APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1989, p. 1818–1822
0099-2240/89/071818-05$02.00/0

Vol. 55, No. 7

Selective Recovery of 16S rRNA Sequences from Natural Microbial Communities in the Form of cDNA

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Received 28 December 1988/Accepted 10 April 1989

Cloning of cDNA obtained from 16S rRNA (16S rcDNA) selectively retrieves species-specific sequence information useful for analyzing the composition and structure of natural microbial communities. With this technique we obtained recombinant 16S rcDNA libraries from *Escherichia coli* and from a model hot-spring cyanobacterial-mat community. The recombinant plasmids contained exclusively 16S rRNA-derived inserts. This selective approach is independent of biasing culture techniques and eliminates the laborious screening required to locate 16S rRNA gene-bearing recombinants in genomic DNA libraries obtained from natural communities.

It is well established that enrichment cultures and other isolation procedures will recover only a minor portion of microbial community members from natural habitats (1, 3; D. M. Ward, *in* W. P. Charaklis, and P. A. Wilderer, ed., *Structure and Function of Biofilms*, Dahlem Konferenzen, Berlin, in press). Pace et al. (18) have suggested the use of 16S rRNA sequence information for ecological applications. The 16S rRNA sequence is characteristic for nearly every organism, and the information content of the molecule is high enough to allow statistically valid phylogenetic analysis as well as the identification of a species when the sequence is known. It was proposed that the 16S rRNA genes (rDNA) be retrieved by shotgun cloning restriction fragments of DNA isolated from natural communities. As 16S rDNA-bearing recombinants are rare in such a community genomic library (ca. 0.2% or less), they must be identified by extensive hybridization screening before sequence analysis.

We present here a new approach that is also based on the use of 16S rRNA sequences as species-specific biomarkers. However, the 16S rRNA sequences were selectively retrieved by cloning and sequencing cDNA synthesized from the 16S rRNA molecule (16S rcDNA) (Fig. 1). The selectivity of the method results from priming cDNA synthesis with a synthetic oligonucleotide complementary to a universally conserved region in the 16S rRNA molecule. We used the conserved region closest to the 3′ end of the 16S rRNA molecule in order to maximize the amount of sequence obtained for cloning. The cDNA was then cloned into the plasmid vector pBR322. This new protocol resulted exclusively in recombinants which possess 16S rRNA sequences, eliminating the screening step which has made the shotgun cloning approach prohibitive for detailed community analysis. The method was developed with *Escherichia coli* and has been tested on a hot-spring cyanobacterial mat, a natural model community which has been extensively investigated (23).

MATERIALS AND METHODS

All gel analyses of RNA or DNA on formaldehyde gels or neutral or alkaline agarose gels were as described by Maniatis et al. (13). Standard precautions were taken to guard against RNase activity (2).

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Extraction and purification of RNA. *E. coli* was lysed with lysozyme, and the nucleic acids were separated from cellular debris by phenol extractions (14, 21) and ethanol precipitation (22). DNA was digested with RNase-free DNase I (Bethesda Research Laboratories, Inc., Gaithersburg, Md. [BRL]) according to the instructions of the manufacturer. The RNA was prepared for cDNA synthesis by being precipitated in 2 M NaCl (17), washed with 80% ethanol, and suspended in diethylpyrocarbonate-treated water to a concentration of 2 μg/μl.

