

## Bacterial Diversity and Sulfur Cycling in a Mesophilic Sulfide-Rich Spring

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**An artesian sulfide- and sulfur-rich spring in southwestern Oklahoma is shown to sustain an extremely rich and diverse microbial community. Laboratory incubations and autoradiography studies indicated that active sulfur cycling is occurring in the abundant microbial mats at Zodletone spring. Anoxygenic phototrophic bacteria oxidize sulfide to sulfate, which is reduced by sulfate-reducing bacterial populations. The microbial community at Zodletone spring was analyzed by cloning and sequencing 16S rRNA genes. A large fraction (83%) of the microbial mat clones belong to sulfur- and sulfate-reducing lineages within  $\delta$ -Proteobacteria, purple sulfur  $\gamma$ -Proteobacteria,  $\epsilon$ -Proteobacteria, *Chloroflexi*, and filamentous *Cyanobacteria* of the order *Oscillatoria* as well as a novel group within  $\gamma$ -Proteobacteria. The 16S clone library constructed from hydrocarbon-exposed sediments at the source of the spring had a higher diversity than the mat clone library (Shannon-Weiner index of 3.84 compared to 2.95 for the mat), with a higher percentage of clones belonging to nonphototrophic lineages (e.g., *Cytophaga*, *Spirochaetes*, *Planctomycetes*, *Firmicutes*, and *Verrucomicrobiae*). Many of these clones were closely related to clones retrieved from hydrocarbon-contaminated environments and anaerobic hydrocarbon-degrading enrichments. In addition, 18 of the source clones did not cluster with any of the previously described microbial divisions. These 18 clones, together with previously published or database-deposited related sequences retrieved from a wide variety of environments, could be clustered into at least four novel candidate divisions. The sulfate-reducing community at Zodletone spring was characterized by cloning and sequencing a 1.9-kb fragment of the dissimilatory sulfite reductase (DSR) gene. DSR clones belonged to the *Desulfococcus-Desulfosarcina-Desulfonema* group, *Desulfobacter* group, and *Desulfovibrio* group as well as to a deeply branched group in the DSR tree with no representatives from cultures. Overall, this work expands the division-level diversity of the bacterial domain and highlights the complexity of microbial communities involved in sulfur cycling in mesophilic microbial mats.**

Within sulfur- and sulfide-rich environments (e.g., springs, hydrothermal vents, anaerobic zones of lakes, and shallow marine and intertidal systems), utilization and cycling of sulfur species play a major role in energy production and the maintenance of the microbial community (16). Since a wide array of microorganisms are able to oxidize, reduce, and disproportionate sulfur species, the microbial community structure of sulfur-rich habitats is clearly influenced by the prevalent environmental conditions at a specific site, e.g., pH; temperature; sulfide, sulfur, or sulfate concentrations; redox conditions; presence of other electron acceptors; light availability; and organic content.

The microbial community structure has been extensively studied in several sulfur-rich habitats, e.g., in hypersaline lakes in Sinai, Egypt (34, 46, 68, 69), and Guerrero Negro, Mexico (14, 50), and in thermal springs in southwestern Iceland (65), Japan (85), and Yellowstone National Park (55, 82). Under these extreme conditions, microbial mats develop due to the absence of grazing by metazoan predators and represent the most prominent feature in the microbial community (69). Hypersaline mats (e.g., those of Solar Lake) are characterized by

extremely high rates of photosynthesis-driven sulfate reduction (8, 69) and a microbial community of cyanobacteria, sulfate-reducing bacteria, and anoxygenic phototrophs that are extremely well adapted to oxic-anoxic fluctuations, sulfide-oxygen gradients, and diurnal-nocturnal fluctuations in light intensity (69). The composition of thermal mat structures is influenced by temperature as well as by sulfide concentrations and prevailing redox conditions. Microorganism species encountered in thermal mats include members of the filamentous *Cyanobacteria* (81), *Chloroflexi* (26, 65, 83), and purple sulfur bacteria (81) as well as members of the order *Aquificales* (55, 65, 76). Mesophilic mats from sulfide- and/or sulfur-rich environments have been previously studied (16, 49, 54, 59, 66). However, detailed analysis of the microbial community in these mats via modern culture-independent approaches is more poorly documented than that of thermophilic and hypersaline mats.

A spring emerges north of Zodletone Mountain in southwestern Oklahoma. In the Zodletone spring, the dissolved sulfide concentration in the emergent water is high (8 to 10 mM) and maintains anoxic conditions in the water and underlying sediments through the spring. As a result of light exposure and constant high sulfide concentrations, microbial mats are visible throughout the spring. Water emerging at the spring source also contains abundant short-chain gaseous alkanes (methane, ethane, and propane). In this work, we character-

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ized the microbial community at two distinct locations at the Zodletone spring: the microbial mat underlying the water flowing from the spring source (for which we predict that photosynthesis and sulfur cycling are the dominant processes) and the spring source (in which the impact of short-chain alkanes may be important). Our characterization of this ecosystem suggests that sulfide emerging from the spring supports a diverse phototrophic, primary producing community that then provides substrates (in the form of electron donors, electron acceptors, and organic carbon) for sulfate-reducing and other sulfur-cycling bacteria. Also, an extremely diverse bacterial community, with novel division-level diversity, was detected in the anoxic sulfide-rich alkane-impacted sediments at the source of the spring.

#### MATERIALS AND METHODS

**Site description and biogeochemistry.** Zodletone spring, located north of Zodletone Mountain in the Anadarko Basin of western Oklahoma, was first described by Havens (S. Havens, Reconnaissance of ground water in the vicinity of the Wichita Mountains, Southwestern Oklahoma; Oklahoma Geological Survey Circular no. 85, 13 pages, 1983), and its geological and hydrological characteristics were subsequently described in some detail (60). At the source of the spring, brine from deep within the Anadarko Basin is ejected along with petroleum, which occurs in seeps in the general vicinity. The dissolved-sulfide concentration in the emergent springwater is high (8 to 10 mM) and maintains anoxic conditions in the water and underlying sediments.

Water at the spring source emerges at a flow rate of approximately 8 liters/min and degasses methane, ethane, and propane as it emerges. The source is a contained area (approximately 1 m<sup>2</sup>) overlaid by water at a depth of about 50 cm and filled with biomass and soft sediments to a depth of at least 15 cm. The spring flows approximately 20 m before discharging into a nearby creek. Springwater chemistry is anomalous in the region, containing 0.2 M NaCl and minor amounts of fluorine and bromide as well as lithium, boron, strontium, barium (390 μM), and sulfate (60 μM) (L. R. Krumholz, J. M. Senko, B. Campbell, J. R. Henriksen, E. L. Grossman, and T. A. Dewers, Abstr. 101st Gen. Meet. Am. Soc. Microbiol., poster N-182, 2001). Sulfate concentrations remain low (50 to 60 μM) over the course of the spring until approximately the 15-m point, where concentrations increase, eventually reaching 2.2 mM at the confluence with the creek. Dissolved sulfur (zero valent) also increases in concentration with the distance from the source, reaching a maximum of nearly 1 mM prior to confluence with the creek. Purple and green microbial mats overlay the sediments throughout the spring, suggesting the presence of an active photosynthetic community at the site.

**Sampling.** Samples for laboratory incubations and activity studies were obtained from the site by inserting 50-ml syringes (modified by sawing off the flange at the piston end) into the mat and withdrawing an intact core. Cores were removed and sealed in the field by replacing the piston and attaching a sealed needle to the luer lock fitting. These were then stored at 4°C and used for experiments within 8 h. For molecular analysis, intact mat materials were sampled at approximately 15 m from the source, source sediments were collected in Whirlpak bags with a sterile spatula, and the samples were immediately frozen on dry ice and stored at 20°C.

