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Characterization of a Yellowstone Hot Spring Microbial Community by 5S rRNA Sequences

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The microorganisms inhabiting a 91°C hot spring in Yellowstone National Park were characterized by sequencing 5S rRNAs isolated from the mixed, natural microflora without cultivation. By comparisons of these sequences with reference sequences, the phylogenetic relationships of the hot spring organisms to better characterized ones were established. Quantitation of the total 5S-sized rRNAs revealed a complex microbial community of three dominant members, a predominant archaebacterium affiliated with the sulfur-metabolizing (dependent) branch of the archaebacteria, and two eubacteria distantly related to *Thermus* spp. The archaebacterial and the eubacterial 5S rRNAs each constituted about half the examined population.

The description of pure cultures is a foundation of experimental microbiology. It seems possible, however, that much of the biological diversity of the earth has not been, or cannot be, brought into pure culture. We are developing technical strategies for analyzing fundamental aspects of naturally occurring microbial populations without the need for their cultivation. The methods use recent advances in nucleic acid sequencing and recombinant DNA technology to determine nucleotide sequences of 5S or 16S rRNA genes from microbial communities containing multiple organisms. By comparing these sequences with known ones, we can define the phylogenetic status of any organism residing in the communities. Because only the naturally available biomass is required for these methodologies, the analyses project a relatively unbiased picture of an in situ microbial community.

The determination of phylogenetic relationships by quantitative comparison of macromolecular sequences is well established (24). Among biopolymers, the rRNAs are possibly uniquely suited as gauges of phylogenetic relatedness because of their ubiquitous distribution, functional homology, high conservation of primary structure, and apparent freedom from artifacts of lateral transfer (8). The direct isolation and sequencing of the 5S rRNAs from environments of reasonably plentiful biomass and limited complexity offers a relatively convenient determination of the dominant community members. We have used this approach to characterize bacteria symbiotically associated with certain marine invertebrates in sulfide-rich habitats (20), and we now apply it to a Yellowstone hot spring microbial community.

The presence of microbial life in near-boiling waters has long been recognized (2, 18); the hydrothermal systems of Yellowstone National Park have provided a rich area for the study of thermophily. One of the more intensively studied thermal habitats in the park is Octopus Spring (3). Here, most effort has been applied to the cooler effluent waters. Although microbial accumulation in the 91°C source of Octopus Spring is evident, there are no reports of the cultivation of characteristic microorganisms. Indeed, the inhabitants have resisted attempts at cultivation (3), a common theme with natural microbial populations. As detailed in this report, sequence analysis of the naturally available 5S rRNAs establishes the presence of a complex community with three dominant members: two representatives of eubacteria distantly related to *Thermus* spp. and one representative of the archaebacteria.

MATERIALS AND METHODS

Sample collection. To obtain sufficient microbial biomass for analysis, we devised a simple collection device consisting of cotton or glass fiber batting sandwiched between nylon screening. This collector was immersed in the main pool of Octopus Spring for 1 week and then harvested by scissoring into conveniently sized segments and freezing on dry ice.

5S rRNA isolation and sequences. Total low-molecular-weight RNA was isolated directly from the fiber batting. After several freeze-thaw cycles in the presence of sodium dodecyl sulfate, total nucleic acids were extracted by using hot phenol and sodium dodecyl sulfate. In our experience, this method offers good yields of the low-molecular-weight species (5S and 4S). The nucleic acids partition in the aqueous phase and are recovered as ethanol precipitates. The 3′ termini of total nucleic acids were labeled by the RNA ligase-catalyzed appendages of [5′-^{32}P]pCp, and the total, labeled, low-molecular-weight RNA population (5S and 4S) was fractionated on either one- or two-dimensional gels (20). Bands corresponding in approximate size to 5S rRNA were excised and eluted for sequence analysis by both enzymatic and chemical protocols (6, 16).

