

MICROBIAL DIVERSITY AND SULFUR CYCLING
IN AN EARLY EARTH ANALOGUE:
FROM ANCIENT NOVELTY TO MODERN COMMONALITY

By

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Abstract: Life emerged and diversified in the absence of molecular oxygen. The prevailing anoxia and unique sulfur chemistry in the Paleo-, Meso- and Neoproterozoic, and early Proterozoic eons may have supported microbial communities that differ from those currently thriving on the earth's surface. Zedler Spring in southwestern Oklahoma represents a unique habitat where spatial sampling could substitute for geological eons: from the anoxic, surficial light-exposed sediments simulating a preoxygenated earth, to overlaid water column where air exposure simulates oxygen intrusion during the Neoproterozoic. We document a remarkably diverse microbial community in the anoxic spring sediments, with 340/516 (65.89%) of genomes recovered in a metagenomic survey belonging to 200 bacterial and archaeal families that were either previously undescribed or that exhibit an extremely rare distribution on the current earth. Such diversity is underpinned by the widespread occurrence of sulfite-, thio-sulfate, tetrathionate-, and sulfur-reduction, and paucity of sulfate-reduction machineries in these taxa; hence greatly expanding lineages mediating reductive sulfur cycling processes in the tree of life. Analysis of the overlaying oxygenated water community demonstrated the development of a significantly less diverse community dominated by well-characterized lineages and a prevalence of oxidative sulfur cycling processes. Such transition from ancient novelty to modern commonality underscores the profound impact of the great oxygenation event on the earth's surficial anoxic community. It also suggests that novel and rare lineages encountered in current anaerobic habitats could represent taxa once thriving in an anoxic earth, but have failed to adapt to earth's progressive oxygenation. Life on earth evolved in an anoxic setting, however, the identity and fate of microorganisms that thrived in a pre-oxygenated earth is poorly understood. Collectively, such patterns strongly suggest that microbial diversity and sulfur cycling processes in a pre-oxygenated earth were drastically different from the currently observed patterns, and that the Great Oxygenation Event has precipitated the near extinction of a wide range of oxygen-sensitive lineages and significantly altered the microbial reductive sulfur-cycling community on earth.

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CHAPTER I

INTRODUCTION

Sulfur is one of the most abundant elements on earth, exhibiting a wide range of oxidation states (-2 to +6). Microorganisms have evolved a plethora of genes and pathways for exploiting sulfur-redox reactions for energy generation. Reductive processes employ sulfur oxyanions or elemental sulfur as terminal electron acceptors in anaerobic respiratory schemes linked to heterotrophic or autotrophic growth. Oxidative processes, on the other hand, employ sulfides or elemental sulfur as electron donors, powering chemolithotrophic and photosynthetic growth.

Thermodynamic considerations limit reductive sulfur processes to habitats where oxygen is limited. This is reflected in the global distribution of sulfate (SO_4^{2-}), sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), tetrathionate ($S_4O_6^{2-}$), and elemental sulfur (S0) reducing microorganisms (henceforth collectively referred to as SRM) in permanently and seasonally anoxic and hypoxic habitats in marine [48, 103, 109], freshwater [39], terrestrial [90], and subsurface [28] ecosystems. Sulfate is highly abundant on the current earth, and hence sulfate-reduction dominates reductive processes in the global sulfur cycle; although reduction and disproportionation of the intermediate sulfur species, e.g. sulfur [45, 98], sulfite [45], thiosulfate and tetrathionate [65, 115] could be significant in localized settings.

The history of earth's sulfur cycle is a prime example of a geological-biological feedback loop, where the evolution of biological processes is driven by, and dramatically impacts, the earth's biogeochemistry. The earth's surface was completely anoxic during the first two billion years of its history, and the availability and speciation of various sulfur species greatly differed from their current values. Sulfate levels were significantly lower when compared to current values in oceanic water (28 mM), with estimates of $< 200\mu M - 1mM$ from the Archean up to the Paleoproterozoic (2.3 Gya) [13, 14, 33, 87]. On the other hand, intermediate sulfur species appear to have played an important role in shaping the ancient sulfur cycle [32]. Modeling suggests that mM levels of SO_3^{2-} were attained in the Archean anoxic shallow surficial aquifers as a result of dissolution of the volcanic SO_2 prevailing in aquatic habitats [87]. Isotopic studies have demonstrated the importance of elemental sulfur, sulfite, and thiosulfate reduction in the Archean [32, 81].

The evolution of life (3.8 to 4.0 Gy) in the early Archean era and the subsequent evolution of major bacterial and archaeal clades in the Archean and early Proterozoic eons [67] occurred within this background of anoxia and characteristic sulfur-chemistry. As such, it has been speculated that organisms using intermediate forms of sulfur were likely more common than sulfate-reducing organisms [32]. However, while isotopic fractionation, modeling, and microscopic studies could provide clues on prevailing sulfur speciation patterns and prevalent biological processes, the identity of microorganisms mediating such processes



Figure 1.1: Zodletone Spring Source Sediments and Overlaid Water

is unknown. This is mostly due to constraints on preservation of nucleic acids and other biological macromolecules, with the oldest successful DNA sequenced sample being only 1.2 M years old [102].

Investigation of the microbial community in modern ecosystems with conditions resembling those prevailing in the ancient earth could provide important clues to the nature and identity of microorganisms that thrived under conditions prevailing prior to earth's oxygenation. In Zodletone spring, a surficial anoxic spring in southwestern Oklahoma, anoxic, surficial, light-exposed conditions are maintained in the sediments by constant emergence of sulfide-saturated water at the spring source from anoxic underground water formations in the Anadarko basin, along with gaseous hydrocarbons, which occur in seeps in the general vicinity. These surficial anoxic conditions also support a sulfur chemistry characterized by high levels of sulfide, sulfite, sulfur (soluble polysulfide), thiosulfate, and a low level of sulfate, as previously reported [12, 21, 97]. Microbial diversity using 16S rRNA amplicon surveys have reported higher level of phylogenetic diversity in the anoxic spring sediments, the affiliation of a fraction of the spring community with previously recognized sulfur-metabolizing lineages, as well as the high proportion of phylogenetically novel taxa in the spring anoxic sediments [21, 113, 112]. As such, the prevailing conditions at the spring source are reminiscent of ancient metabolic capacities prevailing on the earth's surface in the late Archean/early Proterozoic eons, as previously noted [12].

Further, the sediments at the source of the spring are overlaid by an air-exposed water column, and prior microsensor measurements and detailed geochemical analysis [12, 97] demonstrated that oxygen intrusion leads to a vertical oxygen gradient (from oxic in the top

1 μm , to hypoxic in the middle, to anoxic in deeper layers overlaying the sediments) (Figure 1.1). As such, contrasting communities between the anoxic sediments and the oxygen-exposed water column could provide a glimpse on how oxygen evolution has altered such communities. Here, we combined metagenomic, metatranscriptomic, and amplicon-based approaches to fully characterize the microbial community in Zodletone spring. Our results provide a glimpse of the community mediating the ancient sulfur cycle, significantly expand the overall microbial diversity by the description of a wide range of novel lineages, and greatly increase the number of lineages documented to mediate reductive sulfur processes in the microbial tree of life.

CHAPTER II

MATERIALS AND METHODS

2.1 Site description and geochemistry

Zoletone spring is located in the Anadarko Basin of western Oklahoma (N34.99562° W98.68895°). The spring arises from underground, where water is pumped out slowly along with sediments. Sediments settled at the source of the spring, a boxed square $1m^2$ (Figure 1.1), are overlaid with water that collects and settles in a concrete pool erected in the early 1900s. The settled water is 50-cm deep above the sediments and is exposed to atmospheric air. Water and sediments originating from the spring source are highly reduced due to the high dissolved sulfide levels (8-10 mM) in the spring sediments.

Microsensor measurements show a completely anoxic (oxygen levels $< 0.1 \mu\text{M}$) and highly reduced source sediments. Oxygen levels slowly increase in the overlaid water column from 2–4 μM in the 2 mm above the source to complete oxygen exposure at the top of the water column [12]. The spring geochemistry has regularly been monitored during the last two decades [12, 21, 92] and is remarkably stable. The spring is characterized by low levels of sulfate (50-94 μM), with higher levels of sulfite (0.21 mM), elemental sulfur (0.1 mM), and thiosulfate (0.52) [92, 97].

2.2 Sampling

Samples were collected from the source sediments and standing overlaid water in sterile containers and kept on ice until brought back to the lab (2h drive), where they were immediately processed. For metatranscriptomics, samples were collected at three different time points: morning (9:15 am), afternoon (2:30 pm), and evening (5:30 pm) in June 2019; stored on dry ice until transferred to the lab where they were stored at -80°C until processed for RNA extraction within a week.

2.3 Nucleic acid extraction

DNA was directly extracted from 0.5 grams of source sediments. For water samples, water was filtered on 0.2 μm sterile filters. DNA was directly extracted from filters (20 filters, 10 L of water samples). Extraction was conducted using the DNeasy PowerSoil kit (Qiagen, Valencia, CA, USA). RNA was extracted from 0.5 g sediment samples using RNeasy PowerSoil Total RNA Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

2.4 16S rRNA gene amplification, sequencing, and analysis

Triplicate DNA extractions were performed for both sediment and water samples from the Zodletone spring. To characterize the microbial diversity based on 16S rRNA gene sequences, we used the Quick-16S™ NGS Library Prep Kit (Zymo Research, Irvine CA), following the manufacturer’s protocol. For amplification of the V4 hypervariable region we used a mix of modified versions of primers 515F-806R [15], tailored to provide better coverage for several under-represented microbial lineages. They included 515FY (5’GTGYCAGCMGC-CGCGGTAA) [75], 515F-Cren (5’ GTGKCAGCMGCCGCGGTAA, for Crenarchaeota) [82], 515F-Nano (5’GTGGCAGYCGCCRCGGKAA, for Nanoarchaeota) [82], 515F-TM7 (5’ GT-GCCAGCMGCCGCGGTCA for TM7/Saccharibacteria) [18] as forward mix and 805RB (5’ GGA CTACNVGGGTWTCTAAT) [108] and 805R-Nano (5’GGAMTACHGGGGTCTC-TAAT, for Nanoarchaeota) [82] as reverse mix. Purified barcoded amplicon libraries were sequenced on an Illumina MiSeq instrument (Illumina Inc., San Diego, CA) using a v2 500 cycle kit, according to manufacturer’s protocol. Demultiplexed forward and reverse reads were imported as paired fastq files into QIIME2 v. 2020.8 [10] for analysis. The DADA2 plugin was used to trim, denoise, pair, purge chimeras and select amplicon sequence variants (ASVs), using the command “qiime dada2 denoise-paired”. Between 44k and 194k non-chimeric sequences were obtained for the individual samples. The ASVs were taxonomically classified in QIIME2 using a trained classifier built based on Silva-138-99 rRNA sequence database. The ASVs were assigned to 1643 taxonomic categories corresponding to taxonomic level 7 (species and above) and to 932 genera (level 6). Alpha rarefaction curves indicated saturation of observed sequence features (ASVs) at a sequencing depth of 70-80k sequences.

2.5 Metagenome sequencing, assembly, and binning

Metagenomic sequencing was conducted using the services of a commercial provider (Novogene, Beijing, China) using two lanes of the Illumina HiSeq 2500 system for each of the water and sediment samples. Transcriptomic sequencing using Illumina HiSeq 2500 2×150bp paired-end technology was conducted using the services of a commercial provider (Novogene Corporation, Beijing, China). Metagenomic reads were assessed for quality using FastQC followed by quality filtering and trimming using Trimmomatic v0.38 [9]. High quality reads were assembled into contigs using MegaHit (v.1.1.3) with minimum Kmer of 27, maximum kmer of 127, Kmer step of 10, and minimum contig length of 1000 bp. Bowtie2 was used to calculate sequencing coverage of each contig by mapping the raw reads back to the contigs. Assembled contigs were searched for ribosomal protein S3 (rpS3) sequences using a custom hidden Markov model (HMM) built from Uniprot reference sequences assigned to the Kegg Orthologies K02982, and K02984 (corresponding to the bacterial, and archaeal RPS3, respectively) using hmmbuild (HMMER 3.1b2). rpS3 Sequences were clustered at 99% ID using CD-HIT as previously suggested for a putative species cutoff for rpS3 data [50]. Taxonomic affiliations of rpS3 groups were identified using Diamond Blast against the GTDB r95 database [76]. Contigs from the sediment and water assemblies were binned into draft genomes using both Metabat [55] and MaxBin2 [111]. DasTool was used to select the highest quality bins from each metagenome assembly [94]. CheckM was used for estimation of genome completeness, strain heterogeneity, and contamination [77]. Genomic bins

showing contamination levels higher than 10%, were further refined based on the taxonomic affiliations of the binned contigs, as well as the GC content, tetranucleotide frequency, and coverage levels using RefineM [78]. Low quality bins (>10% contamination) were cleaned by removal of the identified outlier contigs, and the percentage completeness and contamination were again re-checked using CheckM.

2.6 Genomes classification

Taxonomic classifications followed the Genome Taxonomy Database (GTDB) release r95 [76], and were carried out using the classify workflow in GTDB-Tk (v1.1.0) [16]. Phylogenomic analysis utilized the concatenated alignment of a set of 120 single-copy bacterial genes, and 122 single-copy archaeal genes [76] generated by the GTDB-Tk. Maximum-likelihood phylogenomic tree was constructed in FastTree using the default parameters [83].

2.7 Annotation and metabolic analysis

Protein-coding genes were predicted using Prodigal [42]. GhostKOALA [54] was used for the functional annotation of every predicted open reading frame in every genomic bin and to assign protein-coding genes to KEGG orthologies (KOs).

2.8 Analysis of sulfur cycling genes

To identify taxa mediating key sulfur-transformation processes in the spring sediments, we mapped the distribution of key sulfur-cycling genes in all genomes and deduced capacities in individual genomes by documenting the occurrence of entire pathways (as explained below in details). This was subsequently confirmed by phylogenetic analysis and examining contiguous genes organization in processes requiring multi-subunit and/or multi-gene. Further, expression data was used from three time points to identify the fraction of the community that is metabolically actively involved in the process. Analysis of S cycling capabilities was conducted on individual genomic bins by building and scanning hidden markov model (HMM) profiles as explained below. To build the sulfur-genes HMM profiles, Uniprot reference sequences for all genes with an assigned KO number were downloaded, aligned using Clustal-omega [95], and the alignment was used to build an HMM profile using hmmbuild (HMMER 3.1b2) [69]. For genes not assigned a KO number (e.g. *otr*, *tsdA*, *tetH*), a representative protein was compared against the KEGG Genes database using Blastp and significant hits (those with e-values < e-80) were downloaded and used to build HMM profiles as explained above. The custom-built HMM profiles were then used to scan the analyzed genomes for significant hits using hmmscan (HMMER 3.1b2) [69] with the option -T 100 to limit the results to only those profiles with an alignment score of at least 100. Further confirmation was achieved through phylogenetic assessment and tree building procedures, in which potential candidates identified by hmmscan were aligned to the reference sequences used to build the custom HMM profiles using Clustal-omega [95], followed by maximum likelihood phylogenetic tree construction using FastTree [83]. Only candidates clustering with reference sequences were deemed true hits and were assigned to the corresponding KO.

2.8.1 Sulfate-reduction

Sulfate reduction capacity was assessed by the presence of genes encoding the enzymes 3'-phosphoadenosine 5'-phosphosulfate synthase [Sat; EC:2.7.7.4 2.7.1.25] for sulfate activation to adenylyl sulfate (APS), the enzyme complex adenylylsulfate reductase [AprAB; EC:1.8.99.2] for APS reduction to sulfite, the quinone-interacting membrane-bound oxidoreductase complex [QmoABC] for electron transfer, the enzyme dissimilatory sulfite reductase [DsrAB; EC:1.8.99.5] and its co-substrate DsrC for dissimilatory sulfite reduction to sulfide, and the sulfite reduction-associated membrane complex DsrMKJOP for linking cytoplasmic sulfite reduction to energy conservation.

2.8.2 Sulfite-Reduction

Sulfite could be utilized by most sulfate-reducing microorganisms [86]. Dedicated sulfite-reduction capacity was assessed by the presence of the dissimilatory sulfite reductase system explained above [17, 35] with the lack of sulfate-activation (Sat) and reduction (Apr) genes. In addition, sulfite-reduction was assessed via the sole or co-occurrence of the anaerobic sulfite reductase (AsrABC) system [106], along with the membrane-bound associated complex (HdrABC) for transfer of electrons to the AsrC subunit [105]. The Asr enzyme has been shown to function in the cytoplasm in *Salmonella typhimurium* to reduce the sulfite released from respiratory reduction of tetrathionate and thiosulfate [86]. However, a scenario where the Asr enzyme is involved in sulfite respiration is possible via electron transfer from a membrane-bound associated complex to AsrC (the physiological partner of AsrAB). A plausible candidate for this membrane complex is the heterodisulfide reductase-related enzymes (HdrABC), analogous to what was suggested for DsrC (the physiological partner of DsrAB) in organisms lacking the sulfite reduction-associated membrane complex DsrMKJOP [106].