**Microbiological incubations.** Mat cores within 50-ml syringes (described above) were incubated at 25°C in the dark or under fluorescent and incandescent lights (103 μmol of quanta/s/cm). Over time, water overlaying the cores was sampled by removing the needle from the 50-ml syringe and withdrawing overlying site water with a 1-ml syringe. Samples for sulfate analysis were centrifuged in a Microfuge and frozen immediately after sampling. For sulfide analysis, 0.5-ml samples were injected into an equal volume of 10% aqueous zinc acetate solution.

**DNA isolation, PCR amplification, cloning, and sequencing.** An indirect DNA extraction procedure (28), which involves using a Percoll gradient to separate cells from sediments, was applied prior to DNA extraction. Briefly, samples (0.5 g) were treated with 1 ml of 0.1% sodium pyrophosphate and vortexed for 3 min to detach separated cells from sediments. The sample was then loaded in a Percoll gradient of 10 ml of Percoll (Amersham Pharmacia, Upsala, Sweden)–5 ml of Tris-EDTA buffer, and the mixture was centrifuged for 15 min at 27,200 × g. This resulted in the separation of cells and cell-size particles from larger sediments. Phase-contrast microscopy indicated that almost no cells were attached to larger sediments present at the bottom of the gradient. DNA isolation

from the Percoll-separated cell fraction was carried out using a lysis bead-beating protocol (15). DNA was amplified from the bulk community DNA in a 50-μl reaction mixture containing (final concentration) 2 μl of a 1:100 dilution of extracted DNA, 1× PCR buffer (Invitrogen), 2.5 mM MgSO<sub>4</sub>, a mixture of 0.2 mM concentrations of each deoxynucleoside triphosphate, 1.5 U of high-fidelity *Taq* polymerase (Invitrogen), and 10 μM concentrations of each of the forward and reverse primers. The primers (Invitrogen Corp., Carlsbad, Calif.) used for amplifying the 16S rRNA genes (16S rDNA) were the universal forward primer 8f (5' AGAGTTTGATCCTGGCTCAG 3') and the *Bacteria*-specific reverse primer 805r (5' GACTACCAGGGTATCTAATCC 3'). Primers used for amplification of the dissimilatory sulfite reductase (DSR) gene were the forward DSR1f and the reverse DSR4r primers (78). PCR amplification was carried out on a Gene Amp PCR system 9700 thermocycler. The 16S rRNA amplification used a protocol involving initial denaturation for 5 min at 94°C and 39 cycles of 92°C for 0.5 min, 50°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 20 min. For amplification of the DSR gene, there was an initial denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1.5 min and a final extension step at 72°C for 20 min. The PCR products obtained were either purified using a gel purification kit (Qiagen Inc., Valencia, Calif.) or directly cloned into a TOPO-TA vector (Invitrogen) with a cloning kit used according to the manufacturer's instructions.

For sequencing, PCR insert-containing subclones were inoculated into 384-well flat-bottom plates containing 80 μl of TB medium, which contains (in grams/liter) KH<sub>2</sub>PO<sub>4</sub> (2.31), K<sub>2</sub>HPO<sub>4</sub> (12.54), Tryptone (12), yeast extract (24), and glycol (4) (supplemented with 100 μg of ampicillin/ml), and incubated in a shaker incubator (HiGro; Gene Machines, Inc.). Cell pellets were collected by centrifugation and frozen at –80°C. DNA was isolated following the modified cleared lysate protocol described in detail at the following website address: [http://www.genome.ou.edu/ds\\_seq\\_template\\_isol\\_hydra.html](http://www.genome.ou.edu/ds_seq_template_isol_hydra.html). The DNA was precipitated by adding 40 μl of isopropanol at room temperature, collected by centrifugation, washed with 50 μl of 70% ethanol, and subsequently dissolved in 2 μl of sterile distilled deionized water. Sequencing was performed using a BigDye terminator kit (catalog no. 402153; Perkin-Elmer Applied Biosystems) as described by the manufacturer and approximately 150 to 200 ng of template DNA, 1 μl of 6.5 μM universal M13 forward (GACGTTGTAAAACGACG GCC) or universal M13 reverse (CACAGGAACAGCTATGACC) primers, dimethyl sulfoxide at a final concentration of 5% (vol/vol), and 2 μl of a 1:16 dilution of ABI BigDye reaction kit premix in each well of the 384-well thermocycler plate (catalog no. 1047-00-0; Robbins Scientific, Inc.). After thermocycling for 60 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 20 s, and extension at 60°C for 4 min, the excess dyes were removed by precipitation with ethanol acetate (95% ethanol–0.12 M sodium acetate) followed by washing with 70% ethanol. After drying and dissolving in 20 μl of distilled-deionized water, the samples were loaded on an ABI PRISM 3700 capillary sequencer. The forward and reverse trace files (i.e., the chromatograms generated from the sequencers for each reaction) of every subclone were paired and analyzed separately using Phred software (20).

**Phylogenetic analysis.** Diversity indices utilized to describe and compare the source and the mat clone libraries for bacterial diversity were determined as described by Martin (45). Collector's curve analysis was performed according to Skirmisdottir et al. (65), and the data shown represent an average of five curves produced by repeated randomization of the order of the addition of the sequences to the data set. For phylogenetic placement, sequences were initially checked using the Basic Local Alignment Search Tool (BLAST) algorithm (1) to roughly determine their phylogenetic affiliations; sequences with more than 98% similarity were considered to be of the same operational taxonomic unit (OTU). The occurrence of chimeras was checked using the chimera-check program in the Ribosomal Database Project (44) as well as by manual inspection of every sequence for the presence of universally conserved regions. Several (six) sequences were identified as possible chimeras and removed from further analysis. Zodletone sequences and sequences downloaded from GenBank were exported to the ARB software environment (<http://arb-home.de>) and aligned using an ARB fast-aligning editor. The aligned files were manually checked and corrected for alignment abnormalities.

Sample trees with no mask (all positions included in phylogenetic analysis), a Lane mask (38), and an empirical mask that filters hypervariable regions (defined as nucleotide positions where less than 50% of the bases in a data set are similar) were constructed. Few topological changes were observed between various trees constructed utilizing the three procedures, and trees generated using the Lane mask are shown in this report. For DSR analysis, trees were constructed from translated amino acid sequences and OTUs were determined for translated amino acid sequences according to a cutoff value of 98% similarity. The amino acid sequences were aligned using ClustalX software (71), and the alignments

were manually checked and corrected using a SeqApp program (D. Gilbert, Indiana University). Evolutionary-distance trees (neighbor-joining algorithm with Jukes-Cantor corrections) were constructed using PAUP 4.01b10 software (Sinauer Associates, Sunderland, Mass.). A novel candidate division was defined as one containing sequences unaffiliated with any of the previously described divisions or candidate divisions (30). Each new candidate division must have at least two sequences that are reproducibly monophyletic upon the application of various tree-building algorithms as well as the alteration of the composition and size of the data set used for phylogenetic analysis (13).