To eliminate band compression and rearrangement sequencing artifacts caused by structures not denatured on the sequencing gels, the 5S rRNAs were also modified with bisulfite under conditions which quantitatively convert all cytidine residues to uridine (11). Gels of unmodified RNA run at 60°C resolved many, but not all, of these sequencing artifacts. The GU duplex structure is much less stable than the GC duplex, and thus sequencing gels of RNA lacking cytosine residues displayed more uniform mobility shifts with successive nucleotide additions. From 10^6 to 10^7 cpm of each 5S rRNA was incubated at 90°C for 45 min in a sealed capillary containing 26 μl of 3.2 M sodium bisulfite–1 mM EDTA (pH 5.6) with NaOH and 10 μg of tRNA. After incubation, the reaction mix was desalted on a 0.5-ml P-6 (Bio-Rad Laboratories) column, and the peak fractions were combined. The bisulfite adduct was removed by the addition

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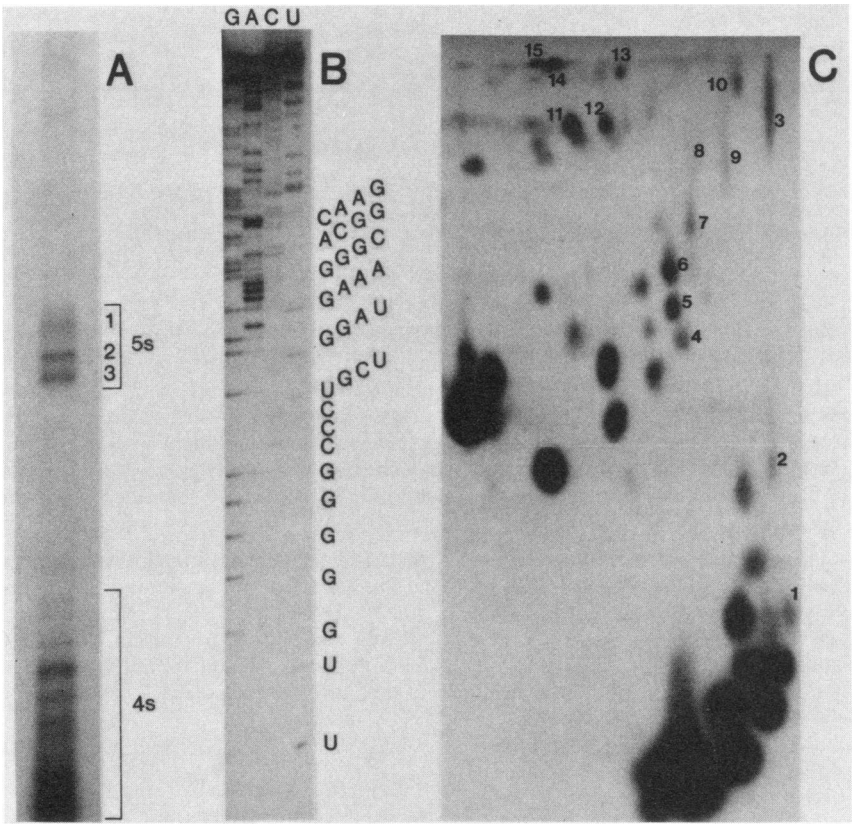

FIG. 1. Sequence and abundance analysis of the Octopus Spring 5S rRNAs. (A) Autoradiograph of total 3′-end-labeled low-molecular-weight RNAs separated on a high-resolution 10% polyacrylamide gel. (B) A representative sequencing gel autoradiograph of an Octopus Spring 5S rRNA subjected to base-specific partial chemical cleavages. (C) Autoradiograph of a two-dimensional fractionation (fingerprint) of RNase T_1 generated and 5′-end-labeled oligonucleotides derived from total 5S-sized rRNAs.

of 1/50 volume of 28% NH_4OH and incubation for 15 min at room temperature. The solution was then neutralized with acetic acid, ethanol precipitated, suspended in 5 M urea, and fractionated on a 10% sequencing gel to remove breakdown products generated by the harsh reaction conditions. The main band was eluted and sequenced by standard enzymatic protocols.

