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Uncultivated Cyanobacteria, *Chloroflexus*-Like Inhabitants, and Spirochete-Like Inhabitants of a Hot Spring Microbial Mat

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Analysis of 16S rRNA sequences retrieved as cDNA (16S rcDNA) from the Octopus Spring cyanobacterial mat has permitted phylogenetic characterization of some uncultivated community members, expanding our knowledge of diversity within this microbial community. Two new cyanobacterial 16S rRNA sequences were discovered, raising to four the number of cyanobacterial sequence types known to occur in the mat. None of the sequences found is that of the cultivated thermophilic cyanobacterium *Synechococcus lividus*. A new 16S rRNA sequence characteristic of green nonsulfur bacteria and their relatives was discovered, raising to two the number of such sequences known to exist in the mat. Both are unique among the 16S rRNA sequences of cultivated members of this group, including an Octopus Spring isolate of *Chloroflexus aurantiacus* and *Heliothrix oregonensis*, whose sequences we report herein. Two spirochete-like 16S rRNA sequences were discovered. One can be placed in the leptospira subdivision of the spirochete group, but the other has such a loose affiliation with the spirochete group that it might actually belong to an as yet unrecognized subdivision or even to a new eubacterial line of descent.

The thermal (50 to 55°C) Octopus Spring cyanobacterial mat is a well-studied model community (3, 6, 25). Several species, including a cyanobacterium, green nonsulfur bacteria, aerobic and anaerobic chemoorganotrophs, a sulfate reducer, and a methanogenic archaebacterium, have been identified as probable community members by the classical culture-dependent approach. Recently, we have begun to analyze the community composition of this mat by using 16S rRNA sequences as biomarkers (24, 26, 27, 33). The initial results of this culture-independent approach confirmed our suspicion that the community harbors many more bacterial species than have been identified by traditional techniques.

In our previous work, the phylogenetic characterization of these uncultivated species has been complicated by the retrieval of relatively short fragments of the 16S rRNA sequence. Here we report the analysis of several long 16S rcDNA fragments, which we have retrieved by preparing a cDNA library by selective priming (32) of RNA obtained from purified small ribosomal subunits (33) and selecting long rcDNA products. The sequence information has permitted the recognition of several previously uncharacterized community members. Analysis of the shorter rcDNA products reveals that size selection can cause a bias against the recovery of certain sequences.

In the course of this work, we have also analyzed the 16S rRNA sequences of a pure culture of the green nonsulfur bacterium *Chloroflexus aurantiacus* (strain Y-400-fl), which was isolated from the Octopus Spring mat (20). In addition, we have obtained a partial 16S rRNA sequence for *Heliothrix oregonensis*, a novel filamentous photosynthetic bacterium, suspected on the basis of its 5S rRNA sequence to be related to *C. aurantiacus* (21). It has so far been possible to grow *H. oregonensis* only in coculture together with the thermophilic chemoorganotroph *Isosphaera pallida* (5), but we were able to retrieve its 16S rRNA sequence by selective cDNA synthesis and cloning (32).

MATERIALS AND METHODS

All procedures for cloning and analysis of 16S rcDNAs were described previously (references 32 and 33 and references cited therein). The rcDNA libraries OS-V-L and OS-V-S were obtained by separately cloning long (ca. 900 to 1,400 bp) and short (less than 900 bp) cDNA fragments, respectively, which resulted from selective priming of cDNA synthesis (32) from small ribosomal subunit rRNA (Fig. 2 of reference 33 shows the two size classes). The cDNA size classes were separated with a Sephacryl S-400 column (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.).

The rRNA from the coculture of *H. oregonensis* and *I. pallida* (kindly provided by S. Giovannoni and R. Castenholz, University of Oregon) was obtained by standard methods (14, 22) after cell lysis with a French press (20,000 lb/in^2). The cDNA synthesis and cloning were done as described before (32). Partial sequence analysis of six clones revealed five sequences identical to the *I. pallida* 16S rRNA sequence, which we had determined previously by the reverse transcriptase method (27). The sixth clone had a different sequence, which we assumed to be that of *H. oregonensis*.

RNA from a pure culture of *C. aurantiacus* Y-400-fl (kindly provided by M. Madigan, Southern Illinois University) was obtained after lysis with a French press (20,000 lb/in^2). The 16S rRNA sequence data were obtained by a modification (2) of the reverse transcriptase method (12).

