul, S. F., T. F. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Amann, R., and W. Ludwig.** 2000. Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. FEMS Microbiol. Rev. **24:**555–565.
4. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
5. **Cannone, J. J., S. Subramanian, M. N. Schnare, J. R. Collett, L. M. D'Souza, Y. Du, B. Feng, N. Lin, L. V. Madabusi, K. M. Muller, N. Pande, Z. Shang, N. Yu, and R. R. Gutell.** 2002. The Comparative RNA Web (CRW) Site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. BMC Bioinformatics **3:**2.
6. **Distefano, T. D., J. M. Gossett, and S. H. Zinder.** 1992. Hydrogen as an electron donor for dechlorination of tetrachloroethene by an anaerobic mixed culture. Appl. Environ. Microbiol. **58:**3622–3629.
7. **Distefano, T. D., J. M. Gossett, and S. H. Zinder.** 1991. Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. Appl. Environ. Microbiol. **57:**2287–2292.
8. **Felsenstein, J.** 1989. PHYLIP - phylogeny inference package (version 3.2). Cladistics **5:**164–166.
9. **Fennell, D. E., A. B. Carroll, J. M. Gossett, and S. H. Zinder.** 2001. Assessment of indigenous reductive dechlorinating potential at a TCE-contaminated site using microcosms, polymerase chain reaction analysis, and site data. Environ. Sci. Technol. **35:**1830–1839.
10. **Fuchs, B. M., G. Wallner, W. Beisker, I. Schwippl, W. Ludwig, and R. Amann.** 1998. Flow cytometric analysis of the in situ accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. Appl. Environ. Microbiol. **64:**4973–4982.
11. **Haston, Z. C., and P. L. McCarty.** 1999. Chlorinated ethene half-velocity coefficients (Ks) for reductive dehalogenation. Environ. Sci. Technol. **33:** 223–226.
12. **Hendrickson, E. R., J. A. Payne, R. M. Young, M. G. Starr, M. P. Perry, S. Fahnestock, D. E. Ellis, and R. C. Ebersole.** 2002. Molecular analysis of *Dehalococcoides* 16S ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe. Appl. Environ. Microbiol. **68:**485–495.
13. **Holliger, C., G. Wohlfarth, and G. Diekert.** 1999. Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol. Rev. **22:** 383–398.
14. **Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei.** 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics **17:**1244–1245.
15. **Loeffler, F. E., Q. Sun, J. Li, and J. M. Tiedje.** 2000. 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. Appl. Environ. Microbiol. **66:**1369–1374.
16. **Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese.** 1997. The RDP (Ribosomal Database Project). Nucleic Acids Res. **25:**109–111.
17. **Maymo-Gatell, X., Y. T. Chien, J. M. Gossett, and S. H. Zinder.** 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science **276:**1568–1571.
18. **Richardson, R. E., V. K. Bhupathiraju, D. L. Song, T. A. Goulet, and L. Alvarez-Cohen.** 2002. Phylogenetic characterization of microbial communities that reductively dechlorinate TCE based upon a combination of molecular techniques. Environ. Sci. Technol. **36:**2652–2662.
19. **Wintzingerode, F., U. B. Gobel, and E. Stackebrandt.** 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21:**213–229.
20. **Yang, Y., and P. L. McCarty.** 1998. Competition for hydrogen within a chlorinated solvent dehalogenating anaerobic mixed culture. Environ. Sci. Technol. **32:**3591–3597.
21. **Zarda, B., D. Hahn, A. Chatzinotas, W. Schoenhuber, A. Neef, R. I. Amann, and J. Zeyer.** 1997. Analysis of bacterial community structure in bulk soil by in situ hybridization. Arch. Microbiol. **168:**185–192.
22. **Zarda, B., G. Mattison, A. Hess, D. Hahn, P. Hoehener, and J. Zeyer.** 1998. Analysis of bacterial and protozoan communities in an aquifer contaminated with monoaromatic hydrocarbons. FEMS Microbiol. Ecol. **27:**141–152.