Determination of relative 5S rRNA abundance. From a broad region of a preparative acrylamide gel, the RNA roughly corresponding in size to 5S rRNA was eluted, digested to completion with RNase T_1, and 5′-^{32}P end labeled with [γ-^{32}P]ATP and polynucleotide kinase. After labeling, an excess of 3′-UMP and an additional 0.7 U of polynucleotide kinase were added, and the incubation was continued to scavenge remaining [γ-^{32}P]ATP. The end-labeled oligonucleotides were resolved by two-dimensional electrophoresis according to the method of Sanger and Brownlee (17) and were located by autoradiography. All spots were excised, and their radioactive contents were determined by scintillation counting. Spots destined for sequence determination were eluted from the paper with either 1 M NH_4HCO_3 or 1 M formic acid adjusted to pH 4.3 with pyridine. The eluted materials were repeatedly lyophilized and hydrated to remove residual salt. Sequencing of the eluted oligonucleotides was by partial enzymatic digestions as outlined in the sequencing description and resolution on acrylamide gels or by polyethyleneimine thin-layer chromatography with the above pyridine-formate solvent.

Determination of phylogenetic relationships. The 5S rRNA nucleotide sequences were aligned according to a common secondary structure and by universal positions in the primary structure, as summarized by Erdmann et al. (7). Regions of base pairing, as defined by the 5S rRNA consensus secondary structure, were labeled according to Stahl et al. (21) (see Fig. 2). The corresponding phylogeny was deduced by the matrix method essentially as described by Hori and Osawa (12). Regions of terminal length variation were omitted from homology calculations, base-paired positions were given half the weight of unpaired positions, and alignment gaps were given half the weight assigned to a nucleotide in the same position. In determining the optimal tree, we weighted the difference between the estimated evolutionary distance separating each pair of sequences and the corresponding tree distance by the statistical uncertainty of the distance estimate (12).

RESULTS

Our initial interest in the Octopus Spring microbial community was prompted by observations of visible microbial biomass accumulation in this hot spring (3). This accumulation, previously described as pink tufts or streamers, is attached to the substratum in the source effluent. Objects immersed in the source overflow are rapidly and visibly colonized. There is one reported analysis of pink tuft accumulations for lipid composition (1) but no reported cultivation of characteristic microorganisms from these materials.

We were unable to extract appreciable nucleic acid from pink tufts collected from Octopus Spring, apparently because the bulk of this material is dead or moribund. Probably only a surface lamella of the fibrous pink tuft accumulations