2.8.3 Polysulfide reduction

In addition to sulfate and sulfite, Zodletone spring is euxinic with extremely high levels of zero valent sulfur, available as soluble polysulfide. Respiratory polysulfide reduction was assessed via the identification of the membrane-bound molybdoenzyme complex PsrABC, which reduces polysulfides with electrons obtained from either a hydrogenase or a formate dehydrogenase through a quinone electron carrier [20]. In addition to the membrane-bound Psr system, representatives of the cytoplasmic sulfurhydrogenase I (HydABCD system), and/or II (ShyABCD system) were identified. However, although these enzymes have been shown to be dissimilatory in the archaeon *Pyrococcus furiosus* [8, 64], their involvement in an ETS-associated respiration is currently unclear.

2.8.4 Thiosulfate reduction/ disproportionation

Thiosulfate occurs in natural environments as a result of the reaction of sulfite with bisulfide (HS-) [115]. Thiosulfate is relatively stable at neutral pH and is present in high levels in Zodletone spring, Thiosulfate contains two sulfur atoms: a sulfone-sulfur (oxidation state +5), and a sulfane-sulfur (oxidation state -1). As such, thiosulfate can be disproportionated where the sulfone-sulfur is reduced (serves as an electron acceptor), and the

sulfane-sulfur is oxidized (serves as an electron donor), with the products being hydrogen sulfide, and sulfite, respectively. We searched for genes encoding the three known pathways for thiosulfate-disproportionation. First, in pure cultures of several sulfate reducers in the *Desulfobacterota* and *Firmicutes*, e.g. *Desulfovibrio*, *Desulfotomaculum*, thiosulfate disproportionation is known to occur via a cytochrome c-dependent thiosulfate reductase [EC: 1.8.2.5] [4, 5, 23, 43, 44, 46, 47, 61]. Second, in pure culture members of the family Enterobacteriaceae (*Gammaproteobacteria*), thiosulfate disproportionation is known to occur via the quinone-dependent membrane-bound molybdopterin-containing thiosulfate reductase PhsABC [36]. Finally, thiosulfate disproportionation to sulfite and hydrogen sulfide can also occur via a rhodanase-like enzyme [EC: 2.8.1.1 or EC: 2.8.1.3], as shown for several bacterial lineages [2, 22, 52, 79, 88], although this could be part of a thiosulfate assimilatory pathway as recently shown in *E. coli* [57]. Following the disproportionation of thiosulfate to sulfite and hydrogen sulfide, microorganisms differ in the fate of the produced sulfite. Some microorganisms reduce the released sulfite to sulfide via a Dsr or Asr dissimilatory sulfite reductase [36]), leading to complete reduction of one thiosulfate molecule to two sulfides (thiosulfate-reduction). Others oxidize the released sulfite to sulfate via the reversal of the sulfate reduction pathway [24, 61], or via the sulfite dehydrogenases SorAB or SoeABC [26], leading to the final conversion of one thiosulfate molecule to one sulfide and one sulfate molecules. The distribution of all thiosulfate disproportionation capacities was assessed by the occurrence of one of the three pathways described above, and the fate of sulfite in genomes mediating the initial disproportionation steps was assessed as described above.

2.8.5 Tetrathionate reduction

Tetrathionate has two sulfur atoms in oxidation state of 0 while the other two are in oxidation state of +5. In nature, tetrathionate is formed via the biotic or abiotic oxidation of thiosulfate under anoxic conditions [115]. Some microorganisms are capable of tetrathionate respiration via membrane-bound tetrathionate reductases that will reduce tetrathionate to thiosulfate serving as the terminal oxidase in a short electron transport system. Enzymes mediating such process include octaheme tetrathionate reductase (*otr*) [71], as well as the guanylyl molybdenum cofactor-containing tetrathionate reductase (*ttrABC*) [37]. The produced thiosulfate could be metabolized through disproportionation as described above.

2.8.6 Oxidative sulfur processes

The versatile sulfur oxidation (SOX enzyme complex) system was assessed in all genomes. The SOX system mediates the oxidation of a wide range of reduced sulfur compounds (sulfide, sulfite, thiosulfate, and elemental sulfur) directly to sulfate. Sulfide oxidation to sulfur was also assessed by the presence of the sulfide dehydrogenase FccAB [EC: 1.8.2.3] and/or the sulfide:quinone oxidoreductase Sqr [EC: 1.8.5.4], both known to oxidize sulfide to sulfur or polysulfide. Sulfur/polysulfide oxidation to sulfite was assessed via the reversal of the Dsr system (encompassing the full Dsr system *dsrAB+dsrC+dsrMKJOP*, in addition to the genes *dsrEFH*, *tusA*, and *rhDA*). Sulfite oxidation to sulfate was assessed via the reversal of *AprAB+QmoABC* system, the sulfite dehydrogenase (quinone) *SoeABC* [EC: 1.8.5.6], or the sulfite dehydrogenase (c-type cytochrome) *SorAB* [EC: 1.8.2.1]. Thiosulfate oxidation

to tetrathionate was assessed via the thiosulfate dehydrogenase *tsdA* [EC: 1.8.2.2], or the thiosulfate dehydrogenase (quinone) *doxAD* [EC: 1.8.5.2]. Tetrathionate generated could be cleaved using tetrathionate hydrolase (*tetH*) [53] that is known to cleave tetrathionate to thiosulfate, sulfur, and sulfate, or converted to sulfite using the rDSR system.

2.9 Expanding lineages mediating reductive sulfur cycling processes

Expanding lineages mediating reductive sulfur cycling processes in the tree of life. Annotree 42 [68] was queried at the family level for the presence of sulfate reduction genes (Figure 3.8) (combined search for the genes *AprA*, *AprB*, *Sat*, *QmoA*, *QmoB*, *QmoC*, *DsrA*, *DsrB*, and *DsrC*), sulfite reduction genes (Figure 3.9) using both the DSR system (combined search for the genes *DsrA*, *DsrB*, and *DsrC*, excluding all duplicate hits from (Figure 3.8) and excluding all hits from phyla known to use the system in the oxidative direction (Proteobacteria, Nitrospira, and Chlorobiaceae), and the ASR system (combined search for the genes *AsrA*, *AsrB*, *AsrC*, *HdrA*, *HdrB*, and *HdrC*), polysulfide reduction genes (Figure 3.10) (combined search for the genes *PsrA*, *PsrB*, and *PsrC*), thiosulfate reduction (Figure 3.11) (combined search for the genes *PhsA*, *PhsB*, and *PhsC*, combined with either *DsrA*, *DsrB*, and *DsrC*, or *AsrA*, *AsrB*, and *AsrC*) and thiosulfate disproportionation (combined search for the genes *PhsA*, *PhsB*, and *PhsC*, combined with either *AprA*, *AprB*, and *Sat*, or *SoeA*, *SoeB*, and *SoeC*, or *SorA*), and tetrathionate reduction (Figure 3.12) (combined search for the genes *TtrA*, *TtrB*, and *TtrC*).

For each of these searches, all hits were downloaded and sorted and parsed to keep one representative from each family with a hit. The Gtdb accession IDs for all such representatives were used to extract their 120 single-copy-protein concatenated alignment available from the Gtdb downloads repository [16]. These were then combined with the concatenated alignments for family-level representatives in Zodletone with genomic evidence of the corresponding sulfur reductive. The maximum likelihood trees were constructed in FastTree 40 [83]. The branches represent family-level taxonomy and are color coded by phylum. Zodletone lineages starting with ZN depict novel lineages as follows: ZNC, novel class; ZNO, novel order; and ZNF, novel family. The track around the tree represents the distribution of each family level representative as follows: only encountered in Annotree, black; only encountered in Zodletone spring sediment, blue; and encountered in both, red. By comparing the number of lineages with the blue track (only encountered in Zodletone) to the combined number of lineages with the black and red tracks, the level of expansion of lineages mediating reductive sulfur cycling processes in the tree of life can be deduced as follows: with regards to sulfate reduction (Figure 3.8), and as stated in the results section of the main text, lineages encountered in Zodletone spring sediment encoded minimal sulfate reduction capacities. Only 7 new lineages were added to an existing list of 90 lineages in the bacteria tree of life (increase by 7.7%).

On the other hand, with regards to sulfite reduction (Figure 3.9), representatives of 71 new families were added to a list of 76 families already known to encode this function (increase by 93.4%). For polysulfide reduction (Figure 3.10), thiosulfate reduction/disproportionation (Figure 3.11), and tetrathionate reduction (Figure 3.12), increases of 11.8% (12 new lineages added to 102 already known), 30.4% (7 new lineages added to 23 already known), and 34.7% (43 new lineages added to 124 already known), respectively, were encountered.

2.10 Phylogenetic affiliation and contig organization of selected sulfur reduction proteins

Alignments were created in Mafft [56] and maximum likelihood trees were constructed in RaxML [60]. Bootstrap support values are shown as bubbles for nodes with >50% support. Branches and branch labels are color coded by phylum for Zodletone sequences. Branch labels depict classification to family level followed by the NCBI genome accession number. Reference sequences are shown in black with the Uniprot accession numbers. Contig organizations of the DSR and ASR loci in selected Zodletone genomes are shown to the right of the trees in A and B. Genes are color coded as shown in the top right corner. Unrelated genes are shown by grey arrows. Gene maps were created in R using the package genoplots [31]. Phylum/class classification is depicted to the right of the trees in Figures 3.15, 3.16, 3.17.

2.11 Phylogenetic analysis and operon organization of S cycling genes

The phylogenetic affiliation of the S cycling proteins AsrB, Otr, PhsC, PsrC, and DsrAB was examined by aligning Zodletone genome predicted protein sequences to Uniprot reference sequences using Mafft [56]. The DsrA and DsrB alignments were concatenated in MEGA X [62]. All alignments were used to construct maximum likelihood phylogenetic trees in RAxML [60]. The R package genoPlotR [31] was used to produce gene maps for the DSR and ASR loci in Zodletone genomes using the Prodigal predicted gene starts, ends, and strand direction.

2.12 Transcription of sulfur cycling genes

A total of 21.4 M, 27.9 M, and 22.5 M 150-bp paired-end reads were obtained from the morning, afternoon, and evening RNA-seq libraries. Reads were pseudo-aligned to all Prodigal-predicted genes from all genomes using Kallisto with default settings [11]. The calculated transcripts per million (TPM) were used to obtain total transcription levels for genes identified from genomic analysis as involved in S cycling in the spring.

2.13 Additional metabolic analysis

For all other non-sulfur related functional predictions, combined GhostKOALA outputs of all genomes belonging to a certain order (for orders with 5 genomes or less; n=206), or family (for orders with more than 5 genomes; n=85) were checked for the presence of groups of KOs constituting metabolic pathways (additional file 1). The list of these 291 lineages is shown in Table S3. The presence of at least 80% of KOs assigned to a certain pathway in at least one genome belonging to a certain order/family was used as an indication of the presence of that pathway in that order/family. Such criteria were used for the prediction of autotrophic capabilities, as well as catabolic heterotrophic degradation capabilities of sugars, amino acids, long-chain fatty acids, short chain fatty acids, anaerobic benzoate degradation, anaerobic short chain alkane degradation, aerobic respiration, nitrate reduction, nitrification, and chlorophyll biosynthesis. Glycolytic, and fermentation capabilities were predicted by

feeding the GhostKOALA output to KeggDecoder [30]. Proteases, peptidases, and protease inhibitors were identified using Blastp against the Merops database [89], while CAZymes (glycoside hydrolases [GHs], polysaccharide lyases [PLs], and carbohydrate esterases [CEs]) were identified by searching all ORFs from all genomes against the dbCAN hidden Markov models V9 [41] (downloaded from the dbCAN web server in September 2020) using hmmscan. FeGenie [27] was used to predict the presence of iron reduction and iron oxidation genes in individual bins.

CHAPTER III

RESULTS

3.1 Novel phylogenetic diversity in Zodletone sediments

3.1.1 Overall Sequencing Overview and Diversity Patterns

Metagenomic sequencing of the spring sediments yielded 281 Gbp, 79.54% of which assembled into 12 Gbp contigs, with 6.8 Gbp contigs longer than 1Kbp. 1,848 genomes were binned, but only 683 passed quality control criteria, and 516 remained after dereplication (Table 3.1). These metagenome-assembled genomes (MAGs) represented 64 phyla or candidate phyla (53 bacterial and 11 archaeal), 127 classes, 198 orders, and 300 families (Figure 3.1). Diversity assessment utilizing small subunit ribosomal protein S3 from assembled contigs (n=2079), as well as a complementary 16S rRNA Illumina sequencing effort (n=309,074 amplicons), identified a higher number of taxa (82 phyla and 1679 species in the ribosomal protein S3 dataset, and 69 phyla and 1050 species in 16S rRNA dataset) (Figure 3.2). Nevertheless, the overall community composition profiles generated from all three approaches were broadly similar (Figure 3.2), suggesting that the MAG list largely reflects the sediment microbial community.

Assessment of the novelty and degree of uniqueness of sediment MAGs identified a remarkably high number of previously undescribed lineages (1 phylum, 14 classes, 43 orders, and 97 families) as well as Lineages exhibiting Rare Global Distribution (LRD) pattern (11 phyla, 24 classes, 45 orders, and 113 families) in the spring (Figure 3.1, 3.3, 3.4). We define LRD lineages as those represented by 5 genomes or less in the Genome Taxonomy Database release 95 (GTDB r95). At the family level, 132 (25.58%), and 208 (40.03%) genomes clustered into 97 novel and 113 LRD families respectively, bringing the proportion of genomes belonging to novel or LRD families in Zodletone sediments to 65.89%. The high level of novelty in the sediment MAGs is reflected in an average RED value of 0.76, a value that is slightly lower than the median RED value for designation of a novel family (0.77) [76].

3.1.2 Detailed phylogenetic analysis of Zodletone sediment MAGs

The *Chloroflexota* (n=69), *Planctomycetota* (n=47), *Bacteroidota* (n= 43), *Desulfobacterota* (n= 43), *Spirochaetota* (n= 28 genomes), *Patescibacteria* (n=20 genomes), and the archaeal phylum *Nanoarchaeota* (n=21) were the most abundant phyla in Zodletone spring sediments, albeit representing only 52.52% of the total number of recovered genomes (Figure 3.1, 3.4, 3.5). An extreme paucity of genomes belonging to the *Proteobacteria* (6 genomes) and *Firmicutes* (12 genomes), widely distributed and abundant taxa in current biomes [73], and the absence of oxygen-generating *Cyanobacteria* (0 genomes) were observed (Figure 3.1, 3.5).

Within the *Chloroflexota*, 38/69 genomes belonged to 3 novel orders, 5 novel families, and multiple LRD orders (Thermoflexales, 4572-78, and UBA2777) and families (E44-bin32, Fen-1058, J111, RBG-13-53-26, RBG-16-64-43, UBA11579, UBA11858, UBA2029, UBA2162, UBA3940, UBA4811, UBA4823, UBA5620, UBA5760, and UBA6092) (Figure 3A inside 3.1).

Within the *Planctomycetota*, 17/47 genomes belonged to 2 novel orders, 8 novel families, and multiple LRD orders (FEN-1346, SZUA-567, and UBA8890) and families (Fen-1342, SM23-30, UBA1845, UTPLA1, and UBA8108) (Figure 3c inside 3.1).

Within the *Bacteroidota*, 27/43 genomes belonged to 1 novel family, and multiple LRD families (FEN-979, GCA-2748055, NBLH01, UBA10428, UBA12170, UBA5072, UBA6680, and SZUA-365) (Figure 3b inside 3.1).

Within the *Spirochaetota*, 19/28 genomes belonged to one novel class, 2 novel orders, and 9 novel families, as well as multiple LRD families (ARS1246, *Marispirochaetaceae*, and RPPD01) (Figure 3d inside 3.1).

Within the *Desulfobacterota*, 35/43 genomes belonging to 3 novel classes, 10 novel orders, and 7 novel families were identified, as well as multiple LRD families (B25-G16, BuS5, HGW-15, MLS-D, NaphS2, UBA2210, and UBA3084). Only 6/43 genomes belonged to the well-described families *Desulfovibrionaceae*, *Geopsychrobacteraceae*, *Smithellaceae*, and *Syntrophaceae*. (Figure 3e inside 3.1)

Finally, an extremely diverse community of *Patescibacteria* (13 different orders, 3 of which belonging to LRD orders, and 14 different families, including 6 novel and 2 LRD families), and *Nanoarchaeota* (2 orders including the LRD order CG07-land), and 15 different families, including 5 novel and 10 LRD families) were identified in the spring sediments (Figure 3F (Figure 3e inside 3.1)). A similar pattern of high proportion of novel and LRD families was identified throughout all other lineages (Figure 3.1). Therefore, in addition to expanding the number of novel lineages (classes, orders, and families), and greatly enriching available genomes in rare, poorly represented taxa, our results highlight the uniqueness and distinction of the microbial community thriving in Zodletone spring sediments, compared to present earth environments studied so far.

