Dissimilatory sulfate reduction in the archaeon ‘Candidatus Vulcanisaeta moutnovskia’ sheds light on the evolution of sulfur metabolism

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1. Extended Data

Figure #	**Figure title** One sentence only	**Filename** This should be the name the file is saved as when it is uploaded to our system. Please include the file extension. i.e.: *Smith_ED_Fig1.jpg*	**Figure Legend** If you are citing a reference for the first time in these legends, please include all new references in the Online Methods References section, and carry on the numbering from the main References section of the paper.
Extended Data Fig. 1	The microbial community composition of Oreshek Spring	Extended_Data_Figure_1.eps	Analysis by high throughput sequencing of 16S rRNA gene fragments.
Extended Data Fig. 2	Growth of Thermoproteus tenax.	Extended_Data_Figure_2.eps	The error bars are SD from independent growth replicates (n=3).
Extended Data Fig. 3	Growth of Caldivirga maquilingensis.	Extended_Data_Figure_3.eps	Error bars represent standard deviations from independent growth replicates (n = 3).
Extended Data Fig. 4	$H^{35}S^-$ formation from $^{35}SO_4^{-2}$ the binary culture 768-28 and the cultures of hyperthermophilic Crenarchaeota strains earlier claimed to be capable of sulphate reduction, but without experimental evidence.	Extended_Data_Figure_4.eps	Error bars represent standard deviations of independent radioisotopic measurement experiments (n = 3).

Extended Data Fig. 5	Genes related to sulphate reduction.	Extended_Data_Figure_5.eps	The key sulphate reduction genes from genomes of selected sulphate reducing archaea ("Candidatus V. moutnovskia", Caldivirga sp. Obs2 and A. fulgidus) and bacteria (D. vulgaris Hildenborough and A. degensii) are shown, as well as from genomes of archaea not capable of sulphate reduction (V. distributa, V. souniana, Caldivirga sp. MU80, Ca. Methanodesulfokores washburnensis MDKW and unclassified Aigarchaeota JZ bin 15).
Extended Data Fig. 6	Proteomic analysis of the soluble fraction of cells in the binary culture.	Extended_Data_Figure_6.eps	Protein extraction was performed with a non-denaturing detergent. Only the 150 most abundant proteins of "Candidatus V. moutnovskia" are shown. a. Binary culture grown on medium with yeast extract and sulphate as the electron acceptor. b. Binary culture grown on medium with yeast extract and elemental sulphur as the electron acceptor. Protein abundances in mol% were estimated from nemPAI values[62]. Data are shown as the mean of n = 2 independent experiments fitted into a model.
Extended Data Fig. 7	Proteomic analysis of the binary culture after protein extraction with denaturing detergent.	Extended_Data_Figure_7.eps	Only proteins of "Candidatus V. moutnovskia" are shown. The binary culture was grown on medium with yeast extract and sulphate as the electron acceptor. Protein abundances in mol% were estimated from nemPAI values[62]. Data are shown as the mean of n = 3 independent experiments fitted into a model.
Extended Data Fig. 8	Phylogenetic analysis of AprAB.	Extended_Data_Figure_8.eps	Maximum likelihood phylogenetic reconstruction (LG+I+G4) of a. AprA and b. AprB proteins present in

			the selected dataset. Only support values above 50 are shown. The scale bar indicates number of substitutions per site
Extended Data Fig. 9	Phylogenetic analysis of Sat.	Extended_Data_Figure_9.eps	Maximum likelihood phylogenetic reconstruction (LG+I+G4) of Sat proteins within the selected dataset. Only non-parametric support values above 70 are shown. The scale bar indicates number of substitutions per site.
Extended Data Fig. 10	Phylogenetic analysis of HdrA-like proteins.	Extended_Data_Figure_10.eps	Maximum-likelihood reconstruction (LG+I+G4) of the HdrA-domain present in 1,391 non-redundant HdrA, QmoA and QmoB proteins. The "*Candidatus* V. moutnovskia" sequences are marked in red. Expanded insets of the "*Candidatus* V. moutnovskia" QmoA (a) and QmoB (b) clades (marked in blue) are shown in Fig. 5. The scale bar indicates number of substitutions per site.

