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Assessment of the Diversity, Abundance, and Ecological Distribution of Members of Candidate Division SR1 Reveals a High Level of Phylogenetic Diversity but Limited Morphotypic Diversity▽†

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We used a combination of 16S rRNA gene clone library surveys, quantitative PCR (qPCR) analysis, and fluorescent in situ hybridization to investigate the diversity, abundance, and distribution of members of candidate division SR1 in multiple habitats. Using SR1-specific 16S rRNA gene primers, we identified multiple novel SR1 lineages in four different anaerobic environments: sediments from Zodletone Spring, a sulfide- and sulfur-rich spring in southwestern Oklahoma; inner layers of microbial mats obtained from Sperm Pool, a high-temperature, low-pH pool (55°C, pH 2.5) in Yellowstone National Park; fresh bovine ruminal contents; and anaerobic freshwater pond sediments (Duck Pond) in Norman, Oklahoma. qPCR analysis indicated that SR1 members constitute a small fraction (<0.01%) of the microbial communities in Duck Pond and ruminal samples but constitute a significant fraction (11.6 and 48.7%) of the total number of bacterial 16S rRNA genes in Zodletone Spring and the inner layers of Sperm Pool microbial mat samples, respectively. By using SR1-specific fluorescent probes, filamentous cells were identified as the sole SR1 morphotype in all environments examined, with the exception of Sperm Pool, where a second bacillus morphotype was also identified. Using a full-cycle 16S rRNA approach, we show that each of these two morphotypes corresponds to a specific phylogenetic lineage identified in the Sperm Pool clone library. This work greatly expands the intralineage phylogenetic diversity within candidate division SR1 and provides valuable quantification and visualization tools that could be used for investigating the ecological roles, dynamics, and genomics of this as-yet-uncultured bacterial phylum.

16S rRNA gene-based surveys conducted during the last two decades have convincingly demonstrated that the scope of bacterial diversity is much broader than previously implied using culture-based approaches (39, 57, 62). Remarkably, many of the novel lineages discovered using 16S rRNA surveys represent deep phylum-level branches within the domain *Bacteria* (21, 37), necessitating coining the term "candidate divisions" to describe such lineages (36). At this time (November 2008), the number of recognized candidate divisions varies in different taxonomical schemes (e.g., between 43 in NCBI taxonomic outline and 61 in Hugenholtz taxonomic outline in Greengenes web server [18]). These estimates will undoubtedly continue to rise with the implementation of novel sequencing technologies in microbial diversity studies (48), as well as with the recent availability of curated databases and rapid alignments and classification tools for 16S rRNA gene clone libraries (17, 18).

With the exception of the 16S rRNA sequences and description of the environment from which they were encountered, little is usually known regarding the physiological properties, energy conservation pathways, and ecological significance of the members of the majority of these novel candidate divisions. This is especially true for members of novel candidate divisions that have always been encountered as a minor component within environmental clone libraries (e.g., candidate divisions AC1, AD3, LD1, NC10, SC3, SC4, SPAM, TM6, OD1, and WS6) (21–23, 44), as well as those that are deposited in public databases but have not yet been described in peer-reviewed publications (e.g., candidate division ctg-CGOF).

Clearly, the development of lineage-specific oligonucleotide 16S rRNA primers and probes could enhance our understanding of the breadth of phylogenetic diversity within these groups (12, 20, 25, 28, 33, 38) and aid in the implementation of targeted genomics investigation (59–61). One of these yet-uncultured lineages is candidate division SR1, which has frequently been reported in 16S rRNA gene clone libraries, especially those derived from anaerobic habitats. Sequences belonging to members of candidate division SR1 as recognized today were first encountered in a survey of a hydrocarbon-contaminated aquifer (21), in which they were classified as members of candidate division OP11 (21, 36). Shortly afterwards, 16S rRNA sequences belonging to candidate division SR1 as recognized today were reported from deep-sea sediments (15, 43), hydrothermal vents (63), oral cavity (GenBank accession number AF125207), termite gut (34), diseased coral tissue (27), near-boiling, silica-depositing thermal springs (7), and a mesophilic sulfide and sulfur rich spring (22). SR1 sequences were referred to in these studies as unknown or unaffiliated (7, 43), candidate division VC2 (22), *Aquificales* related (15), or OP11 (34). Harris et al. (32) recognized the polyphyletic nature of candidate division OP11 as originally proposed (36, 37) and suggested the name candidate division SR1 (after Sulfur River, KY, where additional SR1 sequences were encountered) to describe members of the OP11-4 group. Since then, multiple SR1 sequences have been reported from diverse environments,

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TABLE 1. Oligonucleotide primers or probes used in this study

Primer or probe	*E. coli* positions	Sequence (5′ to 3′)	Reference(s)	Function(s)	Optimized conditions	
					PCR annealing temp (°C)[a]	Formamide concn/NaCl concn[b]
SR1-427F	427–445	GAAGAMGMATGACGGTAC	This study	Clone library, qPCR	52[c]	NA
SR1-427R	427–445	GTACCGTCATKCKTCTTC	This study	FISH	NA	55/20
SR1-668R	668–686	CCACCKGAAATTCCACTA	This study	qPCR	52[c]	NA
SR1-914R	914–932	GYTCCCCCGCCTATCCYT	This study	Clone library	58[d]	NA
SR1-1075R	1075–1093	TTAACYRGACACCTTGCG	This study	Clone library, qPCR, FISH	52[e]	55/20
SR1I-232R	232–249	TAGCTGGTGGTCCGCGCC	This study	FISH	NA	55/20
SR1V-575R	575–592	TATGTCGGGCTACGGACA	This study	FISH	NA	60/14
Non-EUB-338F	320–338	ACATCCTACGGGAGGC	3, 4	qPCR, FISH	54[f]	35/80
EUB-518R	518–537	CGTATTACCGCGGCTGCTGG	53	qPCR	54[f]	NA
Bact-27F	9–27	GAGTTTGATCMTGGCTCAG	41	Clone library	58[d]	NA
Univ1390	1390–1410	GACGGGCGGTGTGTACAA	76	FISH	NA	0/900
Cren499	499–516	CCAGRCTTGCCCCCCGCT	11	FISH	NA	0/900

[a] Optimized PCR annealing temperatures are for primer pairs. NA, not applicable.
[b] The optimum formamide concentration in the hybridization buffer is shown as a percentage (vol/vol), and the corresponding optimum NaCl concentration in the washing buffer is shown in millimolar concentration. NA, not applicable.
[c] Primer pair SR1-427F/SR1-668R.
[d] Primer pair 27F/SR1-914R.
[e] Primer pair SR1-427F/SR1-1075R.
[f] Primer pair EUB-338/518R.

e.g., sulfur-rich springs and caves (31, 46), microbial mats in various hot springs in Yellowstone National Park (50, 68), deep-sea sediments (73), hydrothermal vents (16, 51, 55, 58), and insect and animal guts and alimentary tracts (42, 52, 75). A list of all partial and near-complete SR116S rRNA gene sequences deposited in GenBank is provided as supplemental material (see Table S1 in the supplemental material).