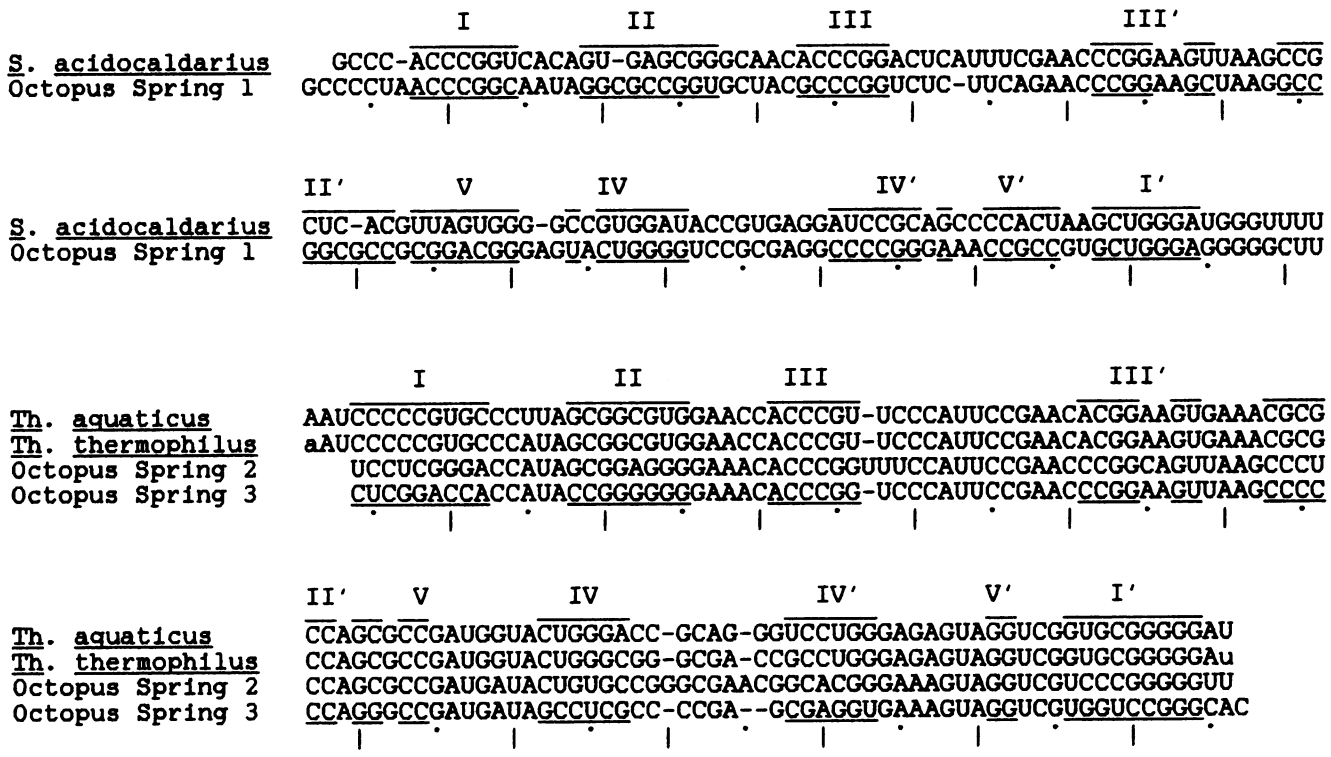

FIG. 2. Alignment of the 5S rRNA sequences isolated from Octopus Spring with those of *Sulfolobus acidocaldarius* and *Thermus* spp. Regions of base pairing as defined by the 5S consensus secondary structure (9, 23) are indicated by horizontal bars and are labeled as previously described (21).

is composed of viable mass. This is consistent with observations by Brock that these accumulations incorporate little or no radioisotopically labeled substrates (3). We therefore used a simple collection device consisting of glass or cotton fiber batting sandwiched between nylon mesh. The basic notion here was to provide an extensive surface area (the fibers) for the development of a microbial film analogous to that seen on contact slides. After immersion for 1 week in the source waters, these devices yielded ample biomass for analysis. Total nucleic acids were extracted directly from mesh segments as described above.

No fractionation or treatment of nucleic acids was carried out before isotopic labeling with RNA ligase and [5'-^{32}P]pCp. Because RNA ligase requires a 3' hydroxyl acceptor, its use (in conjunction with [5'-^{32}P]pCp) favors the labeling of native rRNAs. Most degradation products, such as those that result from random hydrolysis or nonspecific nuclease action, possess 3'-phosphorylated termini and so are not labeled. An example of a preparative fractionation of 5S rRNA from total labeled nucleic acid is shown in Fig. 1. The bands indicated were excised, eluted, and sequenced as detailed above. The sequences of these isolated molecules are shown in Fig. 2. Two of the sequences are eubacterial in nature, and the third characterized is archaebacterial.

The phylogenetic relationships of the Octopus Spring organisms to one another and to better characterized microorganisms are best seen as a phylogenetic tree. The one presented in Fig. 3 spans the purple photosynthetic line of eubacterial descent (10). The inset summarizes most of the lines of eubacterial descent so far defined by partial 16S rRNA sequence characterization and is offered as reference to more familiar organisms. Two of the sequenced 5S rRNAs are of eubacterial descent (Fig. 3), and they most closely resemble the 5S rRNAs of *Thermus* spp., albeit as fairly distant relatives. The 5S rRNA analysis suggests that the represented *Thermus* spp. and the eubacterial Octopus Spring organisms radiated from a common ancestor near the origin of the purple photosynthetic line of descent and should possibly be included in it. No 16S rRNA sequence information is yet available for *Thermus* spp., and because phylogenetic analysis based on 5S rRNA does not satisfactorily bridge longer phylogenetic distances, the suggested placement should be considered tentative until the data base of reference sequences in this region of the phylogenetic map is fleshed out. The phylogenetic affinities of the Octopus Spring organisms for these *Thermus* spp. is, however, clear.