3.2 Hypoxic Waters

3.2.1 Overall Sequencing Overview and Diversity Patterns

Metagenomic sequencing of the oxygen-exposed overlaying water column community yielded 323 Gbp, 80.07% of which assembled into 3.6 Gbp contigs, with 3.1 Gbp contigs >1K. 883 genomes were binned, with only 114 remaining after dereplication. Of these, 62 belonged to families shared with the sediment community, while 52 were water specific. Genomes recovered from the water column belonged to a significantly lower number of phyla (n= 27), classes (n=37), orders (n=52), and families (n=79) when compared to the euxinic sediments (Table 3.1). The community exhibited a much lower level of novelty and rarity at the phylum, class, order, and family levels when compared to the sediment community (Figure 3.3).

3.2.2 Detailed Phylogenetic Analysis of Zodletone Water MAGs

Water-specific genomes (n=52) mostly belonged to well-characterized microbial lineages, e.g. class *Alphaproteobacteria* (5 genomes belonging to *Rhodobacteraceae* and *Rhodospirillaceae*, and 3 belonging to the uncultured lineages NBLK01 and Rs-D84), *Gammaproteobacteria* (9 genomes belonging to the lineages *Thiomicrospiraceae*, *Halothiobacillaceae*, *Acidithiobacillaceae*, *Burkholderiaceae*, *Chromatiaceae*, and *Methylothermaceae*, and 1 belonging to the uncultured lineage UBA9339), phylum *Campylobacterota* (8 genomes, belonging to the families *Sulfurimonadaceae*, *Sulfurovaceae*, and *Sulfurospirillaceae*), *Firmicutes/Firmicutes_A* (4 genomes, and 6 genomes belonging to the *Bacilli*, and *Clostridia* classes, respectively), and well described families in the phyla *Bacteroidota* (*Flavobacteriaceae*, *Prolixibacteraceae*, *Paludibacteraceae*, *Marinilabiliaceae*, *Tannerellaceae*, *Marinifilaceae*, *Balneolaceae*), *Desulfobacterota* (Families *Geopsychrobacteraceae*, *Desulfuromonadaceae*,), and *Sprichoetota* (*Sphaerochaetaceae*, *Treponemataceae*, *Spirochaetaceae_B*) (Figure 3.1, Table 3.1).

Collectively, this demonstrates a pattern where the intrusion of oxygen is associated with a negative impact on novel and LRD lineages that are prevalent in the sediment, and the propagation of communities associated with well-characterized lineages within the bacterial tree of life.

3.3 Reductive sulfur processes dominate Zodletone spring sediment communities

A total of 149 genomes (28.9% of all genomes), belonging to 32 phyla, 51 classes, 69 orders, and 97 families were involved in at least one reductive sulfur processes (Figure 3.6, 3.7, Table 3.2). By comparison, only 21 sediment genomes (4.06% of all genomes) encoded at least one sulfur oxidation pathway (Figure 3.6, 3.7, Table 3.2). The reductive sulfur-community in the spring exhibited two unique traits: First, a majority of genomes encoding such capacities belonged to novel (47 genomes) or LRD (66 genomes) lineages (Figure 3.6, 3.7), and second: sulfite-, polysulfide-, thiosulfate-, and tetrathionate reduction capacities appears to be more prevalent than sulfate-reduction capacities in the sediment genomes.

3.3.1 Sulfate Reduction

Sulfate-reduction capacity was encoded in only 18 sediment genomes (Figure 3.6, 3.7), but exhibited a unique community composition, when compared to well-studied marine and terrestrial habitats [39, 48, 104, 109]. Sulfate-reduction capacities were observed in mostly previously undescribed or LRD lineages within the *Zixibacteria*, *Acidobacteriota* (members of family *UBA6911*, equivalent to *Acidobacteria* group 18), *Myxococcota*, *Bacteroidota*, *Planctomycetota*, candidate phylum *OLB16* (1 genome), as well as rare and novel lineages within the *Desulfobacterota* (Figure 3.6, 3.7, 3.8, Table 3.2). Organization of the sulfate reduction genes differed between different phyla, with *Myxococcota*, *Zixibacteria*, *OLB16*, and *Acidobacteriota* genomes encoding all genes for sulfate activation and reduction, dissimilatory sulfite reduction, as well as energy conservation on one locus, while *Desulfobacterota* genomes encoded genes for sulfate activation and reduction as well as energy conservation on one locus with genes for dissimilatory sulfite reduction on another, as previously observed in cultured

Desulfobacterota [72, 23, 114].

3.3.2 Sulfite Reduction

Sulfite (but not sulfate) reduction via the DsrAB+DsrC+DsrKMJOP system was identified in only 8 genomes belonging to 7 families within the phyla *Planctomycetes*, *Chloroflexota*, *Spirochaetota*, and *Desulfobacterota* (Figure 3.6, 3.7, 3.9, 3.13, Table 3.2). Gene organization of the dsr locus in the above 9 genomes differed between different phyla, with *Chloroflexota*, and *Spirochaetota* genomes encoding all genes for dissimilatory sulfite reduction (dsrABC plus dsrKMJOP) on one locus, while *Planctomycetota* and *Desulfobacterota* genomes showed a split dsr locus with dsrABC on one locus and dsrMKJOP on another (Figure 3.13).

On the other hand, sulfite-reduction capacity within Zodletone spring sediment solely via the Asr/Hdr system was rampant, being encountered in 104 genomes belonging to 28 phyla, 43 (8 novel and 9 LRD) classes, 56 (18 novel, and 12 LRD) orders, and 72 (31 novel and 25 LRD) families (Figure 3.6, 3.7, 3.14, Table 3.2), with a gene organization of the asr locus adjacent to the hdr locus in the majority of genomes (Figure 3.14). Asr-encoding genomes in the sediment included members of previously undescribed and LRD lineages within the *Chloroflexota*, *Desulfobacterota*, *Planctomycetota*, and *Bacteroidota*. The capacity was also rampant in the yet-uncultured bacterial phyla, many of which have fairly limited global distribution (e.g. the candidate phyla CSSED10-310, FCP426, RBG-13-66-14, SM23-31, SZUA-182, UBP14, I, and I). Interestingly, all genomes belonging to the novel phylum Krumholzibacteriota, recently described from the spring sediment [99], encoded complete anaerobic sulfite reductase systems. Zodletone dissimilatory sulfite reductase (Figure S6a) and the anaerobic sulfite reductase (Figure 3.14) sequences clustered with reference sequences from the same phylum, generally showing no evidence of LGT.

3.3.3 Sulfur (polysulfide) reduction

Sulfur (polysulfide) reduction capacities were observed in twenty Zodletone sediment genomes that encoded psrABC genes (Figure 3.6, 3.7, 3.10, 3.15, **Table S2**). These genomes belonged mostly to previously undescribed and LRD families within the phyla *Bacteroidota*, *Desulfobacterota*, *Myxococota*, *Acidobacterota*, *Chloroflexota*, and *Campylobacterota* (Figure 3.7). In addition, representatives of the cytoplasmic sulfurhydrogenase I (HydABCD system), and/or II (ShyABCD system) were identified in 119 Zodletone sediment genomes (Figure 3.6). However, as explained above, direct involvement of these enzymes in an ETS-associated respiration is not yet clear.

3.3.4 Thiosulfate Disproportionation and Reduction

The quinone-dependent membrane-bound molybdopterin-containing thiosulfate reductase PhsABC was encoded in 11 genomes belonging to 6 phyla, with *Bacteroidota* representing the major phsABC-encoding phylum (4 genomes) (Table S2, Figure 3.11). Within these genomes, only two (a *Chloroflexota* family UBA6092 genome, and a *Desulfatiglandales* family HGW15 genome) also encoded a dissimilatory sulfite reductase (the Asr system) akin to the *Gammaproteobacteria* thiosulfate disproportionating pure culture members [84], where

the final products of thiosulfate disproportionation are expected to be only hydrogen sulfide (Figure 3.6, 3.7, 3.11, Table 3.2). On the other hand, 5 of the 11 phsABC-encoding Zodletone genomes also encoded the sulfite dehydrogenase SoeABC system, akin to *Desulfobacterota* and *Firmicutes* pure culture members, where the final products of thiosulfate disproportionation are expected to be both hydrogen sulfide and sulfate [23, 43] (Figure 3.6, 3.7, 3.11, Table 3.2).

In addition to the phsABC system, 14 Zodletone genomes belonging to 6 phyla (*Desulfobacterota*, *Acidobacteriota*, *Chloroflexota*, *Bacteroidota*, *Spirochaetota*, and *Myxococcota*) encoded a rhodanase-like enzyme [EC: 2.8.1.1 or EC: 2.8.1.3] for thiosulfate disproportionation, as well as enzymes for both sulfite oxidation (by means of reversal of sulfate reduction via Sat+AprAB, or the sulfite dehydrogenase SoeABC), and sulfite reduction (via the dissimilatory sulfite reductases Dsr or Asr), where the final products of thiosulfate disproportionation are expected to be both hydrogen sulfide and sulfate (Figure 3.6, 3.7, 3.11, Table 3.2).

3.3.5 Tetrathionate Reduction

Tetrathionate reduction capacities were identified in 105 sediment genomes. Seventy-three Zodletone sediment genomes encoded the octaheme tetrathionate reductase (OTR) enzyme. These genomes belonged to 14 phyla with major contribution from *Bacteroidota* (30 genomes), *Chloroflexota* (10 genomes), and *Desulfobacterota* (10 genomes) (Table 3.2, Figure 3.6, 3.7, 3.17). In addition to Otr, 68 Zodletone genomes encoded the Ttr enzyme system. These genomes belonged to 14 phyla with major contribution from *Chloroflexota* (22 genomes), and *Desulfobacterota* (20 genomes) (Table 3.2, Figure 3.6, 3.7, 3.12). As shown previously in *Salmonella typhimurium* [84], in presence of means for thiosulfate disproportionation/reduction and sulfite reduction, the thiosulfate produced as a result of tetrathionate reduction could be further reduced to sulfide. Out of the 105 sediment genomes encoding the Otr, and/or Ttr enzymes, only 12 genomes also encoded thiosulfate and sulfite reduction enzymes. These genomes belonged to the phyla *Acidobacteriota*, *Chloroflexota*, *Desulfobacterota*, *Myxococcota*, and *Spirochaetota* (Table 3.2).

3.4 Substrates supporting sulfidogenic capacities at Zodletone Spring

Within lineages mediating reductive sulfur processes in Zodletone sediments (n=98), a wide range of substrates supporting sulfidogenesis were identified (Table 3.3, Figure 3.7). These included hexoses (26-87% of sulfidogenic lineages), pentoses (30-41% of sulfidogenic lineages), amino acids and peptides (39% of lineages), short chain fatty acids, e.g. lactate, propionate, butyrate, and acetate (22-73% of lineages), long chain fatty acids (29% of lineages), aromatic hydrocarbons (3% of lineages), and short chain alkanes (6% of lineages). Autotrophic capacities with hydrogen as the electron donor were identified in 28% of sulfidogenic lineages.

3.5 Transcriptomic analysis

Transcriptional expression of genes involved in S-species reduction/disproportionation was analyzed, and the identity of the active sulfur-reducing community in the spring sediment was

examined (Figure 3.6). All S-species reduction/ disproportionation genes discussed above were identified in the metatranscriptomic dataset, and transcripts belonging to 51 different phyla were identified. Analysis of the spring sediment revealed the transcription of both the Dsr and Asr systems for sulfite reduction with contributions from *Chloroflexota*, *Planctomycetota*, *Desulfobacterota*, *Bacteroidota*, *Fermentibacterota*, *Acidobacteriota*, *CSSED10-310*, *Actinobacteriota*, *Spirochaetota*, *WOR-3*, and *Fibrobacterota* (Asr), and *Desulfobacterota*, *Acidobacteriota*, *Zixibacteria*, and *Myxococcota* (Dsr). Sulfate reduction genes (Sat, AprAB, and QmoABC) were also transcribed with major contribution from *Desulfobacterota*, *Myxococcota*, *Zixibacteria*, and *Acidobacteriota*.

Total transcription levels of the Asr system were 4-times higher than the Dsr system, consistent with the higher number of Zodletone sediment genomes encoding the Asr system compared to the Dsr system. Transcription of the thiosulfate disproportionating rhodanese-like enzyme [EC: 2.8.1.1 or EC: 2.8.1.3] was detected in the phyla *Desulfobacterota*, *Actinobacteriota*, *Firmicutes_A*, *Chloroflexota*, *Fibrobacterota*, *Planctomycetota*, *Spirochaetota*, *Bacteroidota*, *Halobacteriota*, and *Acidobacteriota*, while the transcription of the thiosulfate reductase phsABC was detected in the phyla *Actinobacteriota* and *Bacteroidota*. Transcription of the tetrathionate reduction genes ttrABC was detected in the phyla *Desulfobacterota*, *Actinobacteriota*, *Bacteroidota*, *Chloroflexota*, *Acidobacteriota*, and *Spirochaetota*, while the octaheme tetrathionate reductase otr transcription was detected in *Desulfobacterota*, *Bacteroidota*, *Myxococcota*, *UBP7A*, and *Chloroflexota*.

Finally, the transcription of psrABC for polysulfide reduction was detected majorly in the phyla *Bacteroidota*, *Desulfobacterota*, and *Campylobacterota*, while transcription of the cytoplasmic sulfurhydrogenases I and II (hyd/shy systems) was identified in the phyla *Actinobacteriota*, *Chloroflexota*, *Planctomycetota*, *Myxococcota*, *Acidobacteriota*, *Bacteroidota*, and *Desulfobacterota*.

3.6 Oxidative sulfur processes dominate Zodletone water community

In contrast to sediment communities, reductive sulfur-processes were identified in only 25 (21.92%) water genomes, as opposed to 149 (29%) sediment genomes (Figure 3.6, 3.7, Table 3.2). Dissimilatory sulfate reduction to sulfide capacity was completely absent in water genomes. The capacity for dissimilatory sulfite reduction via the Dsr system was absent, and the Asr system was only encoded in 7 water genomes. Thiosulfate reduction/disproportionation capacity to sulfide and sulfate (PhsABC + SoeABC, and/or Rhodanase + Dsr/Asr + SoeABC/SorAB) was encoded in only four genomes, all of which also encoded the capacity for tetrathionate reduction (via Otr and/or ttrABC). Finally, respiratory polysulfide reduction (via PsrABC) was encoded in 19 genomes. In all cases, the reductive sulfur community in water was a subset of the sediment community.

In contrast, oxidative sulfur processes dominated the water community, with pathways encoding sulfide, sulfur, thiosulfate, tetrathionate, and/or sulfite oxidation to sulfate present in 59/114 genomes (51.8% of all water genomes) belonging to 13 phyla, 16 classes, 25 orders, and 43 families. The oxidative sulfur community in the water belonged to mostly well-characterized lineages (Figure 3.6, 3.7, Table 3.2). Only 8 and 10 genomes involved in oxidative sulfur processes belonged to novel, and LDR families, respectively.

A complete SOX system, putatively mediating oxidation of a wide range of reduced sulfur-species to sulfate was encoded in genomes belonging to well-characterized families within the *Proteobacteria* (11 genomes total belonging to families *Acidithiobacillaceae*, *Burkholderiaceae*, *Halothiobacillaceae*, *Rhodobacteraceae*, and *Thiomicrospiraceae*) and *Campylobacterota* (3 genomes in the family *Sulfurimonadaceae*) (Figure 3.6, 3.7, Table 3.2).

3.6.1 Sulfide oxidation to sulfur and sulfite

Thirty nine water genomes encoded the sulfide dehydrogenase fccAB [EC: 1.8.2.3] and/or the sulfide:quinone oxidoreductase Sqr [EC: 1.8.5.4] both known to oxidize sulfide to sulfur/ polysulfide (Figure 3.6, 3.7, Table 3.2). These genomes belonged to the phyla *Bacteroidota* (14 genomes in the well characterized families *Chlorobiaceae*, *Prolixibacteraceae*, and *Paludibacteraceae*, as well as the uncultured families NBLH01, UBA1556, DTU049, and F082 in the order *Bacteroidales*), *Proteobacteria* (13 genomes in the families *Acidithiobacillaceae*, *Burkholderiaceae*, *Chromatiaceae*, *Halothiobacillaceae*, *Methylothermaceae*, *Rhodobacteraceae*, *Thiomicrospiraceae*), *Campylobacterota* (8 genomes in the families *Sulfurimonadaceae*, *Sulfurospirillaceae*, *Sulfurovaceae*), in addition to three genomes in the families *Anaerolineaceae*, *Geopsychrobacteraceae*, and *UBA2242* within the phyla *Chloroflexota*, *Desulfobacterota*, and *Marinisomatota*, respectively, and one genome belonging to a novel *Thermodesulfovibrionales* family (*Nitrospirota*). Only two of the above thirty-nine genomes (one *Proteobacteria* genome and one *Nitrospirota* genome) encoded the capacity to further oxidize the sulfur/polysulfide to sulfite via the reversal of the Dsr system (encompassing the full Dsr system dsrAB+dsrC+dsrMKJOP, in addition to the genes dsrEFH, tusA, and rhdA).