In this study, we developed multiple SR1-specific primers and probes based on SR1 sequences currently available in public databases and used them for the following: (i) an in-depth investigation of the intralineage phylogenetic diversity within candidate division SR1 in multiple habitats; (ii) enumeration of SR1 16S rRNA genes using quantitative PCR (qPCR); and (iii) visualization of SR1 cells using fluorescently labeled probes and linking the observed morphologies to specific SR1 lineages encountered in this study using a full-cycle 16S rRNA approach. We describe multiple novel lineages and a high level of phylogenetic diversity within candidate division SR1. We also demonstrate that in contrast to this high phylogenetic diversity, SR1 cells appear to have limited morphotypic diversity, with only two morphotypes identified in all ecosystems examined.

MATERIALS AND METHODS

Site description, sampling, and geochemical measurements. The diversity, abundance, and morphology of members of candidate division SR1 were investigated in multiple habitats: (i) sediments from an anaerobic, sulfide- and sulfur-rich spring (Zodletone Spring) in southwestern Oklahoma (see references 22 and 66 for a detailed description of the spring); (ii) the middle layer (1 cm) of a multispecies microbial mat (approximately 5 cm thick) collected from the outfall channel of Sperm Pool, a high-temperature (55°C), acidic (pH 2.5) pool in Yellowstone National Park; (iii) fresh bovine ruminal contents from grass-fed fistulated cows in Oklahoma State University Animal Nutritional Physiology Center (Stillwater, OK); (iv) sediments from an anaerobic, mesophilic freshwater pond (Duck Pond) in Norman, OK; (v) soil samples from Kessler farm biological station in central Oklahoma (see reference 23 for description of the site and soil properties); and (vi) two crude oil-impacted soil samples and one pristine aerobic surface soil sample collected from a tall grass prairie preserve in Osage County in Oklahoma from a site adjacent to an oil pipeline break.

Samples collected for 16S rRNA gene analysis and qPCR analysis from Zodletone Spring, Kessler farm biological station soil, Duck Pond, and cow ruminal contents were stored on ice or dry ice until being transferred to the laboratory where they were frozen at −20°C till further analysis. Sperm Pool and prairie soil samples were kindly provided by Babu Fathepure (Oklahoma State University) and Kathleen E. Duncan (University of Oklahoma), respectively. These samples were shipped on ice and stored in the laboratory at −20°C. Samples for fluorescent in situ hybridization (FISH) from Zodletone Spring, ruminal contents, and Duck Pond were fixed on-site (1:3) in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) (154 mM NaCl, 1.69 mM KH_2PO_4, 5 mM Na_2HPO_4) and stored on ice. Yellowstone microbial mat samples were shipped on dry ice and fixed upon arrival to the laboratory as described above.

For geochemical measurements, samples for sulfide analysis were added directly to an equal volume of 10% zinc acetate solution on-site to fix sulfide. Sulfide was quantified as previously described (35). Sulfate and nitrate were quantified by ion chromatography as described before (66). Zero-valent sulfur was determined as previously described in detail in reference 66. Briefly, samples were acidified to pH 1.5 to 2 on-site to precipitate sulfane sulfur and were quantified as sulfide following a Cr(II) extraction procedure (72).

Primers and probe design. Oligonucleotides selectively targeting members of candidate division SR1 were designed using the probe design function on SR1-affiliated sequences available in Greengenes May 2007 database in ARB software package (45). Candidate oligonucleotides obtained were further evaluated against a more detailed SR1 list that includes, in addition to sequences available in the ARB database, partial SR1 sequences (<1,200 nucleotides) available in GenBank database, and SR1 sequences that were recently deposited (from June 2007 to October 2008) in GenBank (see Table S1 in the supplemental material). Redundancies were added to increase coverage when necessary, and the probes obtained were tested for specificity using BLASTnr search (2), as well as RDP probe match function within the Ribosomal Database Project web server (13). Four SR1-specific oligonucleotides were obtained and used as primers for diversity analysis, qPCR analysis, and/or as FISH probes in this study (Table 1).

Probes specific to SR1 subgroup I and SR1 subgroup V lineages (see Results below) were designed in our attempt to link observed cell morphologies to certain phylogenetic lineages in Sperm Pool samples using a full cycle 16S rRNA gene approach (see Results below) (4). Lineage-specific probes were designed by importing Sperm Pool community sequences into the ARB database and using ARB probe design function (45). The two probes obtained (Table 1) were evaluated for specificity and coverage as described above.

DNA extraction, construction, and sequencing of 16S rRNA gene clone libraries. DNA was extracted from all environments using the FastDNA spin kit for soil (MP Biomedicals, Solon, OH). 16S rRNA gene of members of candidate division SR1 was selectively amplified using the *Bacteria*-specific forward primer 27F and the SR1-specific primer 914R (Table 1). Compared to other primer

combinations (SR1-427F/SR1-668R, SR1-427F/SR1-1075R, and 27F/SR1-1075R), this primer pair combination gave a fairly long amplicon length (ca. 900 bp), and had absolute specificity for SR1 sequences. PCR was conducted in a 50-μl reaction mixture containing the following (final concentrations are given): 2 μl of extracted DNA, 1× PCR buffer (Promega), 2.5 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphate mixture, 2.5 U of GoTaq Flexi DNA polymerase (Promega, Madison, WI), and 10 μM of each of the forward and reverse primers. PCR amplification was carried out according to the following protocol: initial denaturation for 5 min at 95°C, followed by 30 cycles, with 1 cycle consisting of denaturation at 95°C for 45 s, annealing at 52°C for 45 s, and elongation at 72°C for 1.5 min. A final elongation step at 72°C for 15 min was included. PCR products obtained were cloned into a TOPO-TA cloning vector according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA), and sequenced as previously described (22).