RNA from the hot-spring cyanobacterial mat (50 to 55°C; shoulder region of Octopus Spring, Yellowstone National Park, Wyo. [6]) was extracted in the field. About 10 to 15 cm^3 of the upper 4 mm of the mat was homogenized in a Wheaton tissue grinder in freshly prepared lysis buffer (10 mM Tris [pH 7.6], 0.5 M NaCl, 1% sodium dodecyl sulfate, 30 mM EDTA). Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to a concentration of 60 μg/ml; the homogenate was incubated at 50°C for 20 min and then mixed with an equal volume of STE (13)-saturated phenol-chloroform-isoamyl alcohol (25:24:1) to stop all enzymatic activities. This mixture was placed on dry ice, transported to the laboratory, and stored at −70°C until needed. Further extraction of the RNA was done by standard protocols (13, 14, 21, 22). Added to these protocols was a treatment with polyvinylpolypyrrolidone (Sigma Chemical Co., St. Louis, Mo.) to absorb compounds (10, 12) coextracted from the Octopus Spring mat, which inhibited cDNA synthesis. A 2-ml volume of a diethylpyrocarbonate-treated slurry of the insoluble polymer (about 25 g/100 ml of STE) was added to 10 ml of the aqueous supernatant after the first phenol extraction. After incubation at room temperature for 1 h, the polyvinylpolypyrrolidone was removed by centrifugation. Purification of the extracted RNA with a Qiagen column (Qiagen, Inc., Studio City, Calif.) resulted in material of sufficient purity for cDNA synthesis.

cDNA synthesis from 16S rRNA. To 5 to 10 μg of RNA in 10 μl of diethylpyrocarbonate-treated water, 1.1 μl of 0.1 M methyl mercury (II) hydroxide (Alfa Products, Morton Thiokol, Inc. Division, Danvers, Mass.) (extremely toxic; use fume hood) was added to reduce secondary structure in the template and to prevent self-primed synthesis from 23S rRNA (our unpublished observation). After 10 min, 2.0 μl of 0.7 M 2-mercaptoethanol (Sigma) was added to quench the

