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16S rRNA Sequences of Uncultivated Hot Spring Cyanobacterial Mat Inhabitants Retrieved as Randomly Primed cDNA

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Cloning and analysis of cDNAs synthesized from rRNAs is one approach to assess the species composition of natural microbial communities. In some earlier attempts to synthesize cDNA from 16S rRNA (16S rcDNA) from the Octopus Spring cyanobacterial mat, a dominance of short 16S rcDNAs was observed, which appear to have originated only from certain organisms. Priming of cDNA synthesis from small ribosomal subunit RNA with random deoxyhexanucleotides can retrieve longer sequences, more suitable for phylogenetic analysis. Here we report the retrieval of 16S rRNA sequences from three formerly uncultured community members. One sequence type, which was retrieved three times from a total of five sequences analyzed, can be placed in the cyanobacterial phylum. A second sequence type is related to 16S rRNAs from green nonsulfur bacteria. The third sequence type may represent a novel phylogenetic type.

The use of rRNA sequences for the classification of microorganisms is by now well established (20, 30), and several methods for the retrieval of 16S rRNA sequence information from complex microbial communities have been developed (10, 22, 29). For microbial ecologists, the main advantage of a direct molecular analysis of the community is the circumvention of biasing culture-dependent approaches. As we have demonstrated previously, a well-studied hot spring cyanobacterial mat community contained the 16S rRNA sequences of many eubacteria which have never been cultured from this habitat (25, 26). If 16S rRNA-based methods are to provide an accurate accounting of the members of natural microbial communities, care must be taken to avoid the introduction of new biases. Examples of possible sources of bias are the incomplete lysis of community members before the extraction of nucleic acids or the selectivity of priming strategies used in either cDNA synthesis from 16S rRNA (29) or amplification of 16S rRNA genes by means of the polymerase chain reaction (10). We have recently developed an approach involving the cloning of cDNA prepared from community 16S rRNA, by priming from a region of the 16S rRNA molecule that is believed to be universally conserved (25, 26, 29). Early applications of this method often resulted in the synthesis of cDNAs of 500 bases or less, apparently due to premature termination of reverse transcription from some 16S rRNA molecules. For the sake of statistically valid phylogenetic analyses of the 16S rcDNAs, the retrieval of longer sequence stretches is preferable. We report a modification of the 16S rcDNA cloning approach which can result in the retrieval of longer cDNAs from those 16S rRNA molecules which block the reverse transcriptase reaction. The new approach involves priming with random hexanucleotides to enable the initiation of cDNA synthesis anywhere along the 16S rRNA molecule and, most importantly, downstream from possible reverse transcriptase termination sites. To preserve the selectivity of the cloning procedure for the recovery of only 16S rRNA sequences, ribosomes are isolated from the natural community and the rRNA is extracted from the small subunit (SSU) of the ribosome. Random priming also eliminates the dependency of the 16S rcDNA method on the presence of a universally conserved region in the 16S rRNA molecule. Reliance on such a specific priming site could bias the recovery of SSU rRNA sequences against those which might have evolved a different nucleotide composition in that region (5). The new method was applied to recover naturally occurring 16S rRNA sequences from the cyanobacterial mat located in Octopus Spring, Yellowstone National Park, which we have extensively studied as a simple and stable model system for understanding microbial community ecology.

MATERIALS AND METHODS

Sources of rRNA. Octopus Spring cyanobacterial mat samples (50 to 55°C; top 4 mm of the shoulder region, Yellowstone National Park, Wyo. [7]) were frozen in liquid nitrogen, placed on dry ice, transported to the laboratory, and stored at −70°C. An 80-cm^3 sample was thawed out, ground with a mortar and pestle in buffer I (20 mM Tris [pH 7.4], 150 mM KCl, 15 mM $MgCl_2$, 2 mM dithiothreitol), and subjected to two cycles through a French press minicell at 20,000 lb/in^2 before isolation of ribosomes (see below). Total cellular RNA from *Escherichia coli* Q 358 was obtained by standard methods (16, 17) after lysis of cells with lysozyme (Sigma Chemical Co., St. Louis, Mo.). *E. coli* MRE 600 SSU rRNA was prepared as described by Tapprich and Hill (23). A cell pellet of a pure culture of *Thermoplasma acidophilum* was kindly provided by Tom Langworthy (University of South Dakota). The cells were lysed by resuspension in neutral pH buffer (20 mM Tris [pH 7.8], 25 mM $MgCl_2$, 40 mM NaCl, 0.1 mM EDTA, 0.01% Triton X-100), and the RNA was extracted by standard protocols.

