APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1993, p. 682–686
0099-2240/93/030682-05$02.00/0

Vol. 59, No. 3

Monitoring the Enrichment and Isolation of Sulfate-Reducing Bacteria by Using Oligonucleotide Hybridization Probes Designed from Environmentally Derived 16S rRNA Sequences

MATTHEW D. KANE,[1] LARS K. POULSEN,[1]† AND DAVID A. STAHL[1,2]*

Departments of Veterinary Pathobiology[1] and Microbiology,[2] University of Illinois, 2001 South Lincoln Avenue, Urbana, Illinois 61801

Received 20 August 1992/Accepted 5 January 1993

A fluorescently labeled version of a population-specific oligonucleotide hybridization probe was used to monitor the enrichment and isolation of a sulfate-reducing bacterium from a multispecies anaerobic bioreactor. The organism was originally identified as a molecular isolate that was phylogenetically related to *Desulfovibrio vulgaris* by amplification and sequencing of part of its 16S rRNA sequence. The sequence, in turn, was used to design a population-specific probe. The anaerobic medium used for the organism's enrichment and isolation was based on the physiological properties of the its closest relatives as identified by sequence comparisons. Of 30 isolates examined, only 3 hybridized with the probe. Nearly complete 16S rRNA sequences determined for each of these three isolates (i) had no mismatches with the probe target site, (ii) were identical to the amplified partial sequence of about 500 nucleotides and to one another in all other positions, and (iii) were 93.9% similar to that of *D. vulgaris*. In addition, one isolate chosen for further study (strain PT-2) had a substrate specificity comparable to that of *D. vulgaris*. These results confirmed that polymerase chain reaction amplification of 16S rRNA sequences from environmental samples can be accurate and can also provide phylogenetic information from which aspects of a population's physiology can be inferred.

The use of molecular methods to describe microbial populations in natural communities has attracted considerable interest (for a recent review, see reference 17). In particular, the direct amplification of 16S rRNA genes from environmental samples by using the polymerase chain reaction (PCR) has proven to be an attractive technique for characterizing members of complex microbial communities and assemblages, because it avoids the well-known biases of culture methods (3, 4, 8, 15, 20). Similarly, oligonucleotides complementary to specific regions of the rRNAs (or their genes) have been used as hybridization probes in determinative and quantitative studies of microbial populations within complex communities (1–5, 7, 16, 19).

Recently, we used these two rRNA-based approaches in concert to examine gram-negative sulfate- and/or sulfur-reducing bacteria (SRB) in multispecies biofilm communities established in laboratory bioreactors (4). Phylogenetic analysis of partial 16S rRNA sequences selectively amplified from SRB that were present in an anaerobic, sulfidogenic bioreactor revealed the presence of two SRB-like population types. Population type 1 was a relative of *Desulfuromonas* spp., and population type 2 was a relative of *Desulfovibrio vulgaris*. These sequences were then used to design population-specific oligonucleotide hybridization probes. Fluorescent versions of these probes and epifluorescence microscopy were used to visualize members of population types 1 and 2 in biofilm material that had colonized glass coverslips placed in the bioreactor. However, the accuracy (PCR fidelity) of SRB-specific 16S rRNA sequence information derived from such a complex environmental sample was still somewhat uncertain. It has been demonstrated that PCR amplification may introduce sequence errors or even result in the formation of hybrid sequences (chimeras), different parts of which derive from distinct populations (10). The present study was undertaken to address these concerns and also to examine the utility of using oligonucleotide hybridization probes to monitor the enrichment and isolation of specific bacterial populations from the environment. For this report, we used a fluorescent oligonucleotide probe specific for population type 2 (*D. vulgaris* relative) to monitor the enrichment and isolation of this organism and then determined the nearly complete 16S rRNA sequences of three isolates which hybridized with the population type 2-specific probe.

(A preliminary report of this work has been presented previously [12a].)

MATERIALS AND METHODS

Bioreactors. Biofilm communities were developed in completely mixed fixed-bed anaerobic bioreactors as previously described (4).