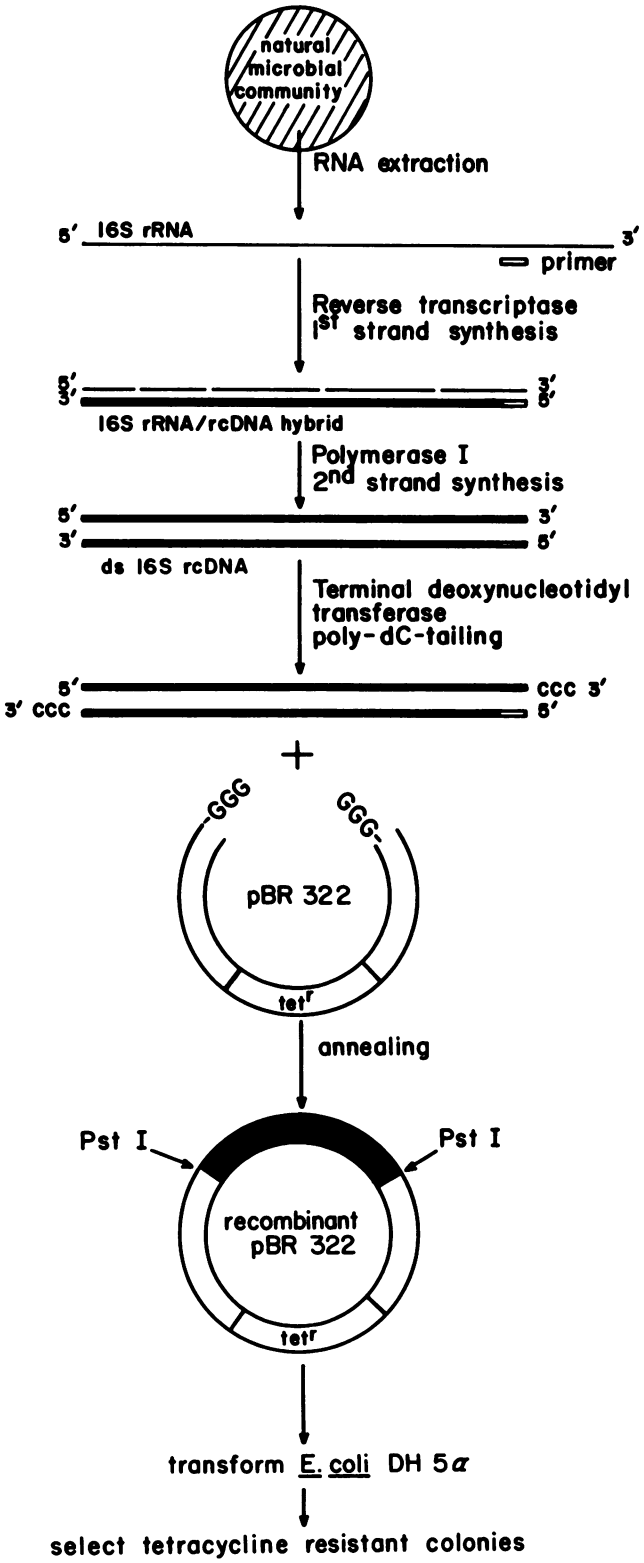

FIG. 1. Outline of 16S rcDNA cloning approach for the selective recovery of rRNA sequences. ds, Double stranded.

methyl mercury. Thirty nanograms of a synthetic oligonucleotide complementary to nucleotides 1392 through 1406 in *E. coli* and 20 μl of diethylpyrocarbonate-treated water were added. The primer was annealed to 16S rRNA by raising the temperature of the mixture to 80°C for 2 min and then cooling it to room temperature for 10 min. cDNA synthesis was performed with the BRL cDNA synthesis system, except that first-strand reaction products were extracted with phenol-chloroform and ethanol precipitated before second-strand synthesis, and second-strand reaction products were treated with DNase-free RNase A (13) to digest residual RNA. The final reaction products were phenol extracted, ethanol precipitated, and purified on a Sephacryl S-400 column in pH 8.0 STE (13).

Tailing of 16S rcDNA. Double-stranded cDNA was tailed with deoxycytidine. A 200-ng amount of the cDNA was treated for 4 to 10 min at 37°C with 25 U of terminal transferase (Boehringer Mannheim Biochemicals) in the presence of 5 to 10 μM dCTP with the buffer recommended by Boehringer Mannheim Biochemicals. The reaction was stopped by the addition of 2 μl of 0.1 M EDTA and heat inactivation of the enzyme at 65°C for 5 min. The reaction products were extracted once with phenol-chloroform and purified on a Sephacryl S-400 column in STE at pH 8.0. To assess the efficiency of the reaction and to obtain some information about the extent of the deoxycytidine addition, purified tailed *E. coli* 16S rcDNA was digested with the restriction endonuclease *Cfo*I (BRL) in the buffer provided. The fragments were separated on a 3% agarose gel (ultrapure DNA-grade agarose; Bio-Rad Laboratories, Richmond, Calif.) in Tris-borate buffer (134 mM Tris, 44 mM boric acid, 2.7 mM EDTA).

Cloning. The tailed cDNA was annealed to the poly(dG)-tailed plasmid vector pBR322 (BRL) by incubation at 65°C for 5 min and then at 57°C for 2 h in annealing buffer (13). The mixture was stored at −20°C until transformation could be performed. Transformation of *E. coli* DH 5α (BRL) with 5 μl of the annealing reaction mixture was done as recommended by BRL.

Analysis of the 16S rcDNA libraries. Plasmid DNA from recombinant cells selected on tetracycline-supplemented medium was isolated by standard protocols (13). Isolated plasmids were digested with the restriction endonuclease *Pst*I (Boehringer Mannheim Biochemicals) to excise the cloned insert. The resulting DNA fragments were separated on 0.7% agarose gels and transferred to nitrocellulose filters (19) for hybridization probing as described below.