Isolation of Octopus Spring mat ribosomes and SSU RNA. Ribosomes were prepared by a modification of standard methods (23). After cell debris was removed by centrifugation (48,000 × *g*, 1 h), the supernatant was layered over a 40% sucrose cushion (wt/wt, in buffer I) and spun for 2.2 h at

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370,000 × *g* to separate phycobilisomes and other materials from the ribosomes, which were collected in the sucrose cushion. The resuspended pellet and the sucrose layer were diluted with buffer I, reloaded onto a 40% sucrose cushion, and centrifuged at 370,000 × *g* for 17 h to pellet the ribosomes. The pellet was resuspended in buffer II (20 mM Tris [pH 7.4], 100 mM KCl, 1.5 mM $MgCl_2$) to dissociate SSU and large ribosomal subunits (LSU), and the subunits were separated on a 5 to 30% sucrose gradient (wt/wt, in buffer II) at 250,000 × *g* for 2.5 h. The RNA was extracted from pooled fractions containing SSU or LSU (as judged by A_{260}) with phenol-chloroform and purified on a column (Qiagen, Inc., Studio City, Calif.).

Elution of *T. acidophilum* 16S rRNA from urea-agarose gels. About 100 μg of *T. acidophilum* RNA was separated on a 1% agarose gel containing 6 M urea and 0.025 M sodium citrate buffer (pH 3.5). The RNA bands were visualized by UV shadowing (16), and the 16S rRNA band was excised. The gel piece was melted completely at 80°C, the agarose was diluted to less than 0.1% with buffer A (50 mM 3-[*N*-morpholino]propanesulfonic acid [MOPS], 400 mM NaCl) preheated to 80°C, and the mixture was cooled on ice for several minutes. Resolidified agarose was collected by centrifugation, and the supernatant was concentrated and purified by using a Qiagen column.

cDNA synthesis. For random priming of cDNA synthesis, 4 to 5 μg of purified SSU RNA was mixed with 20 to 25 μg of deoxyhexanucleotides (Pharmacia-LKB Biotechnology Inc., Piscataway, N.J.) in a total of 16 μl of water. The mixture was heated to 95°C for 2 min and then transferred to a 37°C water bath. A premixed reaction cocktail with the remaining components of a cDNA kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was added, and the reaction was allowed to proceed for 45 min. cDNA synthesis with the specific primer complementary to *E. coli* positions 1392 to 1406 (termed primer C), second-strand synthesis, purification of 16S rcDNA, and tailing were as described before (29). For the analysis of cDNA products on alkaline agarose gels (16), 4 μCi of ^{35}S-labeled [α-thio]dATP (Dupont, NEN Research Products, Boston, Mass.) was included in parallel reactions.

Cloning of 16S rcDNAs. All procedures were essentially as described before (29), except for the following modifications. The 16S rcDNA products were size fractionated on a Sephacryl S-400 column (10 by 0.6 cm) equilibrated with STE buffer (16). The fractions containing the longest cDNA products were pooled and concentrated by ethanol precipitation. The cloning vector pGEM −3Zf(+) (Promega Biotec, Madison, Wis.) was cut with the restriction enzyme *Pst*I, and a guanosine homopolymer tail was added (29). The transformed cells were plated onto solid LB medium supplemented with 50 μg of ampicillin (Sigma) per ml as well as Bluo-Gal (Bethesda Research Laboratories) and isopropylthio-β-galactoside (Bethesda Research Laboratories) (16). Well-isolated colonies were grown in 150 ml of LB broth containing ampicillin, and plasmid DNA was isolated and purified with a Qiagen Plasmid Kit.

Sequencing. Cloned 16S rcDNAs were sequenced by the protocol supplied with the enzyme Sequenase (United States Biochemical Corp., Cleveland, Ohio), using oligonucleotide primers complementary to highly conserved regions in the 16S rRNA sequence (9, 14). Sequencing gels were run and processed as previously described (3). Sequence data are available from GenBank under the accession numbers M62774 to M62776; aligned sequences may be obtained from the authors upon request.