Phylogenetic analysis. To check the phylum level affiliations of clones obtained in this study, sequences were initially compared to entries in GenBank database using BLASTnr (2). In addition, sequences were aligned in Greengenes NAST aligner to a 7,862-character global alignment (17) and run through Greengenes classifier (18). In addition to Greengenes classifier output, the NAST-aligned sequences were imported to Greengenes May 2007 ARB database and added to the ARB universal dendrogram using the ARB parsimony function to determine their position in the global phylogenetic tree (45).

For operational taxonomic unit (OTU) assignment and phylogenetic tree construction, SR1 sequences were aligned using ClustalX program (71), and the alignments were exported to PAUP (version 4.01b10; Sinauer Associates, Sunderland, Mass). A pair-wise distance matrix generated in PAUP was exported to DOTUR (64) and used for assignment of OTUs at 97% sequence similarity cutoff. Basic diversity measurements, e.g., Shannon-Weiner diversity index, average nucleotide diversity, and Good's coverage were calculated as previously described (29, 47, 49). Phylogenetic trees were constructed using SR1 OTUs from this study and representative closely related reference sequences. Distance neighbor-joining trees with no corrections, F-84 corrections, and Jukes-Cantor corrections were constructed using PAUP and gave similar tree topologies.

qPCR. We used qPCR to quantify members of SR1 in multiple environments by using a MyiQ thermocycler (Bio-Rad Laboratories, Hercules, CA) and B-R SYBR green SuperMix for iQ (Quanta Bioscience, Inc., Gaithersburg, MD). The primer pair SR1-427F/SR1-668R was used for SR1 quantification, and the primer pair EUB-338/UNI518R (23, 24) was used to amplify the total bacterial community. Specificity of primer pair SR1-427F/SR1-668R was initially confirmed by cloning the PCR product obtained and sequencing 12 clones, all of which were affiliated with SR1 (data not shown).

The 25-μl PCR reaction mixture contained 0.3 μM of each forward and reverse primers (final concentration), 3 μl extracted template DNA, and 12.5 μl B-R SYBR green SuperMix. The reactions were heated at 95°C for 3 min, followed by 55 cycles, with one cycle consisting of 20 s of 95°C and 30 s at 52°C or 54°C. A pCR 4-TOPO (Invitrogen) plasmid with an SR1 16S rRNA gene insert generated using primer pair 27F and 914R was used as a positive control, as well as to generate a standard curve for both reactions. The efficiency of the amplification of the standards (E) was calculated from the slope of the standard curve using the formula $E = (10^{-1/\text{slope}}) - 1$.

FISH. (i) Sample preparation, sediment removal, and fixation. Paraformaldehyde-fixed samples were centrifuged at high speed (14,000 × *g* for 10 min) to remove the fixant. For Zodletone Spring and Duck Pond sediments, the pellet was resuspended in PBS containing 100 mM sodium pyrophosphate followed by vigorous shaking for 10 min at room temperature. Large sediment particles were removed by centrifugation at 2,000 × *g* for 10 min. The supernatant containing the cells and finer sediment particles was subjected to a higher speed centrifugation (6,000 × *g* for 5 min) to remove fine sediment particles followed by high-speed centrifugation at 14,000 × *g* for 10 to 15 min to collect cells. The pellet was then resuspended in a minimal volume of PBS, and the presence of cells was confirmed by phase-contrast microscopy. Fixed cells were stored at −20°C in PBS-ethanol (1:1) until their use for FISH. For the Sperm Pool mat samples, which contained no sediments, paraformaldehyde was removed by centrifugation at 14,000 × *g* for 10 to 15 min. The microbial mat was resuspended in PBS and dispersed using VDI 12 sonicator (VWR Corp., West Chester, PA) pulses (two or three pulses [each pulse 30 s [) at position 2 and then stored in PBS-ethanol (1:1) at −20°C until use.

(ii) Hybridization and visualization. Alexa Fluor 488-labeled probes S-P-SR1-0427-a-A-18 (1) (SR1-427) and S-P-SR1-1075-a-A-18 (SR1-1075), targeting all members of candidate division SR1, as well as Alexa Fluor 488-labeled probes S-P-SR1I-0232-a-A-18 (SR1-232), and probe S-P-SR1II-0575-a-A-18 (SR1-575), which target subgroups I and V in BD2-14 lineage within SR1, respectively, were synthesized by Invitrogen. All probes are named after the probe nomenclature scheme by Alm et al. (1). All hybridizations were done on Cel-Line slides (Thermoscientific, Portsmouth, NH) with Adcell bioadhesive coating. Six-well slides were used for independent sample positioning. Hybridizations were carried out as previously described (3). Briefly, 10-μl aliquots of fixed cells in PBS were put into individual wells and allowed to air dry. Cells were then dehydrated in an increasing series of ethanol solutions (50, 80, and 100%) for 3 min in each solution. Hybridization buffers contained 900 mM NaCl, 0.1% sodium dodecyl sulfate, 20 mM Tris HCl, the required percentage of formamide (see below), and a final concentration of 5 ng/μl of oligonucleotide probe (when two probes were used, a final concentration of 5 ng/μl of each was used) in a final volume of 10 μl. All hybridizations were carried out at 46°C for 3 h in humid chambers. Slides were then rinsed with prewarmed (48°C) washing buffer, followed by a 25-min immersion in washing buffer at 48°C. Washing buffers contained 0.1% sodium dodecyl sulfate, 20 mM Tris HCl, and depending on the formamide concentration in the hybridization buffer, between 10 and 900 mM NaCl (Table 1). Slides were then rinsed in nanopure water, allowed to air dry, followed by a 10-min incubation at room temperature in the dark with the DNA-binding dye 4′,6′-diamidino-2-phenylindole (DAPI) (final concentration of 10 μg/ml). Excess DAPI was then rinsed off, and the slides were air dried in the dark and then mounted in 4% *n*-propyl gallate in 90% (vol/vol) glycerol in PBS.

