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Permalink

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Journal

The ISME journal, 12(7)

ISSN

1751-7362

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Publication Date

2018-06-01

DOI

10.1038/s41396-018-0078-0

Peer reviewed



Expanded diversity of microbial groups that shape the dissimilatory sulfur cycle

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Received: 11 October 2017 / Revised: 10 January 2018 / Accepted: 13 January 2018 / Published online: 21 February 2018
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Abstract

A critical step in the biogeochemical cycle of sulfur on Earth is microbial sulfate reduction, yet organisms from relatively few lineages have been implicated in this process. Previous studies using functional marker genes have detected abundant, novel dissimilatory sulfite reductases (DsrAB) that could confer the capacity for microbial sulfite/sulfate reduction but were not affiliated with known organisms. Thus, the identity of a significant fraction of sulfate/sulfite-reducing microbes has remained elusive. Here we report the discovery of the capacity for sulfate/sulfite reduction in the genomes of organisms from 13 bacterial and archaeal phyla, thereby more than doubling the number of microbial phyla associated with this process. Eight of the 13 newly identified groups are candidate phyla that lack isolated representatives, a finding only possible given genomes from metagenomes. Organisms from *Verrucomicrobia* and two candidate phyla, *Candidatus* Rokubacteria and *Candidatus* Hydrothermarchaeota, contain some of the earliest evolved *dsrAB* genes. The capacity for sulfite reduction has been laterally transferred in multiple events within some phyla, and a key gene potentially capable of modulating sulfur metabolism in associated cells has been acquired by putatively symbiotic bacteria. We conclude that current functional predictions based on phylogeny significantly underestimate the extent of sulfate/sulfite reduction across Earth's ecosystems. Understanding the prevalence of this capacity is integral to interpreting the carbon cycle because sulfate reduction is often coupled to turnover of buried organic carbon. Our findings expand the diversity of microbial groups associated with sulfur transformations in the environment and motivate revision of biogeochemical process models based on microbial community composition.

Electronic supplementary material The online version of this article (<https://doi.org/10.1038/s41396-018-0078-0>) contains supplementary material, which is available to authorized users.

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Introduction

The cycling of sulfur is one of Earth's major biogeochemical processes. Sulfate reduction in conjunction with sulfur

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disproportionation may be an early evolved microbial metabolism, given evidence for biological fractionation of sulfur isotopes around 3.5 billion years ago [1, 2], and it remains an important energy metabolism for anaerobic life [3]. In natural ecosystems, human microbiomes, and engineered systems, this process is important because the product hydrogen sulfide (H₂S) can be toxic [4], can corrode steel [5], and sour oil reservoirs [6]. Overall, sulfate reduction is a primary driver in the carbon cycle, and is responsible for a large part of the organic carbon flux to CO₂ in marine sedimentary environments [7, 8] and in wetlands [9]. Importantly, the coupling of sulfate/sulfite reduction to oxidation of H₂, small chain fatty acids, or other carbon compounds limits the availability of these substrates to other organisms like methanogens and alters the energetics via syntrophic interactions [10, 11]. All of these processes also impact methane production. Given the many reasons why the biological conversion of sulfate/sulfite to sulfide is important, it is vital that we understand which organisms can carry out the reactions and the pathways involved.

The canonical microbial pathway for dissimilatory sulfate reduction involves the initial reduction of sulfate to sulfite by a combination of sulfate adenylyltransferase (Sat) and adenylyl-sulfate reductase (AprBA) followed by reduction of sulfite by sulfite reductases. Sulfite reductase genes catalyze the rate-limiting steps in the global sulfur cycle [12, 13] and confer bacteria and archaea the ability to grow via reduction of sulfite, and can function in reverse in some organisms that disproportionate or oxidize elemental sulfur [14–16]. Four different groups of sulfite reductases function in dissimilatory sulfur metabolism. Of these, siroheme-dependent dissimilatory sulfite reductase (*dsr*), siroheme-dependent anaerobic sulfite reductase (*asr*) genes, and octaheme cytochrome *c* sulfite reductase (*mccA*) catalyze the reduction of sulfite to sulfide, while reverse dissimilatory sulfite reductase genes (*rdsr*) are involved in sulfur oxidation. All of these sulfite reductases except for *mccA* constitute an ancient lineage of enzymes that may predate the separation of Bacteria and Archaea [17].

The taxonomic distribution of dissimilatory sulfite reductases has been considered to be restricted to organisms from selected bacterial and archaeal phyla [18]. Only organisms from nine microbial phylum-level lineages, namely *Deltaproteobacteria*, *Firmicutes*, *Thermodesulfobacteria*, *Actinobacteria*, *Nitrospirae*, *Caldiserica*, *Euryarchaeota*, *Crenarcheota*, and *Aigarchaeota* are known to possess the genetic capacity to reduce sulfite to sulfide using the *dsr* system. The *asr* enzymes have a far more limited distribution and are known to be present only in organisms from four phylum-level lineages, *Gamma-proteobacteria*, *Firmicutes*, *Spirochaetes*, and

Fusobacteria. The distribution of *MccA* enzymes is restricted to organisms from *Epsilonproteobacteria* [19] and *Gamma-proteobacteria* [20]. Finally, the *rdsr* enzyme complex for sulfur oxidation is associated with organisms from five phylum-level lineages including *Alphaproteobacteria*, *Betaproteobacteria*, *Gamma-proteobacteria*, *Deltaproteobacteria*, and *Chlorobi*. This diversification of sulfite reductases was likely driven by speciation and functional divergence, and to a lesser extent, lateral gene transfer (LGT) [21].

The recent availability of thousands of genomes from organisms belonging to many newly sampled phyla has provided the opportunity to test for the presence of sulfite reductase genes in bacteria and archaea that have not previously been associated with dissimilatory sulfur metabolism [22]. Here we use shotgun metagenomic sequencing and recovery of metagenome-assembled genomes (MAGs) from a diverse set of marine and terrestrial environments to show that organisms from novel lineages contain sulfite reductases that implicate them in the dissimilatory cycling of sulfur. In total, we more than doubled the number of microbial lineages that can catalyze dissimilatory sulfate/sulfite reduction or sulfur oxidation in the environment. We shed light on the complicated evolutionary history of dissimilatory sulfite reductases and show that LGT of catalytic sulfite reductase genes is much more common than previously thought.

Materials and methods

Sample collection and data processing

Details of sample collection (Sampling, DNA Extraction), individual sample geochemical measurements, and data processing (DNA sequencing, assembly, annotation, binning, genome completion estimates) are described in detail elsewhere [23–27].