Bacteria. Population type 2 (*D. vulgaris*-like [4]) bacteria were obtained in enrichment culture by incubating three biofilm-covered glass beads from a sulfidogenic bioreactor in serum-stoppered glass bottles (100-ml volume) containing 50 ml of anoxic PDP-1 medium under 100% N_2 at a pressure of 1 atm (ca. 100 kPa). The bottles were kept stationary and incubated vertically at 30°C. The bottles were samples periodically and examined for the presence of population type 2 cells by fluorescent-probe hybridization and epifluorescence microscopy (see below).

Medium. Aqueous PDP-1 medium contained (grams per liter) NH_4Cl (2.0), K_2HPO_4 (0.5), Na_2SO_4 (2.0), $FeCl_2$ (0.01), $CaCl_2 \cdot 2H_2O$ (0.2), $MgSO_4$ (2.0), yeast extract (1.0), and

* Corresponding author.
† Present address: Department of Microbiology, The Technical University of Denmark, Building 221, DK-2800 Lyngby, Denmark.

resazurin (10^{-4}). In addition, PDP-1 medium contained 1 ml of a mineral salts solution per liter (24). The pH of the solution was adjusted to 7.2 with NaOH, and the solution was heated to boiling and then cooled under an O_2-free atmosphere of N_2 and dispensed by using anaerobic techniques into appropriate bottles or tubes, which were then autoclaved. Cysteine-sulfide (final concentration of an equimolar mixture of cysteine · HCl and $Na_2S \cdot 9H_2O$, 10^{-4} g/liter) and appropriate carbon sources were prepared and sterilized separately before addition to tubes or bottles containing PDP-1 medium. PDP-2 medium was identical to PDP-1, except that it was prepared under an N_2-CO_2 atmosphere (80/20 [vol/vol]), $NaHCO_3$ (4.0 g/liter) and elemental sulfur (2.0 g/liter) were prepared and added separately, and cysteine-sulfide was left out. Anaerobic roll tubes were prepared by using PDP-1 medium solidified with 2% agar. Isolated colonies were picked and transferred to PDP-1 broth, and then liquid samples were examined by hybridization with fluorescently labeled, population type 2-specific oligonucleotide hybridization probes and epifluorescence microscopy. Cultures were considered pure after two successive passages in roll tubes had been made. Microscopic examination confirmed the presence of one morphological type, all of whose cells hybridized with the population-specific probe.

Growth studies. To evaluate the utilization of substrates by population type 2 cells, various organic compounds were presterilized and added separately to serum-stoppered tubes (18-mm diameter) containing 10 ml of PDP-1 medium. The final concentrations of the organic substrates tested for growth were 5 to 10 mM. To evaluate the ability of cells to grow autotrophically with H_2 plus CO_2 as the substrate, PDP-1 medium was prepared in the usual manner but dispensed under an O_2-free atmosphere of H_2-CO_2 (80/20 [vol/vol]). The growth of cells was measured by determining the A_{600} of cultures with a Bausch & Lomb Spectronic 20 colorimeter.

Cell fixation, hybridization, and photomicroscopy. Cells were fixed and loaded into one of six hybridization wells contained on a gelatin-dipped, Teflon-coated slide, and hybridizations were carried out and visualized by epifluorescence microscopy as described elsewhere (2), except that following hybridization reactions the slides were washed in hybridization buffer (15 min, 37°C) rather than in the wash buffer that was used previously. The fluorescently labeled oligonucleotide hybridization probes specific for bioreactor population types 1 and 2 have also been previously described (4), as has the hybridization of radioactive probes to phylogrid membranes and the organisms and nucleic acids used for such analyses (7).