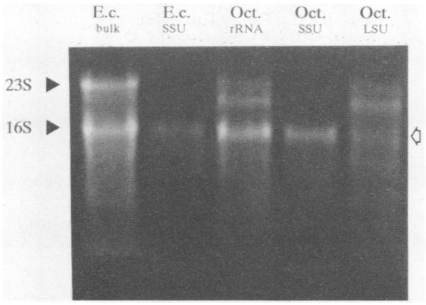

FIG. 1. rRNAs extracted from *E. coli* (E.c.) and the Octopus Spring cyanobacterial mat (Oct.). The rRNAs isolated from intact ribosomes (rRNA) or from SSU or LSU were separated on a 1.4% agarose-formaldehyde gel. Total cellular RNA (bulk) and SSU from *E. coli* serve as size markers. The arrow points at the presence of a 16S-sized fragment in LSU rRNA from Octopus Spring.

Comparative sequence analysis. The sequences were aligned manually to the *E. coli* sequence for the best fit of primary and secondary structural elements. The sequences were compared as described previously (2). For all phylogenetic analyses, a restricted sequence comparison, applying a universal mask (19), was used to block out regions of uncertain alignment in the 16S rRNA sequences. To correct for the possible effects of thermophilic convergence, positions within the universal mask which showed variability in more than 50% of the eubacterial sequences were excluded from the sequence comparisons in one analysis (see below) (27).

RESULTS

SSU rRNA extracted from the Octopus Spring cyanobacterial mat was of the expected size (Fig. 1). However, the rRNA from the LSU migrated in several bands, indicating partial degradation or the possible presence of organisms with posttranscriptionally processed LSU rRNAs (8, 18). Due to a 16S-sized 23S rRNA product (arrow in Fig. 1), the 16S-sized band in the rRNA from whole ribosomes is composed of a mixture of 16S rRNA and 16S-sized 23S rRNA fragments.

Specific priming of cDNA synthesis from *E. coli* SSU rRNA resulted in mostly full-length 16S rcDNA (i.e., 1,400 bases long) (Fig. 2). However, full-length 16S rcDNAs could not be obtained from SSU rRNA from every organism. For example, cDNA produced upon specific priming of gel-purified 16S rRNA from the archaebacterium *T. acidophilum* seldom reached a length over 400 to 500 bases. Apparently, 16S rcDNA synthesis is terminated at one particular location in the 16S rRNA about 400 to 500 bases from the priming site. The advantage of using random hexanucleotides is readily demonstrated as randomly primed 16S rcDNA synthesis products from *T. acidophilum* reached lengths of at least 900 to 1,000 bases. Specific priming of cDNA synthesis from Octopus Spring mat SSU rRNA resulted in 16S rcDNAs of two sizes. The larger size corresponded to full-length 16S rcDNA, while the smaller size was equivalent to the major product of the specifically primed *T. acidophilum* 16S rcDNA synthesis reaction. Hexamer-primed cDNA synthesis resulted in 16S rcDNAs about 100 to 1,500 bp long. After size fractionation of the hexamer-primed Octopus Spring mat 16S rcDNA reaction products to exclude the smaller 16S rcDNAs (i.e., those which were

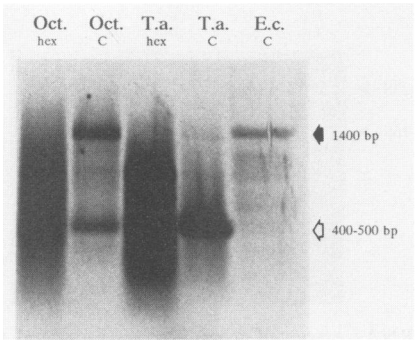

FIG. 2. Autoradiograph of ^{35}S-labeled first-strand 16S rcDNA reaction products, after priming with random hexanucleotides (hex) or with the 1400-region primer (C), from SSU rRNA obtained from the Octopus Spring cyanobacterial mat (Oct.), *T. acidophilum* (T.a.), or *E. coli* (E.c.), separated on a 1.5% alkaline agarose gel. The unshaded arrow points at the 400- to 500-bp fragment. The size estimate of this band is based on the presence of an equivalent band in gels run with lambda size markers (29).

initiated close to the 3′ end and were prematurely terminated), library OS-VI-L was constructed. The 16S rcDNA inserts in this library ranged in size from about 500 to 1,000 bp.