Identification of selected sulfate/sulfite reduction genes

Genome-specific metabolic potential for sulfate/sulfite reduction was determined in an iterative manner by (A) searching all predicted ORFs in a genome with HMM profiles for *dsrA* and *dsrB* from TIGRFAM [28], and *dsrD* from Pfam [29] using hmmscan v3.1b2 [30], and (B) searching against custom hmm profiles for *dsrA*, *dsrB*, and *dsrD* using hits generated from step (A) and searching all predicted ORFs again for the above genes. (C) Identification of anaerobic sulfite reductase genes was conducted by searching all predicted ORFs against *asrA*, *asrB*, and *asrC* hmm databases from TIGRFAM [28]. (D) Identification of genes for the reduction of sulfate to

sulfite was conducted by searching all predicted ORFs against *aprA*, *aprB*, and *sat* hmm databases from TIGRFam [28].

Taxonomic confirmation

The taxonomy of organisms represented by the 123 identified genomes was determined by using a concatenated RP tree. Briefly 16 RPs (L2, L3, L4, L5, L6, L14, L15, L16, 18, L22, L24, S3, S8, S10, S17, S19) were each aligned separately using MUSCLE v3.8.31 [31]. All alignments were end trimmed manually and all columns with >95% gaps were trimmed. The individual alignments were concatenated and the phylogenetic tree was inferred by RAxML v8.0.26 [32] implemented by the CIPRES Science Gateway [33]. This analysis sampled a total of 204 bootstrap replicates before being stopped by the autoMRE algorithm. The complete RP tree is presented as Data File S1.

RAxML was called as follows:

```
raxmlHPC-HYBRID -s input -N autoMRE -n result -f a -p 12345 -x 12345 -m PROTCATLG
```

Other *dsr* genes

To study the *dsr* operon structure in the newly identified organisms, we identified other *dsr* genes, namely C, E, F, H, M, K, J, O, P, N, L, R, and S. These genes were identified in the vicinity of *dsrAB* using domain similarity identified by hits to TIGRFam [28], COG [34], and Pfam databases [29], as well certain key traits and conserved residues in specific genes.

Sequence alignment and phylogeny

Phylogenetic analyses were performed as follows:

Each individual gene (*dsrA*, *dsrB*, *dsrC*, *dsrD*, *aprA*, *aprB*, *dsrT*, *qmoA*, *qmoB*, *sat*) was aligned along with reference sequences using MUSCLE [31] with default parameters. All alignments were manually refined by trimming the start and ends and removing all columns with >95% gaps. For generation of concatenated alignments (*dsrAB*, *qmoAB*, and *aprBA*), individual alignments were concatenated in Geneious version 7 [35]. In construction of the concatenated *qmo* tree, only subunits A and B were used since subunit C is not universally present in sulfate/sulfite-reducing organisms. All phylogenetic analyses were inferred by RAxML v8.0.26 [32] implemented by the CIPRES Science Gateway [33]. RAxML was called as follows:

```
For AsrABC, DsrD, Sat, DsrT, QmoAB, DsrEFH trees:
raxmlHPC-PTHREADS -s input -N 1000 -n result -f a -p 12345 -x 12345 -m PROTGAMMAGTR.
```

```
For DsrAB, AprBA trees:
```

```
raxmlHPC-HYBRID -s input -N autoMRE -n result -f a -p 12345 -x 12345 -m PROTGAMMAGTR.
```

The complete DsrAB tree is presented as Data File S2.

Conserved residues and motifs

Conserved residues and motifs in DsrA, DsrB, DsrC, AsrC, DsrD, and DsrE proteins were identified by aligning the identified genes from all 123 genomes in this study with reference proteins [36–38]. All conserved residues identified by us were also compared with the model sulfate-reducing organism, *Desulfovibrio vulgaris* [39].

Rooting of *dsrA/dsrB* tree

A reference alignment was calculated de novo using MAFFT based on a non-redundant (90% identity clustering with uclust [40]) set of length-filtered (300–500 nt) sulfite reductase superfamily sequences from this study [18], and additional sequences collected from UniProt and UniParc [41] using the eggNOG COG2221 and TIGRFAM DsrA/DsrB HMM models. The reference alignment was end trimmed and filtered with noisy [42]. Selected full-length DsrA, DsrB, and outgroup sequences were reference-aligned and used for phylogenetic inference using Phylo-Bayes with the CAT-GTR model [43].

Trees for inferring LGT by comparison of 16S rRNA and reductive-type DsrAB

16S rRNA genes were aligned with the SINA aligner [44] with default parameters. Alignments were then manually refined by trimming the start and ends and removing all columns with gaps >95%. DsrA and DsrB sequences were aligned as described above. 16S rRNA and DsrAB were sourced from the same organisms where possible. In the case of MAGs not containing 16S genes, the closest related organisms with 16S rRNA genes were chosen for this analysis. Organisms with oxidative-type DsrAB were excluded from this analysis. 16S rRNA trees were calculated using maximum likelihood (RAxML) and neighbor-joining (Geneious) methods. The neighbor joining tree was constructed using the Jukes-Cantor Genetic distance model with 1000 bootstrap replicates.

RAxML was run as follows:

```
raxmlHPC-PTHREADS -f a -s input -n result -m GTRGAMMA -x 12345 -# autoMRE -p 12345 -T 4.
```

The 16S rRNA consensus tree was constructed using the majority (extended) consensus rule setting using CON-SENSE and branch lengths were adjusted using DNAML implemented in the PHYLIP package [45]. Similarly, DsrAB trees were combined into a consensus tree by using the majority (extended) consensus rule in CONSENSE and

Table 1 Details of lineages involved in dissimilatory sulfur cycling as identified in this study

Phylum-level lineage	No. of genomes reported	Potential contribution to sulfur cycle	Mechanism	Source	Electron donor		
					Hydrogen	Fatty acid metabolism	Organic C
<i>Acidobacteria</i>	3	Sulfate/sulfite reduction	dsr	A, D	Yes	Yes	Yes
<i>Actinobacteria</i>	3	Sulfate/sulfite reduction, or sulfur oxidation, or both	dsr	–	Yes	No	Yes
<i>Armatimonadetes</i>	1	Sulfate/sulfite reduction	dsr	C	Yes	No	Yes
<i>Candidatus Desantisbacteria</i>	4	Sulfate/sulfite reduction	dsr	B, C	Yes	No	Yes
<i>Candidatus Falkowbacteria</i>	8	Unknown	Unknown	A	Yes	No	Yes
<i>Candidatus Hydrothermarchaeota</i>	4	Sulfate/sulfite reduction, or sulfur oxidation, or both	dsr	E	Yes	No	Yes
<i>Candidatus Lambdaproteobacteria</i>	5	Sulfite reduction, or sulfur oxidation, or both	dsr	A	Yes	Yes	Yes
<i>Candidatus Muproteobacteria</i>	14	Sulfur oxidation	rdsr	A	Yes	Yes	Yes
<i>Candidatus Omnitrophica</i>	2	Sulfite reduction	asr	A, B	Yes	Yes	Yes
<i>Candidatus Riflebacteria</i>	4	Sulfite reduction	asr	A, B	Yes	Yes	Yes
<i>Candidatus Rokubacteria</i>	8	Sulfur oxidation	dsr	A	No	Yes	Yes
<i>Candidatus Schekmanbacteria</i>	1	Sulfate/sulfite reduction	dsr	A	No	Yes	Yes
<i>Candidatus Zixibacteria</i>	2	Sulfate/sulfite reduction	dsr	B	Yes	Yes	Yes
<i>Chloroflexi</i>	2	Sulfate/sulfite reduction	dsr	A	Yes	Yes	Yes
<i>Deltaproteobacteria</i>	34	Sulfate/sulfite reduction	dsr	A, C	Yes	Yes	Yes
<i>Ignavibacteria</i>	5	Sulfate/sulfite reduction	dsr	A, B	Yes	Yes	Yes
<i>Nitrospinae</i>	3	Sulfur oxidation	rdsr	A	Yes	Yes	Yes
<i>Nitrospirae</i>	2	Sulfur oxidation	rdsr	A	No	Yes	Yes
<i>Nitrospirae</i>	19	Sulfate/sulfite reduction	dsr	A, C	Yes	No	Yes
<i>Planctomycetes</i>	1	Sulfate/sulfite reduction	dsr, asr	A, B	Yes	Yes	Yes
<i>Verrucomicrobia</i>	1	Sulfate/sulfite reduction, or sulfur oxidation, or both	dsr	F	Yes	No	Yes