16S rRNA sequencing and phylogenetic analysis. Nucleotide sequences were determined primarily by the dideoxynucleotide method, by using reverse transcriptase and 16S rRNA as the template (9). Nearly complete sequences were obtained by initiating reactions with one of seven oligonucleotide primers complementary to high conserved regions of the bacterial 16S rRNA (23). The primer sequences were as follows (the numbers in parentheses indicate the positions on *Escherichia coli* 16S rRNA, and the letters indicate the priming direction [R, reverse; F, forward]): CTGCTGCCTC CCGTA (357 to 343, R), ACCGCGGC(G/T)GCTGGC (531 to 517, R), TACGCATTTCACTCCT (703 to 687, R; this primer sequence specifically complements the 16S rRNAs of members of the genus *Desulfovibrio* [6, 7]), GCCCCCG(T/C) CAATTCCT (930 to 915, R), TGGGTCTCGCTCGTTG (1,115 to 1,100, R), ACGGGCGGTGTGT(G/A)C (1,406 to 1,392, R), and TACCTTGTTACGACTT (1,512 to 1,496, R). Direct sequencing of the PCR amplification products (16S rDNA) was also used to confirm and extend sequences first determined by the reverse transcriptase method. PCR reactions were carried out as described by Shyamala and Ames (14) by using the following primers: GTTTGATCCTGGCT CAG (complementary to positions 11 to 28 on the *E. coli* 16S rRNA) and TACCTTGTTACGACTT (complementary to positions 1,512 to 1,496 on the *E. coli* 16S rRNA). Sequences were then determined by using Sequenase (U.S. Biochemical, Cleveland, Ohio), asymmetric PCR products as the template for reactions, and the following sequences as primers: GCAACGAGCGCAACCC (1,099 to 1,114, F) and TGCTACACACCGCCCGT (1,391 to 1,406, F). For phylogenetic analyses, sequence similarities were calculated and a phylogenetic tree was inferred as described previously (4).

Chemicals. All chemicals were reagent grade and were purchased from commercial sources.

Nucleotide sequence accession number. The 16S rRNA sequence of strain PT-2 has been deposited with GenBank under accession no. M89496.

RESULTS

Enrichment and isolation of SRB. To obtain cultures of bacterial population types 1 and 2, enrichments were initiated by inoculating three biofilm-covered glass beads from a sulfidogenic bioreactor into 50 ml of appropriate medium. Procedures for the enrichment of *Desulfuromonas* spp. and *D. vulgaris* were used for population types 1 and 2, respectively (11, 12), because these were the closest relatives of the two bioreactor SRB-like populations, as identified by their partial 16S rRNA sequences (4). Although a number of different medium variations to enrich for population type 1 (*Desulfuromonas* spp.-like) bacteria (i.e., cells that oxidize acetate and reduce sulfur [11]) were attempted, there was no observed sulfur reduction in enrichment bottles, and no cells in such enrichments hybridized with the fluorescently labeled population type 1-specific probe. However, when PDP-1 medium and lactate were used as the carbon source, enrichments for population type 2 (*D. vulgaris*-like) bacteria resulted in considerable production of a black precipitate (presumably FeS) and a highly enriched culture of cells which hybridized to the population type 2-specific probe (Fig. 1A and B). Subsequently, 30 isolated colonies from PDP-1 agar roll tubes were picked and separately inoculated into tubes containing fresh PDP-1 broth medium. Three of these isolates contained cells which hybridized with the fluorescently labeled population type 2-specific probe (Fig. 1C and D). The three isolates were morphologically identical, gram-negative, motile, curved rods measuring 2.0 to 8.0 by 0.5 to 1.5 μm. The strains were then passaged a second time through roll tubes to ensure that each culture was pure.

Analysis of 16S rRNA sequences. Approximately 1,500 positions of the 16S rRNAs of the three population type 2 isolates were sequenced. As expected, the 16S rRNA of each had no mismatches with the population type 2 probe target sequence of UGGACUUGAGUUCGGGAGA (positions 647 to 665, [*E. coli* numbering]). Similarly, the 16S rRNA sequences of all three isolates had no mismatches with the approximately 500 nucleotides of the population type 2 sequence that had been amplified and cloned from the bioreactor (positions 385 to 907) [*E. coli* numbering; 4]). Moreover, the sequences were identical to one another at the remaining ~1,000 positions as well. This indicated that the three isolates were, in fact, each representative and probably identical members of population type 2. One of