**Analytical methods.** Sulfate was determined by an ion chromatography system (17). Sulfide was quantified using a colorimetric procedure (7). When stable-isotope-ratio analysis was to be carried out, zinc sulfide was further transformed into silver sulfide by volatilizing the sulfide into a trap containing a solution of 2 M AgNO<sub>3</sub> (29, 75). The AgS was washed with water and dried prior to analysis. Stable isotope ratios were determined by mass spectroscopy (Coastal Science Laboratories, Austin, Tex.). To quantify and localize the sulfate-reducing activity in the microbial mats, mat cores with overlying water were cut once with a razor blade, 5  $\mu$ Ci of <sup>35</sup>SO<sub>4</sub><sup>2-</sup> was injected into the cut, and a 1- by 5-cm silver foil was inserted. Incubation were performed in light at 30°C for 1 h. Foil preparation and analysis by autoradiography were as described by Krumholz et al. (37). The presence of alkanes and aromatic hydrocarbons in springwater samples was tested by headspace analysis according to previously described procedures (18, 24, 33).

**Nucleotide sequence accession numbers.** Sequences obtained in this study have been deposited in GenBank under accession numbers AY327150 to AY327246.

## RESULTS

**Evidence for sulfur cycling in Zodletone spring.** The prevailing permanent high levels of sulfide (8 to 10 mM) encountered in the shallow waters of Zodletone spring led us to hypothesize that the microbial mat community in the spring gains energy by phototrophic oxidation of sulfide to sulfate. Experiments showed that sulfide was oxidized to sulfate by mat microorganisms in incubations in the light but not in the dark (Fig. 1A). This suggests that anoxygenic sulfide-oxidizing phototrophic bacteria are abundant in these mats. Interestingly, there was a slight lag in sulfate production relative to sulfide depletion, probably as elemental sulfur (S<sup>0</sup>) or polysulfide (products of anoxygenic photosynthesis) accumulated before being further oxidized. The formation of S<sup>0</sup> is important, as S<sup>0</sup> is known to be used for energy production by several groups of microorganisms. Isotopic fractionation of sulfide was assessed in these incubations and revealed an enrichment of <sup>32</sup>S<sup>2-</sup> ( $\delta^{34}$ S of 27.8 to 24.3‰), while little fractionation was observed in the dark incubations. This pattern of isotopic fractionation of sulfur is consistent with that associated with biological sulfide oxidation to sulfur reported by Fry et al. (22).

Using a silver-foil autoradiography technique, sulfate-reducing bacteria were determined to be most active within the top layer of the mat (near the mat-water interface) (Fig. 1B) and maximal sulfate reduction occurred in the same location where maximal rates of photosynthesis would likely occur. The proximity of sulfate-reducing and sulfide-oxidizing bacteria suggests a direct interaction in which sulfate-reducing bacteria consume electron donors and acceptors produced by the phototrophic, sulfide-oxidizing organisms.

**Composition and comparative diversity of mat and source clone libraries.** An overall summary regarding the composition of the source and mat 16S clone libraries is shown in Table 1. The Shannon-Weiner index indicates that the source bacterial community has a higher overall diversity than the mat community. The source also has higher species evenness than the mat,

which might be attributable to the fact that microbial mats are usually composed of few dominant microbial species (mat builders). This difference in overall diversity is reflected by the fact that the source clones belonged to 11 different bacterial divisions and candidate divisions and that 18 source clones were unaffiliated with any division while clones from the mat belonged to only 6 bacterial divisions.

Collector curves were constructed to determine to what extent the number of clones sequenced is enough to account for the overall bacterial diversity (98% sequence similarity cutoff value) at the species level (Fig. 2). The collector curve constructed from the mat clone library was closer to the saturation plateau than the collector curve derived from the source clone library. The two curves suggest that it is likely that new species (i.e., species belonging to new OTUs) will be encountered when additional clones are sequenced. However, a curve generated with sequences having >93% similarity and belonging to a single group clearly indicates that using a hypothetical 93% sequence similarity value to differentiate between genera (47), the probability of encountering new genera (Fig. 2) is low.

A breakdown of the division affiliation of both libraries is given in Fig. 3. Most of the clones belonged to only five bacterial lineages: *Cyanobacteria*,  $\gamma$ -*Proteobacteria*,  $\epsilon$ -*Proteobacteria*,  $\delta$ -*Proteobacteria*, and *Chloroflexi*. Selection for a single species was apparent, since three OTUs, ZB43 ( $\epsilon$ -*Proteobacteria*), ZB67 (*Chloroflexi*), and ZB118 (*Cyanobacteria*), accounted for 56% of the total number of clones in the mat library. Clones belonging to lineages exclusive for phototrophic metabolism such as *Cyanobacteria*, *Chromatium* (mesophilic purple sulfur bacteria), and members of group III *Chloroflexi* constituted 76% of the mat clones, while clones belonging to known sulfur- or sulfate-reducing lineages within the  $\delta$ -*Proteobacteria* made up 10% of the mat clone library.

**Phylogenetic analysis of mat and source clone libraries:  $\delta$ -*Proteobacteria*.** The majority of the source clones belonging to  $\delta$ -*Proteobacteria* in the source clone library were closely (96 to 99%) related to *Desulfocapsa thiozymogenes* as well as to clones retrieved from the chemocline of Lake Codogno, Switzerland (73) (Fig. 4). The few species described for the genus *Desulfocapsa* thus far grow chemolithoautotrophically by disproportionating elemental sulfur to sulfide and sulfate but are unable to grow via sulfate respiration (21, 32). The similarity between these clones and *D. thiozymogenes* suggests the importance of sulfur disproportionation in sulfur cycling at the source.

No *Desulfocapsa*-related clones were identified in the mat library. Instead, eight clones were monophyletic with members of the genus *Desulfuromusa* (Fig. 4), species of which grow via sulfur respiration but are unable to reduce sulfate (41). The prevalence of sulfur-reducing and disproportionating bacteria rather than sulfate-reducing bacteria at Zodletone Mountain is a reflection of the abundant sulfur and low sulfate concentrations at Zodletone spring.

**$\gamma$ -*Proteobacteria*.** Altogether, clones clustered within five distinct groups of the  $\gamma$ -*Proteobacteria*. Clones related to the genus *Halomonas* (two clones and one OTU) and the family *Enterobacteriaceae* (three clones and one OTU) were encountered in low numbers in the source clone library, and a single clone most closely associated with members of the genus *Thiomicrospira* was present in the mat clone library (Fig. 4). The