Sampling sources are indicated by letters: A—Aquifer at Rifle, Colorado, USA; B—Deep subsurface in Japan; C—CO₂ geyser at Green River, Utah, USA; D—Glencore Mine, Canada; E—Juan de Fuca ridge flank marine subsurface fluids; F—Natural Peatland in Germany. Newly identified lineages are shown in bold. Contribution to sulfur cycle for DsrAB-containing organisms were decided as described in Table 2.

branch lengths of the consensus tree were inferred by using PROML (JTT model).

Structural models

We selected the DsrT proteins identified in *Desulfovibrio vulgaris* (WP_012611240) and *Candidatus Rokubacteria* CSP1-6 (KRT71371) for structural modeling. Protein models were predicted using the I-TASSER suite [46]. Only the top predicted models out of the top five I-TASSER simulations are shown. Both DsrT proteins used the identical top threading template from the sporulation inhibitor protein pXO1-118 from *Bacillus anthracis* [47].

Analyses of electron donors for sulfate/sulfite reduction

Analyses of putative electron donors and other metabolic potential were centered around identification of genes for

hydrogen oxidation, complex carbon compounds (carbohydrates), fatty acid metabolism, and carbon fixation. For identification of the potential for hydrogen oxidation, hmm searches were conducted by searching all predicted ORFs against individual HMM profiles for nickel-iron hydrogenases from Groups I, IIa, IIb, IIIa, IIIb, IIIc, and IIId, and Fe-Fe hydrogenases. All hits above the noise cutoffs were inspected manually. The details are described in detail previously [24, 26, 48].

For identification of carbohydrate substrates for sulfate/sulfite reduction, all predicted ORFs were searched against the CAZy HMM database [49]. Pre-filtering of hits was conducted using the following cutoffs: coverage: 0.40; e-value: 1e-18. To determine the specificity of enzymes, we established a set of 84 distinct reactions involving 189 enzyme families that allowed us to track specific substrates and products. All hits to glycosyltransferases (GT) and Carbohydrate Binding Modules (CBM) were excluded from this analysis due to high incidence of false

positives and/or difficulty in determining substrate specificity.

Generation of custom hmm models

For generation of custom HMM profiles, reference sequences and identified genes from the 123 genomes in this study were aligned using MUSCLE [31] with default parameters followed by manually trimming the start and ends of the alignment. The alignment was converted into Stockholm format and databases were built using hmmscan [30]. Individual noise and trusted cutoffs for all HMMs were determined by manual inspection and are built into the custom HMM profiles.

Results and discussion

To investigate the diversity of microorganisms that contain sulfite reductases involved in dissimilatory sulfate/sulfite reduction or sulfur oxidation in the environment, we analyzed genomes reconstructed from metagenomic sequence datasets recovered from six distinct terrestrial and marine subsurface environments where geochemical conditions have suggested active microbial sulfur cycling. Our sampling sites included an aquifer adjacent to the Colorado River, USA [24, 26], a deep subsurface CO₂ geyser in Utah, USA [27], a deep borehole in Japan [23], an acidic sulfide mine waste rock site in Canada, deep seafloor basaltic crustal fluids of the hydrothermally active Juan de Fuca ridge flank in the Pacific Ocean [25], and an acidic peatland in Germany [50]. In total, we searched in excess of 4000 near-complete MAGs for the presence of sulfite reductase genes.

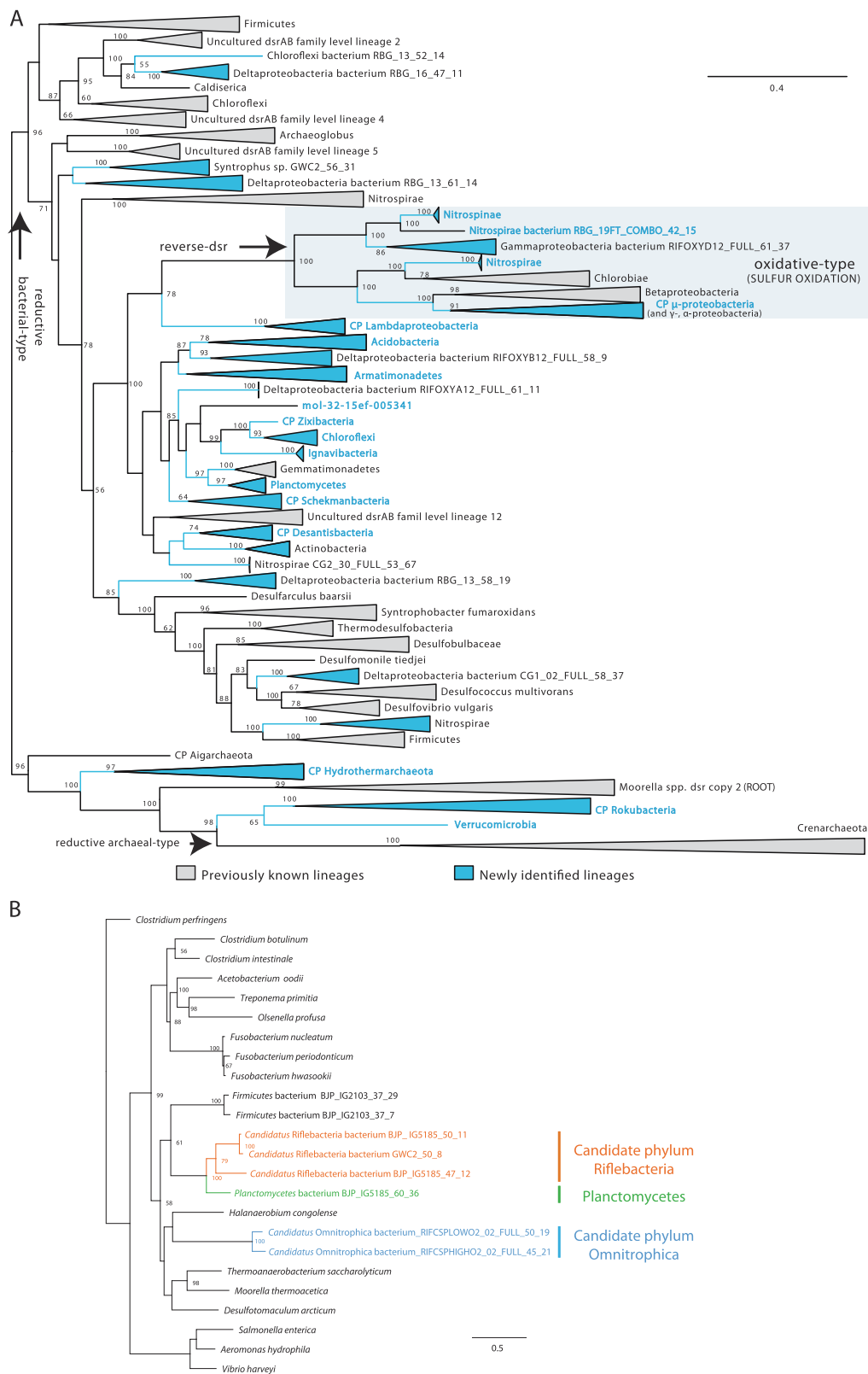
Identification of dissimilatory sulfur cycling organisms from MAGs