Deoxyribooligonucleotide hybridization probes which distinguish cyanobacterial sequence types A and B (21a) were labeled with ^{32}P by using polynucleotide kinase and purified on polyacrylamide gels by standard procedures (22). Target plasmid DNA was extracted and purified by an alkaline lysis miniprep method (7) or with a Qiagen plasmid kit (Qiagen Inc.) and then slot-blotted onto Nytran membranes (Du

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TABLE 1. Octopus Spring cyanobacterial mat 16S rcDNA sequences and 16S rRNA sequences of cultivated hot spring phototrophs reported in this study

Organism or sequence designation	Phylogenetic type[a]	GenBank accession no.	Probe reaction[b]		No. of occurrences
			Type A	Type B	
Cultivated species					
C. aurantiacus Y-400-fl	Green nonsulfur	L04674	ND	ND	
H. oregonensis	Green nonsulfur	L04675	ND	ND	
Library OS-V-L					
Type B	Cyanobacterium	M62776	−	+	1[c]
Type I	Cyanobacterium	L04709	−	−	2
Type J	Cyanobacterium	L04710	−	−	1
Type C[d]	"Green nonsulfur"	M62775	ND	ND	1
OS-V-L-20*[e]	"Green nonsulfur"	L04703	ND	ND	3
OS-V-L-7	Spirochete	L04704	ND	ND	1
Type K	Possible spirochete	L04711	ND	ND	1
Library OS-V-S					
Type B	Cyanobacterium	M62776	−	+	4
Type C	"Green nonsulfur"	M62775	−	−	3
Type N	Unknown	L04712	−	−	1

[a] From Woese (34).
[b] ND, not determined; −, no reaction; +, positive reaction.
[c] A second clone was identical to type B but could not be distinguished from type A, as it did not contain sequence data for the variable region where type A and B sequences differ.
[d] Reported as OS-VI-L-11* in reference 33.
[e] The asterisk indicates that this sequence is a consensus for three clones which have identical sequences in overlapping variable regions.

Pont). Hybridization reactions were carried out in 3× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–10× Denhardt's solution–1% sodium dodecyl sulfate (SDS)–500 μg of polyadenosine per ml (22) at 68°C, and membranes were washed in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS as previously described (24) before autoradiograms were exposed.

Nucleotide sequence accession numbers. All sequence data reported in this article have been submitted to GenBank under accession numbers reported in Table 1.

RESULTS

Library OS-V-L and cultures. Library OS-V-L contained seven distinct sequences, as summarized in Table 1. Each of these was aligned to the *Escherichia coli* 16S rRNA sequence (for an example, see Fig. 1). All were analyzed by pairwise comparison and distance matrix tree analysis (Fig. 2) with a set of eubacterial and archaebacterial 16S rRNA sequences representative of the different major subkingdom lines of descent (34).

Three sequences clearly fall into the cyanobacterial line of descent (Table 2 and Fig. 2). One is identical to the previously described sequence type B (26). The new clone extends this sequence type to encompass positions 197 to 1392, permitting us to recognize that another previously described sequence, OS-VI-L-8* (33), is also likely to be a type B sequence. The other two sequences have not been discovered in earlier Octopus Spring rcDNA libraries and are here designated as new sequence types I and J. The three sequence types show molecular details characteristic of cyanobacterial sequences (34, 36). As shown in Fig. 1, for example, the type I sequence exhibits signature oligonucleotide sequences as well as missing or shortened stem-loop structures around *E. coli* positions 73 to 97, 198 to 219, and 455 to 477, which are characteristic of cyanobacterial sequences. Insofar as the sequence data were available, the same was true for sequence types B and J except that in the type B sequence, an A occurs instead of a U at position 799 and a G occurs instead of an A at position 1233. None of the 16S rRNA sequences of cyanobacteria retrieved from the mat was identical to that of the hot spring cyanobacterial isolate *Synechococcus lividus*.

Two sequences are related to those of members of the green nonsulfur eubacterial line of descent (Table 3 and Fig. 2). One is identical to the previously described type C sequence (26, 33). The other, OS-V-L-20*, cannot be assigned as a new sequence type in the mat community, since for this purpose we use sequence data from the 3′ end of the molecule (see references 24 and 26), which were not obtained for any clones of this kind. Molecular details suggest that both the type C (33) and the OS-V-L-20* sequences are characteristic of members of the green nonsulfur bacteria and their relatives (34, 36), with the single exception that in OS-V-L-20*, a G instead of a C was found at position 903. Neither the type C nor the OS-V-L-20* sequence is identical to any sequence of a cultivated member of the phylogenetic group containing green nonsulfur bacteria and their relatives. This includes the Octopus Spring strain of *C. aurantiacus*, which was found to closely resemble the Japanese strain J-10-fl, and *H. oregonensis*, a more distant relative of *C. aurantiacus*.