2. Supplementary Information:

A. Flat Files

Item	**Present?**	**Filename** **This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. The extension must be .pdf**	**A brief, numerical description of file contents.** **i.e.: *Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.***
Supplementary Information	Yes	Supplementary_Informa	Supplementary Figure 1 and Supplementary Tables 1-3

		tion.pdf	
Reporting Summary	Yes	Report_Summary.pdf	

B. Additional Supplementary Files

Type	**Number** **If there are multiple files of the same type this should be the numerical indicator. i.e. "1" for Video 1, "2" for Video 2, etc.**	**Filename** **This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. i.e.: *Smith_ Supplementary_Video_1.mov***	**Legend or Descriptive Caption** **Describe the contents of the file**
Supplementary Table	Table S4	Supplementary_Table_4.xlsx	Genomic dataset used in the analysis including accession codes, taxonomic information and file location in the original database.

3. Source Data

Complete the Inventory below for all Source Data files.

Figure	Filename This should be the name the file is saved as when it is uploaded to our system, and should include the file extension. i.e.: *Smith_SourceData_Fig1.xls,* or *Smith_ Unmodified_Gels_Fig1.pdf*	Data description i.e.: Unprocessed Western Blots and/or gels, Statistical Source Data, etc.

Source Data Fig. 1	Source_Data_Figure1.xlsx	Binary Culture Growth source data
Source Data Fig. 2	Source_Data_Figure2.xlsx	Protein accessions of the hits underlying each symbol in figure 2.
Source Data Extended Data Fig. 2	Source_Data_Extended_Figure_2.xlsx	Raw data and calculations supporting Extended Data Figure 2
Source Data Extended Data Fig. 3	Source_Data_Extended_Figure_3.xlsx	Raw data and calculations supporting Extended Data Figure 3
Source Data Extended Data Fig. 4	Source_Data_Extended_Figure_4.xlsx	Raw data and calculations supporting Extended Data Figure 4
Source Data Extended Data Fig. 5	Source_Data_Extended_Figure_5.xlsx	Raw data and calculations supporting Extended Data Figure 5
Source Data Extended Data Fig. 6	Source_Data_Extended_Figure_6.xlsx	Raw data and calculations supporting Extended Data Figure 6
Source Data Extended Data Fig. 7	Source_Data_Extended_Figure_7.xlsx	Raw data and calculations supporting Extended Data Figure 7

Dissimilatory sulphate reduction in archaeon *Candidatus* Vulcanisaeta moutnovskia sheds light on evolution of sulphur metabolism

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Abstract

Dissimilatory sulphate reduction (DSR), an important reaction in the biogeochemical sulphur cycle, has been dated to the Paleoarchean using geological evidence, but its evolutionary history is poorly understood. Several lineages of Bacteria carry out DSR but in archaea only *Archaeoglobus*, which acquired DSR genes from Bacteria, has been proven to catalyse this reaction. We investigated substantial rates of sulphate reduction in acidic hyperthermal terrestrial springs of the Kamchatka Peninsula and attributed DSR in this environment to Crenarchaeota in the *Vulcanisaeta* genus. Community profiling, coupled with radioisotope and growth experiments and proteomics, confirmed DSR by "*Candidatus* Vulcanisaeta moutnovskia", which has all the required genes. Other cultivated Thermoproteaceae were briefly reported to use sulphate for respiration but we were unable to detect DSR in these isolates. Phylogenetic studies suggest that DSR is rare in archaea and that it originated in *Vulcanisaeta*, independently of *Archaeoglobus*, by separate acquisition of *qmoABC* genes phylogenetically related to bacterial *hdrA* genes.