The remaining 5S rRNA isolated from the source waters is archaebacterial; its phylogenetic position is depicted in Fig. 4. Although there currently are no 5S rRNAs in our sequence collection which can be considered close relatives of this organism, it clearly belongs with an assemblage recently defined as the sulfur-metabolizing branch of the archaebacteria (K. O. Stetter and W. Zillig, *in* C. R. Woese and R. S. Wolfe, ed., *The Bacteria*, Vol. 8, in press). This group includes the *Thermoproteales* and organisms similar to *Sulfolobus* spp. (*Sulfolobales*).

The relative abundances of these major Octopus Spring 5S rRNAs and hence some appreciation of the relative abundance of the resident microorganisms were established by quantitation of oligonucleotides unique to each representative 5S rRNA. Different 5S rRNAs label at their termini with different efficiencies, so the relative incorporation of radioactivity into the various members of a population of intact 5S rRNAs does not necessarily correlate well with the relative abundances of the donor organisms. To ameliorate this bias, the total collection of 5S-sized material was first digested to

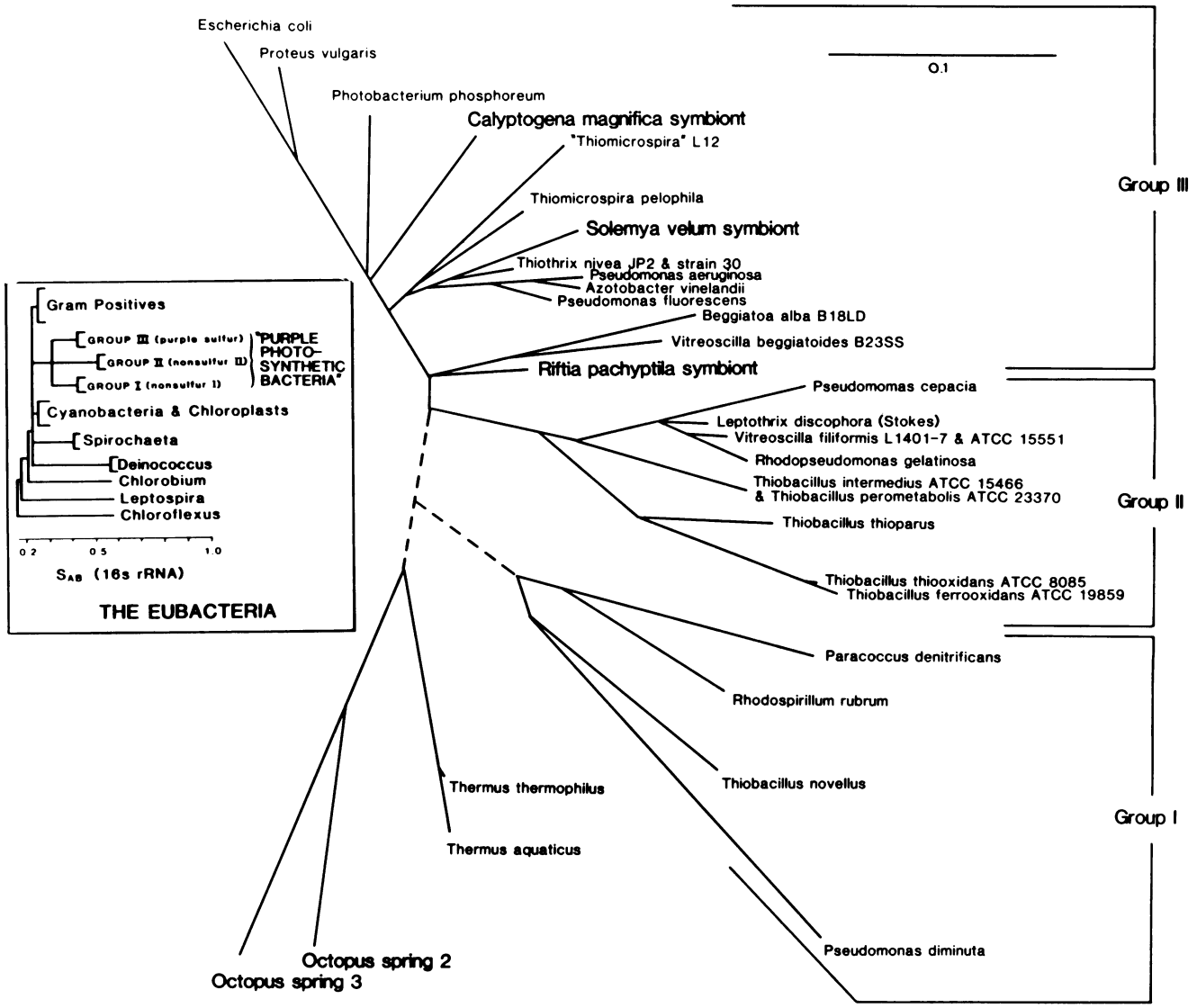

FIG. 3. Phylogeny of representative purple photosynthetic bacteria and *Thermus* spp. 5S rRNA sequences (20; D. A. Stahl, D. J. Lane, D. Heller, and N. R. Pace, unpublished data). The line of descent including the *Thermus* spp. and the Octopus Spring eubacterial 5S rRNAs (in boldface) is not necessarily included in the purple photosynthetic assemblage (see text). The scale bar represents an evolutionary distance of 0.1 nucleotide change per sequence position. The root of the tree, based on relative distances within the tree, is suggested to lie within the dotted segments. The inset (19) illustrates most of the major phylogenetic groupings (phyla) so far defined within the eubacteria. For a discussion of the symbionts (also in boldface), see reference 20.