A more detailed tree analysis was performed to attempt to better resolve the relationships of the type C and OS-V-L-20* sequences with those of green nonsulfur bacteria and their nonphototrophic relatives (Fig. 3). By comparing only members of this group, we could be more certain of alignment in variable regions and thus include more sequence data in the analysis. The OS-V-L-20* sequence formed a cluster with the sequences of the two phototrophs, *C. aurantiacus* J-10-fl and *H. oregonensis*. The type C sequence fell deeper in the tree, between the sequences of the two

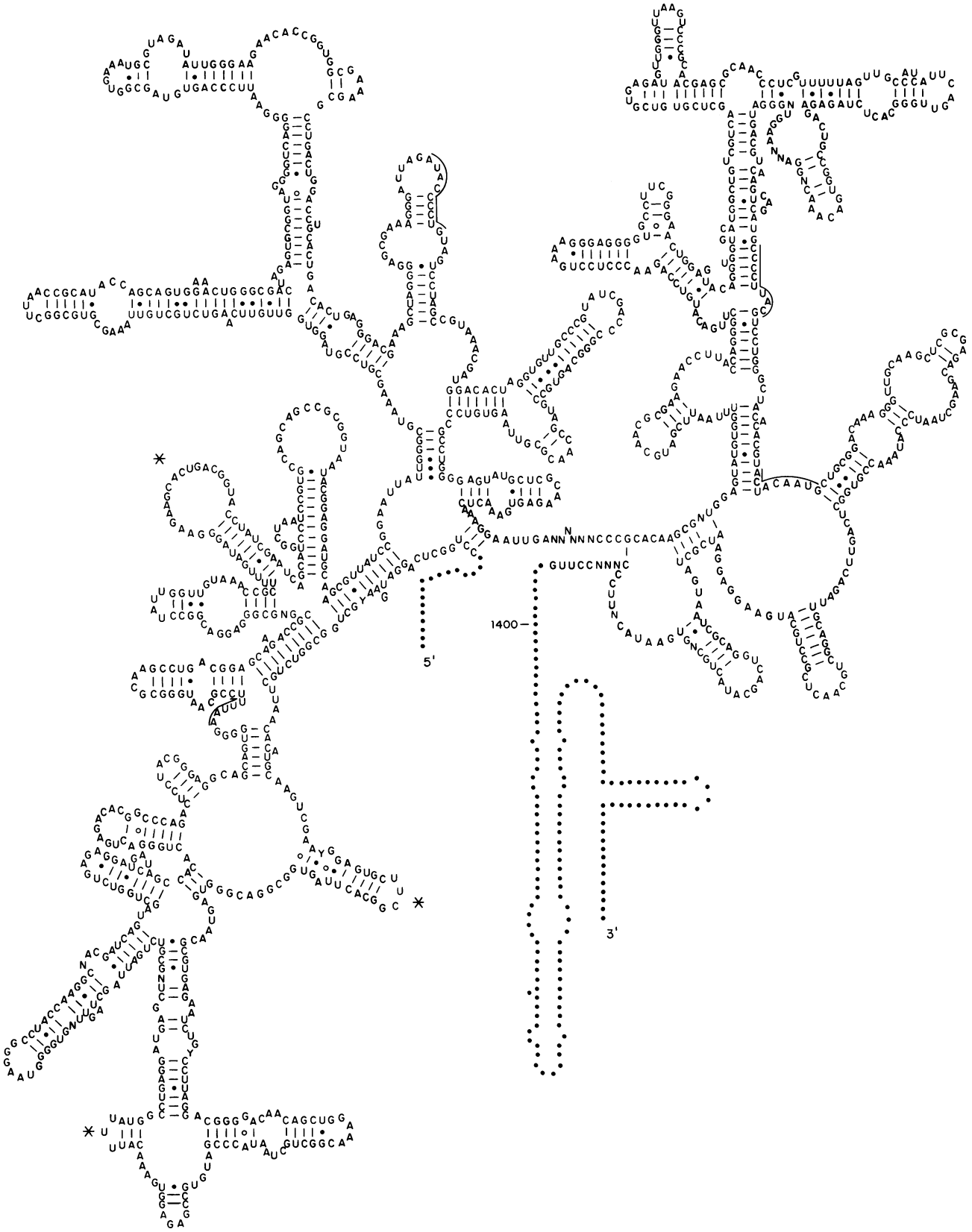

FIG. 1. Possible secondary structure of the Octopus Spring type I 16S rcDNA sequence, which is of cyanobacterial phylogeny. The structure was obtained by superimposing the 16S rcDNA sequence over a secondary structure proposed for the *E. coli* 16S rRNA sequence (11). —, Watson-Crick base-pairing; •, non-Watson-Crick base-pairing. Dots between the 3′ or 5′ end and the sequence data indicate regions of the sequence not present in the 16S rcDNA. Asterisks indicate structural differences between the *E. coli* and cyanobacterial sequences. Portions of sequence highlighted by lines are characteristic cyanobacterial signature sequences.

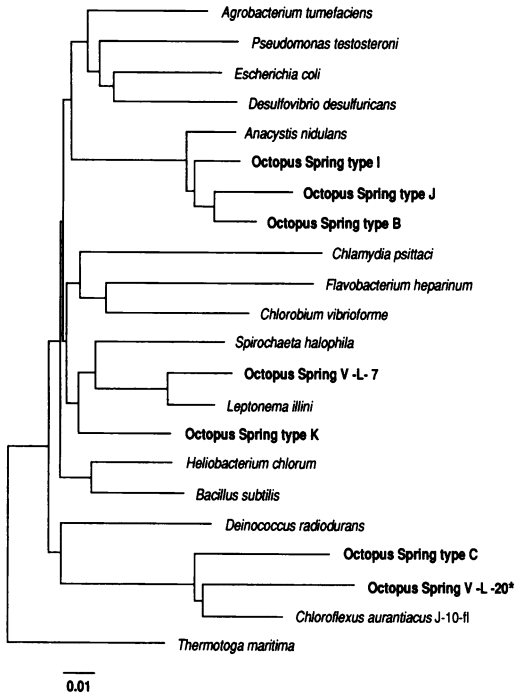

FIG. 2. Distance matrix phylogenetic tree showing the placement of 16S rcDNA sequences retrieved from the Octopus Spring cyanobacterial mat relative to those of representatives of the major eubacterial lines of evolutionary descent. This tree was established by analysis of restricted sequence data (see Table 2, footnote *b*). Published sequences are from references 1, 4, 9, 16–18, 23, 26, 27, 29–31, 35, and 37. The tree was rooted with the 16S rRNA sequence of the archaebacterium *Methanobacterium formicicum* (13). The bar indicates the number of fixed point mutations per nucleotide.