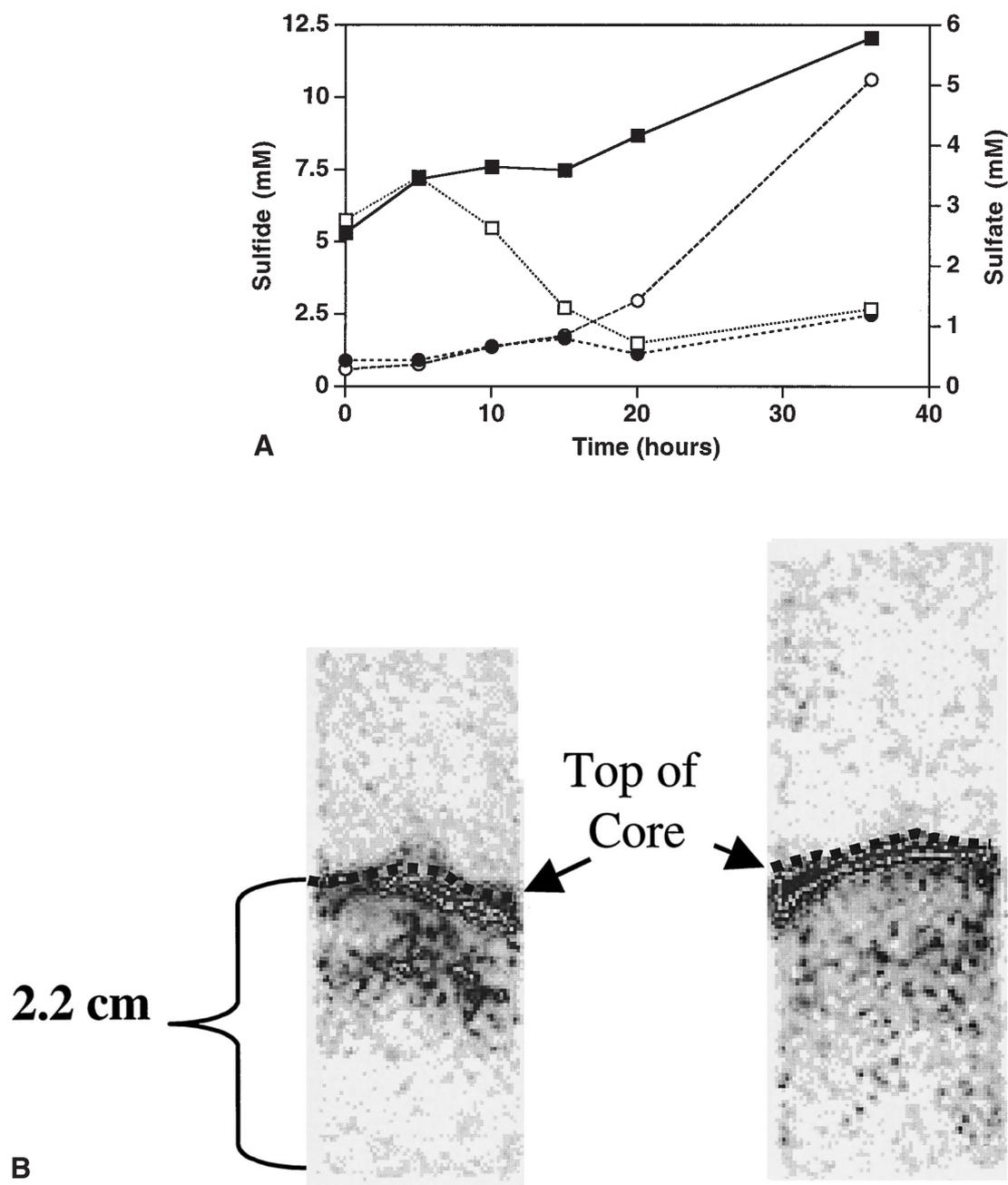


FIG. 1. (A) Oxidation of sulfide (□ and ■) to sulfate (○ and ●) in Zodletone mat and groundwater incubations. Open shapes represent concentrations in cores incubated in the light, and closed shapes represent concentrations in cores incubated in the dark. (B) Autoradiogram of silver foils incubated with sediment cores. Darker regions indicate greater sulfate-reducing activity. The figure shows two foils. Dashed lines represent the sediment-water interface; cores were collected to a depth of 2.2 cm.

majority of clones of  $\gamma$ -Proteobacteria, however, belonged either to the family Chromatiaceae (mesophilic purple sulfur bacteria) or to a novel group of  $\gamma$ -Proteobacteria. A total of 8 mat and 13 source clones belonged to twelve OTUs within the family Chromatiaceae. OTUs with >95% similarity to members of the genera *Chromatium*, *Rhabdochromatium*, *Halochromatium*, and *Thiocystis* were encountered in both libraries; however, several OTUs (e.g., ZB80, ZB82, ZB83, ZB94, and ZB103 [Fig. 4]) were less than 93% similar (using an em-

pirical 93% cutoff for the genus level [47]) to their closest database match, indicating the presence of novel genera of purple sulfur bacteria at the site. Finally, 11 clones (three OTUs) belonged to a novel monophyletic group (OTUs ZB77, ZB78, and ZB81) present only in the mat clone library (Fig. 4). The closest pure-culture relatives to this group were members of the aerobic sulfur- and thiosulfate-oxidizing genus *Halothiobacillus* (36, 64) (85 to 88% sequence similarity). This large difference in sequence simi-

TABLE 1. Composition and diversity of the mat and the source clone libraries

| Library | No. of clones sequenced | No. of OTUs | No. of divisions or candidate divisions | Shannon-Weiner index (98% similarity cutoff) | Maximum possible value for Shannon-Weiner index | Species evenness | Maximum possible value for species evenness | % Clones with <95% similarity to closest cultured representative | % of unaffiliated sequences |
|---------|-------------------------|-------------|---|--|---|------------------|---|--|-----------------------------|
| Mat     | 96                      | 40          | 6                                       | 2.95   | 4.56  | 0.81             | 1.24  | 54   | 0                           |
| Source  | 116                     | 66          | 11                                      | 3.84   | 4.73  | 0.65             | 1.13  | 70   | 16                          |

larity clearly indicates that clones ZB77, ZB78, and ZB81 represent a novel group within the  $\gamma$ -*Proteobacteria*. However, the physiology of this group and its ecological role in the Zodletone Mountain spring can only be speculated on.

**$\epsilon$ - and  $\alpha$ -*Proteobacteria*.** A single OTU (ZB43) was present in both libraries but made up 10% of the clones in the mat clone library, suggesting that it is an integral component of mat communities (Fig. 4). ZB43 belongs to the epsilon I subgroup of *Proteobacteria*. The closest relatives to ZB43 are clones retrieved from a cold sulfurous spring (59), a deep-sea trench of Japan (40), and Guaymas Basin (70). In addition, three OTUs (ZB45, ZB49, and ZB52) formed a separate, bootstrap-supported novel lineage within the  $\epsilon$ -*Proteobacteria*.

$\alpha$ -*Proteobacteria* represented only a minor fraction of the total clones. The majority belonged to the  $\alpha$ -3 lineage, with their closest relatives in the *Rhodobacter-Rhodovulum-Rhodobaca* group (Fig. 4). This close affiliation with phototrophic members of the purple nonsulfur bacteria suggests an anoxygenic photosynthetic mode of metabolism.

**Anoxygenic green bacteria and cyanobacteria.** The photosynthetic community at Zodletone Mountain was also comprised of clones belonging to the *Chloroflexi* (green nonsulfur), *Chlorobium* (green sulfur), and *Cyanobacteria* (Fig. 3). Cyanobacteria and species of *Chloroflexi* were a major component of the Zodletone spring mat, representing 51% of the clones sequenced. These two groups, however, were present in lower numbers (only 19%) in the source. Green sulfur bacteria were present only in the source clone library (4%).

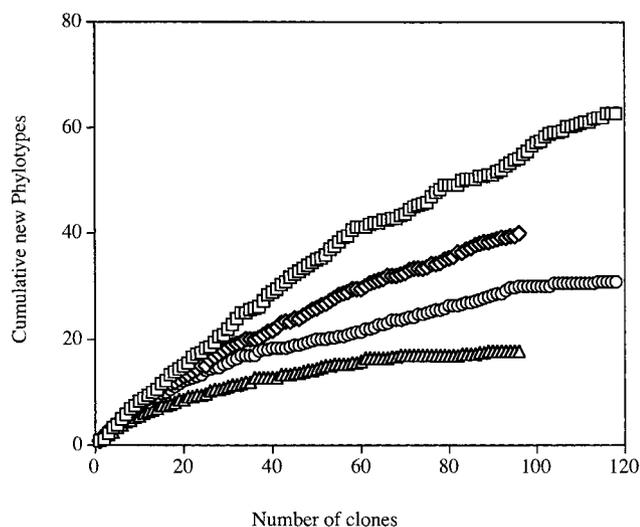


FIG. 2. Collector curve analysis for the source and mat clone libraries analyzed with 98 and 93% cutoff values.  $\diamond$ , mat (cutoff value, 98%);  $\square$ , source (98%);  $\Delta$ , mat (93%);  $\circ$ , source (93%).