3.6.2 Sulfite oxidation to sulfate

A total of twenty-six water genomes encoded the capacity for sulfite oxidation to sulfate via the reversal of AprAB+QmoABC system (1 *Bacteroidales* genome), the sulfite dehydrogenase (quinone) SoeABC [EC: 1.8.5.6] (22 genomes belonging to the order *Bacteroidales*, and the families *Acidithiobacillaceae*, *Burkholderiaceae*, *Chromatiaceae*, *Dethiosulfatibacteraceae*, *Halothiobacillaceae*, *Methylothermaceae*, *Rhodobacteraceae*, *Thiomicrospiraceae* within *Proteobacteria*, the families *Sulfurimonadaceae*, *Sulfurospirillaceae*, *Sulfurovaceae* within *Campylobacterota*, the *Syntrophales* family *UBA3084*, and a novel *Thermodesulfovibrionales* family (*Nitrospirota*)), or the sulfite dehydrogenase (cytochrome) SorAB [EC: 1.8.2.1] (3 genomes total within the families *Chromatiaceae*, *Halothiobacillaceae* (*Proteobacteria*), and *UBA12059* (*Spirochaetota*)) (Figure 3.6, 3.7, Table 3.2).

3.6.3 Thiosulfate Oxidation

Eight water genomes encoded thiosulfate to tetrathionate oxidation capacities via either the thiosulfate dehydrogenase tsdA [EC: 1.8.2.2] (7 genomes belonging to the families *Sulfurimonadaceae* within *Campylobacterota*, and *Rhodobacteraceae*, *Burkholderiaceae*, *Halothiobacillaceae*, *Thiomicrospiraceae* within *Proteobacteria*), or the thiosulfate dehydrogenase (quinone) doxAD [EC: 1.8.5.2] (1 *Flavobacteriaceae* genome) (Figure 3.6, 3.7, Table 3.2). Two of these 8 genomes (1 *Rhodobacteraceae*, and 1 *Halothiobacillaceae* genomes) also encoded tetrathionate hydrolase (tetH) [53] that is known to cleave tetrathionate to thiosulfate,

sulfur, and sulfate (Figure 3.6, 3.7, Table 3.2). Simultaneous identification of the SOX system and both forms of sulfide dehydrogenase (fccAB and Sqr) imply that these two genomes encode the capacity for complete thiosulfate oxidation to sulfate.

3.6.4 Tetrathionate oxidation

In addition to the above two genomes, ten other water genomes encoded tetrathionate hydrolase, but with no other means of thiosulfate and sulfide oxidation capacities (Figure 3.6, 3.7, Table 3.2). Surprisingly, tetH (without other means of thiosulfate or sulfide oxidation) was also encoded in 100 sediment genomes, belonging to 26 phyla, 37 classes, 49 orders, and 66 families (including 23 novel families). Only nine of these genomes (belonging to 9 families including 4 novel ones) showed tetH transcriptional levels above 1 (Figure 3.6, 3.7, Table 3.2). However, the exact function of tetrathionate hydrolase in these organisms is not entirely clear, as the subsequent steps of oxidation could not be identified.

3.7 Additional metabolic capacities in Zodletone spring sediments

In addition to reductive sulfur processes, strict fermentative capacities were highly prevalent in sediment genomes (Table 3.3) being identified in 100 of the 291 lineages studied. On the other hand, a dearth of aerobic (only 38 lineages), nitrate (only 65 lineages encoded dissimilatory nitrite reduction to ammonium, with 2 of which also encoding the suite of genes for denitrification), Fe³⁺ respiration (8 lineages), or chemolithotrophic nitrifying (only 1 lineage encoded the combination of ammonia monooxygenase and hydroxylamine dehydrogenase), and photosynthetic capacities were identified (Figure 3.1.a, Table 3.3). Strict fermentative lineages mediate the degradation a wide range of substrates, e.g. sugars (89 of the 100 fermentative lineages), amino acids (85 of the 100 fermentative lineages), short chain fatty acids (37 of the 100 fermentative lineages), complex carbohydrates (36 of the 100 fermentative lineages), long chain fatty acid oxidation (2 lineages), and short chain alkanes (1 lineage) (Table 3.3), producing a wide range of fermentative end products including lactate, formate, acetate, ethanol, succinate, and hydrogen. Primary productivity in the spring sediments appears to be mostly mediated via hydrogen utilization coupled to either sulfur-cycle intermediates reduction (27 lineages, Table 3.3), or to CO₂ fixation by hydrogenotrophic methanogens and acetogens using the Wood-Ljungdahl pathway (8 lineages, Table 3.3).

Table 3.1: List of all genomes analyzed in this study
 List of all genomes analyzed in this study with their NCBI Assembly accession numbers, taxonomic classification, sequencing statistics, and general genomic features

Table S1. List of all genomes analyzed in this study with their NCBI Assembly accession numbers, taxonomic classification, sequencing statistics, and general genomic features.

Genome	assembly number	% completeness	sequencing %	taxonomy	Phylum	Class	Order	Family	Genus	Species	Size (Mb)	Number of contigs	Median contig length (bp)	Number of genes	Number of CDSs	Assembly sN50 (bp)	Assembly N50 (bp)	Repeat coverage	GC %	Number of rRNA	Number of tRNA	Avg GC content (%)	Number of rRNA genes	Number of tRNA genes	Number of rRNA genes	Number of tRNA genes	Quality
Genome 1	NCBI:GCA000000000	99.5	99.5	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodospirillaceae	Rhodobacter	Rhodobacter ruber	1.5	1000	1000	1000	1000	1000	1000	1000	50	10	50	50	50	50	50	50	50
Genome 2	NCBI:GCA000000001	99.5	99.5	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodospirillaceae	Rhodobacter	Rhodobacter ruber	1.5	1000	1000	1000	1000	1000	1000	1000	50	10	50	50	50	50	50	50	50
Genome 3	NCBI:GCA000000002	99.5	99.5	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodospirillaceae	Rhodobacter	Rhodobacter ruber	1.5	1000	1000	1000	1000	1000	1000	1000	50	10	50	50	50	50	50	50	50

BLAST values (relative evolutionary divergence value calculated for each genome as GTRM-TL; No BLAST values were obtained when a genome is classified down to the species level).
Family distribution in CTRM: Families were classified according to how abundant they were in CTRM based on the available number of genomes. Abundance score from 1 genome are available in CTRM for the family; None, 2 genomes or less are available in CTRM for the family; None, no genomes were available in CTRM (those correspond to novel families based from Zeddisine springs).
Median genome coverage: Median assembly coverage for each MAG calculated based on the number of reads (127bp) mapped to each contig and the length of each contig as follows: $\text{coverage} = (\text{Number of reads mapped to the contig} \times 127) / \text{contig length}$.
Number of contigs: Number of contigs identified for each genome.
Assembly N50: Calculated using the median contig N50; 3.3 database at <https://metajob.org/assembly/medianN50/>.
Assembly size (Mb): refers to the size of the assembled contigs for each genome.
Repeat genome size (Mb): genome size excluding repeats of the genome.
GC%: Percentage G+C for each genome calculated using as in <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC270490/>.
Number of genes: Total number of protein-coding genes as predicted by Prodigal (<https://github.com/dahmed/prodigal>), 10/10/2019.
Coding %: Percentage of genome that is gene-coding. Calculated as cumulative size of all predicted genes/ genome size x 100.
Average gene length: Average size of all genes (bp).
Number of rRNA genes: Predicted number of rRNA genes as determined using Barrnap 0.8 (<https://github.com/jeffleung/barrnap>).
Number of tRNA genes: Predicted number of tRNA genes as determined using tRNAscan-SE (<https://github.com/tRNAscan-SE>), 12/10/2019.
Quality rate: Genomes were classified as medium-quality draft (MQD) or high-quality draft (HQD) based on the criteria set forth by [Shawara, 2017 #129](https://doi.org/10.1093/bioinformatics/bty112).

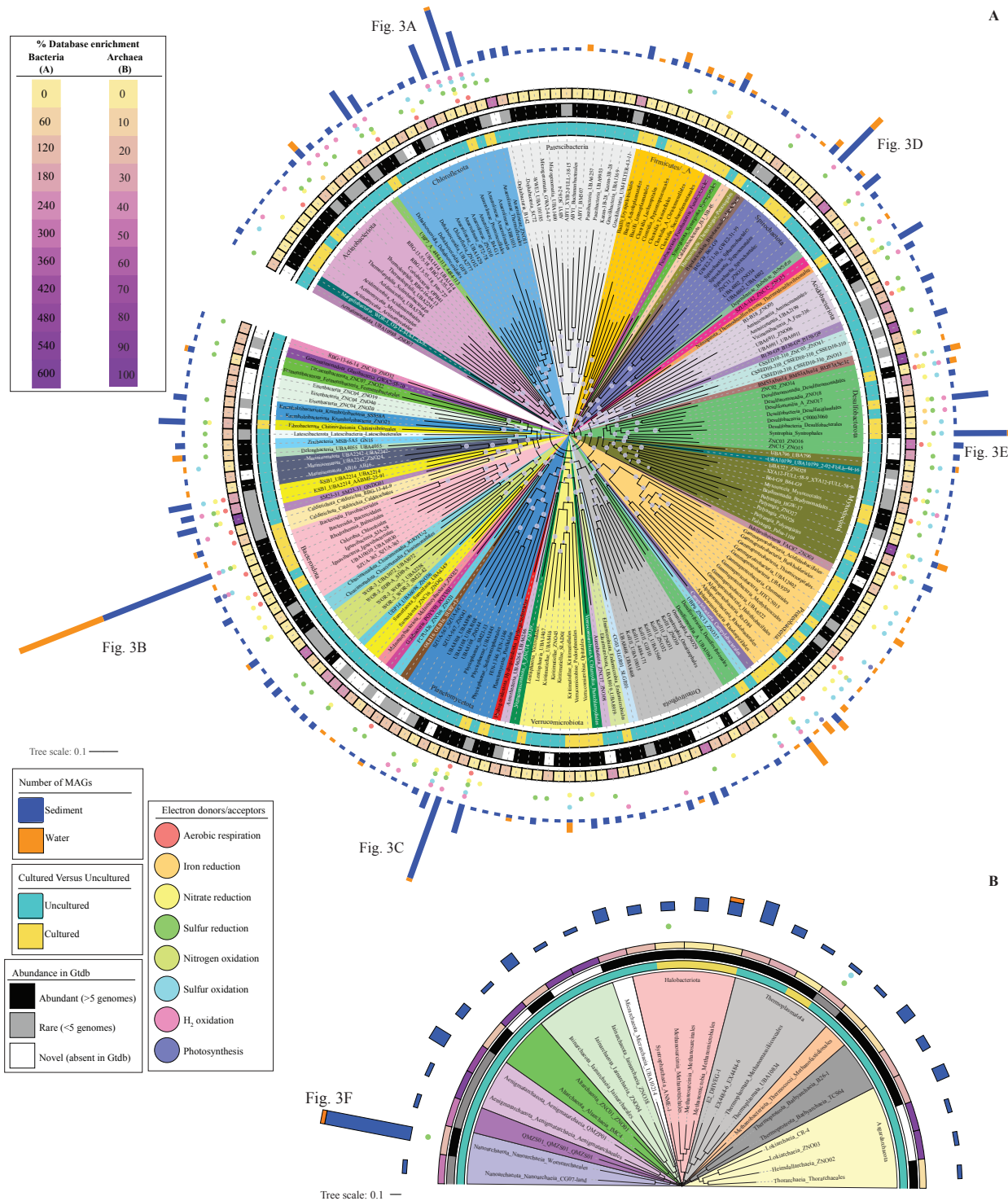


Figure 3.1: Phylogenomics of the bacterial and archaeal genomes

Figure 3.1: Detailed caption of Figure 3.1

Phylogenomics of the 516 bacterial (A), and 114 archaeal (B) genomes analyzed in this study. The maximum likelihood trees were constructed in FastTree [83] based on the concatenated alignments of 120 (bacterial), and 122 (archaeal) housekeeping genes obtained from GTDB-TK (85). The branches represent order-level taxonomy and are color coded by phylum. For phyla with 4 orders or less branches are labeled as Phylum_Class_Order. For phyla with more than 4 orders, the phylum is shown at the base of the colored wedge and the branches are labeled as Class_Order. Lineages starting with ZN depict novel lineages (ZNC = novel class; ZNO = novel order). Bootstrap support values are shown as bubbles for nodes with >70% support. Tracks around the tree represent (from innermost to outermost): cultured status at the order level (cultured versus uncultured), abundance in GTDB based on the number of available genomes (abundant with more than 5 genomes, rare with 5 genomes or less, and novel with no genomes in GTDB), percentage database enrichment (calculated as number of genomes belonging to a certain order binned in the current study as a percentage of the number of genomes belonging to the same order in GTDB), energy conservation capabilities depicted by colored circles (salmon, aerobic respiration; orange, Fe³⁺ respiration; yellow, nitrate/nitrite reduction; dark green, reductive sulfur processes; lime green, nitrogen oxidation; cyan, oxidative sulfur-processes; pink, respiratory hydrogen oxidation; and purple, photosynthesis), and the number of MAGs belonging to each order binned from the sediment (blue bars) and the water (orange bars). For orders with 20 or more genomes, the family-level delineation is shown in Figure 3. These orders are: *Anaerolineales* (Fig. 3A), *Bacteroidales* (Fig. 3B), *Sedimentisphaerales* (Fig. 3C), *Spirochaetales* (Fig. 3D), *Syntrophales* (Fig. 3E), and *Woesearchaeales* (Fig. 3F).

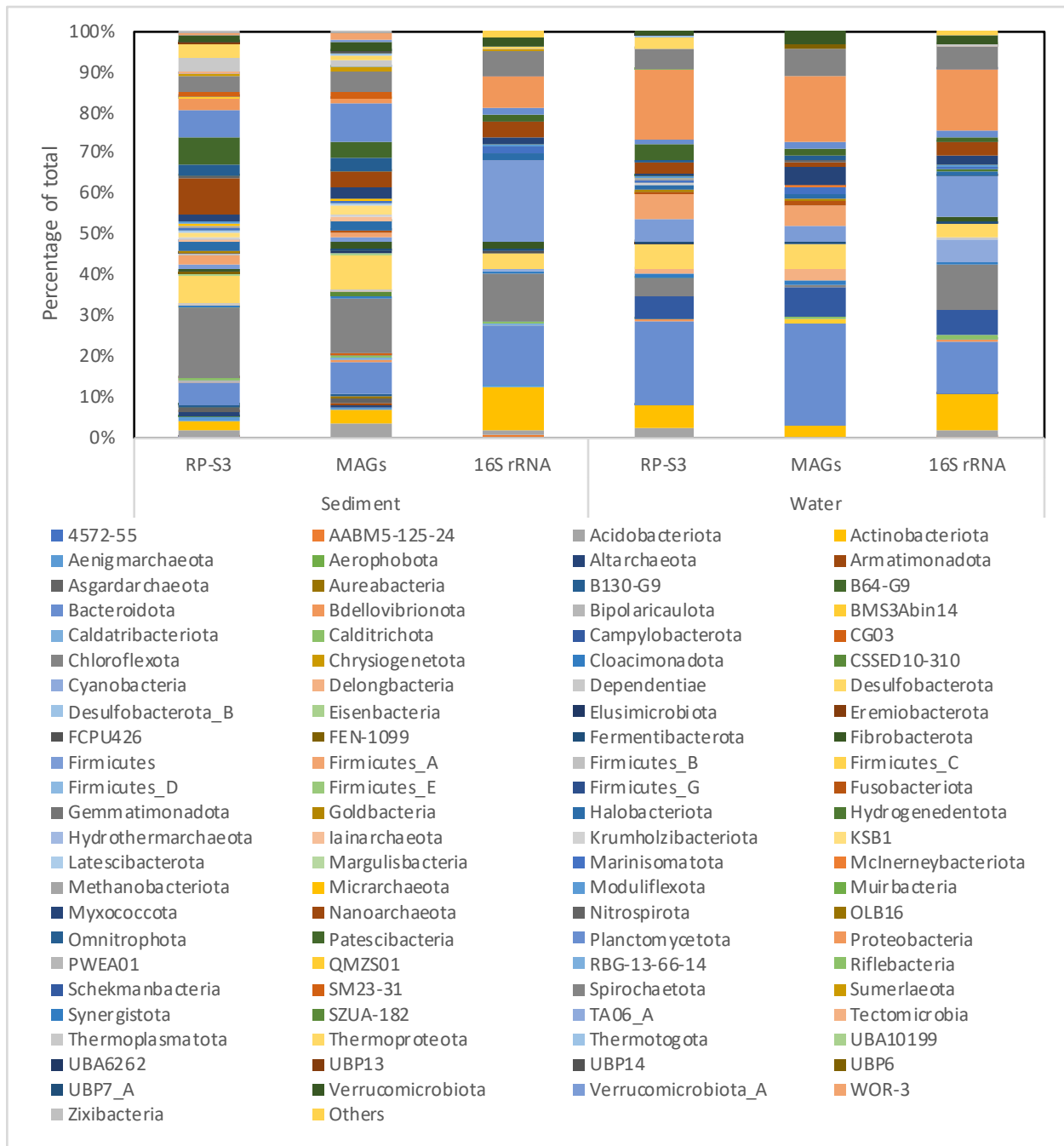


Figure 3.2: Zodletone spring phylum-level community composition

Zodletone spring phylum-level community composition based on ribosomal protein S3 (RP-S3), binned genomes (MAGs), as well as the gene for 16S rRNA for both the sediment and the water samples.