Hybridization probing. RNAs from *E. coli* and *Thermoplasma acidophilum* (extracted as described above) were separated on a denaturing polyacrylamide-urea gel (13). The band corresponding to 16S rRNA was excised, twice equilibrated in 5 ml of bicarbonate buffer (50 mM $NaHCO_3$, 2 mM Na_2CO_3, 1 mM EDTA [pH 9.0]) on ice for 1.5 h and incubated at 90°C for 45 min to cause limited alkaline hydrolysis. 16S rRNA fragments were eluted in 1 ml of 1 M Tris hydrochloride (pH 7.4) and 5 ml of Maxam-Gilbert buffer (0.5 M ammonium acetate, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% sodium dodecyl sulfate) by rotating the mixture at 4°C overnight. The slurry was filtered (filter 591-A; Schleicher & Schuell, Inc., Keene, N.H.) to remove gel particles, and the fragments were precipitated with ethanol at −20°C. The 16S rRNA fragments were labeled with photoactivatable biotin (Clontech Laboratories, Inc., Palo Alto, Calif.) and purified as recommended by Clontech.

Nitrocellulose filters with bound DNA (see above) were prehybridized for 6 h (0.75 M NaCl, 50 mM Na_2PO_4 [pH

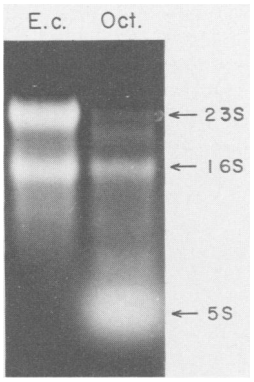

FIG. 2. RNAs isolated from *E. coli* (E.c.) and the Octopus Spring cyanobacterial mat (Oct.) separated on a 1.4% agarose–formaldehyde gel. *E. coli* RNA was salt precipitated; Octopus Spring RNA was purified with a Qiagen tip-20.

6.8], 50% deionized formamide, 5 mM EDTA, 0.5% sodium dodecyl sulfate, 10 μg of poly(A) per ml [Schwarz/Mann Biotech, Cleveland, Ohio], 10× Denhardt solution [as described in reference 13 except that bovine serum albumin was omitted]) before biotinylated 16S rRNA fragments were added to 200 ng/ml. Hybridization occurred overnight in a water bath initially set at 60°C, which was allowed to slowly cool to room temperature. The filters were washed for 15 min each in the presence of 0.1% sodium dodecyl sulfate in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (13) at room temperature, then in 1× SSC at room temperature, and finally in 0.1× SSC at 53°C. Detection of the biotinylated probes with an avidin-alkaline phosphatase conjugate was as recommended by Clontech.

RESULTS

Agarose-formaldehyde gel analysis of the RNAs extracted from *E. coli* and the Octopus Spring cyanobacterial mat showed the expected pattern (23S, 16S, and 5S rRNAs) (Fig. 2). The RNA extracted from the Octopus Spring mat consistently showed an additional band between the 23S and 16S rRNAs, possibly a degradation product of 23S rRNA. The 23S band appeared relatively weak in comparison with 16S and 5S bands. As expected, 5S rRNA was absent from the salt-precipitated *E. coli* RNA. First-strand cDNA synthesis from 16S rRNA resulted mainly in full-length (i.e., 1,400-base-pair-long) cDNA (Fig. 3). Second-strand synthesis (Fig. 4) produced full-length double-stranded cDNA but resulted also in shorter 16S rcDNA molecules (especially for Octopus Spring 16S rcDNA). Oligo(dC) tailing of both ends of the purified 16S rcDNA molecules could readily be achieved, as demonstrated by the shift to larger sizes of both ends of the *E. coli* double-stranded 16S rcDNA digested with the restriction endonuclease *Cfo*I (Fig. 5). The lengths of oligo(dC) tails were a function of the time of incubation in the presence of terminal transferase. Transformation with 5 ng of annealed DNA (plasmid plus 16S rcDNA) resulted in about 5×10^4 and 1×10^5 recombinant cells per μg of DNA for *E. coli* and Octopus Spring mat 16S rcDNAs, respectively. Of the recombinant plasmids analyzed so far (24 from the *E. coli* and 66 from the Octopus Spring mat 16S rcDNA library), 100% contained 16S rRNA-derived inserts, as illustrated by hybridization with labeled 16S rRNA fragments (Fig. 6). Uncut supercoiled as well as partially digested