Introduction

Microbial dissimilatory sulphate reduction (DSR) is the main driver of the modern sulphur cycle, and an important actor in the carbon cycle[1,2]. Although dissimilation of sulphur compounds was present on the early Earth[3], there is dispute regarding the onset of DSR. The Paleoarchean barite deposits of 3.5, 3.4 and 3.2 Ga in Western Australia, India and South Africa support a well-mixed, large scale, marine sulphate pool[4], with low micromolar concentrations[5,6]. This sulphate was produced by atmospheric photolysis of volcanic SO_2[7] and, as observed in modern environments[8,9], the low levels of sulphate did not preclude active DSR. However, this metabolism did not imprint a strong mass-dependent fractionation on Archean sedimentary records probably due to a low level of biological activity and an atmospherically-derived elemental sulphur pool that masked isotopic evidence for DSR[10]. Nevertheless, the isotopic and microfossil evidence for Paleoarchean DSR[11,12] has been confirmed by recent high-resolution quadruple sulphur isotope measurements[4,6,13], strongly supporting the existence of sulphate-reducing organisms at 3.55 Ga. However, the evolutionary history of sulphate reducers is poorly understood.

DSR capacity is found in several phyla of Bacteria, including Proteobacteria, Firmicutes, Thermodesulfobacteria, and Nitrospirae[1], and possibly also in newly identified uncultured lineages[14–18]. Among Archaea, DSR has been reported in the hyperthermophilic marine *Archaeoglobus* genus of the Euryarchaeota, and for some hyperthermophilic terrestrial members of the Thermoproteaceae family from the Crenarchaeota, including *Caldivirga maquilingensis*[19], *Vulcanisaeta distributa*[20], *Vulcanisaeta souniana*[20], *Thermoproteus tenax*[21], and *Thermoproteus thermophilus*[22]. However, conclusive experimental proof of DSR, namely quantitative data on sulphide accumulation from growth with sulphate, has not been reported for the Thermoproteaceae. DSR is mediated by a specific set of proteins, which include sulphate adenylyl transferase (Sat), adenosine phosphosulphate reductase (AprAB) and the QmoABC membrane complex essential for sulphate reduction to sulphite[23,24], as well as the dissimilatory sulphite reductase (DsrAB), its substrate DsrC, and the membrane DsrMK(JOP) complex, involved in sulphite reduction to sulphide[24,25]. The presence of DSR in both Archaea and Bacteria could indicate an ancient origin for this metabolism[26]. However, phylogenetic analyses of *Archaeoglobus aprAB* and *dsrAB* genes suggest their acquisition via horizontal gene transfer (HGT) from Bacteria[27,28]. In contrast, the Thermoproteaceae *dsrAB* genes represent a deep archaeal lineage distinct from bacterial sulphate reducers and sulphur oxidisers, supporting the origin of DsrAB as a reductive enzyme[14,16,26,27]. Thus, the Thermoproteaceae need to be further investigated to elucidate the evolutionary history of DSR.

Here, we studied terrestrial thermal acidic environments in which DSR was previously detected[29]. Using community profiling, culture studies and radioisotope measurements, coupled with comparative genomics, proteomics, and phylogenetics, we conclude that among the reported archaeal sulphate reducers with sequenced genomes, only *Archaeoglobus* spp. and "*Candidatus* Vulcanisaeta moutnovskia" are capable of DSR. In "*Candidatus* V. moutnovskia" (as in *Archaeoglobus* spp.) this activity correlates with the presence of functional (full-length) *qmoABC* genes, which are absent or truncated in the genomes of other Thermoproteaceae. Furthermore, the *qmoABC* genes of "*Candidatus* V. moutnovskia" and *Archaeoglobus* spp. are of different evolutionary origin (albeit in both cases bacterial). These observations suggest that DSR arose twice in archaea, both times by acquisition of bacterial genes, and seems to be dependent on functional *qmoABC* genes needed for energy conservation. In contrast, dissimilatory sulphite reduction appears to be more ancient and may have arisen early within the Thermoproteaceae.