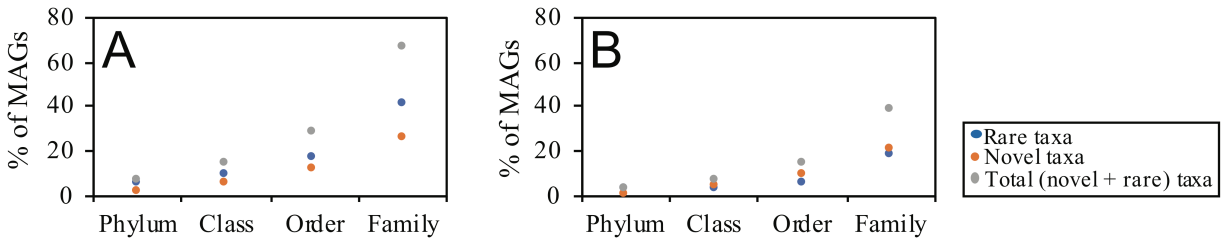


Figure 3.3: Novelty, rarity, and phylum-level makeup in Zodletone

Novelty, rarity, and phylum-level makeup in Zodletone sediment and water communities. Genomes belonging to novel (orange), and LRD (blue) lineages are shown as a percentage of total binned genomes in the sediment (A) and the water (B) communities. The sum of novel and LRD genomes percentages is shown in grey.

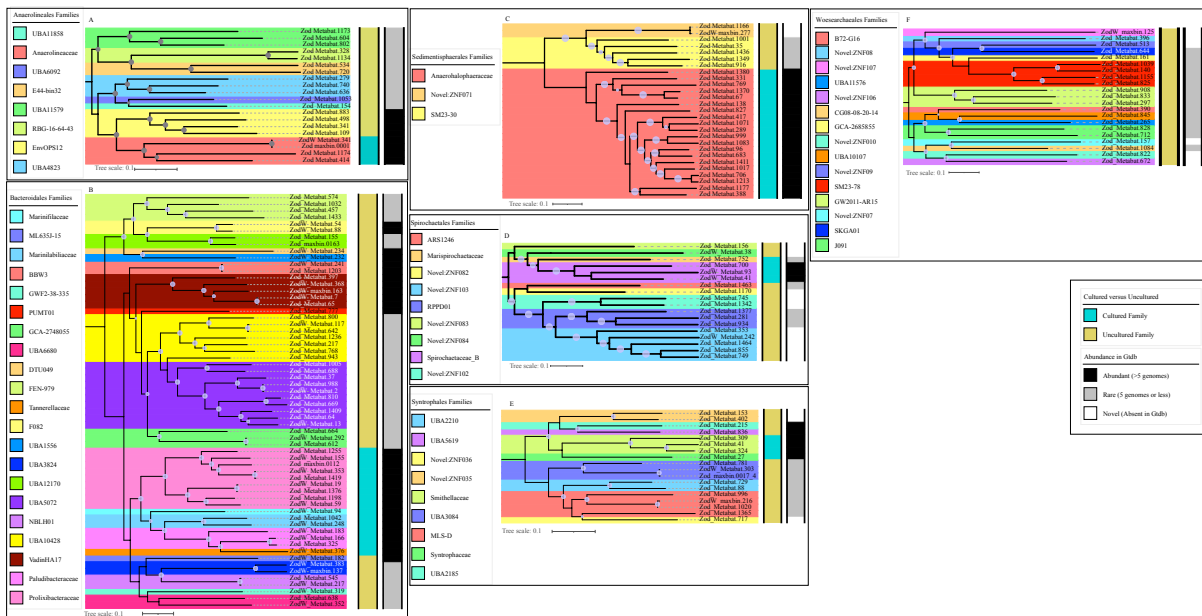


Figure 3.4: Family-level delineation for orders

Family-level delineation for orders with 20 or more genomes. The maximum likelihood trees were constructed in FastTree [83] based on the concatenated alignments of 120-, and 122- single copy genes, respectively, obtained from GTDB-TK (85). Bootstrap support values are shown as bubbles for nodes with >70% support. Families are color-coded. To the right of the trees, tracks are shown for cultured status at the family level (cultured versus uncultured), and abundance in GTDB based on the number of available genomes (abundant with more than 5 genomes, rare with 5 genomes or less, and novel with no genomes in GTDB).

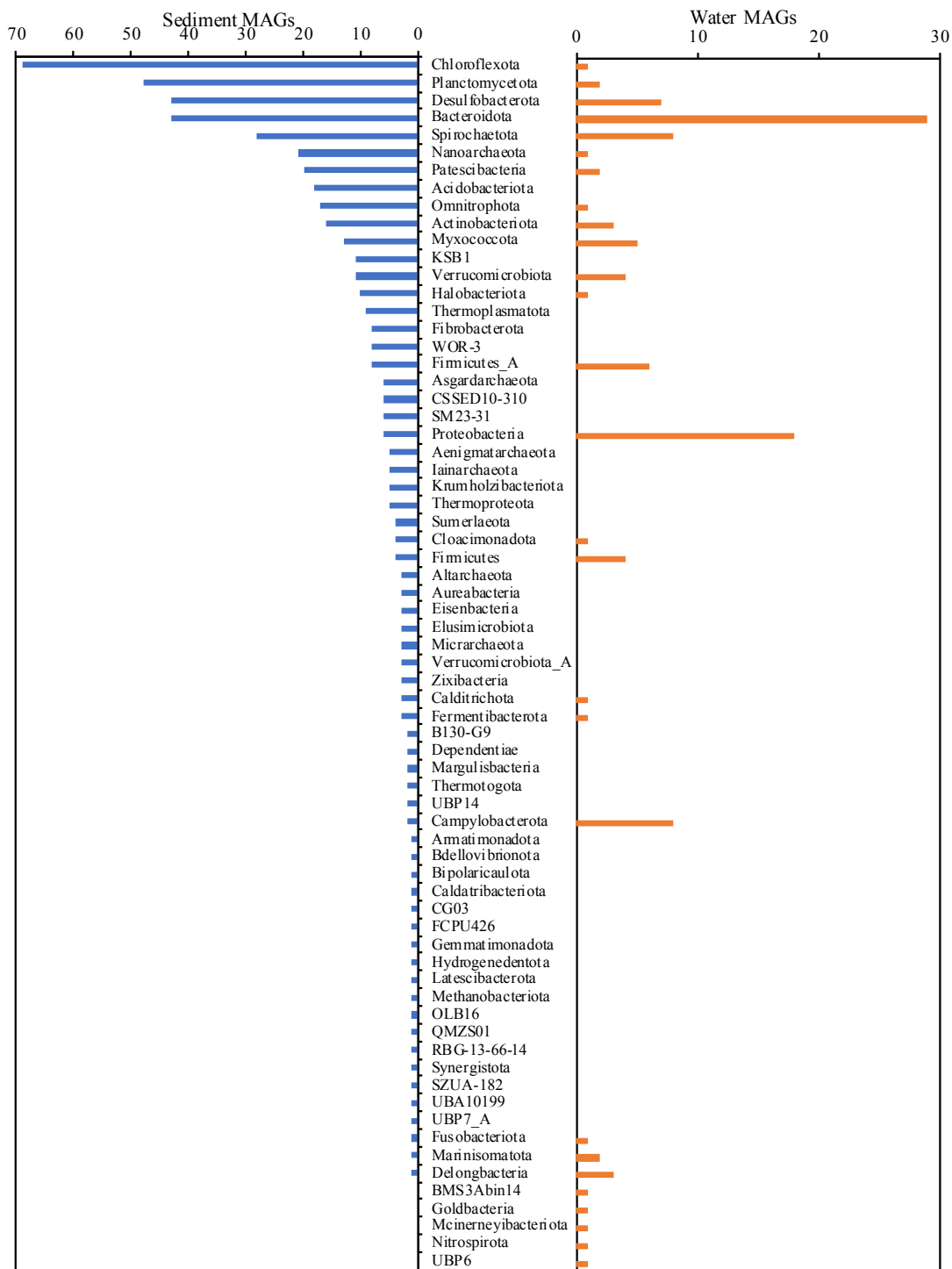


Figure 3.5: Phylum-level affiliation for sediment versus water genomes

Number of genomes belonging to each phylum is shown for the sediment (blue bars on the left) and the water (orange bars on the right).

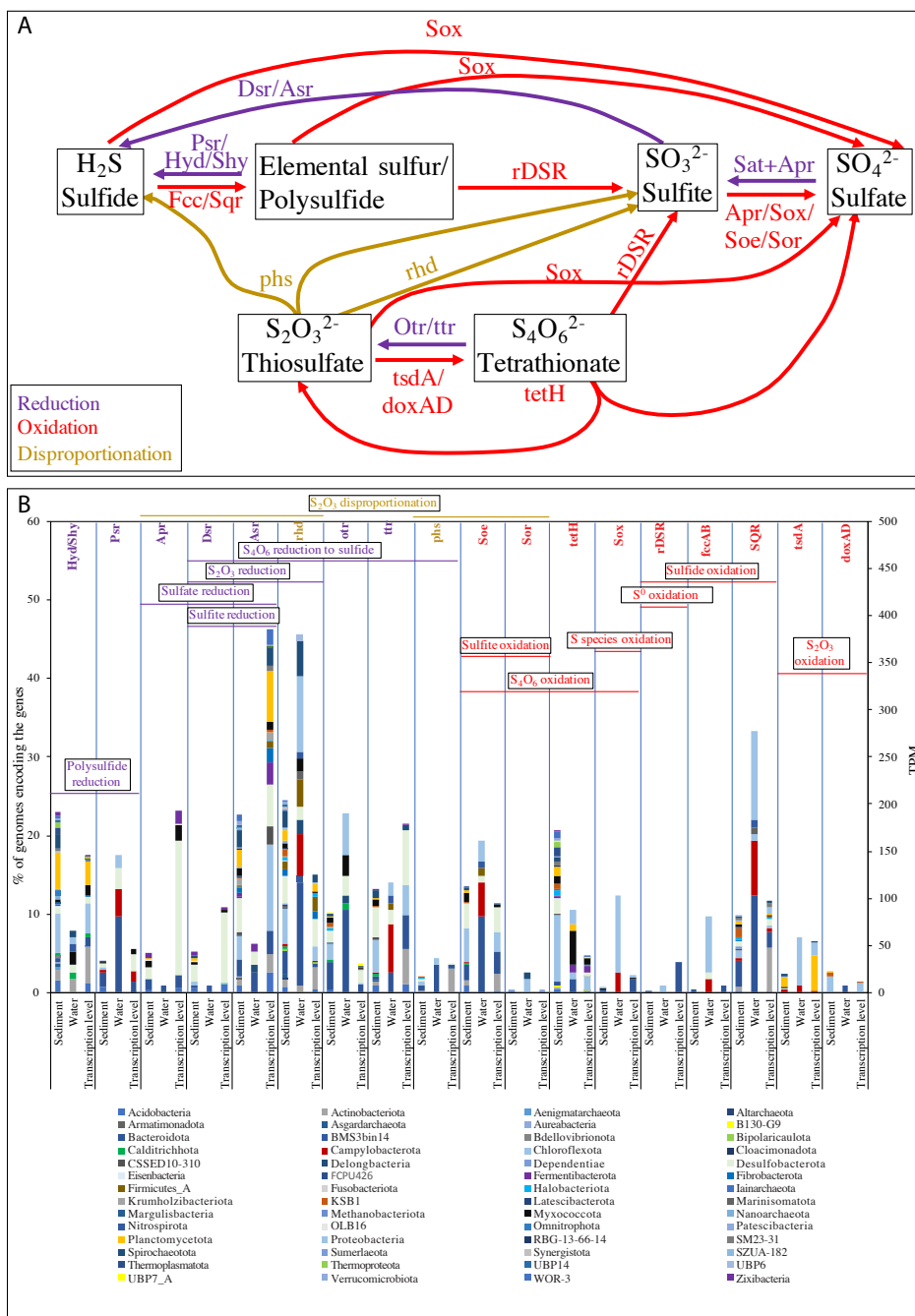


Figure 3.6: Sulfur cycle in Zodletone spring continued

(A) Diagram of sulfur transformations predicted to take place in the spring. Different sulfur species are shown in black boxes. Reduction reactions are depicted by purple arrows, oxidation reactions are depicted by red arrows, while disproportionation reactions are depicted by golden brown arrows. The gene(s) names are shown on the arrows. (B) Phylum-level distribution of the S-cycling genes shown at the top of the figure in sediment and water genomes, as well as the transcriptomic dataset. Processes involving more than one gene are highlighted by horizontal bars and are color coded by reduction (purple), oxidation (red), or disproportionation (golden brown), with the name of the process shown on top of the horizontal bar. RNA-seq reads were pseudo-aligned to the S-cycling genes predicted in Zodletone genomes to detect exact matches using Kallisto (95). The transcripts per million are shown on the secondary Y-axis for the gene/ group of genes depicted at the top of the figure.

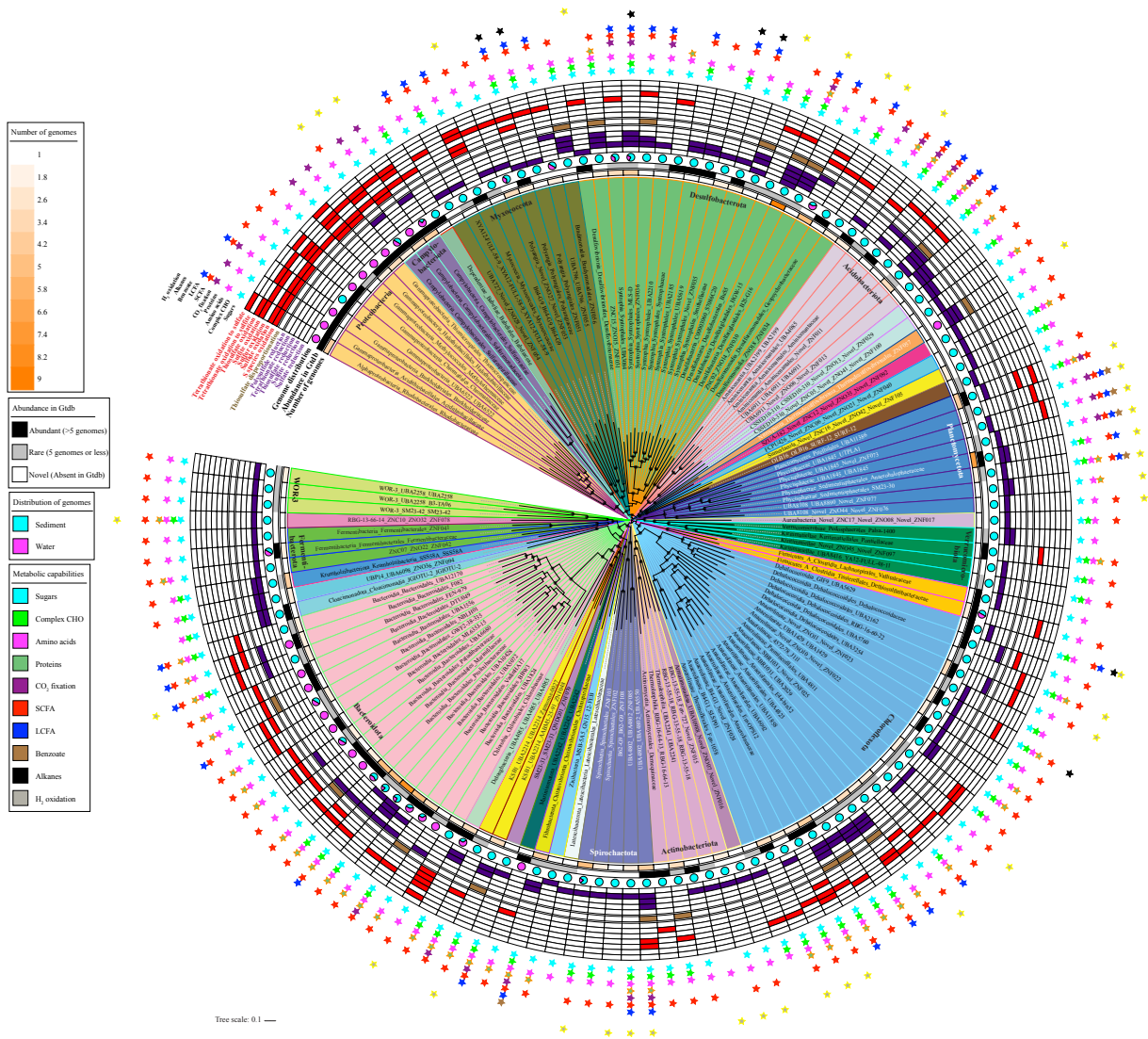


Figure 3.7: Family-level distribution of the genomes involved in S cycling

Figure 3.7: Family-level distribution of the genomes involved in S cycling continued

The maximum likelihood tree was constructed in FastTree 40 based on the concatenated alignments of 120 single-copy genes obtained from Gtdb-TK 41. The branches represent family-level taxonomy and are color coded by phylum. For phyla with 2 families or less involved in S cycling, branches are labeled as Phylum_Class_Order_Family. For phyla where 3 or more families are involved in S cycling, the phylum is shown at the base of the colored wedge and the branches are labeled as Class_Order_Family. Lineages starting with ZN depict novel lineages as follows: ZNC, novel class; ZNO, novel order; and ZNF, novel family. Bootstrap support values are shown as bubbles for nodes with >70% support. Tracks around the tree represent (from innermost to outermost): heatmap for the number of genomes in each family, abundance in Gtdb based on the number of available genomes (abundant with more than 5 genomes, rare with 5 genomes or less, and novel with no genomes in Gtdb), pie charts of the breakdown of the number of genomes in the sediment (cyan) versus water (magenta), sulfur reduction pathways (5 tracks in purple), thiosulfate disproportionation pathways (1 track in golden brown), sulfur oxidation pathways (7 tracks in red), and substrates predicted to support growth depicted by colored stars (cyan, sugars; lime green, complex carbohydrates; magenta, amino acids; orange, proteins; purple, CO₂ fixation; red, short-chain fatty acids (SCFA); blue, beta oxidation of long-chain fatty acids; brown, anaerobic benzoate/aromatic hydrocarbon degradation; black, anaerobic alkane degradation; and grey, Hydrogen oxidation).