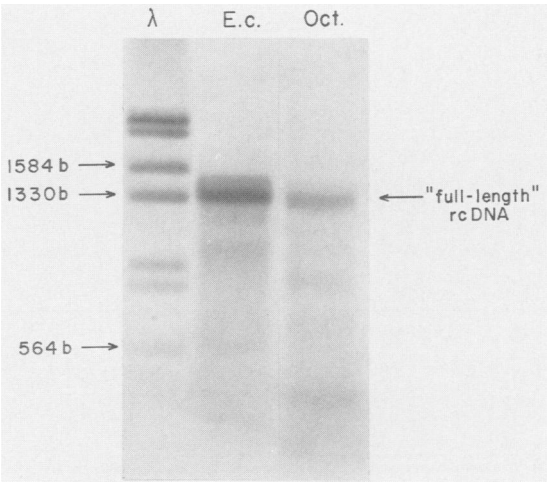

FIG. 3. First-strand 16S rcDNA reaction products for *E. coli* (E.c.) and Octopus Spring mat (Oct.) separated on a 1.5% alkaline agarose gel, blotted to nitrocellulose, and probed with biotinylated 16S rRNA fragments from *E. coli* and *T. acidophilum*. λ, Biotinylated lambda *Hin*dIII-*Eco*RI digest size markers. b, Bases.

forms of the recombinant plasmids also gave a positive hybridization response. Both libraries contain recombinant plasmids with inserted 16S rcDNA of a variety of molecular weights, as expected from the size distribution of the second-strand reaction products.

DISCUSSION

Selective recovery of 16S rRNA sequences as 16S rcDNA has several advantages over methods previously proposed (16, 18) for applying molecular techniques to questions of microbial ecology. The suggested shotgun cloning of natural DNA results in a huge library, so that a detailed community analysis will require the screening of several hundred thousand recombinants to obtain a reasonable number of 16S rDNA-bearing recombinants. A search for 16S rDNA from

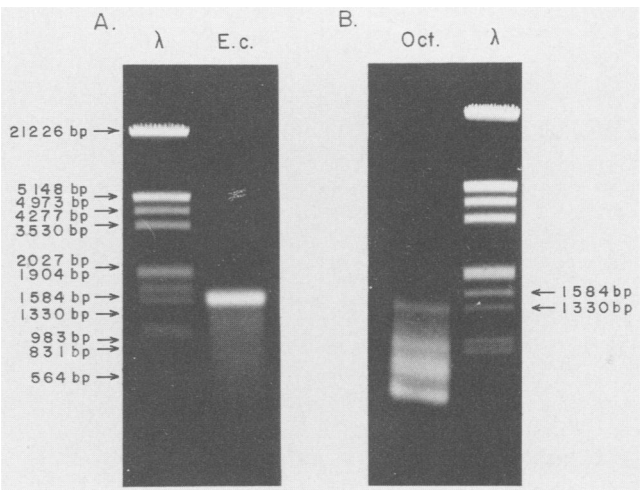

FIG. 4. Second-strand 16S rcDNA reaction products for *E. coli* (E.c.) (A) and Octopus Spring mat (Oct.) (B) analyzed on a 0.8% agarose gel. λ, Lambda *Hin*dIII-*Eco*RI digest size markers. bp, Base pairs.