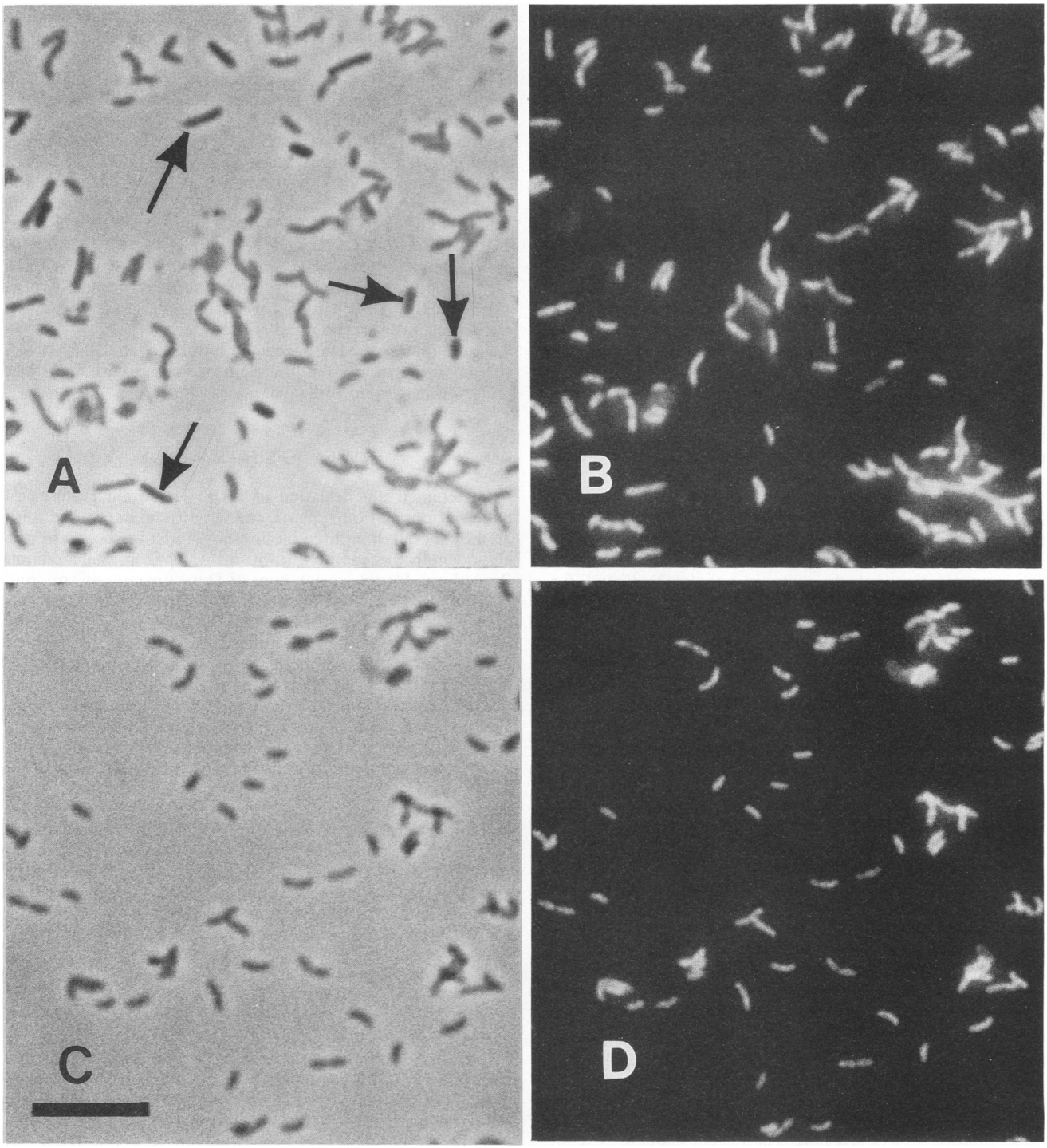

FIG. 1. Phase-contrast (A and C) and epifluorescence (B and D) micrographs of enrichment (A and B) and pure (C and D) cultures of population type 2 cells hybridized with the fluorescently labeled population type 2-specific probe. Arrows indicate cells in the enrichment culture which failed to hybridize with the population type 2-specific probe. (All cells hybridized with a universal probe that binds to the 16S-like rRNAs of all organisms [7 and data not shown]). Bar in C, 10 μm.

these isolates was chosen for further study and was designated strain PT-2.