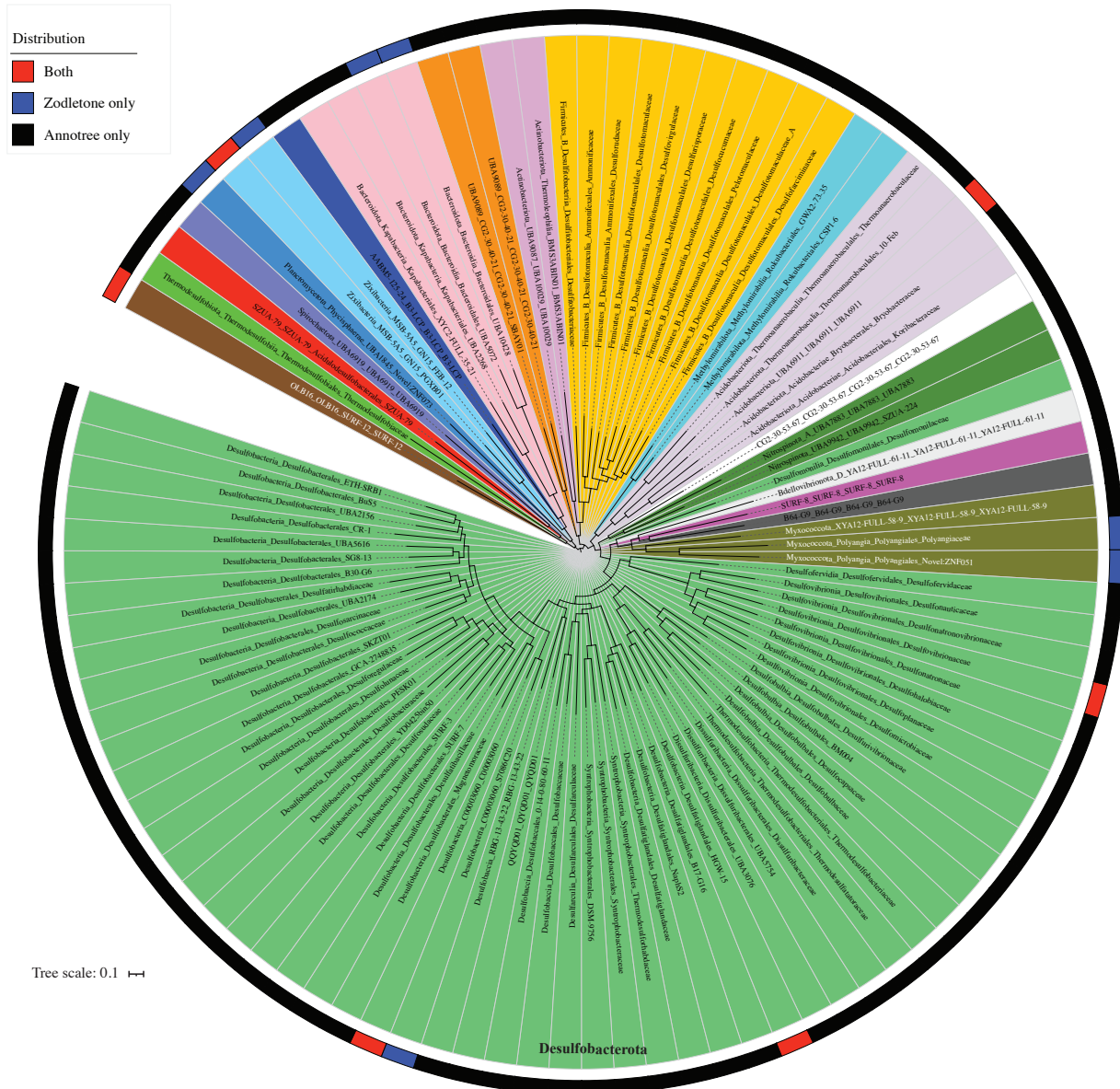


Figure 3.8: Expanding Lineages with sulfate reduction genes

Expanding lineages mediating reductive sulfur cycling processes in the tree of life. Annotree 42 was queried at the family level for the presence of sulfate reduction genes (combined search for the genes AprA, AprB, Sat, QmoA, QmoB, QmoC, DsrA, DsrB, and DsrC).

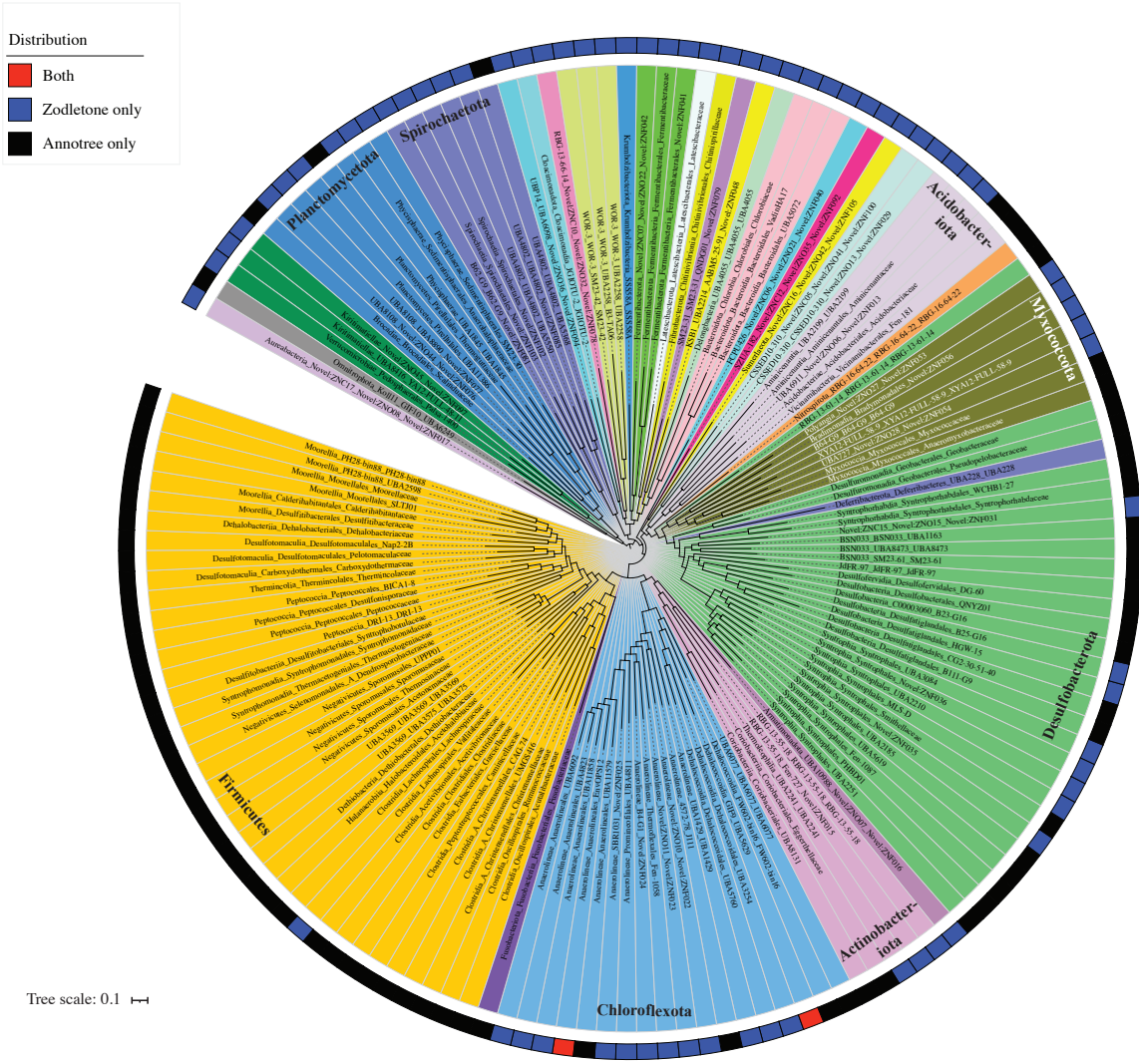


Figure 3.9: Expanding Lineages with sulfite reduction genes

Expanding lineages mediating reductive sulfur cycling processes in the tree of life. Annotree 42 was queried at the family level for the presence of sulfite reduction genes using both the DSR system (combined search for the genes DsrA, DsrB, and DsrC, excluding all duplicate hits from (A) and excluding all hits from phyla known to use the system in the oxidative direction (Proteobacteria, Nitrospira, and Chlorobiaceae), and the ASR system (combined search for the genes AsrA, AsrB, AsrC, HdrA, HdrB, and HdrC).

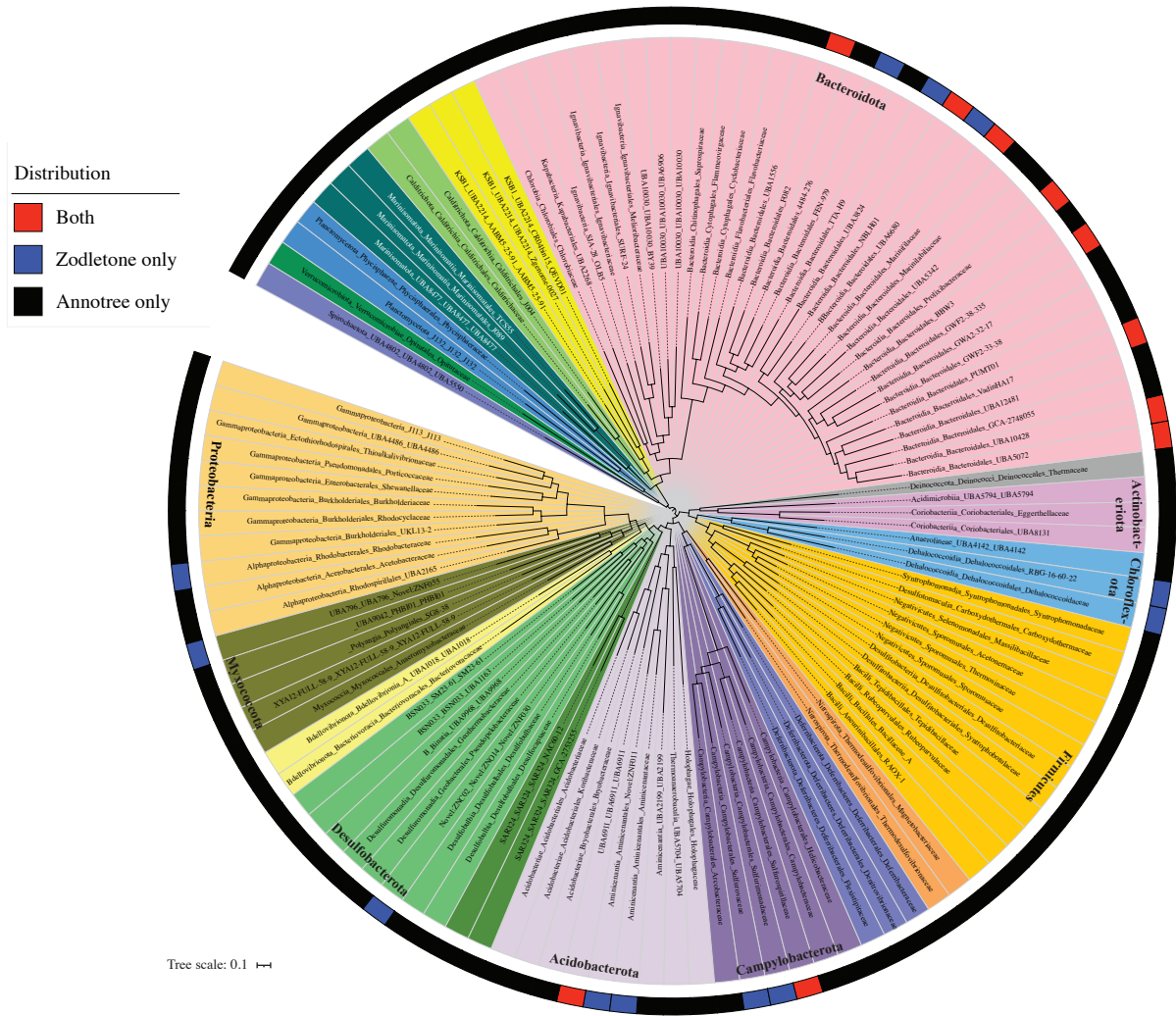


Figure 3.10: Expanding Lineages with polysulfide reduction genes

Expanding lineages mediating reductive sulfur cycling processes in the tree of life. Annotree 42 was queried at the family level for the presence of polysulfide reduction genes (combined search for the genes *PsrA*, *PsrB*, and *PsrC*).

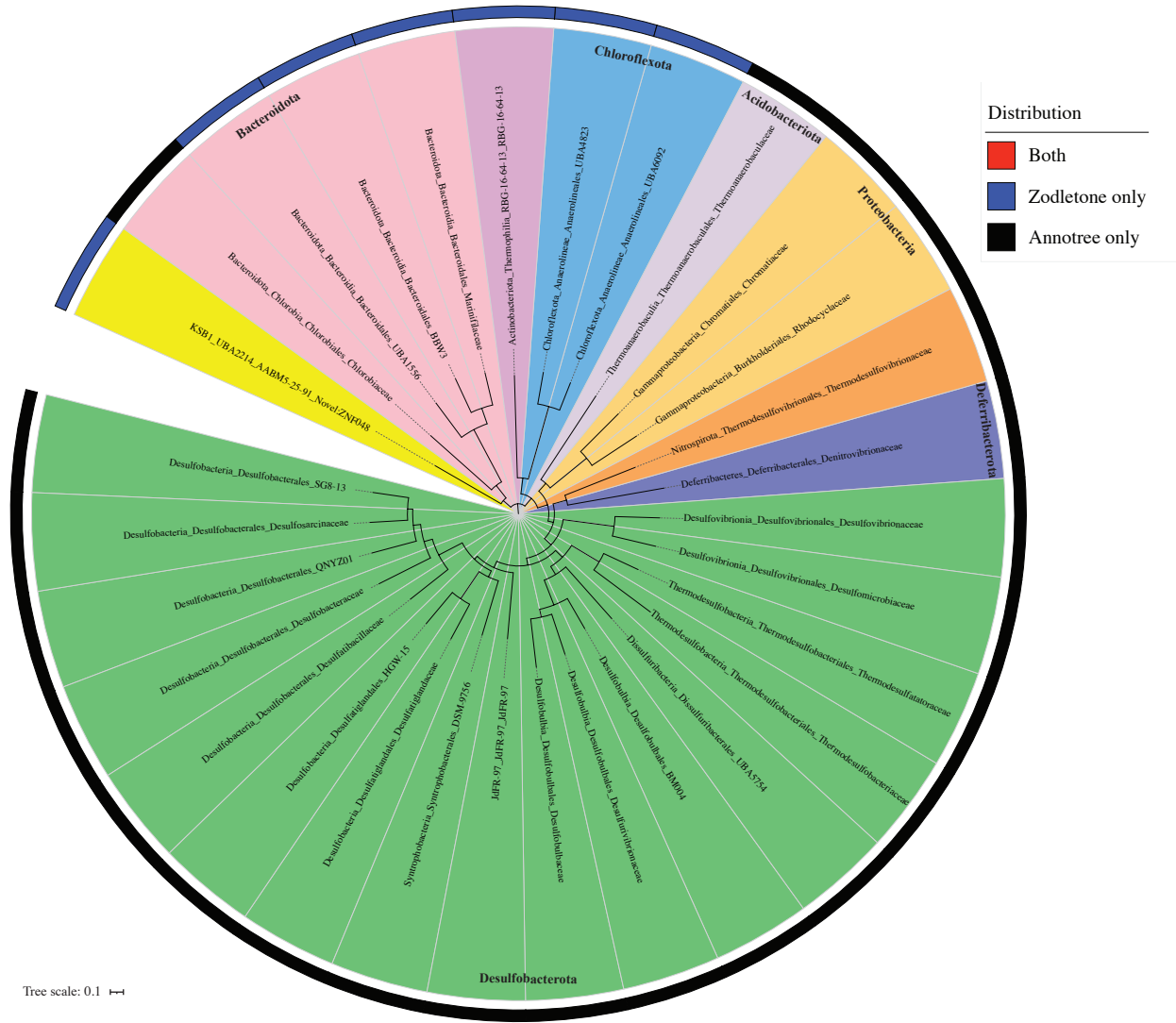


Figure 3.11: Expanding Lineages with thiosulfate reduction genes

Expanding lineages mediating reductive sulfur cycling processes in the tree of life.