Five 16S rcDNAs were sequenced and further analyzed. All five sequences spanned the 16S rRNA molecule between positions 15 and 20 (*E. coli* position numbers) and 900 and 920 (for example, see Fig. 3). Three sequences were judged to be from the same organism (99.4 to 100% similar in unrestricted analysis of 256 to 434 nucleotides, including data from three variable regions). A single base difference in one sequence may have been due to the incorporation of a nonmatching nucleotide during cDNA synthesis (25). The consensus sequence constructed from these three 16S rcDNAs (847 nucleotides) is designated OS-VI-L-8*. Another sequence was identical (100% similar in unrestricted analysis of 348 nucleotides, including a variable region) to a sequence obtained from another library constructed from the full-length 16S rcDNA products of the specific priming reaction shown in Fig. 2. This longer sequence (960 bases), designated OS-VI-L-11*, was used in phylogenetic analyses to allow a more accurate phylogenetic placement. The sequence data obtained from the fifth 16S rcDNA, designated OS-VI-L-4, covered the entire 16S rcDNA (852 bases). All sequences were readily alignable to both primary and secondary structure features of the *E. coli* 16S rRNA sequence (Fig. 3).

Pairwise comparison of these Octopus Spring cyanobacterial mat sequence types to 16S rRNA sequences from the pure-cultured mat community members (2, 3, 25) revealed that all were from organisms not yet cultured from this community. The highest similarities in restricted analyses with cultured community members were found between OS-VI-L-8* and the cyanobacterium *Synechococcus lividus* (92.0%) and between OS-VI-L-11* and the green nonsulfur bacterium *Chloroflexus aurantiacus* (88.9%). Similarity values relating the 16S rcDNA sequences to representatives of the 10 major eubacterial phyla (30, 31) are given in Table 1. The OS-VI-L-8* sequence shows the highest similarity to the 16S rRNA from the cyanobacterium *Anacystis nidulans*. The OS-VI-L-4 sequence shows high similarity to the 16S rRNA sequences from the proteobacterium *Pseudomonas testosteroni* and the cyanobacterium *A. nidulans*.

In phylogenetic treeing analyses, the OS-VI-L-8* sequence clearly falls into the cyanobacterial phylum (as represented by *A. nidulans* and *S. lividus* sequences in Fig. 4). This placement is supported by the presence of a signature oligonucleotide at position 365 (31) (Fig. 3), found only in cyanobacteria and green nonsulfur bacteria, as well as by a truncated helix between positions 184 and 193 (*E. coli* numbering) which occurs rarely among eubacteria but is found in cyanobacterial sequences (30). The OS-VI-L-11* sequence is placed in the green nonsulfur phylum, as represented by the *C. aurantiacus* sequence. This placement is strengthened by the presence of oligonucleotides diagnostic for green nonsulfur bacteria at positions 910 and 1225. The phylogenetic affiliation of the OS-VI-L-4 sequence is uncertain. Its placement in the tree analysis shown in Fig. 4 between the cyanobacteria and the proteobacteria is consistent with similarity results (Table 1). However, its position in trees is unstable, shifting into the β-subdivision of the proteobacteria (represented by the *P. testosteroni* sequence) when different cyanobacterial sequences are used, when sequence data are restricted to bases common to all sequences compared, or when variable bases which might cause thermophilic convergence are eliminated. Although the sequence has a short helix between positions 184 and 193, characteristic of both cyanobacteria and β-subdivision proteobacteria, it lacks diagnostic features of the β-subdivision (oligonucleotides at position 50 and 555; extended helix between positions 198 and 219) and of the cyanobacteria (oligonucleotides at position 365 and 795).