Community profiling of Oreshek Spring

The microbial community of Oreshek Spring (Moutnovsky Volcano, Kamchatka Peninsula, Russia, temperature of 91ºC, pH 3.5, sulphate concentration of 538 mg/l), which displays significant rates of sulphate reduction (1.094 nmol $H_2S/cm^3 \cdot day$)[29], was analysed by sequencing 16S rRNA gene fragments. After quality filtering, we obtained 28152716S rRNA gene fragments corresponding to 434 operational taxonomic units (OTUs) defined with 0.98 similarity threshold. This allowed a coverage of 0.825 for the microbial community, estimated by determining Chao1 richness (Chao1=526). Such coverage does not permit a complete description of phylogenetic diversity, but allows identification of all major groups. The prokaryotic diversity in Oreshek Spring was estimated by the Shannon index, which amounted to 3.37. Archaea dominated the microbial community, accounting for 85.0% of 16S rRNA genes obtained. Bacterial sequences accounted for 13.0%, and 2.0% of sequences could not be affiliated. The archaeal sequences represented three phyla: Crenarchaeota (75% of all library sequences), Thaumarchaeota (2.0%) and Euryarchaeota (8.0%). Among the archaeal representatives, three genera of putative sulphate reducers were detected: *Vulcanisaeta*, *Thermoproteus* and *Caldivirga* (20.0%, 9.0% and 0.9% of library sequences). Among bacterial sequences, no taxa representing known sulphate reducers were detected. Thus, sulphate reduction at Oreshek Spring seems to be performed mainly by archaea, among which *Vulcanisaeta* spp. predominate (**Extended Data Figure 1**).

Two sulphate-reducing cultures were previously enriched from hot springs at Moutnovsky Volcano, both containing archaea of the genera *Vulcanisaeta* and *Thermoproteus.*

The *Vulcanisaeta* component of culture 768-28 was given the tentative species name "*Candidatus* V. moutnovskia"[29–31]. We designed specific primers for the detection of "*Candidatus* V. moutnovskia" *dsrAB* genes and used them for qPCR analysis of an environmental sample from Oreshek Spring. The primers specificity was confirmed by amplicon sequencing. A significant number of "*Candidatus* V. moutnovskia" *dsrAB* genes were detected (**Supplementary Table 1**). In the same sample, we assessed the abundance of archaea and bacteria using qPCR with 16S rRNA gene-specific primers. Comparison of the qPCR results with 16S rDNA- and *dsrAB*-specific primers showed that archaea represented 95% of all prokaryotes in the community, and of them, 40% were archaeal sulphate reducers related to *"Candidatus* V. moutnovskia*"* (**Supplementary Table 1**).

Sulphate reduction by Crenarchaeota

For studies of DSR we used the binary culture 768-28[30] (hereafter referred to as the binary culture), which contains "*Candidatus* V. moutnovskia" and *Thermoproteus uzoniensis* (all attempts to isolate the pure cultures were unsuccessful). Using liquid medium with sulphate as electron acceptor and yeast extract as energy source, we tested the DSR capacity of the binary culture and of the pure cultures of putative Thermoproteaceae sulphate reducers *T. tenax, V. distributa, V. souniana,* and *C. maquilingensis.* The binary culture was able to grow by DSR with yeast extract, showing sulphate consumption and sulphide accumulation (**Fig. 1**). Among tested electron donors, only peptone and yeast extract supported growth with sulphate (**Supplementary Table 2**). In sulphate-reducing conditions "*Candidatus* V. moutnovskia*"* predominated in the binary culture, while *T. uzoniensis* was present only as a minor component. qPCR with 16S rRNA gene-specific primers showed that in DSR conditions the share of *T. uzoniensis* was 0.01 to 0.1%, while in sulphur-reducing conditions it increased to 50%. This suggests that DSR is a capacity of "*Candidatus* V. moutnovskia*"*, while *T. uzoniensis* is likely incapable of sulphate reduction, as supported by genomic and proteomic analyses (see below).

Cultures of *T. tenax, V. distributa, V. souniana* and *C. maquilingensis*, obtained from validated collections, showed good growth on medium with elemental sulphur as electron acceptor (authenticity of the grown cultures was checked by 16S rRNA gene sequencing), but no growth by DSR with any electron donor (**Supplementary Table 2**). Neither consumption of sulphate nor accumulation of sulphide were observed after 10 days of incubation, and there was either no increase in cell number or sometimes only a very slight increase (2-fold). This could be due to a small amount of sulphur formed from oxidation of sulphide used as medium reductant, or a small growth stimulation due to assimilation of sulphate. The former hypothesis was confirmed in further experiments where similar small biomass increments were produced in the

presence of sulphate and in the absence of externally added electron acceptors (**Extended Data Figure 2 and Extended Data Figure 3)**.