aerobic chemoorganotrophic isolates *Thermomicrobium roseum* and *Herpetosiphon aurantiacus*.

Two 16S rRNA sequences exhibit possible relationships with those of members of the spirochete line of descent (Table 4 and Fig. 2). The OS-V-L-7 sequence shows a strong affiliation with that of *Leptonema illini*. Its molecular signature is in good agreement with that characteristic of the spirochete eubacterial subkingdom line of descent (18, 34, 36) but is not exactly like that reported for leptospiras. Some characteristic features are present (e.g., a C at positions 392, 556, and 812, a U at position 358, a G at position 369, and oligonucleotide sequence near position 365), and some are absent (e.g., no G at position 976 and inappropriate oligonucleotide sequence near position 1210). Because of the lack of data for the 3′ end of this 16S rcDNA, OS-V-L-7 cannot be assigned as a new sequence type in the mat community.

The other spirochete-like sequence has not been reported previously and is here designated Octopus Spring sequence type K. In tree analysis, it is consistently placed at the base of the spirochete subdivision, but pairwise similarity values with sequences of cultivated spirochetes are not obviously greater than those with members of other eubacterial lines of descent (Table 4). Signature analysis also fails to show a definite relationship to the spirochetes, since the diagnostic nucleotides at positions 45, 47, 50, 52, 53, 358 to 361, and 396 are different from those expected (18). It is interesting that 16S rRNA sequence type K does show a close relationship with a short sequence previously retrieved from the Octopus Spring mat, type H (26), which itself shows high similarity with the 16S rRNA sequence of *Spirochaeta halophila* (Table 4).