We identified sulfite reductase genes in 123 near-complete microbial genomes (Supplementary Table 1). Phylogenetic analyses using a set of 16 concatenated ribosomal proteins (RP) and the small subunit ribosomal (SSU) RNA gene show that these genomes belong to organisms from 20 distinct phylum-level lineages (Table 1), 13 of which were not known to have *dsr* genes [18]. In addition, we identified anaerobic sulfite reductase (*asr*) genes required for sulfite reduction in three bacterial groups not previously reported to have this capacity [51]. All of the identified catalytic protein subunits (*DsrA*, *DsrB*, and *AsrC*) contained all conserved sulfite reductase residues and secondary structure elements for the formation of α helices and β sheets [36] (Supplementary Figs. 1–3).

Dissimilatory sulfite reductase containing organisms

Given our interest in identifying organisms with the capacity to produce sulfide, we initially searched the genomes for operons that contained genes encoding *DsrD* [52]. This gene was considered a marker for sulfite reduction because it is absent in bacteria that use the *rdsr* pathway for sulfur oxidation [53]. It is however important to note that the *dsrD* gene is present and highly expressed in sulfur disproportionating organisms like *Desulfurivibrio alkaliphilus* that cannot be distinguished from canonical sulfate-reducing bacteria using gene synteny or other genomic features [16]. Although the exact function of the *DsrD* protein is unclear, the presence of winged-helix domains in its structure and its association with other core proteins of the *dsr* complex (*dsrABC*) suggest a regulatory role in bacterial sulfite reduction [37]. We identified 78 genomes that encode at least *dsrABCD* (Supplementary Fig. 4). A multiple alignment of *DsrD* sequences confirmed highly conserved residues, indicating that the proteins are likely active (Supplementary Fig. 5). These putative sulfate/sulfite-reducing microorganisms affiliate with eight distinct phyla not previously reported to be capable of these processes. Four are phyla with isolated representatives (*Acidobacteria*, *Armatimonadetes*, *Ignavibacteria*, *Planctomycetes*) and four are uncultivated candidate phyla (*Candidatus Zixibacteria*, *Candidatus Schekmanbacteria*, *Candidatus Desantisbacteria*, *Candidatus Lambdaproteobacteria*) (Fig. 1a).

Importantly, organisms from *Verrucomicrobia* and two candidate phyla, *Candidatus Rokubacteria* and *Candidatus Hydrothermarchaeota*, lack *dsrD* genes and their *dsrAB* sequences form completely novel lineages outside the four known main phylogenetic *DsrAB* clusters, namely the reductive bacterial-type, the oxidative bacterial-type, the reductive archaeal-type, and the second *dsrAB* copies of *Moorella* species (Fig. 1a). To determine the earliest evolved and most basal lineages in the *DsrAB* tree, we performed paralogous rooting analysis on a representative subset of sequences. In accordance with previous reports, our results show that the second copies of *dsrAB* in *Moorella* spp. likely represent the most basal *DsrAB* branch [18]. This was followed by the newly identified sequences from *Candidatus Rokubacteria*, *Verrucomicrobia*, and *Candidatus Hydrothermarchaeota* (Fig. 2). Interestingly, *Candidatus Hydrothermarchaeota* sequences were not monophyletic with one sequence (JdFR-18 JGI24020J35080_1000005) clustering with *Verrucomicrobia* and *Candidatus Rokubacteria*, while the remaining were affiliated with bacterial-type *DsrAB*. Other organisms lacking *dsrD* genes cluster together with organisms known to be sulfur oxidizers in the *dsrAB* tree. Based on this clustering, the group implicated in sulfur oxidation using



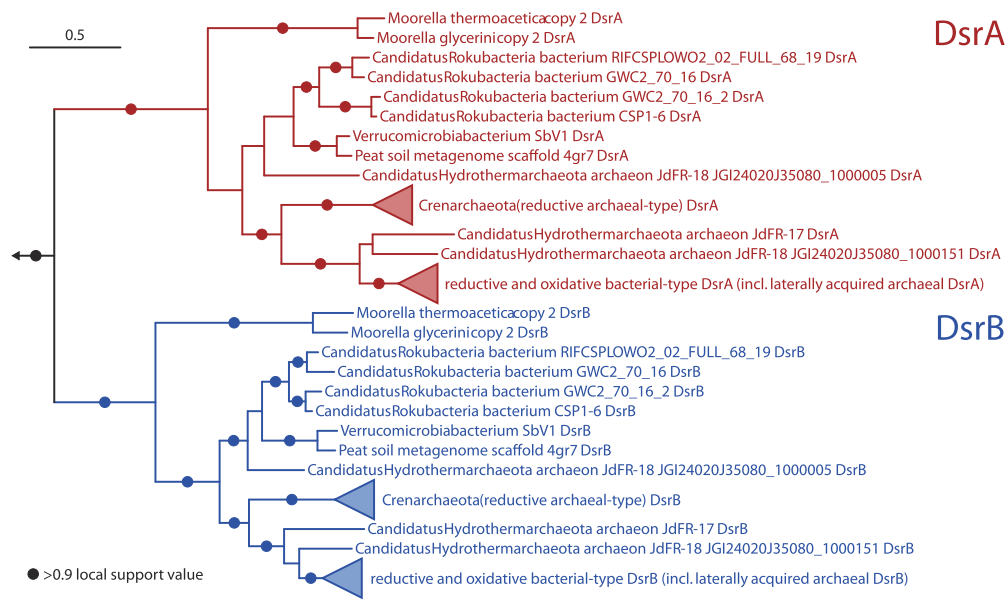


Fig. 2 Paralogous rooting analysis of DsrAB. Bayesian inference tree showing the phylogenetic relationship between DsrA and DsrB (50 sequences, 377 alignment positions). Arrow indicates outgroup of other sulfite, non-DsrAB reductase superfamily (COG2221)

sequences. Branch supports (posterior probability) higher than 0.9 are indicated by black circles. DsrA/DsrB sequences from this study are marked in bold. Assignment of oxidative/reductive, bacterial/archaeal-type DsrAB is according to Müller et al. [18]

the *rdsr* pathway now includes bacteria from three additional phylum-level lineages: *Nitrospirae*, *Nitrospinae*, and *Candidatus* Muproteobacteria (Fig. 1a).