The nearly complete 16S rRNA sequence of strain PT-2 was aligned and compared with those of other representative SRB, and a phylogenetic tree was constructed (Fig. 2). This analysis confirmed preliminary results which indicated that the closest relative to strain PT-2 was *D. vulgaris* (4). In this case, the similarity between the 16S rRNAs of strain PT-2 and *D. vulgaris* was 93.9%, which is in close agreement with a 94.5% similarity value obtained from a comparison re-

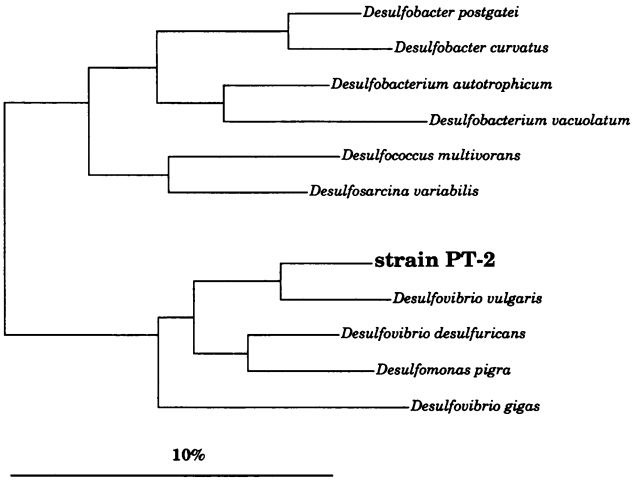

FIG. 2. Phylogenetic tree inferred from comparison of the 16S rRNA sequences of characterized sulfate-reducing bacteria (6) and that of strain PT-2. The bar represents 0.1 estimated changes per nucleotide position.

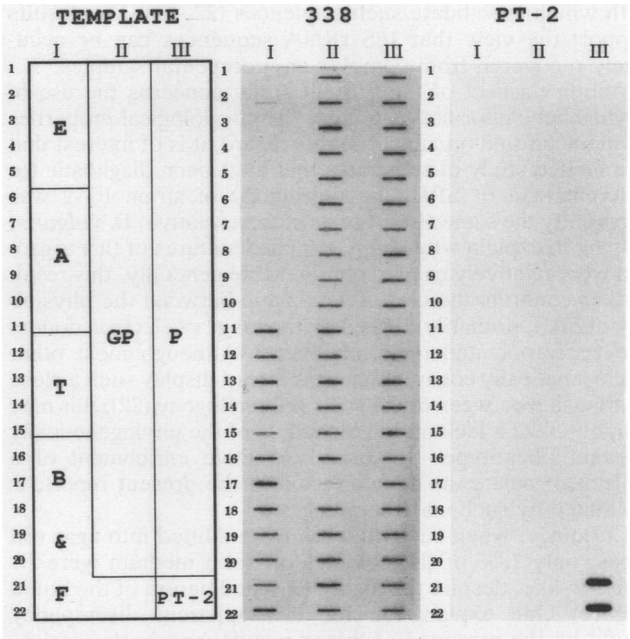

FIG. 3. Phylogrid membrane analysis of population type 2 probe specificity. A template of the membranes' phylogenetic arrangement is provided, with abbreviations as follows: E, Eukarya; A, Archaea; T, *Thermotoga* species, *Spirochaeta* species, and *Chlorobium* species; B & F, *Bacteroides* species and *Flavobacterium* species GP, gram-positive bacteria; P, proteo- (purple) bacteria; PT-2, duplicate blots of strain PT-2. The specific organisms used as sources for nucleic acids have been described previously (7). The two probes used were 338, which binds to all bacteria (but not eukarya or archaea [1]), and the population type 2-specific probe (4).

stricted to the smaller amplified region encompassing approximately 500 positions. (Note that regrettably, in a previous report (4), we incorrectly reported a similarity of 98% between the amplified sequence and that of *D. vulgaris*.)

Phylogrid analysis of probe specificity. The availability of pure cultures enabled us to verify the specificity of the probe which targeted population type 2 by using a phylogrid membrane analysis (7). Phylogrid membranes are nylon membranes that are (slot) blotted with nucleic acids from the targeted organism and from 64 nontargeted taxa which represent a diverse collection of Eukary, Archaea, and bacteria. All nontarget nucleic acids were from organisms with known small subunit rRNA sequences. A phylogrid was hybridized with a radioactive version of the population type 2-specific probe. An autoradiograph of the phylogrid indicated that the probe hybridized only with RNA from strain PT-2, and not with RNAs from any of the other 64 organisms (Fig. 3). The population type 1-specific probe was not tested with a phylogrid membrane, since population type 1-specific nucleic acids were not available as a positive control.