DISCUSSION

We previously reported the retrieval of 16S rRNA sequences from eight uncultured organisms from the Octopus Spring cyanobacterial mat (26). One of the more interesting findings was the multiple recovery of one sequence type (type A) which, on the basis of similarity values, seemed related to the cyanobacteria. Unfortunately, all retrieved sequences were short (118 to 588 bp) and did not lend themselves to more detailed phylogenetic analyses. Especially for the type A sequence, it is noteworthy that all 16S rcDNAs recovered ended upstream of positions 966 and 967, adjacent methylated bases in the *E. coli* 16S rRNA. This modified 16S rRNA site is under functional constraints (it might be involved in the termination of translation [22a]). Thus, it is not surprising that other organisms have evolved modifications of these positions which lead to the premature termination of reverse transcription (13). In 16S rcDNA libraries prepared by priming upstream from such a modification (for example, priming at the universally conserved 1400 region), organisms which feature strong termination sites might be represented by only short (400- to 500-bp) 16S rcDNAs. That this is a legitimate concern is demonstrated in Fig. 2. While cDNA produced from *E. coli* 16S rRNA primed with the 1400-region primer reached ca. 1,400 nucleotides in length, cDNA primed from *T. acidophilum* 16S rRNA seldom exceeded 400 to 500 bases.

We observed two dominant sizes of cDNA products primed from SSU rRNA from a natural community containing many different organisms (Fig. 2). These could be interpreted to represent 16S rcDNAs from organisms of two categories. Long 16S rcDNA might be synthesized from some community members lacking modifications which lead to termination of reverse transcription, while short 16S rcDNAs might originate from other organisms with impeding base modifications. The hexamer priming approach can help