The cyanobacterial populations were completely different between the two locations. The mat community was mainly formed of clones closely (97 to 99%) related to *Oscillatoria terebriformis*, a known mat-building cyanobacterium (5, 8, 9). The source clone library had only one OTU (ZB45; 6 clones) most closely related to the unicellular *Gloeotheca* species (Fig. 5).

Clones belonging to *Chloroflexi* (previously known as green nonsulfur bacteria) were present in both the mat and the source libraries (Fig. 5). A single OTU (ZB67) from a lineage within group III *Chloroflexi* represented 16% of the mat clone library (Fig. 5), suggesting that it is derived from an integral component of the mat community. In addition, a single clone (ZB69), together with a clone from the hypersaline Mono Lake (GenBank accession number AF513960) and one from a marine coast sediment (GenBank accession number AB031632), formed a separate monophyletic division within the *Chloroflexi* which we suggest calling group V *Chloroflexi*.

**Nonproteobacterial, nonphotosynthetic clones at Zodletone spring.** Nonproteobacterial clones belonging to lineages outside the *Chloroflexi*, *Chlorobium*, and *Cyanobacteria* represented 40% of the source clones compared to only 4% of the mat clones (Fig. 3 and 5). These clones belonged to the following divisions: *Cytophaga*, *Spirochaetes*, *Planctomyces*, *Verrucomicrobiae*, *Firmicutes*, *Actinomyces*, and candidate division TM7. At the source, the concentrations of methane, ethane, and propane were high and many of the source clones were closely related to clones from hydrocarbon-rich sites or hydrocarbon-degrading enrichments (15, 53, 61, 62, 70, 77). The presence of hydrocarbons could therefore have an impact on shaping this microbial community. In addition, two OTUs (5 clones) represent a separate lineage within the *Bacteroides-Cytophaga* group (Fig. 5). Clones ZB26 and ZB32 were reproducibly monophyletic and had only 81% similarity to their closest *Cytophaga* relative, adding additional diversity to this group.

**Novel division-level diversity in the source clone library.** Seven OTUs (18 clones) from the source samples did not cluster within any of the previously recognized bacterial divisions or candidate divisions. These seven phylotypes showed high (>25%) levels of sequence divergence from members of previously described divisions or candidate divisions, but together with several phylotypes with published or GenBank-deposited sequences, five of these seven OTUs formed four clusters well supported by bootstrapping (Fig. 5). We propose accommodating these sequences in four novel candidate divisions. (i) Candidate division VC2 contains four sequences, one from Zodletone spring (ZB18), one from a mid-ocean vent (56), one from the human oral cavity (GenBank accession number AF125207), and one from a deep-sea trench of Japan

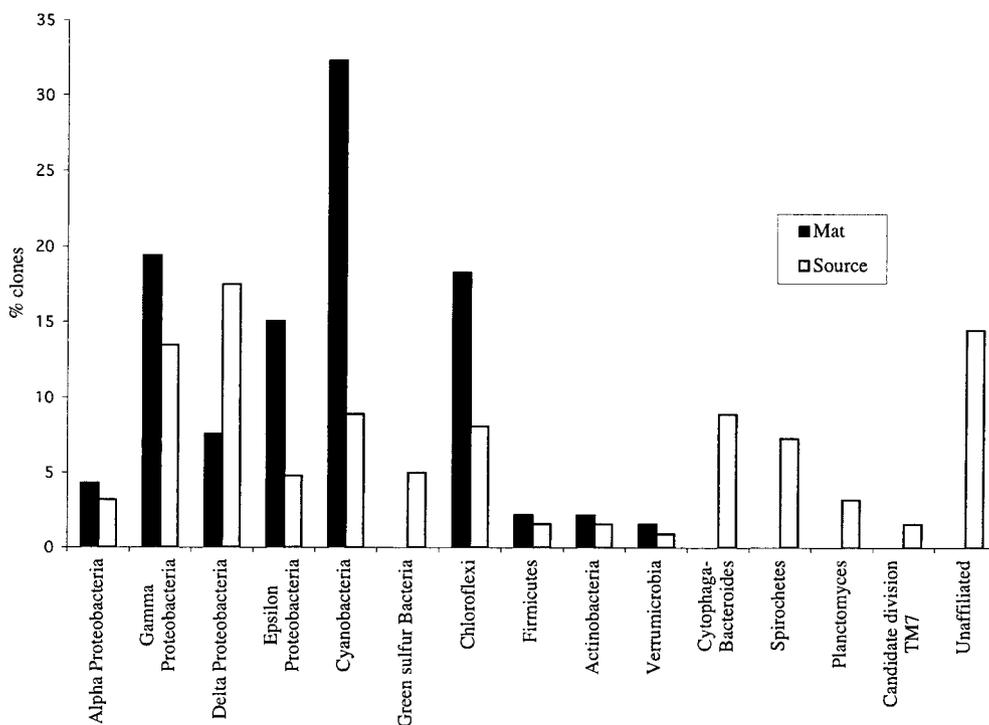


FIG. 3. Distribution of source and mat clones among various bacterial divisions.

(39). The intralinear difference between these four sequences is between 12 to 24%, and the division is named after clone VC2\_1Bac16 (after Reysenbach et al. [56]). (ii) Candidate division ZB1 has, in addition to the ZB16 sequence, sequences retrieved from a denitrifying reactor (19), a phosphorus removal reactor (12), a subseafloor volcanic eruption clone (GenBank accession number AF469402), and an anoxic rice paddy soil clone (27). The intralinear difference is between 5 and 13%. (iii) Candidate division ZB2 is formed of Zodletone spring clone ZB17, an agricultural soil clone (23), and two clones from a deep location in the Sea of Japan (47), with an intralinear difference of 9 to 24%. (iv) Candidate division ZB3 is formed of OTUs ZB15 and ZB21 from Zodletone spring and a clone from the oxic-anoxic interface from hypersaline Mono Lake (GenBank accession number AF507892). The intralinear difference in this group was 16 to 21%.

In addition to these four novel candidate divisions, clone ZB12 was most closely related to two unaffiliated clones isolated from the brine-seawater interface in the Red Sea (GenBank accession numbers AJ347757 and AJ347758) and clone ZB14 appears to be most closely related to members of candidate division VC2. However, these associations were not always supported by bootstrap analysis upon applying various phylogenetic algorithms and upon using different data sets for tree construction. We speculate that ZB12 and ZB14 might represent two new candidate divisions, and future availability of more closely related sequences will help in resolving their phylogenetic affiliations.

**Sulfate-reducing community in Zodletone spring as determined via cloning and sequencing of the DSR gene.** The majority of  $\delta$ -proteobacterial clones retrieved from the Zodletone spring 16S rDNA clone libraries belonged to either sulfur-

disproportionating (*Desulfocapsa*) or sulfur-reducing (*Desulfuromusa*) lineages. To characterize the microbial community responsible for the sulfate-reduction activity observed in the silver foil assay (Fig. 1B), a 1.9-kb fragment of the DSR gene was amplified and cloned and 85 of the source clones as well as 50 of the mat clones were sequenced. By using a 98% amino acid similarity standard, the DSR sequences could be classified as five different OTUs (Fig. 6). Three of these OTUs (ZDSR1, ZDSR2, and ZDSR3) represented 92 and 100% of the clones sequenced from the mat and the source clone libraries, respectively. These three OTUs, which belonged to the *Desulfobacter* group (ZDSR1), the *Desulfococcus-Desulfobacca-Desulfonema* group (ZDSR2), and DSR3, together with another OTU (KYF-135) retrieved from anaerobic marine sediments in Aarhus Bay, Denmark (72), formed a deeply branched cluster within the DSR tree. The less abundant OTUs, DSR4 (two clones) and DSR5 (two clones), were present only in the mat library and belonged to the *Desulfovibrio* and the *Desulfomicrobium* groups, respectively.