Physiology of strain PT-2. Cells of strain PT-2 were strictly anaerobic and were able to use lactate, malate, fumarate, and formate as sources of carbon and electrons in PDP-1 medium, but no growth was observed on acetate, isobutyrate, or glycerol. In addition, poor but consistent growth was observed with ethanol or H_2 plus CO_2 as substrates. On all substrates tested, the cells required the addition of either sulfate or nitrate as an electron acceptor. Although our physiological studies were not exhaustive, the results with the substrates described above (which are normally diagnostic for SRB) indicated that the physiology of strain PT-2 is comparable to that of *D. vulgaris* (21). Cells of strain PT-2 have been deposited with the American Type Culture Collection (ATCC 49975).

DISCUSSION

The limitations of using culture-dependent methods for studying the diversity and ecology of natural microbial communities are well known. As a consequence, the use of more-direct molecular characterizations has become common (for recent reviews, see references 13, 16–18). Among various molecular methods being developed, those directed at using either small or large subunit rRNA sequences have held the greatest promise for the analysis of microbial populations within complex communities (1–5, 7, 8, 15, 19). Within this phylogenetic framework, two molecular tools, rRNA-directed oligonucleotide hybridization probes and PCR amplification of 16S rRNAs, have proven valuable in initial studies of SRB populations in complex communities (4, 7). In the present study, we used both of these rRNA-based approaches in combination with classic enrichment and isolation techniques. Thus, a population identified as a molecular isolate was subsequently obtained in pure culture by exploiting the original molecular information. This enabled us to verify the accuracy of retrieving sequence information from a complex environmental sample and will facilitate further in situ studies (see below). By contrast, in a recent study by Liesack et al. (10), amplification of 16S rRNA genes (16S rDNA) by PCR and cloning and sequencing of amplification products revealed the creation of hybrid molecules from two different bacterial strains that had occurred as an artifact of the PCR. In regard to hybrid amplification product formation, it is worth noting that the DNA used as the template in that study was reported to be substantially degraded during preparation. Thus, the result might be better ascribed to the quality of the DNA template than to a general PCR artifact. In the case of 16S rRNA or rDNA sequences, however, detection of such artifacts is more straightforward than for other sequences, because there is an abundance of information concerning the secondary structure and conservation of the 16S rRNA molecule

with which to validate such sequences (22, 23). Our results support the view that 16S rRNA sequences can be accurately recovered from complex environmental samples.

Another aspect of the present study concerns the use of phylogenetic information to infer the physiological properties of an uncultured organism. In this regard, it is of interest that in a limited study of substrates that have been diagnostic for different taxa of SRB, the physiology of strain PT-2 was apparently the same as that of its closest relative, *D. vulgaris*, helping to explain why highly enriched cultures of this organism were relatively easy to obtain. More generally, this result further confirms the close relationship between the physiology of SRB, primarily defined by the range of electron donors and acceptors, and their phylogeny. Although most other phylogenetically coherent lineages do not display such a close relationship between physiology and phylogeny (22), this may simply reflect a lack of understanding of the phylogenetically relevant phenotype. Successful selective enrichment of a molecular isolate, as demonstrated in the present report, is facilitated by such understanding.

Curiously, when such cultures were diluted into agar roll tubes, only 10% of the colonies on solid medium were *D. vulgaris*-like, despite the highly enriched nature of the liquid culture. One explanation for this apparent discrepancy would be the presence of minor populations in the enrichment which were not as sensitive as strain PT-2 to heat from the agar medium prior to rolling and solidification. However, screening of cultures with the population-specific probe enabled us to easily identify the desired isolates, despite apparent loss of viability during dilution in roll tubes.

In conclusion, these results emphasize the value and complementarity of both molecular and classic microbiological methods. Now that we have been able to identify one of the dominant bioreactor SRB populations by molecular phylogenetic criteria and to subsequently obtain cells of the organism in pure culture, further, studies are possible. For example, we have begun to examine the correlation between rRNA content and the growth rate of strain PT-2 cells so that we might be able to estimate the growth rate of cells within the complex bioreactor community as it experiences changes in environmental conditions.