Slides were visualized using an Olympus BX51 microscope (Olympus, Center Valley, PA), equipped with Brightline fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) filter sets for Alexa Fluor 488 and Alexa Fluor 546 fluorescence, as well as a Brightline DAPI high-contrast filter set for DAPI fluorescence. Photomicrographs were taken with a DP71 digital camera (Olympus). Exposure times were 10 ms for DAPI fluorescence and 100 ms for Alexa Fluor fluorescence.

FISH controls. Due to the unavailability of pure culture representatives of candidate division SR1, we used clone FISH (65) to determine the optimum formamide concentration for probe hybridizations. Clones containing the target sequence for the SR1-427 probe were obtained from our ruminal fluid clone library. Several clones were sequenced to identify a clone with the insert in the forward direction. Since the SR1-1075 probe targets a region that is not amplified with SR1 primers used in this study, we used a synthetic 362-bp 16S rRNA gene segment corresponding to nucleotide positions 810 to 1163 in an uncultured SR1 clone from Sulfur River in Kentucky (32), GenBank accession number AY193201). Gene synthesis was performed by Genscript Corp. (Piscataway, NJ), and the insert (supplied in pUC57 vector that lacks a T7 priming site) was cloned into PCR-4-Topo vector, and subsequently transformed into TOP10 chemically competent host cells (Invitrogen), according to the manufacturer's instructions. Several clones were then sequenced to identify a clone with the insert in the forward direction. These clones, now carrying the target sequence for either the SR1-427 or SR1-1075 probe and a T7 priming site (while the host cells lack a T7 RNA polymerase) could be used for clone FISH following an overnight incubation with chloramphenicol (170 mg/liter) to increase the plasmid copy number and generate high levels of target rRNA through leaky transcription from the T7 priming site (65). The clones were then fixed in 4% paraformaldehyde (overnight at 4°C), washed twice in PBS, and then stored at −20°C in PBS-ethanol until subjected to FISH. Formamide concentrations of 35 to 65% were tested in 5% increments to determine the optimum concentration for use with environmental samples (Table 1).

Negative controls. Using the RDP database probe check, we identified cultured microorganisms with one mismatch to SR1 probes to be used as negative controls. *Desulfotomaculum geothermicum* strain B2T (DSMZ 3669; GenBank accession number AJ621886) has one mismatch to the SR1-427 probe, and *Vulcanisaeta souniana* strain IC059^T (DSMZ 14430; GenBank accession no AB063645) has one mismatch to the SR1-1075 probe. Both microorganisms were obtained from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany) and cultured in the appropriate culturing medium. Cells were fixed and stored as described above until they were used for FISH. To ensure that negative hybridization with the SR1 probes in the negative controls was due to target sequence mismatch and not due to the inaccessibility of the probes to the target site, the Alexa Fluor 546-labeled universal probe Univ1390 (76) and *Crenarchaeota* probe Cren499 (11) were used as FISH-positive controls for *D. geothermicum* and *V. souniana* strains, respectively (the universal probe Univ1390 has a single mismatch with *V. souniana* 16S rRNA gene and hence could not be used). The non-EUB-338 probe (4) was also included as an additional control to monitor nonspecific probe binding. For *V. souniana*, cell wall permeabilization was required (by incubation with proteinase K 4U/ml at 37°C for 1 h; Sigma, St. Louis, MO) due to its protein-rich cell wall (70, 74).

Nucleotide sequence accession numbers. Sequences generated in this study were deposited in GenBank under accession numbers FJ479804 to FJ480103.

TABLE 2. Diversity within SR1 clone libraries generated in this study

Environment	No. of clones	No. of OTUs	Shannon-Weiner diversity index	Maximum sequence divergence	Avg nucleotide diversity (θ)	% Coverage
Zodletone Spring	158	18	2.26	0.25	0.12	98.7
Sperm Pool mat	79	8	1.37	0.26	0.11	94.9
Duck Pond	30	2	0.69	0.21	0.09	100.0
Bovine rumen	33	2	0.14	0.05	0.01	97.0

RESULTS

Phylogenetic diversity of members of candidate division SR1 in multiple anaerobic habitats. SR1 sequences were identified in Zodletone Spring, Sperm Pool microbial mat, Duck Pond, and cow ruminal samples. SR1 sequences, however, were not detected in the soil samples examined in this study. This is in agreement with the fact that SR1-affiliated sequences have never been encountered in any of the thousands of soil clone libraries constructed and analyzed so far (39) and suggests that this is due to the unavailability of suitable conditions in soils, rather than inadequate sampling.

We sequenced a total of 300 clones from these four environments, with the aim of achieving high coverage ($\geq$95%) within each clone library. The number of clones, OTUs, and various sequence diversity estimates per environment (e.g., Shannon-Weiner diversity index, mean sequence divergence, and average nucleotide divergence) are shown in Table 2. Clearly, Zodletone Spring and Sperm Pool microbial mats were the most diverse, while bovine ruminal samples and Duck Pond were the least diverse, with only two OTUs in each of these two libraries (Table 2).

Within the Hugenholtz taxonomic outline in Greengenes database, candidate division SR1 is currently divided into two subphylum level lineages, BH1 and BD2-14, after clones BH1 (GenBank accession number AF352532) and BD2 to BD14 (GenBank accession number AB015542) from Japan deep-sea sediments and Black Pool in Yellowstone National Park, respectively (7, 43). Members of BH-1 lineage have been encountered only in geothermal habitats, mainly hot springs and pools at Yellowstone National Park (7, 50, 56, 68), as well as California (GenBank accession number EU942246), and Tibet (GenBank accession number EF205568). Members of this monophyletic lineage have high sequence divergence (30.0 to 33.7%) from their closest SR1 clones belonging to BD2-14 lineages. Indeed, the BH1 and BD2-14 lineages are not always monphyletic in trees constructed using taxa belonging to phyla loosely related to SR1 (e.g., OP11 and OD1) using parsimony and neighbor-joining algorithms (data not shown). Future availability of sequences belonging to the BH1 lineage might warrant designating this group as an independent candidate division.