completion with RNase T_1, which cleaves specifically after guanosine residues. The oligonucleotides derived from the mixed population of 5S rRNAs were then end labeled at their 5′ termini with [γ-^{32}P]ATP and polynucleotide kinase and fractionated by two-dimensional, high-voltage paper electrophoresis according to the method of Sanger and Brownlee (17). The derived oligonucleotides labeled with uniformly good efficiencies, thus eliminating the uncertainty of differential incorporation into the intact molecules. All spots on the autoradiogram were quantitated by radioactive content, and a selected collection of these oligonucleotides were sequenced. Table 1 displays the sequences and relative abundances of some of the 5S-derived oligonucleotides. By the criterion of specific oligonucleotide abundance, the Octopus Spring archaebacterium possesses about half the extractable 5S rRNA, the remainder being divided nearly equally between the two thermus-like rRNAs. Importantly, there are no dominant oligonucleotides unaccounted for in the nucleotide sequences of the three isolated 5S rRNAs.

DISCUSSION

The use of biochemical markers to define microbial community structure has been largely restricted to cell envelope and membrane components (lipids, carbohydrates, etc.) (15). These markers often offer some categorization of populations, but they are limited to available biochemical descriptions. Novel constituents, representing unknown microbes, cannot be interpreted. We can say with good confidence that the rRNAs are ubiquitous, and a phylogenetic framework based on their evolutionary drift in structure is established. Thus, the analysis of rRNA nucleotide sequences offers a powerful and generally applicable description of any life form. The precision with which an organism of unknown phylogenetic affiliation can be ordered relative

to characterized isolates is limited only by the availability of the corresponding rRNA sequences of the isolates. There are now about 250 5S rRNA sequences available, spanning all three primary kingdoms (eubacteria, archaebacteria, and eucaryotes). The 5S rRNAs extracted from environmental samples, therefore, can be related at some level to those of better characterized microorganisms.