## DISCUSSION

The phylogenetic analysis of the mat community via culture-independent procedures supports our initial hypothesis regarding the important relationship between anoxygenic, phototrophic sulfide oxidation and sulfate reduction in the Zodletone spring microbial mats. However, our analysis suggests that sulfur cycling at this spring is a complex process in which many more groups than we would have predicted interact by oxidizing, reducing, or disproportionating sulfur species to contribute to the overall cycling process. Previous work describing sulfide-rich thermal springs (55, 65, 85) has noted the domi-

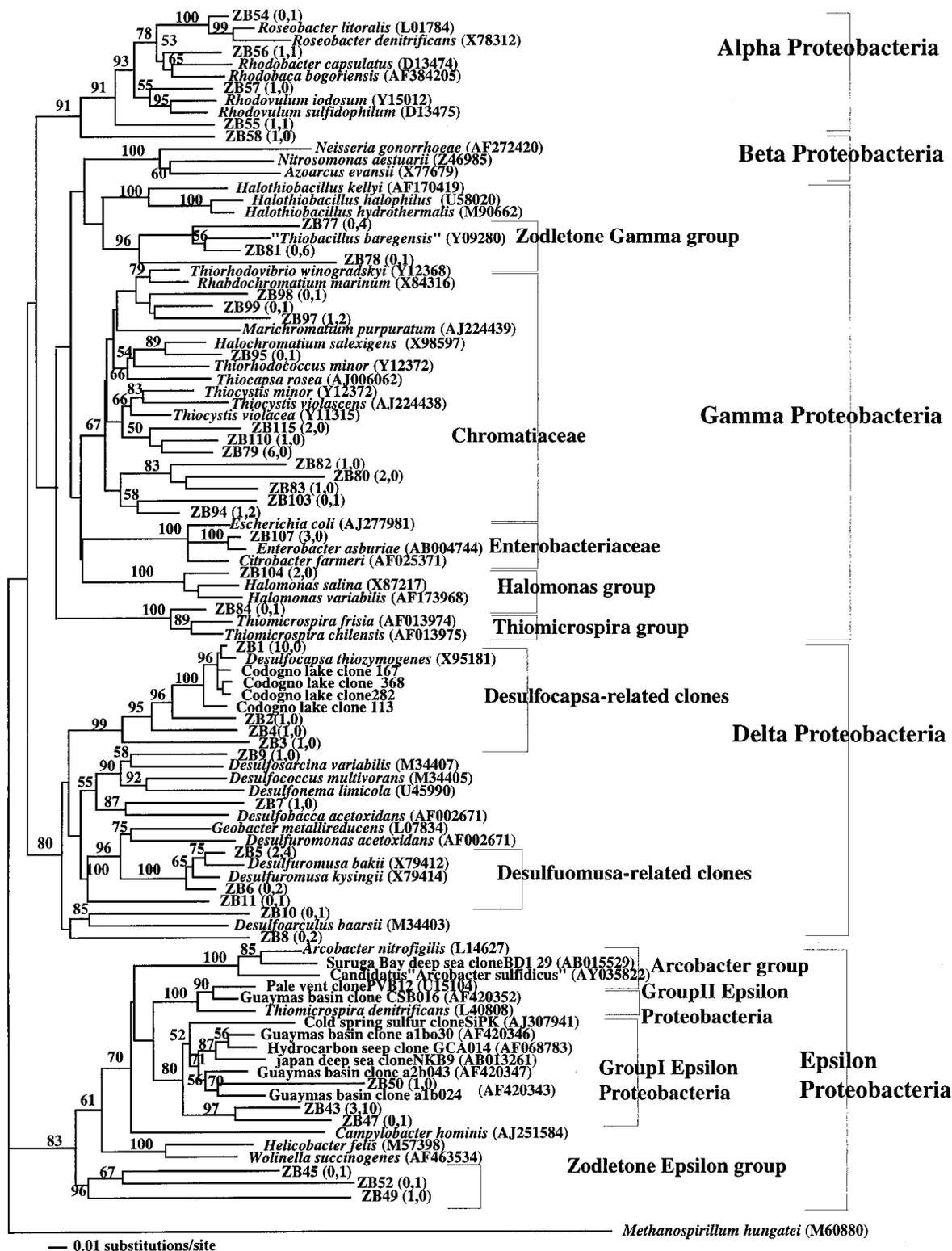
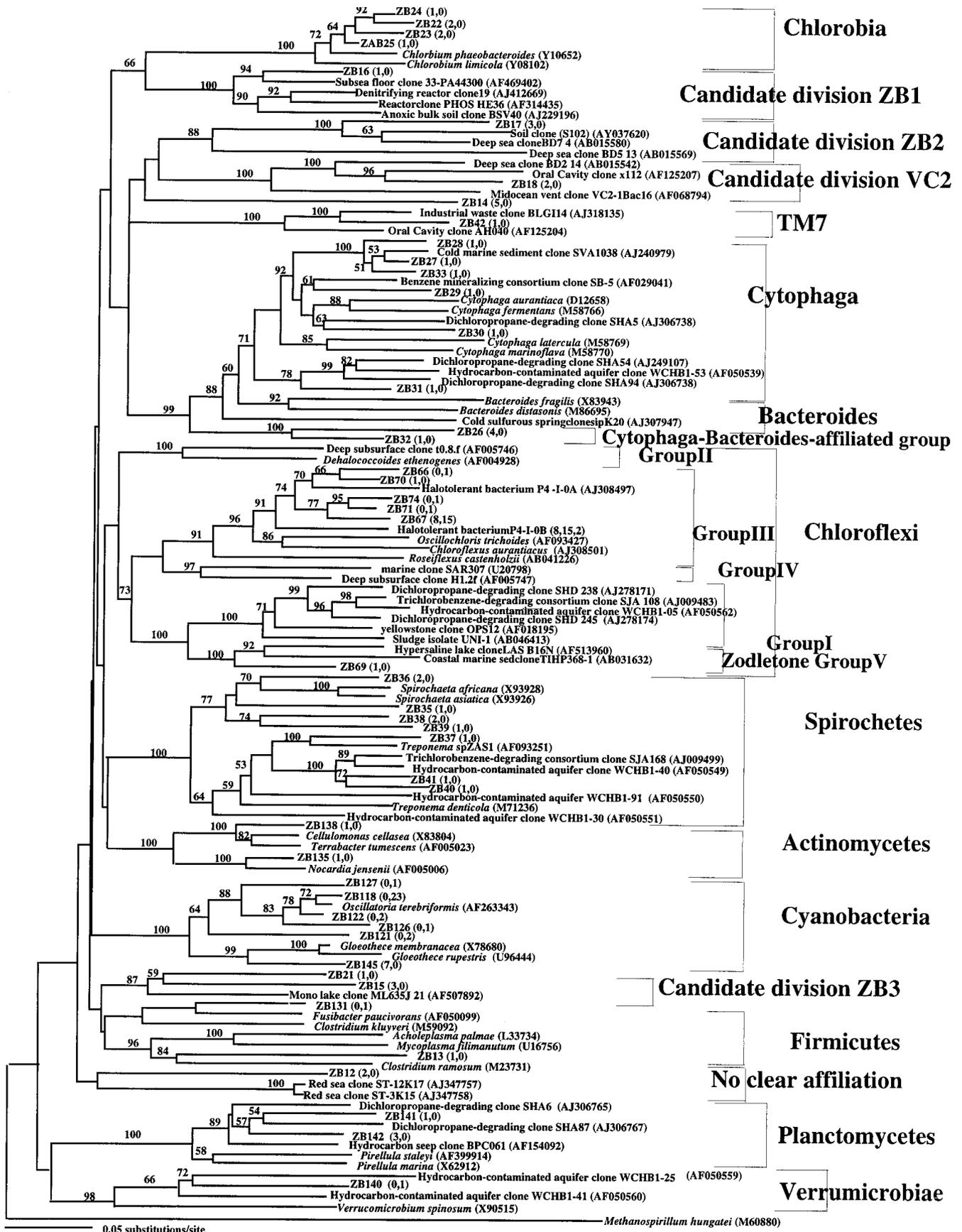