Compared to the relatively limited ecological distribution of the BH-1 lineage, members of the BD2-14 lineage are relatively more widely distributed, being detected in geothermal environments as well as low-temperature terrestrial and marine environments with various degrees of salinity (Fig. 1) (see Table S1 in the supplemental material). All sequences retrieved in this study (157 clones from Zodletone Spring, 71 from Sperm Pool, 30 from cow rumen, and 33 from Duck Pond) belonged to various subgroups within this lineage. Phylogenetic analysis (Fig. 1) identified nine bootstrap-supported subgroups within the BD2-14 lineage. Some of these lineages (subgroups I, VI, and IX) are exclusively represented by sequences retrieved in this study and hence represent novel lineages within candidate division SR1 (Fig. 1).

All bovine ruminal fluid sequences belonged to two OTUs, both of which were closely related and belonged to BD2-14 subgroup III. It is interesting to note that this lineage is exclusively composed of SR1 clones retrieved from human (e.g., oral cavity, esophagus), mammalian (cow rumen, rhinoceros feces), or insect (termite) origins (Fig. 1).

Sequences from Sperm Pool microbial mats (71 clones) belonged to seven OTUs and two distinct SR1 subgroups. One group (34 clones, two OTUs) belonged to subgroup V and was closely related to sequences from Zodletone Spring, Duck Pond, and deep-sea clone BD2-14 (Fig. 1). The second group (37 clones, six OTUs) formed a bootstrap-supported, deep-branching, and distinct novel SR1 lineage (subgroup I), together with a single OTU retrieved from a Duck Pond clone library. Members of this lineage have extremely low sequence similarity (78 to 80%) to their closest SR1 relative outside this group.

The Zodletone Spring SR1 community was clearly the most diverse of all environments tested, judging by nucleotide sequence diversity parameters (Table 2), as well as the fact that Zodletone Spring SR1 sequences belonged to seven out of the nine SRI-BD2-14 lineages (subgroups II, IV, V, VI, VII, VIII, and IX). Three lineages (subgroups II, VI, and IX) are exclusively formed by sequences encountered in Zodletone Spring, either only in this study (subgroups VI and IX), or in this study as well as in a clone library generated using general bacterial primers (subgroup II, clone ZB18 in Fig. 1) in a previous study (22).

Candidate division SR1 quantification. We used primer pair SR1-427F and SR1-668R to quantify SR1 16S rRNA gene copies in all four environments examined in this study. Members of candidate division SR1 have the lowest 16S rRNA gene copy numbers in Duck Pond, followed by bovine rumen, and in both samples, members of SR1 represented a small fraction (less than 0.01%) of the total 16S rRNA gene copy number (Table 3).

In contrast to the low SR1 16S rRNA gene copy numbers in these two environments, SR1 16S rRNA genes were present in much higher numbers and represented a higher fraction of the community in Zodletone Spring sediments and Sperm Pool microbial mat sample (Table 3). These results clearly indicate that under appropriate conditions, members of candidate division SR1 could form a significant fraction and an integral

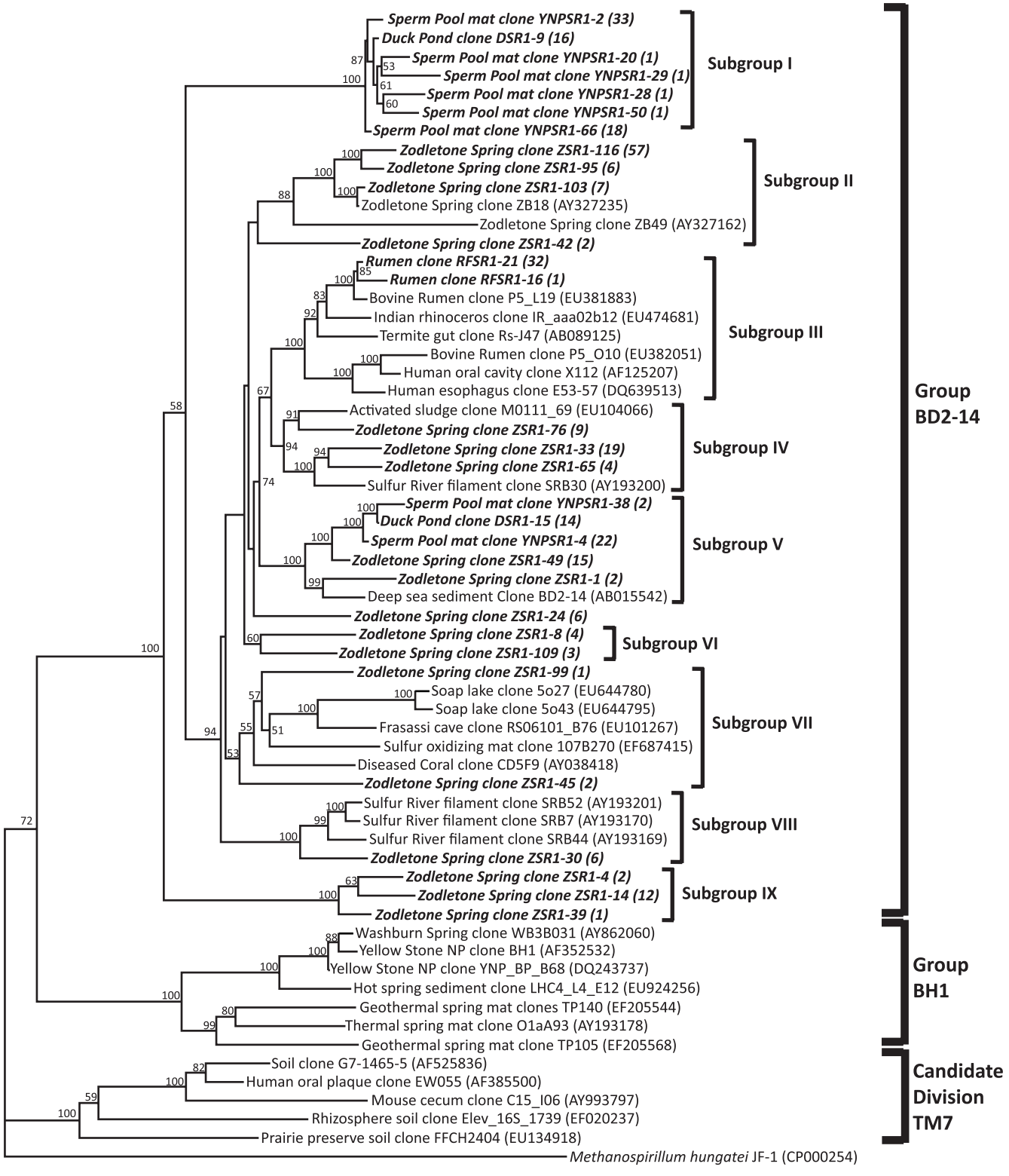

FIG. 1. Phylogenetic tree based on 16S rRNA gene sequences of members of candidate division SR1 encountered in this study. Bootstrap values (expressed as percentages) are based on 1,000 replicates and are shown for branches with bootstrap values of more than 50%. Sequences generated in this study are in boldface type, with the number of clones in each OTU reported in parentheses. GenBank accession numbers are shown in parentheses for other clones. The tree was constructed with the neighbor-joining algorithm with Jukes-Cantor corrections as described in Materials and Methods.