One intent of studying microorganisms in their natural environs is to discern their contributions to geochemical transformations. In principle, phylogenetic placement can be interpreted in terms of physiology. However, our ability to describe physiology based on a phylogenetic assignment is at this time limited both practically and conceptually. The precision with which we can describe the members of natural populations is limited practically by the availability of well-described, phylogenetically close microorganisms in our reference sequence collection. At present, our collection of rRNA sequences is quite limited, and we do not yet understand the phylogenetic significance of important physiological attributes. For example, although sulfur-based chemolithotrophy has frequently been used to define traditional taxonomic hierarchies, heterotrophs and sulfur-based autotrophs may be rather close phylogenetic relatives (20). However, as rRNA sequences are added to our reference collection, we will be better able to discern phylogenetically significant physiological attributes and also to describe more precisely those organisms encountered in natural settings.

We selected Octopus Spring for this first analysis of a mixed microbial habitat by rRNA sequences because it offers a vigorous community of apparently limited complexity. Also, although Brock and colleagues (3, 4) characterized to some extent the in situ activity of the community, characteristic microorganisms had not been obtained in culture. The temperature of the source waters is a nearly constant 91°C and matches the temperature optimum for incorporation of radioisotopically labeled substrates (leucine, lactate, aspartate, phenylalanine, thymidine) (3, 4). The resident microbial population therefore appears to be optimally adapted for growth at the ambient temperature. The slightly alkaline waters are low (ca. 5 μM) in sulfide, and addition of sulfide inhibits the uptake of labeled substances (4), so it seems unlikely that sulfide serves the metabolic needs of the community. These earlier studies also demonstrated that bubbling with CH_4, N_2, CO_2, or air does not stimulate uptake of labeled substrates. Consistent with our observation of an archaebacterium in the Octopus Spring population are transmission electron micrographs that reveal microorganisms lacking the typical eubacterial cell wall structure (3).

TABLE 1. Sequence listing and quantitation of selected RNase T_1-generated oligonucleotides[a]

	Oligonucleotide	Unique 5S rRNA			cpm	Relative abundance
		I	II	III		
1.	CCCCG	1		1	13,329	0.75
2.	AAACCG	1			36,775[b]	2.1
	AACCCG	1	1	1		
3.	AAACACCCG		1	1	26,748	1.5
4.	UCCCG		1		15,320[b]	0.79
	CCUCG			1		
5.	CUACG	1	1		22,956	1.8
6.	CUAAG	1	1		26,716	1.5
7.	CAAUAG	1	1		19,227	1.1
8.	ACCAUAG					
9.	CCCUCCAG				15,551	0.9
10.	CCCCUAACCCG	1	1		9,827	0.6
11.	UUAAG			1	26,668	1.5
12.	AUACUG				14,062	0.8
13.	UCCCAUUCCG			1	6,202	0.37
14.	UCUCUUCAG	1	1		13,322	0.75
15.	UUUCCAUUCCG				6,248	0.35

[a] See Fig. 1C. The quantitation has been normalized in the relative abundance column to reflect the relative proportion of individual oligonucleotides and by inference the parent 5S rRNAs.

[b] Figure applies to both corresponding nucleotides.

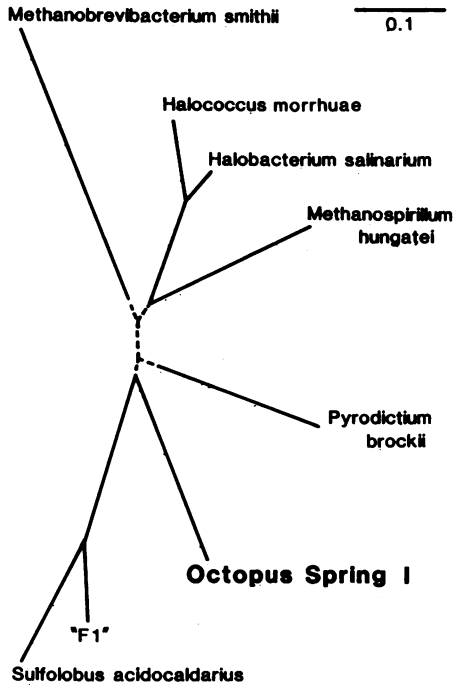

FIG. 4. Phylogeny of the Octopus Spring archaebacterium and other selected archaebacterial 5S rRNA sequences. The format is the same as that of Fig. 3.