Annotree 42 was queried at the family level for the presence of thiosulfate reduction (combined search for the genes PhsA, PhsB, and PhsC, combined with either DsrA, DsrB, and DsrC, or AsrA, AsrB, and AsrC) and thiosulfate disproportionation (combined search for the genes PhsA, PhsB, and PhsC, combined with either AprA, AprB, and Sat, or SoeA, SoeB, and SoeC, or SorA).

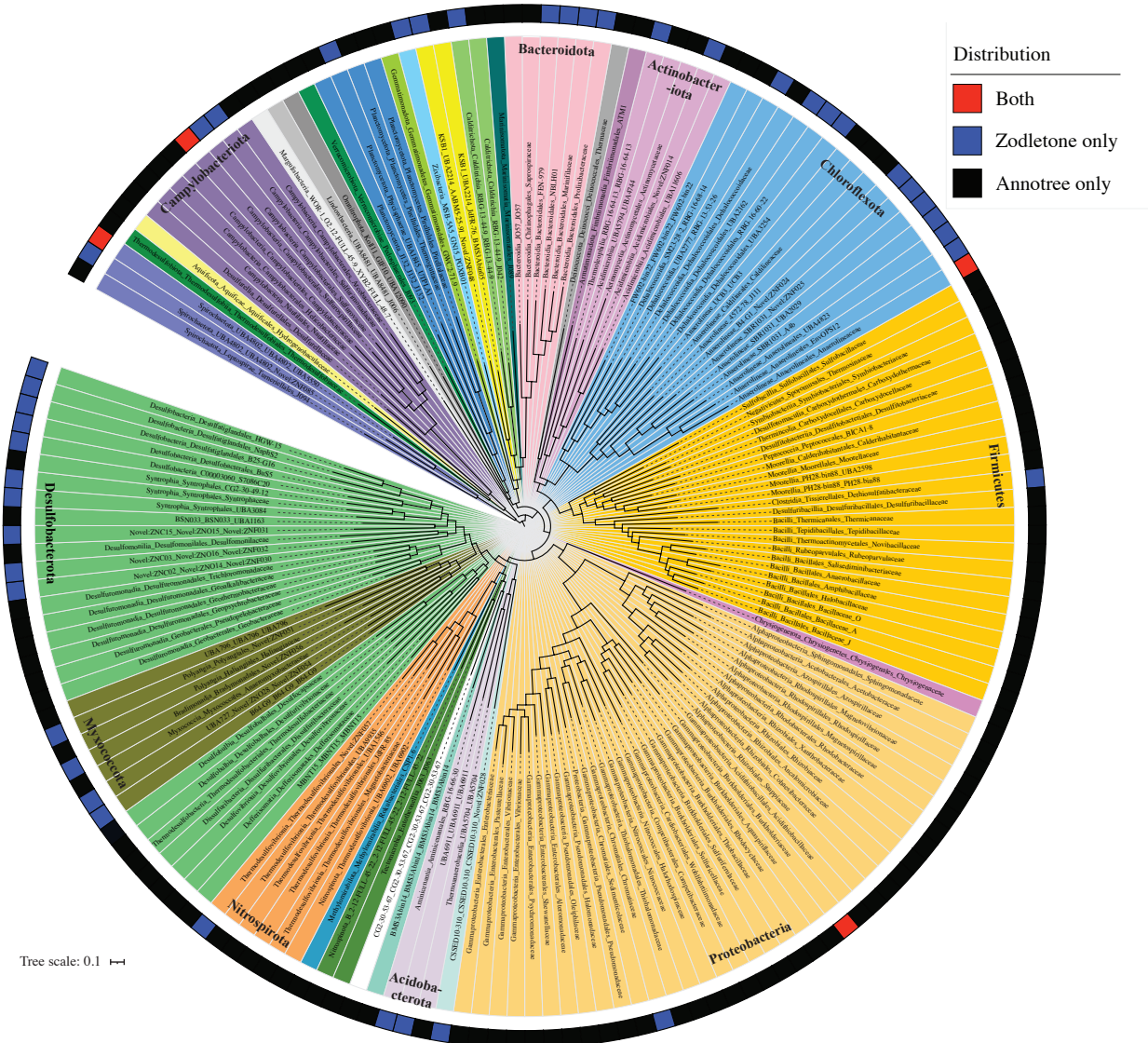


Figure 3.12: Expanding Lineages with tetrathionate reduction genes

Expanding lineages mediating reductive sulfur cycling processes in the tree of life. Annotree 42 was queried at the family level for the presence of tetrathionate reduction (combined search for the genes TtrA, TtrB, and TtrC).

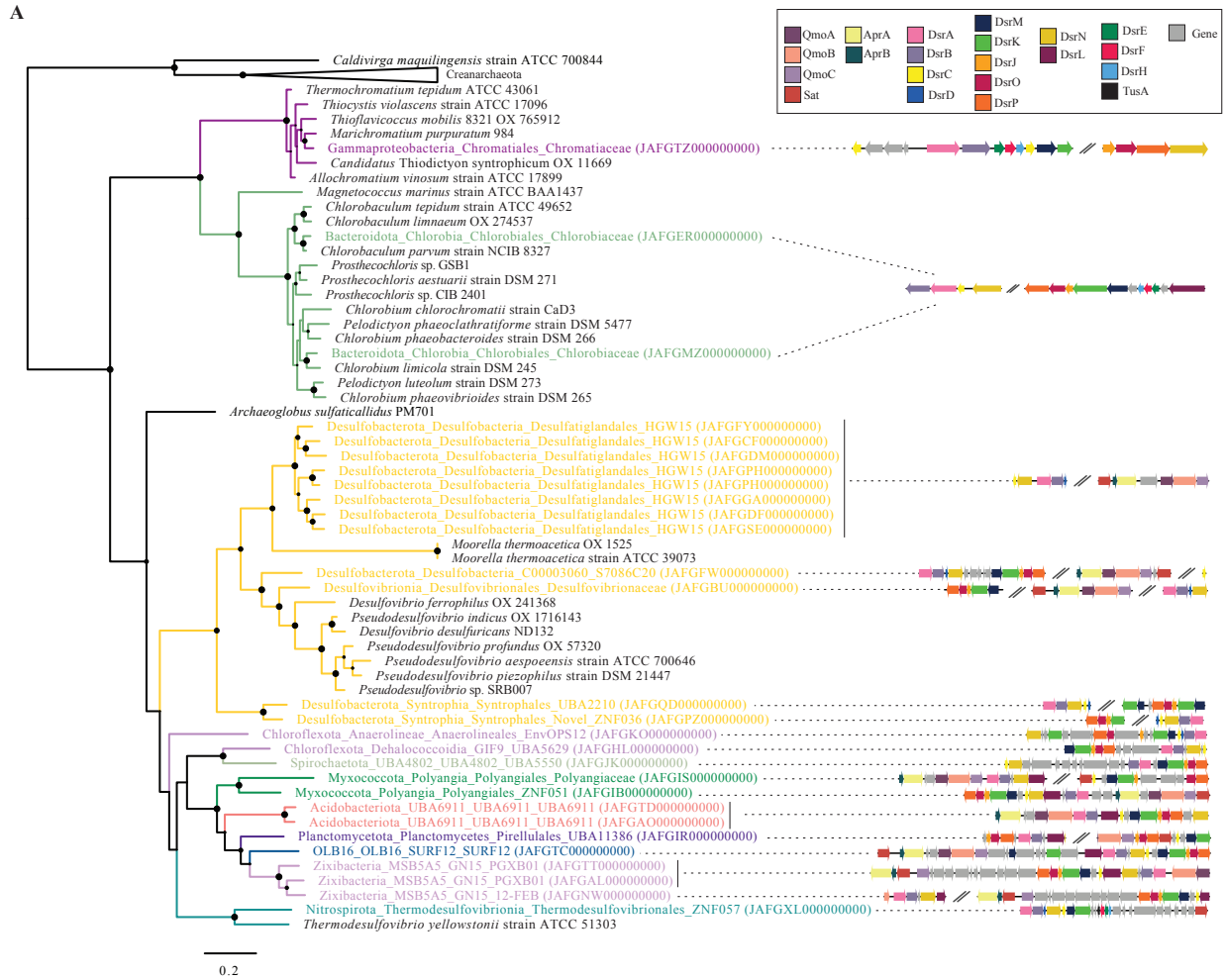


Figure 3.13: Phylogenetic affiliation and contig organization of DsrAB

Phylogenetic affiliation and contig organization of selected sulfur reduction proteins. Phylogeny of the dissimilatory sulfite reductase DsrAB [EC:1.8.99.5] concatenated proteins, anaerobic sulfite reductase subunit B AsrB.

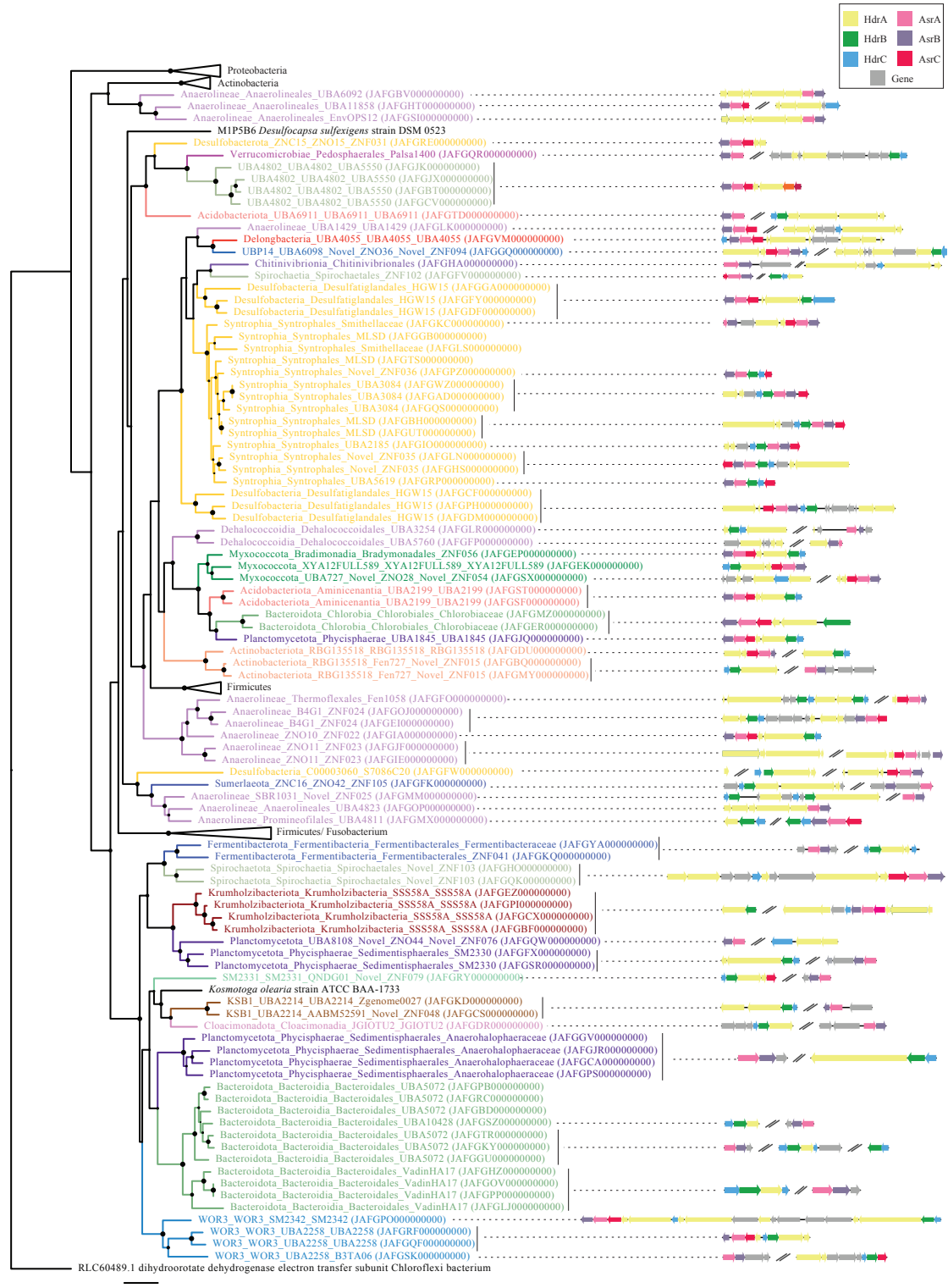


Figure 3.14: Phylogenetic affiliation and contig organization of AsrB

Phylogenetic affiliation and contig organization of selected sulfur reduction proteins.
Phylogeny of the anaerobic sulfite reductase subunit B AsrB.

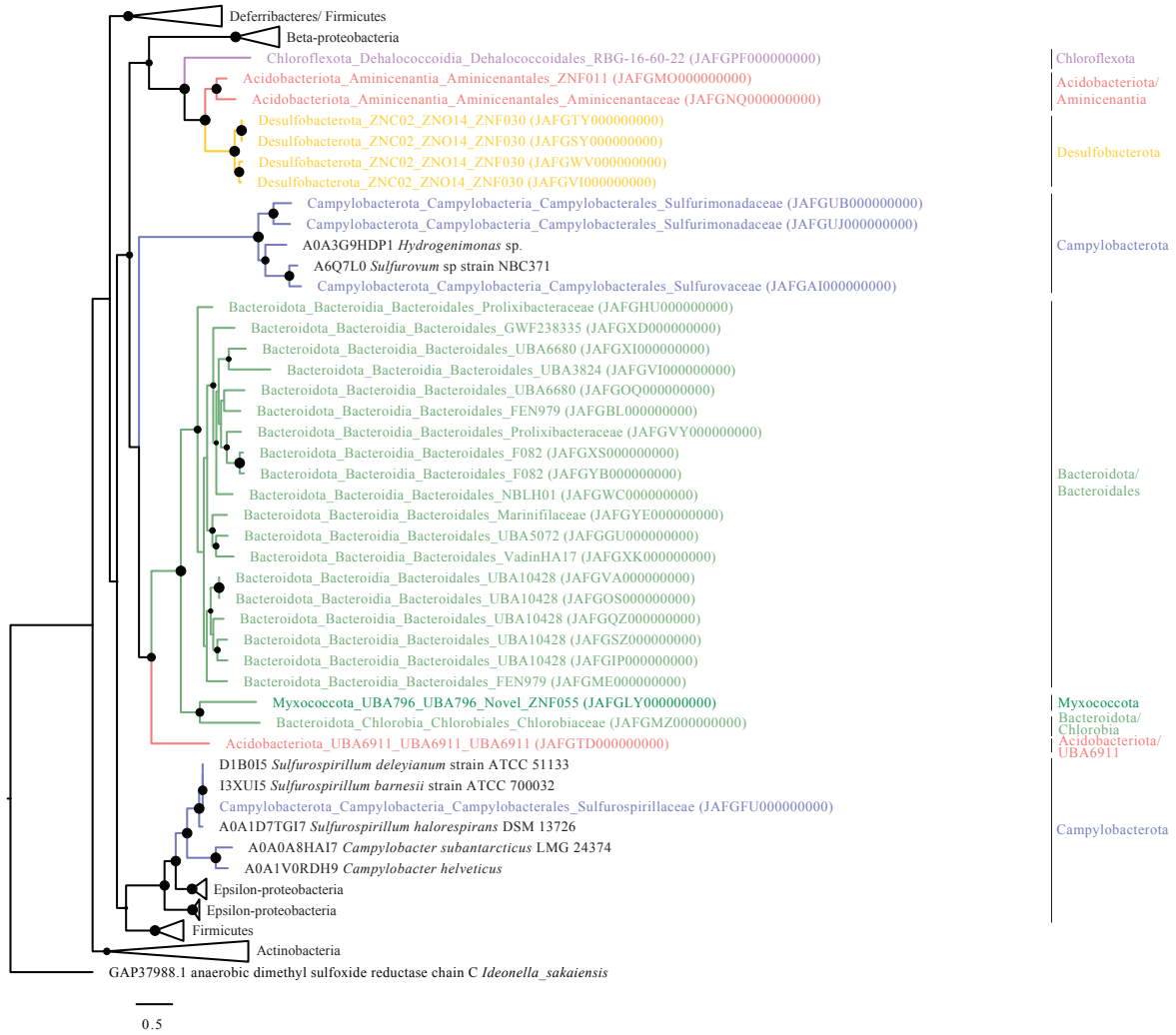


Figure 3.15: Phylogenetic affiliation and contig organization of PsrC

Phylogenetic affiliation and contig organization of selected sulfur reduction proteins. Phylogeny of the ep polysulfide reductase subunit gamma PsrC. , and thiosulfate reductase cytochrome b subunit PhsC.