Anaerobic sulfite reductase-encoding organisms

The *asr* pathway for sulfite reduction was found in three bacterial phyla not previously known to possess this pathway, namely *Planctomycetes* and members of two candidate phyla, *Candidatus* Omnitrphica and *Candidatus* Riflebacteria. Concatenated protein trees of all three subunits *AsrA*, *AsrB*, and *AsrC* showed that sequences from these phyla clustered with those from *Firmicutes*, suggesting that they were acquired by LGT (Fig. 1b). Investigations into the operon structure of the *asr* complex revealed that while organisms from *Planctomycetes* and *Candidatus* Riflebacteria had a canonical gene organization in the order *asrA*, *asrB*, and *asrC*, *Candidatus* Omnitrphica had a fourth gene (*asrD*) as an insertion between *asrB* and *asrC* subunits. Analyses of conserved domains show that *AsrD* is related to the family of formate and nitrite transporters (pfam01226, COG2116, TIGRfam00790). We hypothesize that this may in fact serve as a bisulfide channel associated with dissimilatory sulfite reduction using the *asr* enzyme complex as observed in *Clostridium difficile* [54].

dsrD genes in candidate phyla radiation organisms

Surprisingly, we identified *dsrD* genes in eight genomes of organisms affiliating with *Candidatus* Falkowbacteria,

putatively symbiotic bacteria within the Parcubacteria superphylum of the candidate phyla radiation (CPR) [55]. There is no indication of the presence of other *dsr* genes in these genomes. Given the predicted close physical and metabolic interactions between CPR bacteria and their hosts, we suggest that this small protein could modulate host metabolism, as sometimes occurs with viruses/phage and their hosts [56]. CPR organisms are common in aquifers where conditions oscillate between oxic and anoxic [24, 26]. The predicted Falkowbacteria *DsrD* protein sequences cluster with sequences from well-characterized *Deltaproteobacteria* capable of sulfate reduction, suggesting that *deltaproteobacterial* sulfate reducers served as *dsrD*-donors during LGT to these CPR bacteria (Supplementary Fig. 6). Considering the presence of *dsrD* genes in CPR organisms and putative sulfur-oxidizing/sulfur disproportionating bacteria [16], we propose that *dsrD* is not a good marker for sulfite reduction. Therefore, we suggest an alternate set of rules for utilizing a combination of *dsr* genes to distinguish DsrAB-based sulfite reduction from sulfur oxidation on the basis of genomic features (Table 2).

Lateral gene transfer of DsrAB sulfite reductases

Prior analyses have suggested that LGT has influenced the evolution of *dsrAB* among extant microorganisms but only by comparably few events among major taxonomic lineages [14, 18, 57]. We used a comparison of 16S ribosomal RNA and concatenated DsrAB protein trees to reevaluate the extent to which LGT has influenced the organismal

Table 2 Suggested rules for determination of direction of dissimilatory sulfur metabolism for uncultivated organisms

Contribution to sulfur cycle	Suggested rules
Sulfate reduction (to sulfide)	<i>aprBA</i> , <i>sat</i> are present, DsrAB cluster with reductive DsrAB, <i>dsrD</i> gene is present, and <i>dsrEFH</i> are absent
Sulfite reduction (to sulfide)	DsrAB cluster with reductive DsrAB, <i>dsrD</i> gene is present, and <i>dsrEFH</i> are absent
Sulfate reduction (to sulfite)	<i>aprBA</i> and <i>sat</i> are present
Sulfur oxidation (to sulfite)	DsrAB do not cluster with reductive DsrAB, <i>dsrEFH</i> are present, and <i>dsrD</i> is absent
Sulfur oxidation (to sulfate)	DsrAB do not cluster with reductive DsrAB, <i>dsrEFH</i> are present, and <i>dsrD</i> is absent, <i>aprBA</i> and <i>sat</i> are present
Sulfite reduction, or sulfur oxidation, or both	<i>dsrD</i> and <i>dsrEFH</i> are present.
Sulfur oxidation (to sulfite) as a part of sulfur disproportionation	DsrAB cluster with reductive DsrAB, <i>dsrD</i> gene is present, and <i>dsrEFH</i> are absent

Sulfur disproportionating organisms cannot be differentiated from sulfite-reducing organisms on genomic features alone. Details of individual *dsr* genes are specified in Supplementary Table 6.

distribution of *dsrAB* genes (Fig. 3). Mismatching branching pattern between the two trees indicates that *dsrAB* has been introduced into most of the candidate phyla members by multiple independent LGT events. Our analyses show that organisms from five bacterial and archaeal phyla, *Deltaproteobacteria*, *Nitrospirae*, *Candidatus* Hydrothermarchaeota, *Actinobacteria*, *Chloroflexi* likely acquired *dsrAB* genes in multiple events. Amongst these, *Nitrospirae* and *Deltaproteobacteria* displayed the highest number of LGT involving five independent events spanning across both reductive and oxidative branches of the DsrAB tree. These findings provide evidence for the complex evolutionary history of *dsr* genes. Currently, it may not be possible to identify the specific lineage in which sulfite reduction first appeared; however, our extensive dataset sets the stage for future studies to investigate the evolution of dissimilatory sulfur metabolism.

Sulfate vs. sulfite reduction in organisms

To determine whether these newly identified organisms reduce sulfate vs. sulfite to sulfide we looked for the genes involved in the reduction of sulfate to sulfite, specifically adenylyl-sulfate reductase subunits A and B (*aprBA*), sulfate adenylyl transferase (*sat*), and quinone-interacting membrane-bound oxidoreductase subunits A, B, and C (*qmoABC*) [58–60]. Organisms from three phyla, the *dsr*-containing *Candidatus* Lambdaproteobacteria, and *asr*-containing *Candidatus* Riflebacteria, and *Candidatus* Omnitrophica, lacked genes for the reduction of sulfate to sulfite suggesting that they were sulfite reducers. Sulfite utilized by these organisms may derive from the environment or is produced inside the cell as part of other sulfur metabolism pathways such as tetrathionate or thiosulfate reduction, sulfur disproportionation, or by organosulfonate respiration. This suggests that recent genome-based

observations supporting potential “metabolic handoffs” between organisms (transfer of metabolites associated with energy metabolism) in the oxidative cycle of sulfur [24, 26, 61] likely extend to the reductive cycle as well [62]. Interestingly, *Candidatus* Rokubacteria whose DsrAB sequences represent a novel deep-branching lineage in the DsrAB tree also have *apr*, *sat*, and *qmo* genes that are required for sulfate reduction or sulfite oxidation. Phylogenetic analyses of the individual *dsr* proteins shows that the sulfate reduction system of *Candidatus* Rokubacteria is of mosaic evolutionary origin (Fig. 4) (Supplementary Figs. 7–9).