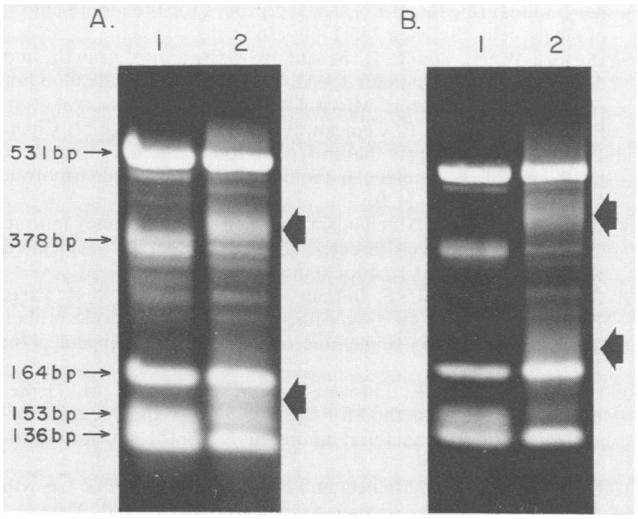

FIG. 5. Oligo(dC)-tailed (lanes 2) and untailed (lanes 1) *Cfo*I-digested *E. coli* 16S rcDNA separated on a 3% agarose gel. The addition of deoxycytidine is visualized by the size increase of *Cfo*I restriction fragments from both ends of the 16S rcDNA molecules. Arrows indicate the positions of the 153- and 378-base-pair fragments after tailing. Note that increase in fragment length is less in the 4-min (A) than in the 10-min (B) treatment with terminal transferase. bp, Base pairs.

community members of low abundance seems impossible. A few recombinants with 16S rDNA inserts have been found in a shotgun-cloned community DNA library derived from the Octopus Spring source pool community (16). Fuhrman et al. (7) reported the construction of a library obtained by shotgun cloning DNA from a natural community but did not report the isolation of 16S rDNA-bearing recombinants. We also succeeded in preparing such a library from a hot-spring microbial mat, but 16S rDNA-bearing recombinants were too rare to be found by hybridization screening. Selective retrieval of 16S rRNA sequence information reduces the problem of molecular analysis of communities to analysis of only the relevant clones.

How completely molecular methods describe natural communities will depend on the efficiency with which nucleic acids can be extracted. Shotgun cloning of DNA fragments requires high-molecular-weight DNA (10,000 to 20,000 kilobase pairs) which can be cut with restriction enzymes. This prohibits the application of cell disruption techniques which shear DNA. The smallness of the 16S-like rRNA (ca. 1,500 to 1,600 bases), its secondary structure, and its association with ribosomal proteins should permit the use of lysis procedures like French pressure cell or bead beater treatment and sonication. These more rigorous cell disruption methods should minimize a bias towards the recovery of nucleic acids from only the more readily lysed community members.

Methods which target rRNAs (e.g., oligonucleotide probing methods and our rcDNA method), rather than the genes encoding them, have the potential to describe a microbial community quantitatively (20; Ward, in press). By investigating the abundance of an essential ribosome component, one measures the protein synthetic capacity of cells with a unique rRNA sequence (i.e., each unique community member). This should be a function of both the number of cells of that type and their physiological status. In our method, one 16S rcDNA molecule per 16S rRNA molecule should result, since the RNase H activity associated with the reverse transcriptase destroys the template molecules (8) upon cDNA synthesis. Thus the relative proportions of organism-specific 16S rRNA molecules should be preserved in the 16S rcDNA library. If it is generally true, as has been shown for *E. coli* (5, 11), that ribosome content is a function of growth rate, the rRNA sequence of the most actively growing members of the community should be represented more often in the library than the sequences of less active or dormant community members. An rRNA gene-dependent method, like shotgun cloning, cannot provide this information, since rRNA gene copy numbers are highly variable among species (16) and are independent of the physiological status of the organisms. As has been pointed out (Ward, in press), more investigation of the regulation of ribosome synthesis in different species and of the fate of rRNA in dormant and dead cells is needed before we will completely understand what these molecular methods tell us about quantitative aspects of natural microbial communities.