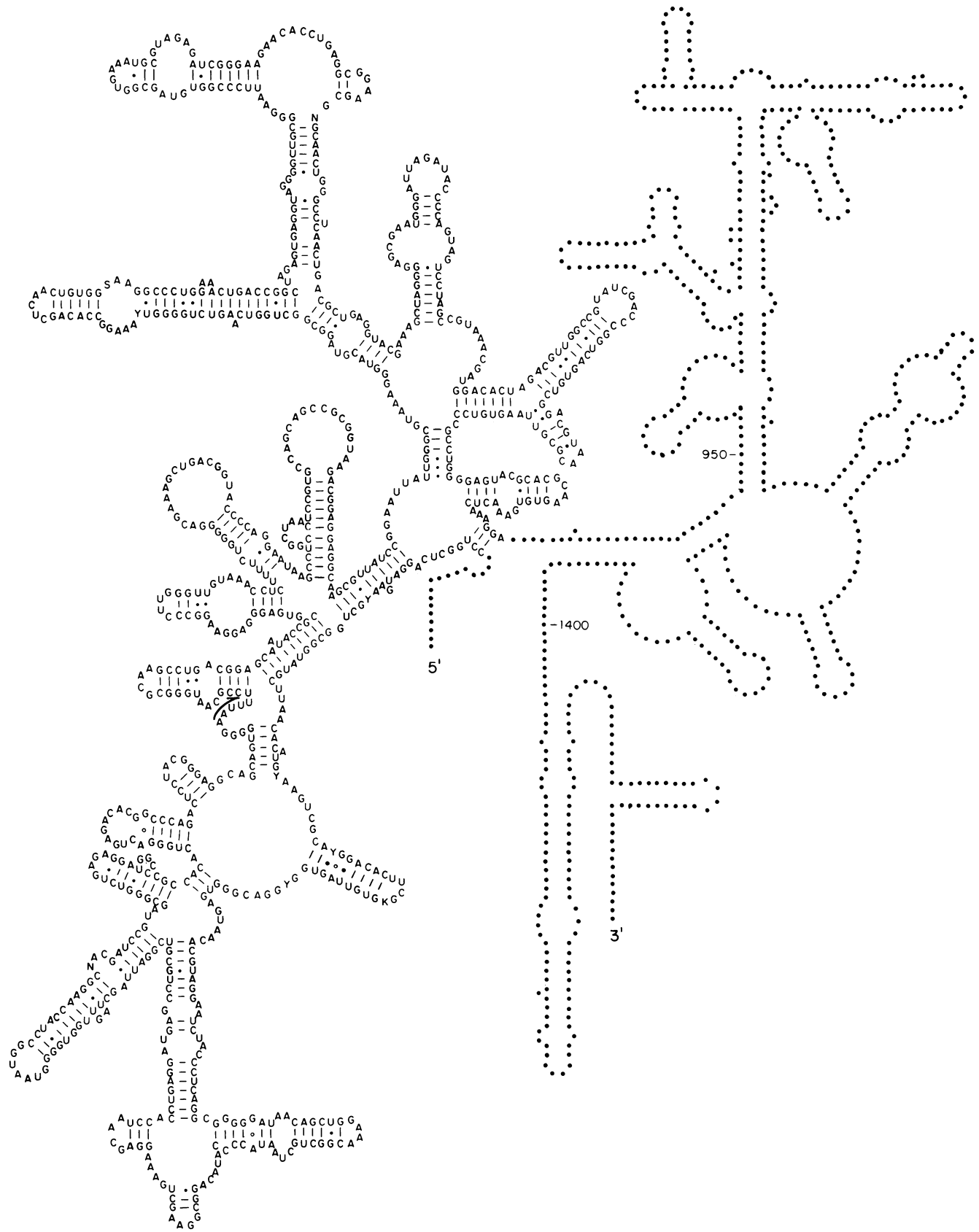

FIG. 3. Secondary structure of the partial 16S rcDNA consensus sequence OS-VI-L-8* superimposed over a proposed secondary structure for the *E. coli* 16S rRNA sequence (12). —, Watson-Crick basepairing; ● and ○, non-Watson-Crick basepairing. Dots between the 3′ end and sequence data indicate regions of sequence not present in the recovered 16S rcDNA. An oligonucleotide characteristic of cyanobacteria is highlighted by the line adjacent to the sequence; positions 950 and 1400 in the *E. coli* sequence are indicated for reference.

TABLE 1. Similarity between 16S rcDNA sequences retrieved from the Octopus Spring cyanobacterial mat community and 16S rRNA sequences from representatives of major bacterial groups[a]

16S rRNA from representative phyla	Percent restricted similarity with 16S rcDNA		
	OS-VI-L-8*	OS-VI-L-11*	OS-VI-L-4
A. nidulans (cyanobacterium)	92.7	81.6	84.9
C. aurantiacus (green nonsulfur bacterium)	81.9	88.9	81.3
C. vibrioforme (green sulfur bacterium)	82.9	83.1	81.6
I. pallida (planctomycete bacterium)	83.4	81.3	77.9
B. subtilis (gram-positive bacterium)	84.5	80.7	82.8
P. testosteroni (proteobacterium)	83.6	81.4	88.1
C. psittaci (chlamydiae)	78.7	78.2	78.0
S. halophila (spirochete)	81.7	80.4	83.1
F. heparinum (flavobacteria/ bacteroides)	79.1	78.3	81.2
D. radiodurans (deinococcus/ thermus)	81.1	83.9	81.8
T. maritima (thermotoga)	82.1	80.6	83.6
M. formicicum (archaebacterium)	70.6	68.3	70.0

[a] The sources of the 16S rRNA sequences are as follows: *Anacystis nidulans* (24), *Chloroflexus aurantiacus* (21), *Isosphaera pallida* (25), *Bacillus subtilis* (11), *Pseudomonas testosteroni* (32), *Spirochaeta halophila* (kindly provided by C. R. Woese, University of Illinois, Urbana), *Flavobacterium heparinum* (28), *Thermotoga maritima* (1), and *Methanobacterium formicicum* (15). The sequences for *Deinococcus radiodurans*, *Chlamydia psittaci*, and *Chlorobium vibrioforme* are unpublished (26a). The sequence comparison was restricted by the use of a universal mask, to exclude regions where alignment is uncertain. Typical interkingdom similarities within the compared regions range from 64.3 to 72% and typical interphylum similarities range between 76 and 89.3%, as calculated from sequences of these pure cultured organisms analyzed with the same mask.

to retrieve longer sequences from such organisms simply by initiating cDNA synthesis downstream from the modified site (Fig. 2). These hexamer-primed 16S rcDNAs can be long enough to carry out phylogenetic analyses.

Of the five 16S rcDNAs analyzed, three sequences which we considered identical showed a clear affiliation with the cyanobacteria (Fig. 4). One sequence type (OS-VI-L-11*) showed a relationship with the green nonsulfur bacteria. This sequence type is 99.5% similar to sequence type C, which we observed in an earlier 16S rcDNA library (26). A fifth sequence (OS-VI-L-4) may either be distantly related to representatives of the proteobacteria or the cyanobacteria or may represent a novel phylogeny. None of the retrieved sequence types was identical to 16S rRNA sequences of organisms cultured from the Octopus Spring mat community (2, 3, 25). All 16S rcDNAs spanned only the 5′ portion of the 16S rRNA molecule (from about *E. coli* position 920 to position 20), downstream from the suspected modification site. This further supports the argument that cDNA synthesis primed upstream of the 966/967 position might be prematurely terminated.