FIG. 4. Distance dendrogram constructed on the basis of the 16S sequences of proteobacterial OTUs encountered in the Zodletone spring mat and source clone libraries. Bootstrap values (percent) were determined on the basis of results for 1,000 replicates and are shown for branches with more than 50% bootstrap support. Each number (other than accession numbers) in parentheses represents the frequency of occurrence of a specific OTU in the source and mat clone libraries, respectively.



nance of a single group. However, this mesophilic spring shows evidence for selection of several groups of microorganisms and clearly contains a great diversity of bacteria.

The occurrence of filamentous cyanobacteria (belonging to the order *Oscillatoria*) in sulfide-rich environments is well documented (5, 9, 10, 11). Their filamentous structures and polysaccharide matrices probably represent the backbone of the microbial mat (76). The metabolic abilities of filamentous members of the *Oscillatoria* order can shed light on the role played by closely related clones encountered in the Zodletone spring mats. *Oscillatoria* species are known to dwell in anaerobic, sulfide-containing habitats (5). At night they can grow by fermenting glycogen and other compounds produced during daytime photosynthesis (57, 69). Some species are also capable of growth in the dark via sulfur respiration (51, 67). Elemental sulfur is produced as an intermediate of anoxygenic photosynthesis and is abundant in the Zodletone spring. Therefore, the anoxic conditions (along with high sulfide and sulfur concentrations) in the Zodletone spring represent an ideal habitat for members of the order *Oscillatoriales*.

Although microbial mats formed entirely by members of the family *Chloroflexi* (filamentous anoxygenic phototrophic bacteria) (4) have been previously reported, especially in thermal habitat, their association with cyanobacteria at mesophilic and thermophilic (up to 72°C) temperatures is also well documented (4). Since clones from Zodletone spring belonging to the family *Chloroflexi* clustered within group III of green nonsulfur bacteria, the phototrophic nature of these microorganisms is likely. When growing with cyanobacteria, species of the family *Chloroflexi* and their relatives can grow either photoheterotrophically, deriving organic carbon from cyanobacteria (81), or photolithotrophically, utilizing sulfide as an electron donor for photosynthesis (4). Purple sulfur bacteria have also been shown to be associated with cyanobacterial mats (76, 81). Together, these three groups of microorganisms are probably responsible for oxidizing the majority of the abundant sulfide in the spring to sulfur and sulfate. Clones belonging to a purple nonsulfur clade of  $\alpha$ -*Proteobacteria* were detected in small numbers in the mat clone library. However, most purple nonsulfur bacteria examined in pure-culture studies have been shown to be inhibited by high sulfide concentrations (31). Therefore, species of the groups *Cyanobacteria* and *Chloroflexi* are likely responsible for oxidizing the majority of the abundant sulfide encountered in Zodletone spring. Sulfur produced via sulfide oxidation may be reduced by *Desulfuromusa*-related clones (and possibly by *Oscillatoria*-related clones) at night. Sulfate may be reduced via the sulfate-reducing populations identified by 16S and DSR analysis.

Diverse physiologies are encountered in cultures of species belonging to the  $\epsilon$ -*Proteobacteria* family. Therefore, assignment of a metabolic function to clones ZB43 and ZB47 (Fig. 4) is not feasible. However, the ability to oxidize various reduced sulfur species is a common feature in various members of the

$\epsilon$ -*Proteobacteria* family (42), and many of the clones belonging to the epsilon I group of the *Proteobacteria* family were retrieved from sulfur-rich environments (e.g., sulfurous caves [2], sulfur springs [59], and tube worms [42]). Given the sulfur-rich environment in Zodletone spring and the abundance of  $\epsilon$ -proteobacterial clones in the mat, a role for these microorganisms in sulfur oxidation is possible.

Another intriguing group encountered in the Zodletone spring mats is represented by 11 clones (three OTUs [ZB77, ZB78, and ZB81] [Fig. 4]) belonging to a novel  $\gamma$ -proteobacterial lineage. There is a large difference between clones belonging to this group and their closest well-described pure-culture relatives (12 to 15% sequence difference between these clones and members of the genus *Halothiobacillus*). However, these Zodletone clones (ZB77, ZB78, and ZB81) are similar to the aerobic sulfoxidizing isolate "*Thiobacillus bargeensis*." No detailed published account regarding this microorganism is available; hence, its physiology and metabolic capabilities are not known. In spite of the anaerobic conditions permanently prevailing in the Zodletone spring, the possibility of a role for aerobic sulfur-oxidizing bacteria (SOB) in sulfur cycling could not be disproved unequivocally. Microaerophilic isolates of SOB that grow at low oxygen thresholds had been previously described (84), and microelectrode studies indicate the possible existence of aerobic SOB at very low oxygen tensions (48, 58). Enumeration of microorganisms in the Zodletone spring capable of aerobic chemolithoautotrophic growth on thiosulfate, an intermediate in the aerobic sulfur oxidation pathway of many SOB (52), yielded  $2.3 \times 10^7$  cells/g of sediment (unpublished data), reflecting the possibility of a role for chemolithotrophic sulfide-oxidizing bacteria in Zodletone spring.

Although anaerobic sulfur cycling between phototrophic sulfide oxidizers and sulfate-reducing bacteria has long been documented, its occurrence at a surficial mat is rarely encountered. Sulfur cycling is known to occur in stratified lakes and ponds, where the sulfide produced by sediment sulfate-reducing populations is utilized by various groups of anoxygenic phototrophs, which position themselves at the appropriate light, oxygen, and sulfide concentrations (43). Also, the close association of aerotolerant sulfate-reducers with either cyanobacteria or aerobic sulfide oxidizers (e.g., *Beggiatoa* spp.) has been well documented in hypersaline lakes (8, 11, 14, 69). Recently, a mesophilic sulfide-rich surface spring was described and detailed microscopic examination of the mat community in that spring suggested the presence of a diverse phototrophic community as well as an abundance of different sulfur forms in the spring (16). However, the role of sulfate-reducing bacteria and various aspects of sulfur cycling in that spring have not yet been investigated.