Prevalence of *dsrT* in *dsrAB*-containing microorganisms

In addition to *dsrD*, we sought evidence for hypothetical genes in proximity to known *dsr* genes that may help in distinguishing between DsrAB-based sulfate/sulfite reduction and sulfur oxidation pathways. We identified a hypothetical gene that encodes for the N-terminal domain of an anti-sigma factor antagonist protein [63] that almost always occurs within the operon encoding *dsr* genes (Fig. 5; Supplementary Fig. 4). This hypothetical protein is part of a protein family that includes the *Bacillus subtilis* RsbT co-antagonist protein rsbRD, which are important components of the stressosome and function as negative regulators of the general stress transcription factor sigma-B [64]. This gene is unique to DsrAB-based sulfite-reducing organisms and is mostly absent in recognized sulfur-oxidizing organisms, except for those within the phylum *Chlorobi* (Supplementary Fig. 10). We refer to this gene as ‘*dsrT*’ in accordance with homologous genes in phototrophic green sulfur bacteria from the phylum *Chlorobi* [65]. This gene always precedes the electron transport components encoded by *dsrMKJOP* genes [66] and is fused with *dsrM* in some

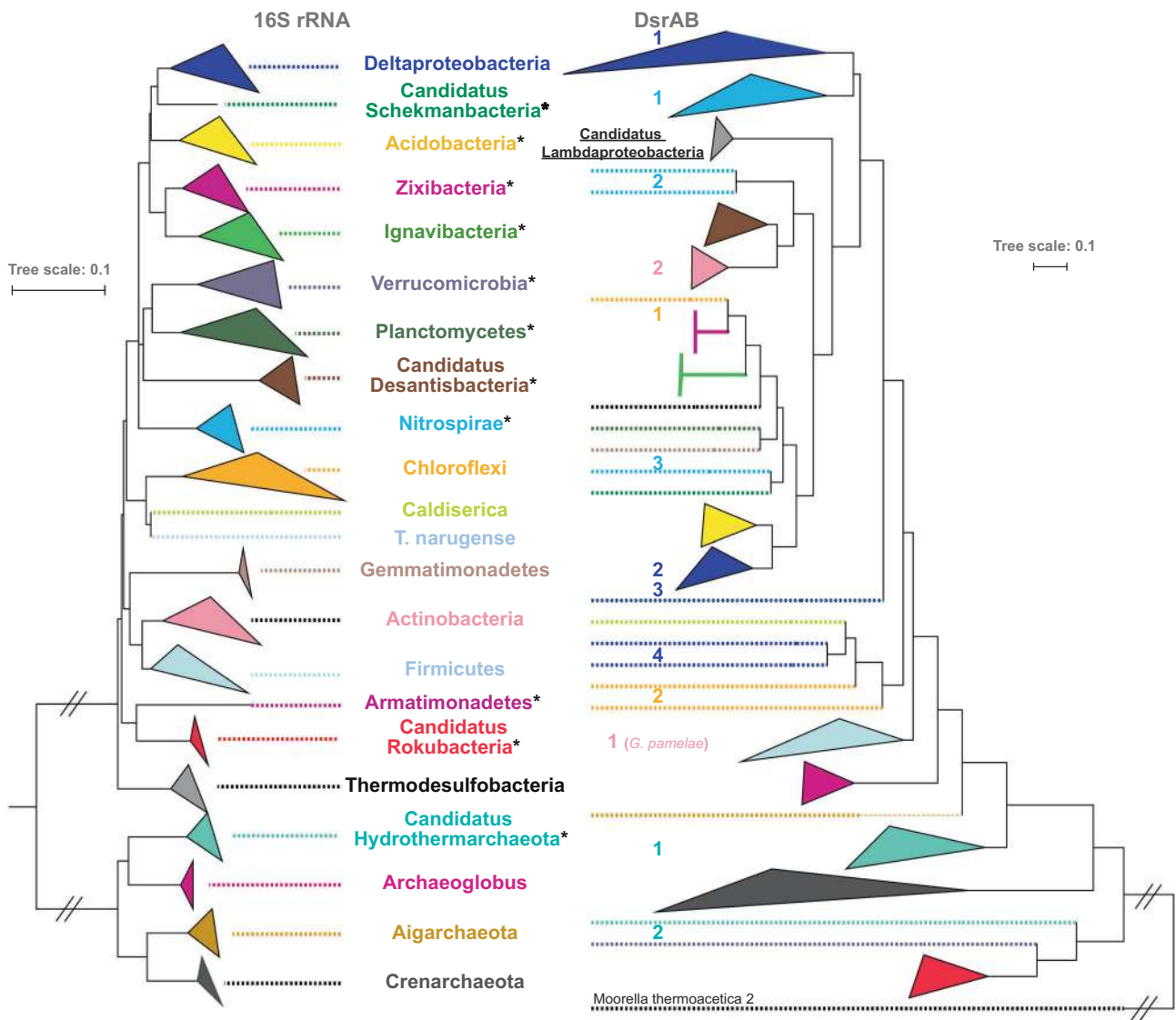


Fig. 3 Comparison of 16S rRNA and concatenated DsrAB trees for sulfate/sulfite-reducing microorganisms. Sequences are grouped at the phylum level. Trees were constructed using a consensus of neighbor-joining and maximum-likelihood phylogenies with 1000 bootstrap resamplings each. Each phylum is colored differently to identify LGT based on inconsistent branching patterns. Phylum names with an

asterisk represent sulfate/sulfite-reducing lineages that were discovered in this study. Numbers indicate number of independent LGT events associated with the specific phylum. Complete phylogenetic trees with bootstrap values are available in Data Files S3–S6. LGT events involving oxidative-type DsrAB for Nitrospirae (2 events) and Deltaproteobacteria (1 event) are not shown

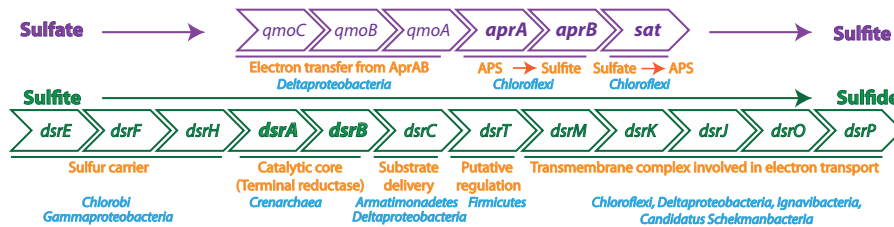


Fig. 4 Dsr operon structure and enzymatic roles of proteins involved in sulfate reduction in *Candidatus Rokubacteria*. Purple: genes involved in sulfate reduction to sulfite. Orange: putative enzymatic roles of genes; blue: microbial lineages with closest homologs as determined

by phylogeny/BLAST against NCBI GenBank. APS refers to adenosine-5'-phosphosulfate. Green: genes involved in sulfite reduction to sulfide. This is the first case in which *dsrE*, *dsrF*, and *dsrH* genes are present in organisms other than sulfur-oxidizing bacteria