The frequent recovery of a cyanobacterial sequence type in the OS-VI-L library parallels the observation made earlier on another specifically primed library (26). This leads to the speculation that the OS-VI-L-8* sequence constitutes the 5′ portion of the 16S rRNA of a cyanobacterium in the mat whose 3′ portion (type A sequence) was previously recovered. The replicate occurrence of a cyanobacterial sequence in 16S rcDNA libraries from a cyanobacterial mat is not unexpected. The libraries should reflect the abundance of rRNAs in the natural community. This rRNA abundance is a function of both the numerical abundance of the organisms and their physiological state (for example, growth rate [see references 6 and 29]). A plausible interpretation is that the two cyanobacterial sequence fragments recovered in different libraries originated from a dominant and very active community member. The specific cyanobacterium contributing this sequence is not *S. lividus*, the organism suspected to dominate this mat community (7). However, the presence of *S. lividus* was only inferred from the microscopic observation of a red-autofluorescing (chlorophyll *a*-containing) organism with a morphology resembling this cyanobacterial species. A culture of the dominating cyanobacterium in the Octopus Spring mat community may not yet have been obtained.

The potential advantages of the hexamer priming strategy go beyond the retrieval of longer sequences from organisms with modified nucleotides. The priming from defined universally conserved regions of the molecule can retrieve only such sequences which possess the complements to these primers. The consensus sequences which made possible the recognition of universally conserved 16S rRNA regions have been established from a rather limited set of sequences from organisms in pure culture. Some more exotic organisms, which so far may have escaped cultivation, might show deviations from these consensus sequences. An extreme example is the mitochondrial SSU rRNA from *Leishmania*

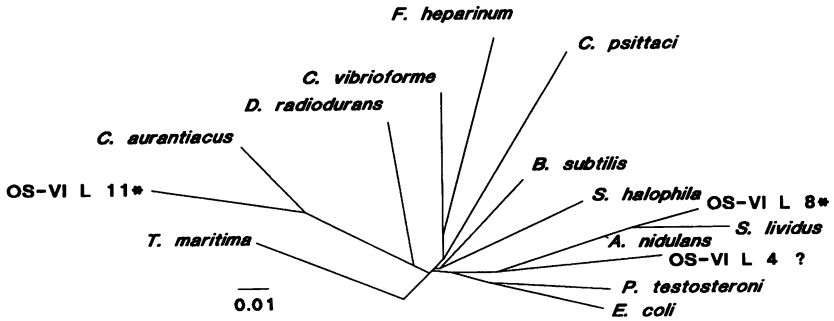

FIG. 4. Distance matrix phylogenetic tree showing placement of 16S rcDNA sequence types OS-VI-L-8*, OS-VI-L-11*, and OS-VI-L-4, retrieved from the Octopus Spring cyanobacterial mat. This tree was established by analysis of all nucleotide positions included in the universal mask. The 16S rRNA sequence from *S. lividus* is from reference 25, and the sequence for *E. coli* is from reference 4. The tree was rooted with the sequence from *Methanobacterium formicicum* (15). The scale bar corresponds to 0.01 fixed point mutations per sequence position. ? indicates that the phylogenetic position of the OS-VI-L-4 sequence is uncertain.

tarantolae (5). The "universally conserved" regions of this 16S-like rRNA shows a very high degree of base substitution. Organisms of ancient lineages or organisms living in tight symbiotic relationships might contain unusual SSU rRNAs, which would not be recovered by the use of defined primers.

In conclusion, we improved the general applicability of the cDNA method developed earlier to recover 16S rRNA sequence information (29). It is now possible to recover long 16S rcDNAs from a greater variety of organisms. These sequences are sufficiently long to result in a meaningful classification. Three sequence types retrieved by this method from the Octopus Spring mat were successfully placed in the proposed phylogenetic tree (30). A frequently recovered cyanobacterial sequence, from a formerly unknown organism, might represent the dominant, mat-building primary producer in this community.

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