Library OS-V-S. Eight clones selected from this library and analyzed by partial sequencing and hybridization probing contained inserts which fell between the priming site (*E. coli* positions 1392 to 1406) and positions near a site of frequent chain termination in cDNA synthesis at positions 966 and 967 (Table 1). Four were shown by oligodeoxynucleotide probe analysis to be of type B. Three were identical to sequence type C. One 16S rRNA sequence has not been encountered so far in any other Octopus Spring rcDNA library and is here designated sequence type N. Because of the short length of this sequence, we did not attempt phylogenetic characterization.

TABLE 2. Pairwise similarity among Octopus Spring mat cyanobacterial 16S rcDNA sequence types and 16S rRNA sequences of cyanobacteria

Sequence[a]	% Similarity (restricted/unrestricted[b]) with:						
	Type A	Type B	Type J	Type I	*S. lividus*	*A. nidulans*	*E. coli*
Octopus Spring							
Type A	—	96.9	88.7	89.4			
Type B	98.2	—	90.2	87.6			
Type J	93.7	93.9	—	87.8			
Type I	94.7	93.7	93.7	—			
Synechococcus lividus	90.6	92.0	92.8	94.2	—		
Anacystis nidulans	93.8	93.2	92.7	95.0	95.4	—	
Escherichia coli	88.3	86.2	84.8	86.1	81.5	85.9	—

[a] Published sequence data from references 4, 23, 26, and 27.
[b] Values above the diagonal include all available sequence data; values below the diagonal include nucleotides present in sequences which align with *E. coli* position numbers 6 to 38, 49 to 63, 105 to 122, 240 to 393, 499 to 580, 655 to 751, 761 to 825, 875 to 990, 1046 to 1114, 1157 to 1250, 1287 to 1409, and 1491 to 1534.

TABLE 3. Pairwise similarities among Octopus Spring mat *Chloroflexus*-like 16S rcDNAs and 16S rRNA sequences of *C. aurantiacus* and its relatives

No. and sequence[a]	% Similarity (restricted)[b] with:							
	1	2	3	4	5	6	7	8
1. Octopus Spring type C	—							
2. Octopus Spring OS-V-L-20*	86.6	—						
3. *Chloroflexus aurantiacus* J-10-fl	89.9	89.3	—					
4. *Chloroflexus aurantiacus* Y-400-fl	89.1	87.6	98.8	—				
5. *Heliothrix oregonensis*	87.2	90.1	91.8	90.5	—			
6. *Herpetosiphon aurantiacus*	86.7	85.5	88.1	85.5	89.7	—		
7. *Thermomicrobium roseum*	84.6	86.2	86.8	83.1	84.5	85.0	—	
8. *Escherichia coli*	82.3	82.3	83.8	81.3	81.6	83.1	83.8	—

[a] Published sequences are from references 4 and 16.
[b] See Table 2, footnote *b*.

DISCUSSION

Including the new sequences reported herein, we have so far recovered four 16S rcDNA sequence types which show a

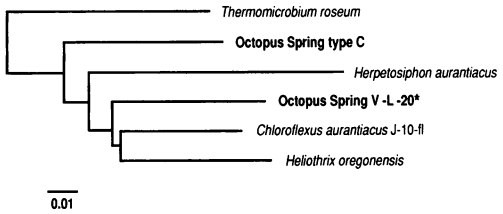

FIG. 3. Distance matrix phylogenetic tree showing the placement of Octopus Spring type C and OS-V-L-20* and *H. oregonensis* 16S rcDNA sequences relative to those of representatives of the eubacterial line of descent, containing *Chloroflexus* spp. and relatives. This tree was established by using nucleotides which align with *E. coli* position numbers 240 to 446, 485 to 489, 491 to 492, 494 to 596, 605 to 633, 642 to 840, 846 to 857, 872 to 901, and 904 to 964. Published sequences are from reference 16. The tree was rooted with the 16S rRNA sequence of *E. coli* (4). The bar indicates the number of fixed point mutations per nucleotide.

clear relationship to the 16S rRNA sequences of cyanobacteria. None of the four sequence types is that of *S. lividus*. The cyanobacteria are known to be a phylogenetically tightly clustered eubacterial group (8). Thus, it seems likely that the four sequence types are contributed by community members which have a cyanobacterial phenotype (i.e., physiology). It is not necessarily the case that each sequence type is contributed by a unique cyanobacterium. However, in all but one case (type A versus type B), the difference between cyanobacterial sequence types exceeds that usually observed between two different small ribosomal subunit rRNA sequences of the same organism (10, 15). In fact, the evolutionary distance among the four cyanobacterial sequences found in the mat is approximately half that observed among all cyanobacterial sequences so far investigated (8). We are thus led to conclude that the diversity within the cyanobacterial population has been underestimated because of its morphologic simplicity. This is not surprising considering current opinions about the shortcomings of morphology for classifying unicellular cyanobacteria (28). Genetically stable "temperature strains" have been cultivated from hot spring microbial mats (19). If such isolates represent the different cyanobacterial 16S rRNA inputs that we have discovered, the designation as strains certainly underestimates the phylogenetic distances among them.