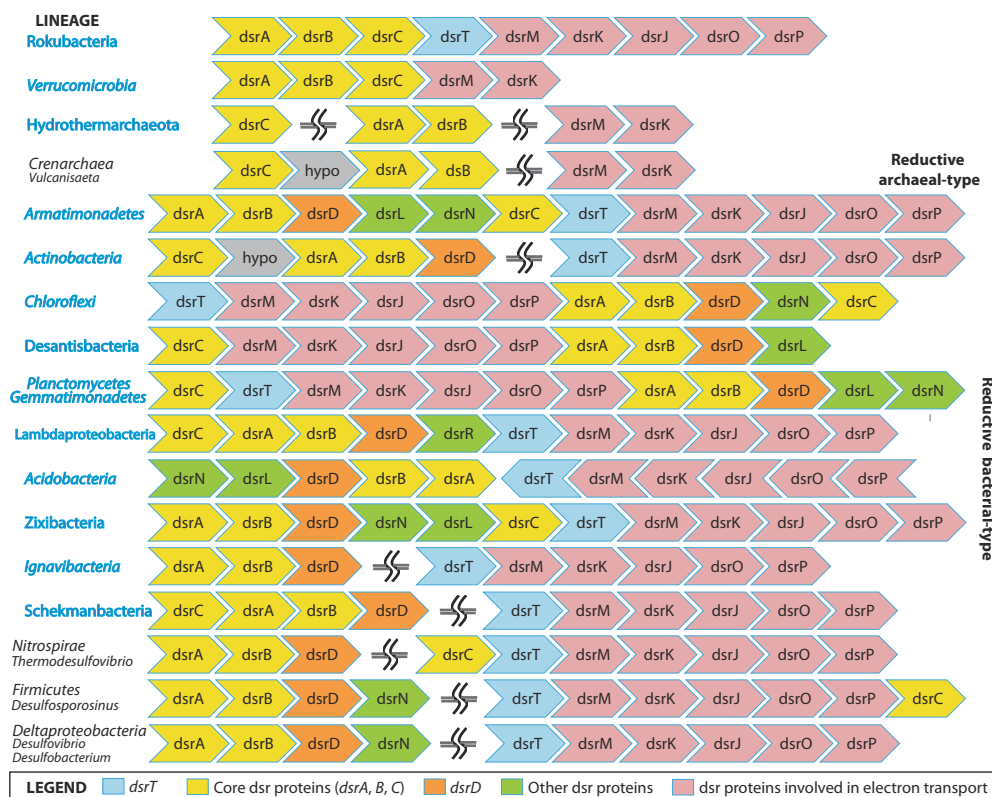


Fig. 5 Dsr operon structure in previously reported (black names) and newly reported groups (blue names). Interestingly, and in contrast to the previously studied organisms for which the operon is interrupted

(==SS==), the entire dsr pathway (including electron transport chain and ancillary proteins) is often encoded in a single genomic region

organisms (Supplementary Fig. 11). Fused *dsrT-dsrM* genes are oriented with *dsrT* in the N-terminal and *dsrM* in the C-terminal, thereby maintaining the gene order observed in canonical dsr operons: *dsrT*, *dsrM*, *dsrK*. From structural predictions and conserved motifs, we hypothesize that it likely performs a regulatory function (Supplementary Fig. 12).

***dsrEFH* in the newly discovered *dsrAB*-containing microorganisms**

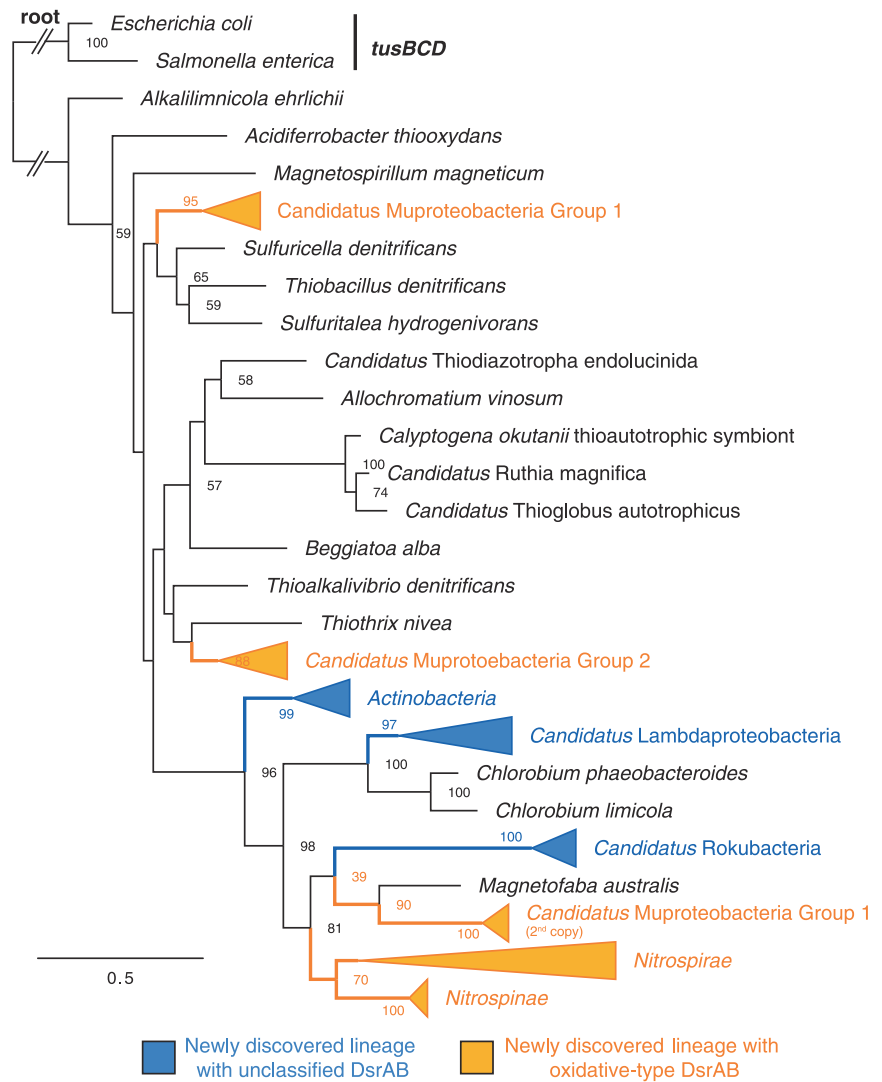
Recent studies looking into the distribution of genes associated with the dsr operon have suggested that *dsrE*, *dsrF*, and *dsrH* are unique to sulfur oxidizing microorganisms and are absent in sulfate/sulfite-reducing and putative sulfur disproportionating microorganisms [16, 38, 67]. In sulfur-oxidizing microorganisms, DsrEFH can serve as an effective sulfur donor for DsrC [68]. On the other hand, collocated *dsrE*, *dsrF*, and *dsrH* genes are present in ~24% of the newly identified DsrAB-encoding microorganisms (30 out of 123 genomes) (Supplementary Fig. 4). These *dsrEFH* genes were identified in organisms from six phylum-level lineages, *Actinobacteria*, *Candidatus Rokubacteria*, *Candidatus Lambdaproteobacteria*, *Candidatus Muproteobacteria*, *Nitrospirae*, and *Nitrospinae*. Phylogenetic