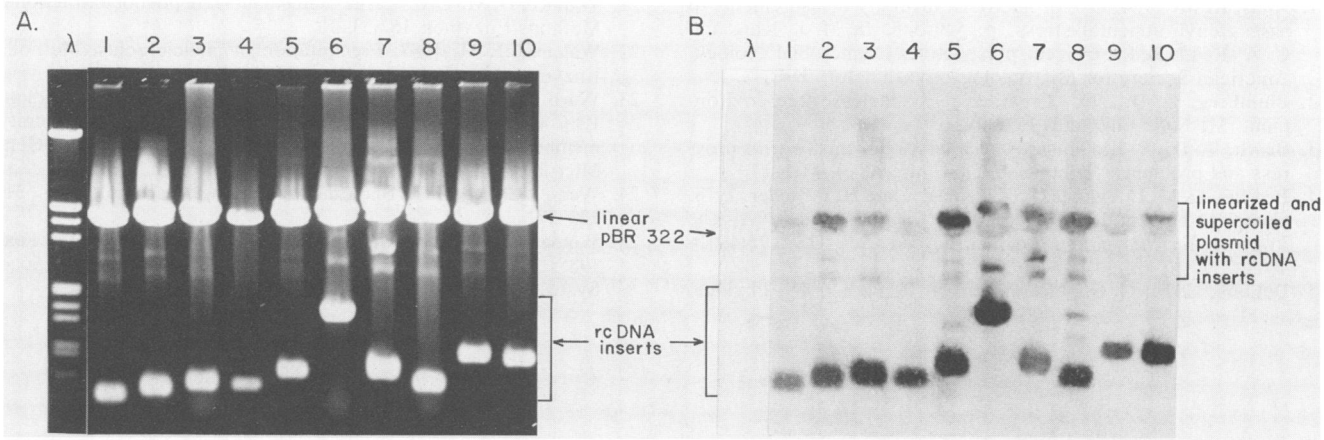

FIG. 6. (A) Analysis of recombinant plasmids from the Octopus Spring mat 16S rcDNA library on a 0.7% agarose gel. Isolated recombinant plasmids were digested with the restriction endonuclease *Pst*I to excise the inserted DNA. (B) The gel was Southern blotted and probed with biotinylated 16S rRNA fragments from *E. coli* and *T. acidophilum*. The sizes of the inserts range from about 400 to 1,400 base pairs and are without exception homologous to 16S rRNA. λ, Lambda *Hin*dIII-*Eco*RI digest size markers.

Minor modifications of the rcDNA method could be used to prepare rcDNA libraries suitable for studying a selected group of microorganisms from a natural environment. This selection would be accomplished with a primer for cDNA synthesis complementing a region which is conserved within a single phylogenetically coherent group (9, 15, 25), rather than universally conserved. The method also provides an easy way to obtain 16S rRNA sequence information for phylogenetic studies of organisms which cannot be grown in axenic cultures.

Preliminary analysis of the Octopus Spring 16S rcDNA library has shown that it contains a diversity of sequences (D. M. Ward, R. Weller, and M. M. Bateson, manuscript submitted for publication). Recombinant plasmids can be sequenced with primers complementary to other universally conserved regions in the 16S rRNA molecule (and their complements to the sequence in the reverse direction) as well as with primers complementing regions of the pBR322 plasmid adjacent to the cloning site. The fidelity of the sequencing is high (e.g., 99% homology between *E. coli* 16S rcDNA and a published *E. coli* sequence [4]). First comparisons of natural sequences with those of Octopus Spring isolates (e.g., M. M. Bateson, J. Wiegel, and D. M. Ward, Syst. Appl. Microbiol., in press) have already revealed the presence of organisms that have never been isolated from this well-studied community (Ward et al., submitted). The sequence data themselves can lead to phylogenetic analysis (24) and the design of oligonucleotide hybridization probes (9, 20) to study the morphology and distribution of organisms contributing such sequences to the community. Independent of inherently biasing culture methods, this and other molecular techniques will allow a more thorough understanding of the structure of many microbial communities.