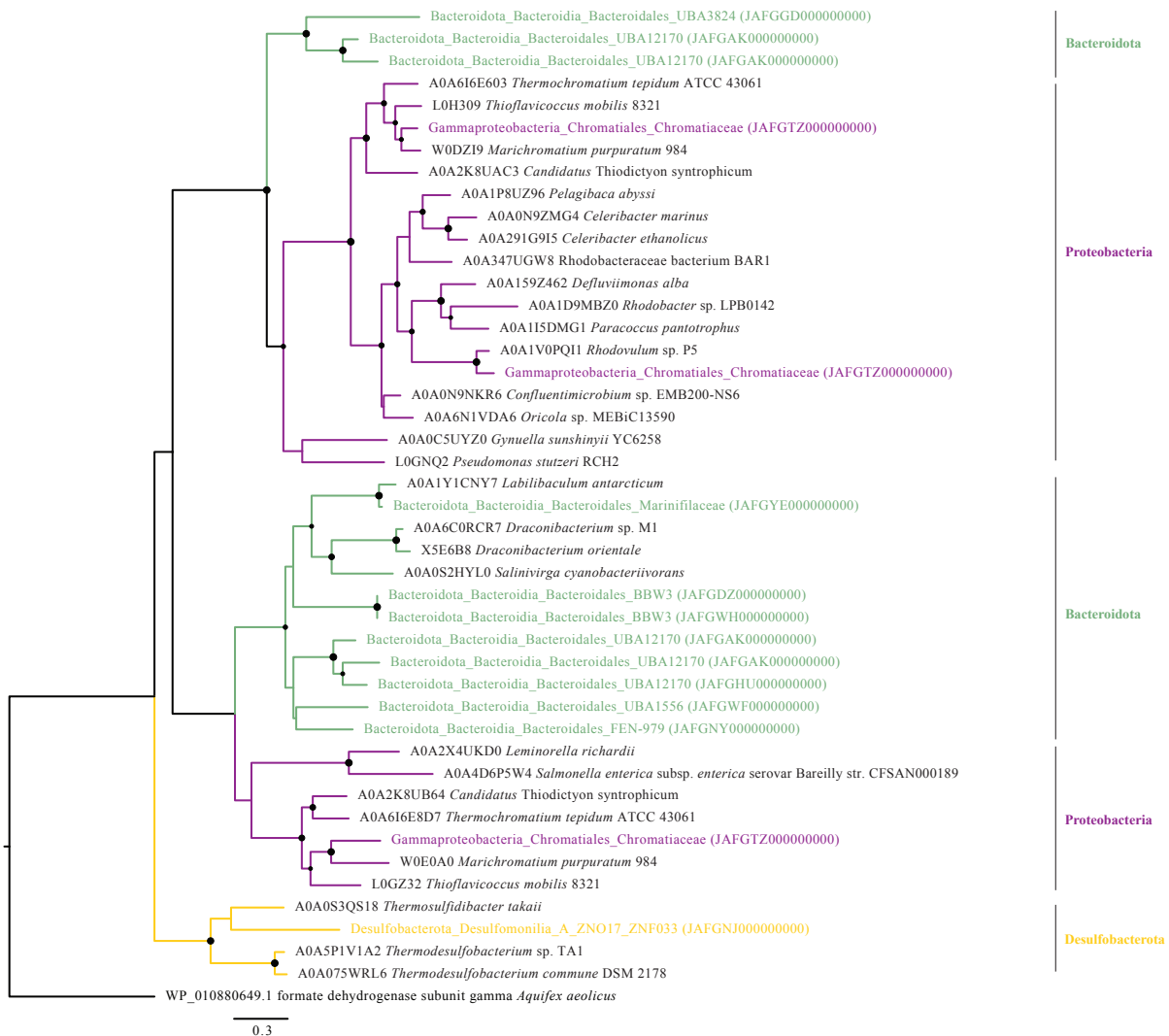


Figure 3.16: Phylogenetic affiliation and contig organization of PhsC
 Phylogenetic affiliation and contig organization of selected sulfur reduction proteins.
 Phylogeny of the thiosulfate reductase cytochrome b subunit PhsC.

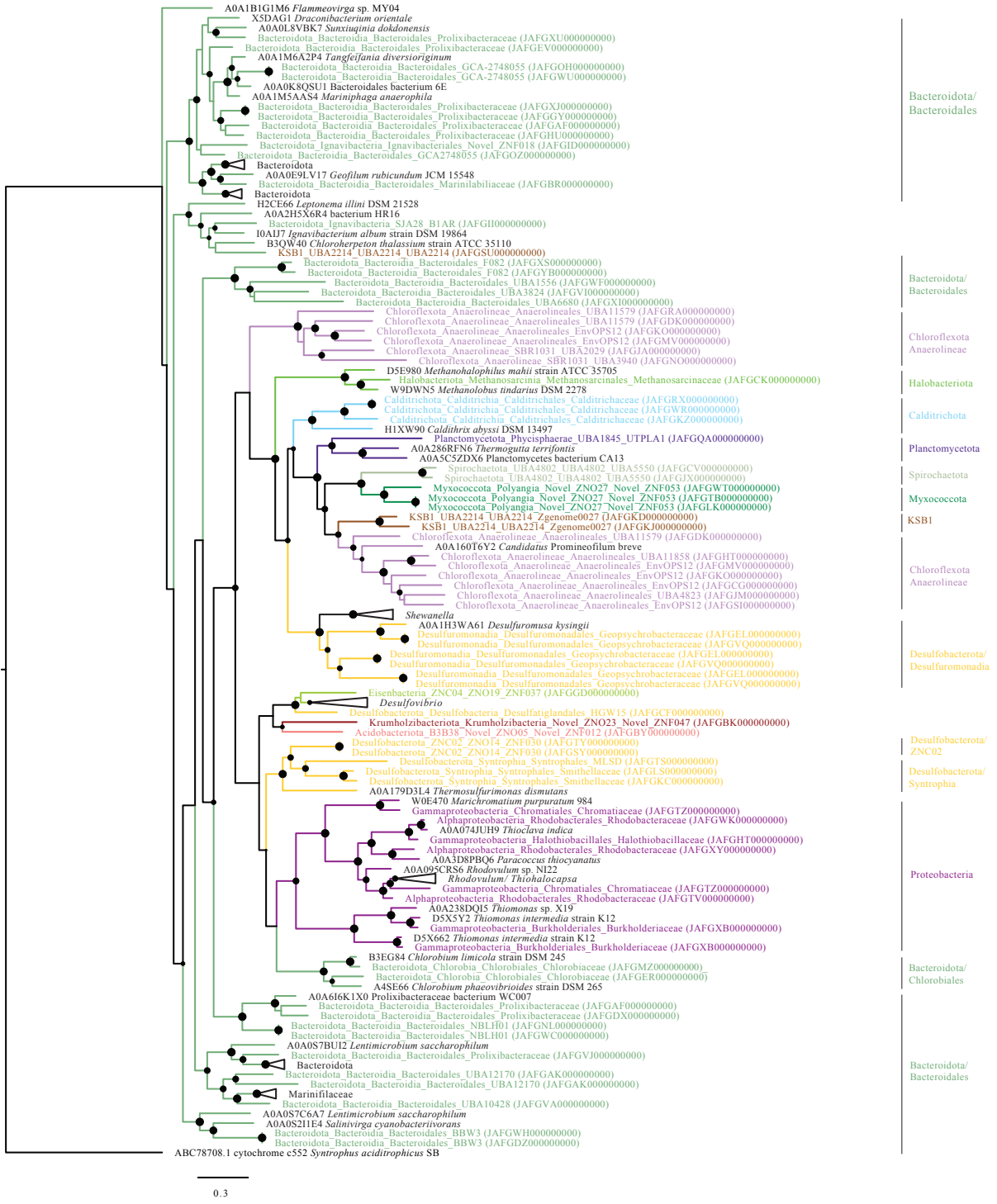


Figure 3.17: Phylogenetic affiliation and contig organization of Otr
 Phylogenetic affiliation and contig organization of selected sulfur reduction proteins.
 Phylogeny of the octaheme tetrathionate reductase Otr.

CHAPTER IV

DISCUSSION

The microbial community in Zodletone spring sediments exhibited a high level of phylogenetic diversity, novelty, and rarity (Figure 3.1, 3.3, 3.4, 3.5). Conversely, representatives of lineages that predominate in most present earth environments, e.g. Proteobacteria, Firmicutes, and Cyanobacteria were absent or extremely sparse within the sediments. The community in the spring sediments was also characterized by a high proportion of SRM, and the prevalence of lineages mediating the reduction of sulfur cycle intermediates (sulfite, thio-sulfate, tetrathionate, and elemental sulfur) over sulfate-reducers. Many of the organisms mediating reductive sulfur-cycling processes belonged to novel and LRD lineages (Figure 3.7), hence expanding the range of SRM within the tree of life (Figure 3.8, 3.9, 3.10, 3.11, 3.12).

What drives the assembly, propagation, and maintenance of such a diverse, novel, and distinct community in the spring sediments? The high level of diversity, novelty, and rarity within Zodletone spring sediment SRM community could be attributed to two main factors. First, the availability of a wide range of sulfur cycle intermediates in concentrations much higher than sulfate, in contrast to sulfate-predominance in current ecosystems [109]. Such pattern selects for a more diverse community of SRM in the spring, when compared to predominantly sulfate-driven marine and freshwater ecosystems (Figure 3.7). Second, additional factors usually constraining SRM growth in several habitats such as diel or seasonal intrusion of oxygen, Fe and NO_3 [1, 29, 48, 70], recalcitrance of available substrates [19, 28, 50], temperature [49, 91], pH [100, 96, 104], salinity [101], and pressure extremes [6, 19], or combinations thereof, are absent in the spring. Therefore, while the reductive global sulfur cycle appears to be dominated by a few sulfate-reducing lineages within the Desulfobacterota, and to a lesser extent the Firmicutes, as well as Thermodesulfobacteria and *Archaeoglobus* in high temperature habitat, the SRM community in Zodletone is extremely diverse, encompassing a wide range of previously undescribed and LRD lineages (Figure 4, S4, S5, S6). Sulfate-reducing organisms are the most prevalent component of the reductive sulfur cycle in most marine and aquatic ecosystems. Aspects of the ecology [109], physiology [85], and biochemistry [7, 80, 110] of dissimilatory sulfate reduction have been extensively investigated [86]. While not the most prevalent process, the sulfate-reducing community in Zodletone spring sediment exhibited a unique composition, with members of the Zixibacteria, Acidobacteriota, Myxococcota, Bacteroidota, Planctomycetota, and candidate phylum OLB16 constituting the major players, as well as rare and novel lineages within the Desulfobacterota (Desulfatiglandales, and Order C00003060), with scarce representation of canonical Desulfobacterota sulfate reducers (1 genome). While the identification of the dissimilatory sulfate reducing machinery in some of these lineages (e.g. Zixibacteria, Aci-

dobacteriota, and Planctomycetota) has been shown before [3, 25, 35], these members rarely appear to be the dominant players in a single ecosystem.

Compared to sulfate-reduction, the ecology and diversity of microbial dissimilatory sulfite reduction has not been extensively studied. The biochemistry of the process has been examined in sulfate-reducers, when grown on sulfite [86], as well as in a few other dedicated sulfite reducers such as *Desulfitobacterium* [107], *Salmonella* [40], *Shewanella* [93], and *Wolinella* [58]. A recent study suggested the importance and ancient nature of sulfite reduction in an extreme thermophilic environment in a limited diversity biofilm [17]. We document a plethora of microorganisms within the phyla Planctomycetes, Chloroflexota, Spirochaetota, and Desulfobacterota encoding the dissimilatory sulfite reductase DSR, as well as 72 additional families (31 novel and 25 LRD) encoding the anaerobic sulfite reductase. These organisms expand the known sulfite reduction capacity within the domain Bacteria (Figure 3.8, 3.9, 3.10, 3.11, 3.12). Further, the novelty or rarity of some of these families is a reflection of the dearth of present-time habitats that could support this mode of metabolism, once predominant on ancient earth.

The bulk of knowledge on thiosulfate reduction and or disproportionation comes from studies in pure cultures, e.g. members of the Desulfobulbaceae (e.g. *Desulfocapsa*) [23, 26] and the genera *Desulfovibrio* and *Desulfomonile* [34, 99, 72] within Desulfobacterota, the gammaproteobacterium *Pantoea agglomerans* [74], members of Thermodesulfobacteria [66] and Firmicutes [43]. Radioisotope tracing of different sulfur atoms showed a significant contribution of thiosulfate disproportionation to the sulfur cycle in marine [46], as well as freshwater sediments [51]. However, the lack of a marker gene for the process hinders ecological culture-independent studies. Similar to sulfite, the high levels, and constant generation of thiosulfate in Zodletone sediments sustains a highly diverse thiosulfate reducing (thiosulfate reductase plus a sulfite reduction complex), or disproportionating (thiosulfate reductase plus both sulfite reduction and sulfite oxidation systems) community with major contribution from novel or rare families in the Acidobacteriota, Chloroflexota, Desulfobacterota, KSB1, Myxococcota, and Spirochaetota. Finally, the extremely high levels of zero valent sulfur, available as soluble polysulfide, result in enriching the community with a plethora of polysulfide-reducing organisms.

As described above, this study infers that the microbial communities thriving in ancient conditions of anoxia and high proportion of sulfur cycle intermediates were extremely diverse. In comparison, communities in the oxygen-exposed water column were markedly less diverse. What drives this drastic shift in diversity and community structure? We argue that oxygen introduction into the system is responsible for such shift, as evident by the shift to oxidative sulfur processes in the water samples. The prevailing conditions in the water column are hence more akin to microbial communities that would thrive in sulfur-rich yet air-exposed habitat on the current earth.

The comparison presented here between both communities could putatively demonstrate how ancient metabolic pathways and lineages mediating them have been curtailed due to oxygen evolution and predominance in the current surficial earth. The evolution of oxygenic photosynthesis has led to the steady and inexorable accumulation of O_2 in Earth's atmosphere (the great oxidation event, GOE), with the rise of atmospheric O_2 to 1-5% of current levels between 2.4 and 2.1 billion years (Gyr) ago, and its accumulation to values comparable to modern values 500-600 Mya [63]. Due to the expected sensitivity and lack of adaptive

mechanisms to cope with atmospheric oxygen in multiple strict anaerobes, as well as the chemical instability of multiple S species in an oxygenated atmosphere, the GOE exerted a profound negative impact on anaerobic surficial life forms (the oxygen catastrophe) leading to the first and arguably most profound extinction event in earth's history [63]. In addition to suppressing anaerobiosis in atmospherically-exposed habitats, the GOE also led to a significant change in the S cycle, from one based on atmospheric inputs to one dependent on oxidative weathering leading to the release of huge amount of sulfate derived from the oxidation of pyrite and the dissolution of sulfate minerals [59], hitherto a minor byproduct of Archean abiotic and biotic reactions [32, 38]. Therefore, it appears that loss of niches associated with geological transformations could be one of the possible explanations for high extinction rates for microorganisms on earth, as well as the constant identification of rare, novel taxa within anaerobic settings. It is notable that phylogenetically novel branches with extremely rare distribution in earth (defined as phyla with 5 genomes or less in GTDB) have been consistently identified in anaerobic habitats.

In summary, by examining microbial diversity in Zodletone spring, we greatly expand the overall diversity within the tree of life via the discovery and characterization of a wide range of novel lineages, and significantly enrich representation of a wide range of LRD lineages. We also describe a unique sulfur-cycling community in the spring that is largely dependent on sulfite, thiosulfate, sulfur, and tetrathionate, rather than sulfate, as an electron acceptor. Given the remarkable similarity to conditions prevailing prior to the GOE, we consider the spring an invaluable portal to investigate the community thriving on the earth's surface during these eons, and posit that GOE precipitated the near extinction of a wide range of phylogenetically distinct oxygen-sensitive lineages and drastically altered the reductive sulfur-cycling community from sulfite, sulfur, and thiosulfate reducers to predominantly sulfate reduction in the current earth.

The full paper can be found here: <https://doi.org/10.1128/mbio.00016-22>

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