TABLE 3. SR1 quantification using qPCR in multiple environments

Environment	No. of 16S rRNA gene copies[a]		% SR1
	SR1	Total	
Zodletone Spring	1.50×10^7	1.29×10^8	11.6
Bovine rumen	1.24×10^6	1.55×10^9	0.08
Sperm Pool mat	1.22×10^{10}	2.51×10^{10}	48.7
Duck Pond	6.28×10^4	6.74×10^8	0.009

[a] Values are expressed as the number of 16S rRNA genes/gram of sample. Values are averages of triplicate measurements.

part of a microbial community and could potentially fulfill an as-yet-unidentified but potentially crucial role(s) within the mat ecosystem.

Visualization of candidate division SR1 using FISH. We used Alexa Fluor-labeled phylum-specific SR1 probes to visualize cells belonging to candidate division SR1 in situ. The persistence of solid particulates unremovable after successive centrifugation, coupled with the low proportion of SR1 cells (Table 3), and the autofluorescence of microeukaryotes within the bovine rumen, prevented us from effectively visualizing SR1 within this habitat. However, members of SR1 were identified in Duck Pond, Zodletone Spring, and Sperm Pool. Clone FISH suggested that a formamide concentration of 55% was ideal for both SR1-427 and SR1-1075 probes. No signal was detected when these two probes were used against pure cultures of *D. geothermicum*, and *V. souniana* at the appropriate formamide concentration. On the other hand, cells of *D. geothermicum* and *V. souniana* were successfully labeled and visualized using Univ1390 and Cren499 probes but not the nonEUB-338 probe (data not shown). These controls ensure that cells identified using SR1-427 and SR1-1075 probes in natural habitats with a formamide concentration of 55% in the hybridization buffer belong to candidate division SR1.

In Zodletone Spring sediments, Duck Pond sediments, and Sperm Pool mats, FISH using both SR1-227 and SR1-1075 probes separately or in combination revealed a filamentous morphotype with a highly variable length of 2.7 to 137.5 μm in Zodletone Spring ($n = 15$), 6.36 to 32 μm in Duck Pond ($n = 32$), and 6.4 to 109 μm ($n = 44$) in Sperm Pool, but a constant cell width of 0.7 to 0.8 μm in all environments (Fig. 2). Cells sometimes appeared segmented, especially with DAPI staining (Fig. 2A). This cell morphology is strikingly similar to TM7 cells previously visualized in the oral cavity (56). The filamentous morphotype described above was the only cell morphology visualized in Zodletone Spring and Duck Pond sediments. However, within Sperm Pool microbial mats, a second SR1 cell morphotype was observed. These cells were bacilli with round ends (2.7 to 5.5 μm in length, 1.8 μm in width) ($n = 28$), which appeared mostly as single cells, but sometimes as doubles or chains (Fig. 2E and F).

It is interesting to note that in addition to observing two SR1 morphotypes in Sperm Pool mats, 16S rRNA analysis indicated that the SR1 community in the mat sample belonged to two distinct lineages (subgroups I and V in subphylum BD2-14 [Fig. 1]). This observation led us to hypothesize that each of these two lineages corresponds to a distinct cell morphotype. To examine this hypothesis, we used Alexa Fluor-labeled probes SR1-232 and SR1-575 to selectively target subgroups I and V, respectively. Using clone FISH, optimum formamide concentrations of 55 and 60% were determined for probes SR1-232 and SR1-575, respectively. Probe SR1-232, targeting subgroup I hybridized only to bacilli, indicating that members of SR1 BD2-14 group I are bacilli, while probe SR1-575 hybridized only to filaments, indicating that members of SR1 BD2-14 group I are filamentous (Fig. 3).

DISCUSSION

In this study, we designed, evaluated, and utilized multiple primers and probes targeting candidate division SR1 to investigate the phylogenetic diversity, abundance, and cell morphologies of this as-yet-uncultured lineage in multiple habitats. We show that the scope of phylogenetic diversity within candidate division SR1 is much broader than previously implied based on abundance of SR1 clones in clone libraries constructed using general bacterial primers. We also successfully developed and implemented qPCR and FISH protocols for quantification and visualization of SR1 cells in situ and demonstrated that members of candidate division SR1 have a limited morphotypic diversity in all environments examined, with either one or two morphotypes encountered in all environments examined.

The development of primers and probes targeting a specific microbial lineage allows for in-depth, targeted diversity surveys of the lineage (5, 6, 9, 38), spatial and temporal monitoring of the target lineage within a specific ecosystem, as well as between various ecosystems (9, 10), and detection of cell morphologies and quantification through FISH and qPCR (8, 24, 25, 38). Further, group-specific oligonucleotides are crucial for targeted genomic and metagenomic-based approaches, e.g., for the screening of metagenomic libraries (61), and the implementation of single-cell-based genomic approaches (60), as well as for targeted enrichment and isolation of novel representatives of these lineages (14, 28, 40, 67, 69). The large amount of information regarding the ecology, metabolic abilities, and growth characteristics of lineages for which primers and probes have been developed and implemented is in stark contrast to the dearth of information regarding lineages for which no similar effort have been made. This study thus represents a useful first step that aims to provide the tools necessary for better targeting SR1 cells and genomic fragments in natural environments, enrichments, as well as in metagenomic libraries.