An interesting feature of many of the clones retrieved from the spring source is their close phylogenetic affiliation to clones retrieved from natural or anthropogenic hydrocarbon-rich anaerobic ecosystems or hydrocarbon-degrading enrichment cul-

FIG. 5. Distance dendrogram constructed on the basis of the 16S sequences of bacterial clones (excluding *Proteobacteria*) encountered in the Zodletone spring mat and source clone libraries. Bootstrap values (percent) were determined on the basis of results for 1,000 replicates and are shown for branches with more than 50% bootstrap support. Each number (other than accession numbers) in parentheses represents the frequency of occurrence of a specific OTU in the source and mat clone libraries, respectively.

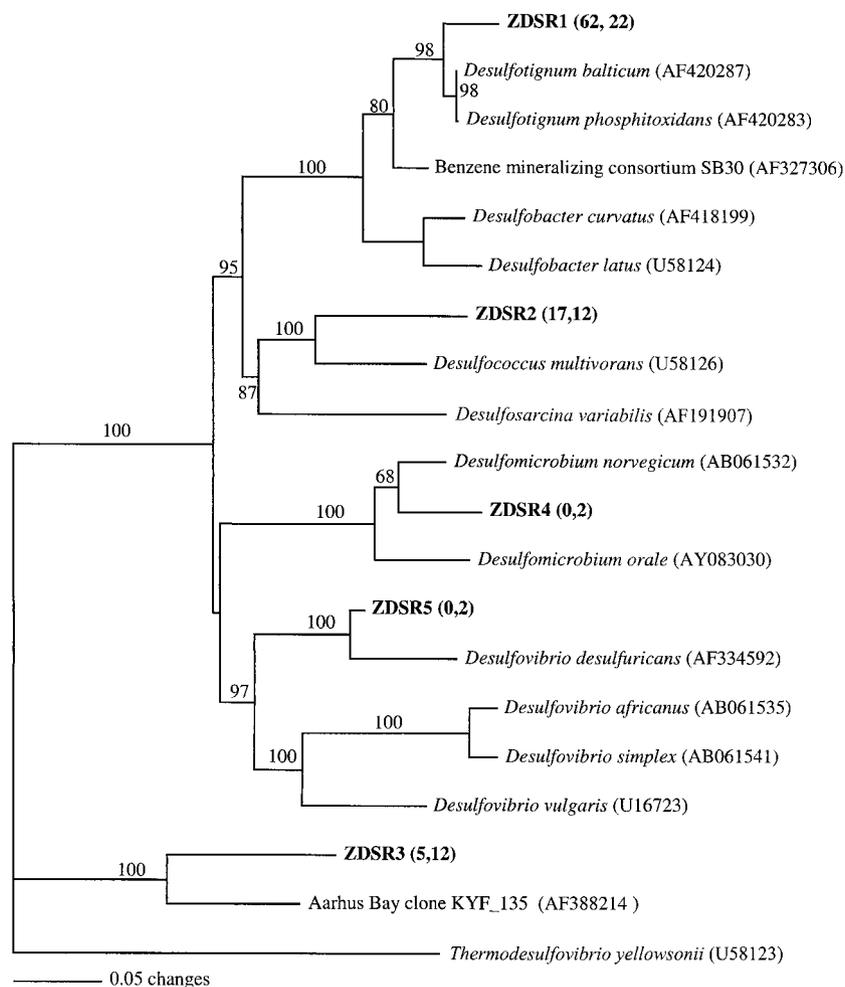


FIG. 6. Distance dendrogram constructed on the basis of translated amino acid sequences of the DSR gene amplified from the mat and the source of the Zodletone spring. Bootstrap values (percent) were determined on the basis of results for 1,000 replicates and are shown for branches with more than 50% bootstrap support. Each number (other than accession numbers) in parentheses represents the frequency of occurrence of a specific OTU in the mat and source clone libraries, respectively.

tures. For example, *Cytophaga* clones ZB29, ZB30, and ZB31 formed two bootstrap-supported clusters with clones retrieved from a benzene-mineralizing consortium (53), a dichloropropane-degrading community in an anaerobic bioreactor (61, 62), a trichlorobenzene-degrading consortium (77), clones from Guaymas Basin methane hydrate sediments (70), and a terrestrial chloroaromatic hydrocarbon-impacted site (15). The closest database relatives of *Planctomyces* clones ZB141 and ZB142 are clones retrieved from a natural hydrocarbon seep and from dichloropropane-degrading enrichments (61, 62). *Spirochaetes* clones ZB40 and ZB41 formed a bootstrap-supported cluster with 3-chlorobenzoate-degrading enrichments (77) and clones from a hydrocarbon-contaminated aquifer (15). Finally, *Verrucomicrobiae* clone ZB140 was also closely related to clones from the previously mentioned aquifer (15). This similarity between clones from different hydrocarbon-rich ecosystems has been previously observed (70, 77), and the reason for the selective enrichment for members of specific groups of the *Bacteria* and *Archaea* in such environments is still unclear. No pure cultures of species belonging to the family

*Cytophaga*, *Planctomyces*, *Spirochaetes*, or *Verrucomicrobiae* and capable of anaerobic degradation of hydrocarbons have yet been isolated, suggesting an indirect role for these microorganisms in hydrocarbon metabolism.

The anoxygenic microbial mats present in Zodletone spring bear an intriguing resemblance to surface ecosystems that might have occurred in ancient geological settings. The late Archean and early Proterozoic environment was characterized by a reduced atmosphere with little or no free oxygen and an abundance of reduced-sulfur or -iron species (80). During this period, sulfide and sulfur are believed to have been the dominant electron donors for phototrophic carbon fixation. Similarly, sulfur and sulfate reduction are believed to have been among the major respiratory processes occurring in this period (35, 78, 79, 80), although recent studies (25) challenge the importance of sulfate reduction in Archean settings. Some investigators have also concluded that methane was much more abundant in Earth's early atmosphere than today (3). Because oxygen is currently abundant in the atmosphere and methane is present only at low concentrations, it is uncommon

to find anoxic surficial environments similar to the Zodletone spring that support anoxic sulfur-based microbial processes and contain abundant methane.

Microfossil studies suggest that surficial mats of phototrophs were a prominent feature of the Archean era, with evidence suggesting the presence of cyanobacteria as a component in these mats (6). Cyanobacteria are known to have existed early in life history, although a significant rise in oxygen concentration (e.g., to 10% of present atmospheric level) in Earth's atmosphere was not achieved until 2.3 gigaannum ago (6, 63, 74). Sulfate reduction is thought to be an important process in Earth's early history, and recent studies suggested the importance and antiquity of sulfate reduction in the early biosphere (74, 78). Therefore, close cooperation between phototrophs and sulfate reducers in ancient microbial mats seems likely. Indeed, Shen et al. (63) recently described an Archean microfossil with an isotopic ratio that suggested the occurrence of sulfate reduction as early as 3.47 gigaannum before the present. Stromatolites were present immediately overlying these rocks, suggesting the presence of a simple but complete ecosystem in which ancient phototrophs provided the sulfate as well as electron donors necessary for the survival of the underlying sulfate-reducing bacteria now evident in the microfossil.

The ecosystem at the Zodletone spring is apparently similar to the system postulated by Shen et al. (63), in which carbon cycling in a highly productive ecosystem is dependent on sulfur cycling. Furthermore, our results suggest a prominent role for sulfur disproportionation in ancient ecosystems and provide evidence of the rich microbial diversity likely to have been present. The ecosystem described above also offers a unique opportunity for the study of the type of surficial anoxic environments that may have dominated the ancient Earth but that have since all but vanished. Our study suggests that these systems were characterized by high primary productivity via anoxygenic photosynthesis, sulfur cycling between sulfate reducers and phototrophs, and high phototrophic as well as overall microbial diversity.

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