We have retrieved two *Chloroflexus*-like sequences from the mat. Neither is that of *C. aurantiacus* (including an Octopus Spring strain) or *H. oregonensis*, a novel hot spring filamentous phototroph related to *C. aurantiacus*. Inferring physiologic type within this line of descent is obviously more difficult because the green nonsulfur bacteria and their relatives exhibit different physiologies. Does the clustering of the OS-V-L-20* sequence with those of *C. aurantiacus* and *H. oregonensis* indicate that this sequence is contributed by a photosynthetic bacterium? Similarly, does the deeper positioning of the type C sequence between the two nonphotosynthetic members of the group indicate that this sequence originates from a nonphotosynthetic *Chloroflexus* relative? Our present knowledge of the correspondence between phylogenetic and phenotypic relationships is insufficient to allow a conclusion to be drawn.

We have microscopically observed spirochetes in this mat (unpublished observations), but to our knowledge none of these has yet been cultivated. Neither of the two spirochete-like 16S rRNA sequences that we recovered matches the sequence of a thermophilic spirochete which was cultivated from the cyanobacterial mat of Hunter's Hot Spring, Oregon (Table 4) (18). The lack of a relationship between the type K sequence and those of spirochetes is intriguing. It may be the case that a closely related spirochete sequence is simply not present in the data base to demonstrate the relationship. However, the type K sequence also failed to show high homology with any of a larger set of 16S rRNA sequences from cultivated species representative of the major spirochete subdivisions (see footnote *d* of Table 4 and reference 18). Perhaps the type K sequence belongs to a spirochete subdivision which is as yet unknown. It is also possible that the type K sequence is not that of a spirochete and may be of novel phylogeny.

The type B and type C 16S rRNA sequences were more prevalent among the smaller rcDNAs (library OS-V-S) than among the larger rcDNAs (library OS-V-L). Apparently, certain 16S rRNA molecules, such as these, have a greater tendency for early termination of rcDNA synthesis. There appears to be no phylogenetic pattern associated with this phenomenon, since other cyanobacterial or *Chloroflexus*-like sequences were not truncated. Such termination does not appear to be an absolute phenomenon, since long type B and C sequences were recovered from the OS-V-L library. The contrast in the compositions of the two libraries points out that size selection can cause a bias in community analysis. The common occurrence of cyanobacterial 16S rRNA sequences in both libraries is, however, consistent with our previous observations (26, 33). We believe that this is probably a consequence of the fact that cyanobacteria are at the base of the mat food chain (24).

TABLE 4. Pairwise similarities among Octopus Spring spirochete-like 16S rcDNAs and 16S rRNA sequences of cultivated spirochetes

No. and sequence[a]	% Similarity (restricted)[b] with sequence:						
	1	2	3	4	5	6	7
1. Octopus Spring OS-V-L-7	—						
2. Octopus Spring type K	89.2	—					
3. Octopus Spring type H	88.3	93.2	—				
4. Thermophilic spirochete H1[c]	87.5	89.1	93.7	—			
5. *Leptonema illini*	94.3	88.2	91.3	88.7	—		
6. *Spirochaeta halophila*	87.4	89.8[d]	94.8	93.1	89.1	—	
7. *Escherichia coli*	87.3	88.6	86.2	85.8	87.4	84.0	—

[a] Published sequences are from references 4, 18, and 26 or were kindly provided by C. R. Woese and W. G. Weisberg (University of Illinois, Champaign).

[b] See Table 2, footnote *b*.

[c] Cultivated from Hunter's Hot Spring (18).

[d] Percent restricted similarity between Octopus Spring type K and *Borrelia burgdorferi*, *Leptospira* sp. (Turtle strain), *Spirochaeta aurantiacus*, *Treponema bryantii*, *T. phagedenis*, and *Serpula hyodysenteriae* ranged from 84.2 to 88.2% (these sequences were kindly provided by B. Paster, Forsyth Dental Clinic, Boston, Mass.).

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