This study greatly expands on the phylogenetic diversity within candidate division SR1 and identifies multiple novel lineages within this candidate division. With the exception of the Sperm Pool microbial mats, which were selectively chosen for this study based on prior knowledge of the high proportion of the SR1 cells within this specific layer of this microbial mat, diversity (as measured by richness, average nucleotide diversity, nucleotide range, and affiliation with SR1 lineages), and abundance (number of 16S rRNA gene copies/gram or milliliter) was highest in Zodletone Spring sediments and lowest in Duck Pond samples (Table 3). Factors controlling diversity and abundance of members of SR1 in various ecosystems have not yet been elucidated. However, it has previously been speculated, based on ecological distribution, that members of SR1 are involved in sulfur transformation (32), e.g., chemolithotrophic sulfide oxidation (58). Sulfide, elemental sulfur, and sul-

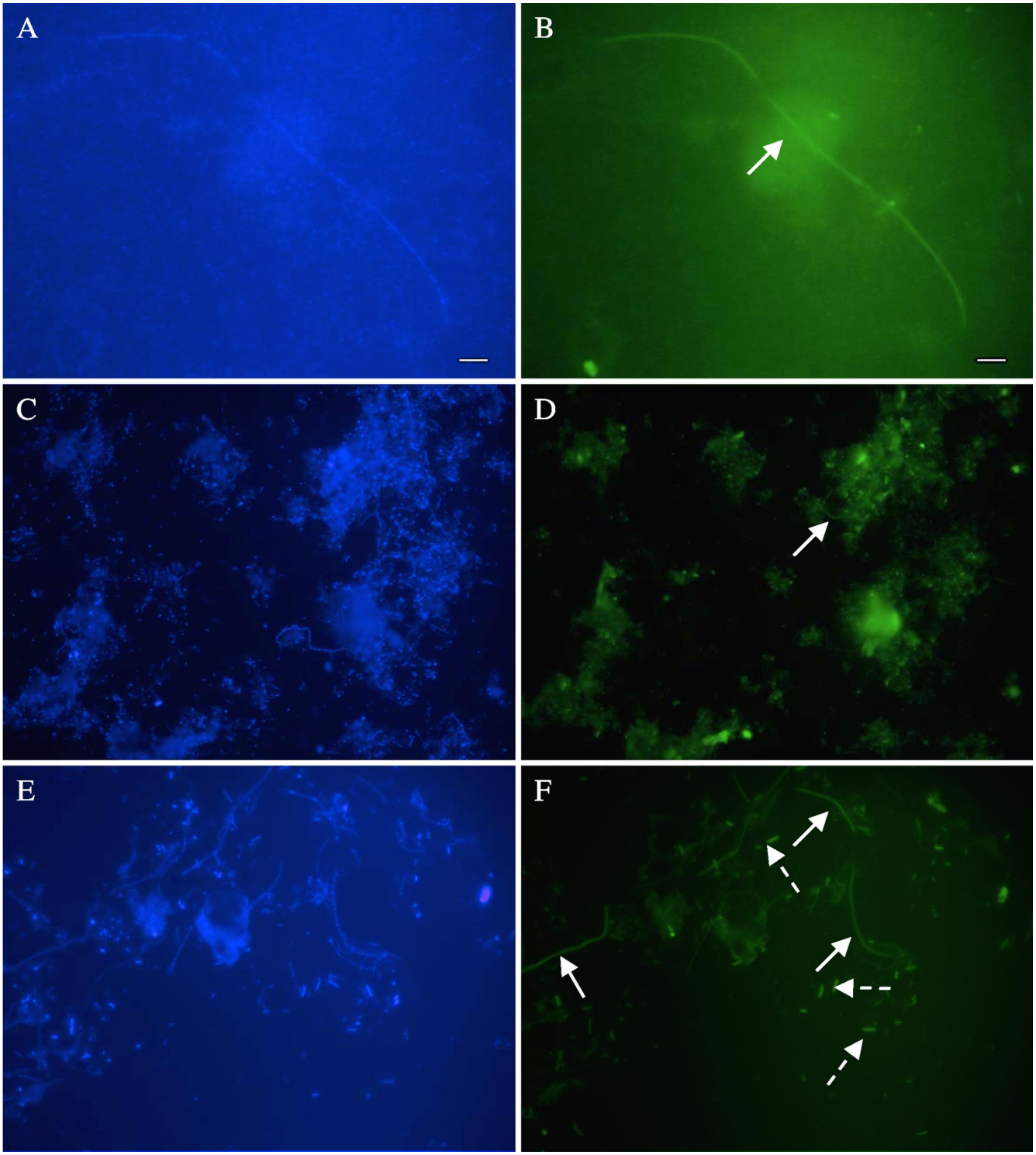

FIG. 2. Whole-cell hybridization of paraformaldehyde-fixed cells with Alexa Fluor 488-labeled candidate division SR1-427 probe. Panels A and B, C and D, and E and F depict DAPI-stained cells (A, C, and E) versus FISH-labeled cells (B, D, and F) of anaerobic sulfur spring (Zodletone Spring) source sediment sample (A and B), anaerobic freshwater sediment sample (Duck Pond) (C and D), and Yellowstone National Park Sperm Pool microbial mat sample (E and F). Note the scarcity of SR1 cells in Zodletone Spring (B) and Duck Pond (D) ecosystems, as opposed to the abundance of SR1 cells in Sperm Pool ecosystem (F). The solid white arrows point to the filamentous SR1 morphotypes in all three environments, while the white broken arrows point to the bacillus morphotype in Sperm Pool. The bars (10 μm) in panels A and B applies to all panels of the figure.