analysis of all identified DsrEFH shows that they cluster with well-characterized sulfur-oxidizing organisms (Fig. 6). The presence of *dsrEFH* genes (with well-known roles in sulfur oxidation) in organisms from *Actinobacteria* and *Candidatus Lambdaproteobacteria* is perplexing since these organisms also possess the *dsrD* gene that is unique to DsrAB-containing sulfite-reducing organisms. Further, the presence of *dsrEFH* genes in organisms within the phylum-level lineage *Candidatus Rokubacteria* (with a novel deep-branching clade of DsrAB) suggests that these organisms are likely involved in sulfur oxidation rather than sulfite reduction. Finally, sequences from *Candidatus Muproteobacteria* formed two distinct clades (Group 1, Group 2) and clustered with two separate groups, *Alphaproteobacteria* and *Gammaproteobacteria* respectively. All *Candidatus Muproteobacteria* with *dsrEFH* sequences in Group 1 also possessed a second copy of these genes that clustered with sequences from *Magnetofaba australis* and *Candidatus Rokubacteria*.

Electron donors and other metabolic potential associated with sulfur cycling

In order to better understand the energy metabolism and ecology of these newly identified organisms, we

Fig. 6 Concatenated DsrEFH protein tree inferred by maximum likelihood. Phylum-level lineages with first report of the presence of *dsrEFH* genes are shown in blue (from organisms with unknown-type DsrAB) and orange (from organisms with oxidative type DsrAB). Homologous TusBCD from *E. coli* and *S. enterica* were used to root the tree. Only bootstrap values >50 are shown



investigated potential electron donors for putative sulfate/sulfite reduction. Specifically, we targeted genes involved in the oxidation of hydrogen [69] (Ni–Fe hydrogenase groups I, IIa, IIb, IIIa, IIIb, IIIc, IIId, Fe–Fe hydrogenase groups A, B1/B2) and transformation of organic carbon compounds (genes involved in breakdown of cellulose, hemicellulose, chitin, pectin, starch, amino sugars, other monosaccharides, and polysaccharides) [49] and short chain fatty acids. Our analyses show that organisms from 12 of the 13 newly identified putative sulfate/sulfite-reducing DsrAB-containing lineages identified in this study possess the ability to utilize hydrogen as an electron donor (Supplementary Table 2). On the other hand, organisms from all 13 lineages possessed the ability to breakdown complex carbon compounds although the diversity of genes encoding for specific carbohydrate-active enzymes varied greatly across phyla (Supplementary Table 3). Organisms from 8 of the 13 putative sulfate/

sulfite-reducing lineages possessed the ability to oxidize short chain fatty acids by beta-oxidation (Supplementary Table 4). In order to understand if these newly identified organisms were heterotrophs or autotrophs, we looked at the carbon fixation potential encoded in the genomes. We identified three different carbon fixation mechanisms, the Calvin–Benson cycle (CBB), the reverse (reductive) tri-carboxylic acid (rTCA) cycle, and the Wood–Ljungdahl pathway in ~50% of all organisms (Supplementary Table 5). In total, 11 organisms contained genes encoding for the CBB cycle with 10 possessing the Form I RuBisCO and 1 organism possessed the Form II RuBisCO. Genes for the Wood–Ljungdahl pathway were encoded in 42 genomes while genes for the rTCA cycle were encoded in 7 genomes. We propose that sulfate/sulfite reduction by organisms from these newly identified lineages likely serves an important control on carbon cycling in the terrestrial and marine subsurface.

Conclusions

By the Proterozoic Eon, sulfate reduction had become a significant biological process in the oceans [70, 71]. Based on phylogenomic arguments and isotopic records, it was suggested that the capacity to reduce sulfite to sulfide emerged in thermophilic archaea around 3.5 billion years ago, and that mesophilic sulfate reducers evolved only after the rise in atmospheric oxygen level [2, 72]. Our findings indicate a complex evolutionary history of this capacity involving extensive LGT of *dsr* genes. Consequently, it may be impossible to constrain the specific lineage in which this metabolism first appeared. The ability for a DsrAB-based dissimilatory sulfur metabolism is now predicted in a much wider diversity of mesophilic bacterial and archaeal groups than was recognized previously. We conclude that many groups of microorganisms now known to have genes involved in dissimilatory sulfur metabolism impact biogeochemical processes in marine and terrestrial sediments, aquifers, wetlands, methane seeps, coastal marshes and estuaries, as well as agricultural and human microbiomes. Many are organisms from well-studied phyla, but still novel at the genus to class levels, but others are organisms from candidate phyla known only based on their genomes. The results underline the value of genomic analyses for prediction of key ecosystem capacities that cannot be made based on rRNA gene surveys and motivate targeted cultivation strategies for organisms currently lacking laboratory tractable representatives. Finally, these findings will better inform future microbial trait-based ecosystem models that can predict the outcomes of global change on biogeochemical processes and planetary elemental cycles [73].

Data availability

NCBI Genbank, BioProject, BioSample, and Taxonomy ID (TaxID) accession numbers for individual genomes are listed in Supplementary Table 1. Genomes are also available through ggKbase: http://ggkbase.berkeley.edu/novel_sulfate_reducers (ggKbase is a 'live' site, genomes may be updated after publication). The JdFR-17, JdFR-18, and JdFR-19 genomes are also available through the Integrated Microbial Genomes and Microbiomes database (IMG) through Genome IDs: 2728369317, 2728369320, 2728369322. Hmm databases used in this study are available from <https://github.com/banfieldlab/metabolic-hmms>. The authors declare that all other data supporting the findings of this study are available within the article and its supplementary information files, or from the corresponding author on request.

Acknowledgements This work was supported by Lawrence Berkeley National Laboratory's Sustainable Systems Scientific Focus Area funded by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under contract DE-AC02-05CH11231 and by the Austrian Science Fund (FWF, P25111-B22 and I2320-B22).

Compliance with Ethical Standards

Conflict of interest :The authors declare that they have no conflict of interest.

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