ACKNOWLEDGMENTS

We are grateful to Walter Hill for providing us with the oligonucleotide primer. We thank Mary Bateson, Cliff Bond, and Linda Mann for many fruitful discussions about experimental details. We also thank the U.S. National Park Service for granting permission to work in Yellowstone National Park.

This work was supported by grant BSR-8506602 from the National Science Foundation.

LITERATURE CITED

1. **Atlas, R. M.** 1984. Use of microbial diversity measurements to assess environmental stress, p. 540–545. *In* M. J. Klug and C. A. Reddy (ed.), Current perspectives in microbial ecology. American Society for Microbiology, Washington, D.C.
2. **Blumberg, D. D.** 1987. Creating a ribonuclease-free environment. Methods Enzymol. **152**:20–24.
3. **Brock, T. D.** 1987. The study of microorganisms *in situ*: progress and problems. Symp. Soc. Gen. Microbiol. **41**:1–17.
4. **Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75**:4801–4805.
5. **DeLong, E. F., G. S. Wickha, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243**:1360–1363.
6. **Doemel, W. N., and T. D. Brock.** 1977. Structure, growth, and decomposition of laminated algal-bacterial mats in alkaline hot springs. Appl. Environ. Microbiol. **34**:433–452.
7. **Fuhrman, J. A., D. E. Comeau, Å. Hagström, and A. M. Chan.** 1988. Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. Appl. Environ. Microbiol. **54**:1426–1429.
8. **Gerard, G. F.** 1981. Mechanism of action of the Moloney murine leukemia virus RNA-directed DNA polymerase associated RNase H (RNase H I). Biochemistry **20**:256–265.
9. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170**: 720–726.
10. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54**:703–711.
11. **Ingraham, L. J., O. Maaløe, and F. C. Neidhardt.** 1983. Growth of the bacterial cell. Sinauer Associates, Sunderland, Mass.
12. **Loomis, W. D., and J. Battaile.** 1966. Plant phenolic compounds and the isolation of plant enzymes. Phytochemistry **5**:423–438.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Marmur, J.** 1963. A procedure for the isolation of deoxyribonucleic acid from microorganisms. Methods Enzymol. **6**:726–738.
15. **McGill, T. J., J. M. Sobieski, M. H. Pickett, C. R. Woese, and G. E. Fox.** 1986. Characteristic archaebacterial 16S rRNA oligonucleotides. Syst. Appl. Microbiol. **7**:194–197.
16. **Olsen, G. J., D. J. Lane, S. J. Giovannoni, and N. R. Pace.** 1986. Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. **40**:337–365.
17. **Pace, B., E. A. Matthews, K. D. Johnson, C. R. Cantor, and N. R. Pace.** 1982. Conserved 5S rRNA complement to tRNA is not required for protein synthesis. Proc. Natl. Acad. Sci. USA **79**:36–40.
18. **Pace, N. R., D. A. Stahl, D. J. Lane, and G. J. Olsen.** 1986. The analysis of natural microbial populations by ribosomal RNA sequences. Adv. Microb. Ecol. **9**:1–55.
19. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
20. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54**:1079–1084.
21. **Wallace, D. M.** 1987. Large- and small-scale phenol extractions. Methods Enzymol. **152**:33–41.
22. **Wallace, D. M.** 1987. Precipitation of nucleic acids. Methods Enzymol. **152**:41–48.
23. **Ward, D. M., T. A. Tayne, K. L. Anderson, and M. M. Bateson.** 1986. Community structure and interactions among community members in hot spring cyanobacterial mats. Symp. Soc. Gen. Microbiol. **41**:179–210.
24. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**: 221–271.
25. **Woese, C. R., E. Stackebrandt, T. J. Macke, and G. E. Fox.** 1985. A phylogenetic definition of the major eubacterial taxa. Syst. Appl. Microbiol. **6**:143–151.