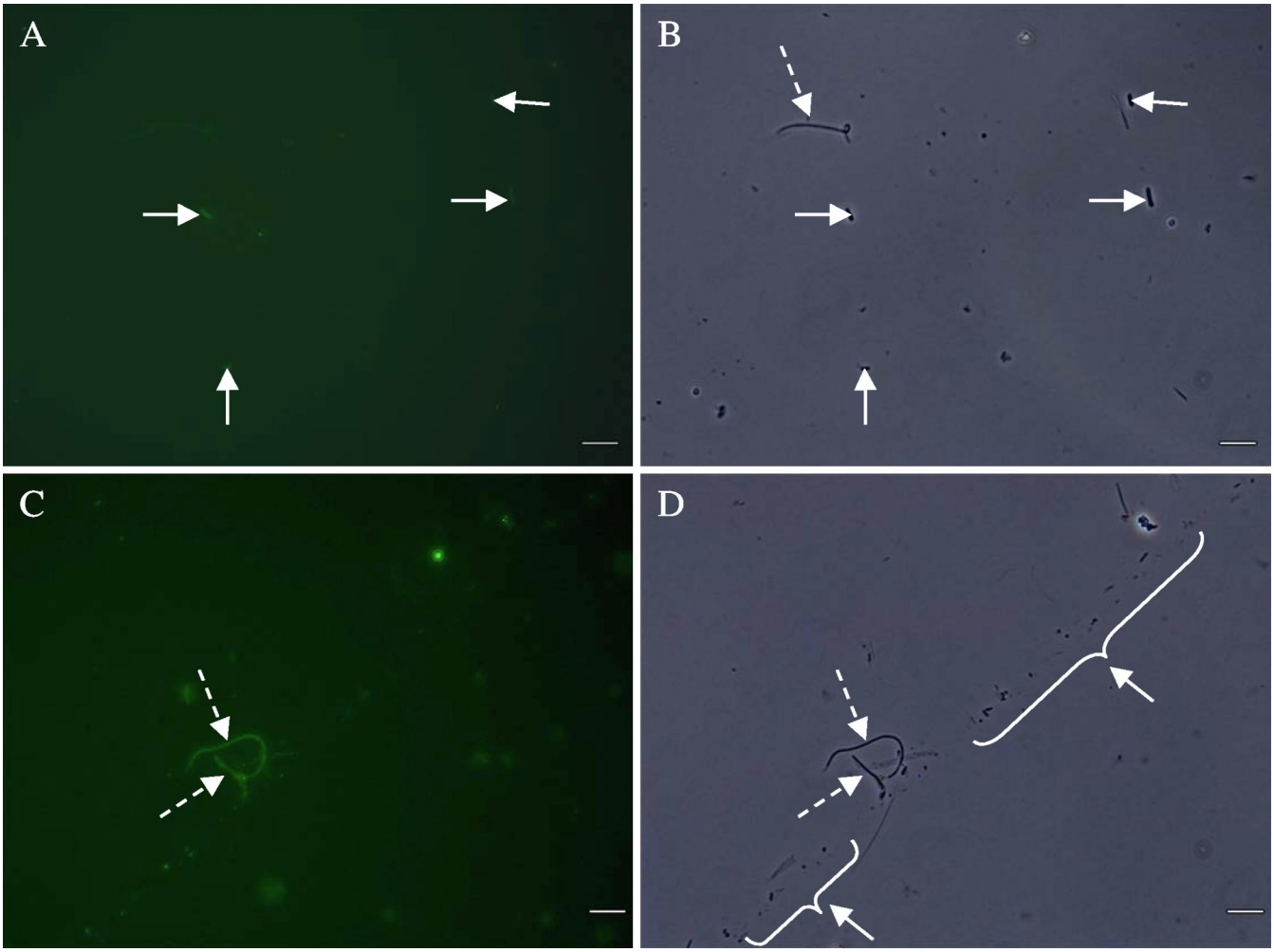

FIG. 3. Whole-cell hybridization of Yellowstone microbial mat paraformaldehyde-fixed cells with Alexa Fluor 488-labeled probe SR1-232 (specific for SR1 BD2-14 subgroup I) (A) and Alexa Fluor 488-labeled probe SR1-575 (specific for SR1 BD2-14 subgroup V) (C). Panels B and D are the phase-contrast images for the same microscopic fields shown in panels A and C, respectively. Solid white arrows point to bacillus-like cells labeled with probe SR1-232 but not with probe SR1-575, while white broken arrows point to filamentous cells labeled with probe SR1-575 but not with probe SR1-232. Bars, 10 μm.

fate measurements (Table 4) in all three environments show that sulfur and sulfide levels (but not sulfate) were also highest in Zodletone Spring than in Duck Pond and bovine ruminal fluid samples. This positive correlation between sulfur and sulfide levels on one side and SR1 numbers further attests to the potential role of members of SR1 in sulfur transformation. Information regarding Sperm Pool geochemistry is not available, but sulfur cycling and high sulfide and sulfur levels are known to be associated with various pools in Yellowstone National Park (7, 50, 68). Indeed, in clone libraries constructed using general bacterial primers from Sperm Pool microbial mats, SR1 sequences were associated with bacteria known to be involved in sulfur transformation within the mat (B. Fathepure, personal communication).

On the basis of this information, we hypothesize that members of candidate division SR1 have lower in situ growth rates than other more-competitive sulfur-metabolizing microorganisms (mainly within the *Proteobacteria*). A constant supply of

TABLE 4. Basic geochemical characteristics of anaerobic ecosystems examined in this study

Environment	Salinity (%)	pH	Temp (°C)	Sulfide concn (mM)	Sulfur concn (mM)	Sulfate concn (mM)	Nitrate concn (mM)
Zodletone Spring	0.2	6.8	20	15.6	4.2	0.04	0
Bovine rumen	3	6	41	0.31	0.18	0.006	0
Sperm Pool mat	ND[a]	2.5	53	ND	ND	ND	0.085
Duck Pond	0	7	20	5.3	0.47	1.1	ND

[a] ND, not determined.

fairly high levels of sulfur and sulfide (e.g., in Zodletone Spring), especially when coupled to extreme conditions, which constrains the growth of many microbial cells (e.g., low pH and high temperature in Sperm Pool), will result in easing the competition between members of SR1 and other sulfur-metabolizing microorganisms and SR1 abundance and diversity will then increase. On the other hand, in environments with limited supply and low levels of sulfate and elemental sulfur (e.g., bovine rumen, Duck Pond), members of SR1 will be outcompeted by more efficient sulfur metabolizers, resulting in lower abundance and diversity. While a sulfur-based metabolism for members of candidate division SR1 appears plausible, the nature of such sulfur-based metabolism (whether members of SR1 are chemolithotrophic sulfide or sulfur oxidizers as suggested by Perner et al. [58] or chemoorganotrophic or autotrophic sulfur reducers) is not yet clear. In addition, it is plausible that various members of candidate division SR1 might have multiple distinct metabolic abilities. Further, it is entirely possible that similar to certain bacterial and archaeal genera (e.g., *Beggiatoa* and *Thermoproteus*), some (or all) SR1 strains might grow mixotrophically, switching between chemolithotrophy and heterotrophy depending on the surrounding environmental conditions (26, 30).

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