ACKNOWLEDGMENTS

We thank P. F. DePropis and S. Stone for their participation in these experiments.

This research was supported by grant no. NOOO14-88-K-0093 from the Office of Naval Research. M.D.K. was the recipient of a National Science Foundation Fellowship in Environmental Biology (BSR-9001832), and L.K.P. was supported by the Danish Center for Microbial Ecology.

M.D.K. and L.K.P. contributed equally to this report.

REFERENCES

1. **Amann, R. I., B. Binder, S. W. Chisholm, R. Olsen, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
2. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
3. **Amann, R. I., N. Springer, W. Ludwig, K.-H. Schleifer, and N. Peterson.** 1991. Identification in situ and phylogeny of uncultured bacterial endosymbionts. Nature (London) **351:**161–164.
4. **Amann, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol. **58:**614–623.
5. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243:**1360–1363.
6. **Devereux, R., M. Delaney, F. Widdel, and D. A. Stahl.** 1989. Natural relationships among sulfate-reducing bacteria. J. Bacteriol. **171:**6689–6695.
7. **Devereux, R., M. D. Kane, J. Winfrey, and D. A. Stahl.** Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. Syst. Appl. Microbiol., in press.
8. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature (London) **345:**60–63.
9. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA **82:**6955–6959.
10. **Liesack, W., H. Weyland, and E. Stackebrandt.** 1991. Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophillic bacteria. Microb. Ecol. **21:**191–198.
11. **Pfennig, N., and H. Biebl.** 1981. The dissimilatory sulfur-reducing bacteria, p. 941–947. *In* M. P. Starr, H. Stolp, H. G. Truper, A. Ballows, and H. G. Schlegel (ed.), The prokaryotes, vol. 1. Springer-Verlag KG, Heidelberg, Germany.
12. **Pfennig, N., F. Widdel, and H. G. Truper.** 1981. The dissimilatory sulfate-reducing bacteria, p. 926–940. *In* M. P. Starr, H. Stolp, H. G. Truper, A. Ballows, and H. G. Schlegel (ed.), The prokaryotes, vol. 1. Springer-Verlag KG, Heidelberg, Germany.

12a. **Poulsen, L. K., M. D. Kane, and D. A. Stahl.** 1992. Abstr. Annu. Meet. Am. Soc. Microbiol. 1992, Q-61, p. 345.

13. **Sayler, G. S., and A. C. Layton.** 1990. Environmental application of nucleic acid hybridization. Annu. Rev. Microbiol. **44:**625–648.
14. **Shyamala, V., and G. F.-L. Ames.** 1989. Amplification of bacterial genomic DNA by the polymerase chain reaction and direct sequencing after asymmetric amplification: application to the study of periplasmic permeases. J. Bacteriol. **171:**1602–1608.
15. **Spring, S., R. Amann, W. Ludwig, K.-H. Schleifer, and N. Peterson.** 1992. Phylogenetic diversity and identification of nonculturable magnetotactic bacteria. Syst. Appl. Microbiol. **15:**116–122.
16. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes in bacterial systematics, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), Sequencing and hybridization techniques in bacterial systematics. John Wiley & Sons, Chichester, England.
17. **Stahl, D. A., and M. D. Kane.** 1992. Methods of microbial identification, tracking and monitoring of function. Curr. Opin. Biotechnol. **3:**244–252.
18. **Steffan, R. J., and R. M. Atlas.** 1991. Polymerase chain reaction: applications in environmental microbiology. Annu. Rev. Microbiol. **45:**137–161.
19. **Tsien, H. C., B. J. Bratina, K. Tsuji, and R. S. Hanson.** 1990. Use of oligonucleotide signature probes for identification of physiological groups of methylotrophic bacteria. Appl. Environ. Microbiol. **56:**2858–2865.
20. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) **345:**63–65.
21. **Widdel, F., and N. Pfennig.** 1984. Dissimilatory sulfate- or sulfur-reducing bacteria, p. 663–679. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
22. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
23. **Woese, C. R., R. Gutell, R. Gupta, and H. F. Noller.** 1983. Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. Microbiol. Rev. **47:**621–669.
24. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238:**2882–2886.