The two characterized eubacterial 5S rRNAs obtained from Octopus Spring most closely resemble the 5S rRNAs of the two representatives of the genus *Thermus* (*T. aquaticus* and *T. thermophilus*) in our reference collection. The members of this genus described thus far have a maximum temperature for growth of about 85°C (13, 14). The rudiments of the *Thermus* phylogeny emerging from 5S sequence comparisons suggest it to be of relatively ancient origin. The *Thermus* line of descent so far defined is at least as ancient as any of the three primary divisions within the purple photosynthetic bacteria. Over such broad phylogenetic distances, credible predictions of physiology cannot be made at this time. Members of the genus *Thermus* are formally described as filamentous, nonsporulating, nonmotile, obligately aerobic, oligotrophic heterotrophs (5). Thermus-like filaments have been observed in flowing thermal environments with organic concentrations as low as 2 ppm (2 μg/liter) and are conspicuous on contact slides immersed in Octopus Spring source waters. Contact slides immersed with the fiber mattings used here for biomass accumulation contained roughly half thermus-like filaments, the remainder being nondescript rods resembling the above-mentioned, probable archaebacterium. This observation is consistent with the quantitation derived from the 5S rRNA oligonucleotide content. Although we have no measured value for the organic contents of Octopus Spring, dense microbial mats (primarily *Chloro-*

flexus and *Synechoccus* species [3]) thriving in the cooler shallows surrounding the source waters probably supply adequate organics to sustain oligotrophic growth. The *Thermus* relatives encountered in the Octopus Spring source may, therefore, adhere to the physiological profile of this genus.

The Octopus Spring archaebacterium which accounts for about 50% of the isolatable 5S rRNA is not closely related to any organism in our sequence collection; the list of available archaebacterial 5S rRNA sequences is scant. It is, however, clearly affiliated with the branch of the archaebacteria designated the sulfur-metabolizing (dependent) branch, a physiologically diverse assemblage. Representatives of the genus *Sulfolobus* grow heterotrophically or as sulfur-oxidizing autotrophs. Other members of this branch grow by sulfur-dependent respiration of hydrogen or organic compounds, either heterotrophically or autotrophically. The discovery reported here of a representative of this assemblage in an alkaline source is noteworthy. To our knowledge, there so far has been no successful isolation of a member of the sulfur-metabolizing archaebacteria from an alkaline environment, and until recently, this line of descent was termed the thermoacidophilic branch of the archaebacteria. With the recognition that many representatives of this collection continue to grow or grow optimally near neutrality, the alternative designation proposed by Stetter and Zillig (in press), based on sulfur utilization, might seem a more comprehensive description. However, we point out that most if not all methanogenic archaebacteria so far characterized have the capacity to substitute sulfur as an electron acceptor for the oxidation of hydrogen (22). Thus, the designation of sulfur utilization may not address a fundamental biochemical difference between these deep branchings of archaebacteria. Instead, the broad distribution of sulfur-metabolizing capabilities may reflect the ancient origin of sulfur-based energetics. Octopus Spring is a low-sulfide (ca. 5 μM) environment, suggesting that this archaebacterium, like the *Thermus* relatives, is growing heterotrophically.

The Octopus Spring source pool microbial community is relatively simple in major constituent numbers and thus is amenable to the direct isolation and fractionation of the 5S rRNAs. The practical limitation on this methodology by community complexity has not yet been established, although the fractionation of 10 or so unique species of 5S rRNA is easily within the range of analysis. However, an alternative approach which does not seem to have limitations of population complexity involves the direct cloning of the rRNA genes from naturally available DNA. Individual population members are represented by unique clones, so that the isolation of unique rDNAs (population members) requires only the sorting of these clones. In concert, these techniques should be generally applicable to questions regarding microbial ecology.

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