To test the ability for DSR with higher sensitivity, we used the radioisotopic approach to measure sulphate reduction in these Thermoproteaceae (**Extended Data Figure 4**). Using the cell numbers at the end of incubation, we calculated the specific sulphate reduction rate by the "*Candidatus* V. moutnovskia" culture to be 1.055 fmol sulphate $cell^{-1}$ day^{-1}. This value falls within the range of 0.16–417 fmol sulphate $cell^{-1}$ day^{-1} reported for pure cultures of sulphate reducers[32,33], while the specific sulphate reduction rates for *T. tenax, V. distributa, V. souniana,* and *C. maquilingensis* were negligible. Thus, of the five organisms tested, only "*Candidatus* V. moutnovskia" seems to be capable of DSR.

Sulphate reduction gene distribution in archaea

The previously sequenced "*Candidatus* V. moutnovskia" genome[31] contains the minimal set of genes necessary for DSR, namely genes for sulphate transporters and pyrophosphatase, *sat*, *aprAB, qmoABC, dsrAB*, *dsrC* and *dsrMK*. In addition, we found no evidence for possible alternative genes that could be involved in this process, such as coding for membrane complexes or HdrA-related proteins (with one exception, see below). This analysis was extended to a large (meta)genomic dataset which showed that, apart from *Archaeoglobus* spp. and "*Candidatus* V. moutnovskia" no other archaeon possesses a complete set of full-length genes necessary for DSR (**Fig. 2 and Extended Data Figure 5**). Among the Thermoproteaceae cultures claimed to be capable of DSR, *T. tenax, V. distributa* and *C. maquilingensis* lack the *qmoABC* genes, and *T. tenax* and *C. maquilingensis* also contain no *hdrA*-like genes. In the *V. souniana* low quality draft genome, all necessary genes for DSR could be found, but several of them appear to be impaired (with an apparent abundance of sequencing artefacts masking possible natural frameshifts). In the genome of *T. uzoniensis,* the second component of the binary culture, the *dsrAB, dsrMK,* and *qmoABC* genes are absent, while *sat*, *aprAB* and *dsrC* are present.

Recently, the presence of *dsrABC* genes has been reported in several uncultivated organisms belonging to the Aigarchaeota[17,34], Korarchaeota[16] and Hydrothermarchaeota[15] candidate phyla. We found that in *Candidatus* Hydrothermarchaeota genomes, no *qmoABC* or bona-fide *aprAB* could be identified, although bona fide *sat, dsrABC* and *dsrMK* are present. Interestingly, in both *Ca.* Methanodesulfokores washburnensis MDKW and uncultivated Aigarchaeota JZ bin 15 genomes, in which the presence of *dsrAB*, *dsrC* and *dsrMK* has been reported[17], *qmoABC* gene candidates were also found, albeit no *aprAB* genes were present (**Fig. 2**). Both metagenomic assemblies have a high level of completeness (above 95%), but it cannot be excluded that *aprAB* genes are located in the missing part of the assembly. Similar *qmoABC*

homologues were also identified in genomes lacking any *dsr* genes, such as in the complete genome of *Ca*. Korarchaeum cryptofilum OPF8 (**Fig. 2**). None of the Asgard lineages, in which Thorarchaeota genomes are included, have the *dsr* genes required for dissimilatory sulphate/sulphite reduction.

Proteomics of the binary culture

We performed proteomic analysis of the binary culture grown anaerobically with sulphate or sulphur as electron acceptors (**Extended Data Figure 6**). For cells grown with sulphate, several of the most abundant proteins detected were "*Candidatus* V. moutnovskia" proteins involved in sulphate reduction (**Supplementary Table S3**), whereas no *T. uzoniensis* proteins that might be involved in sulphate reduction (Sat, AprAB and DsrC) could be identified. The "*Candidatus* V. moutnovskia" QmoAB proteins were detected only in DSR conditions, as well as a related protein with a domain composition similar to a fusion of QmoA and QmoB ("QmoAB-like", VMUT_0635). The latter protein is a distant relative of *Geobacter metallireducens* BamE, a subunit of the Benzoyl CoA-reductase[35], and is not conserved in other sulphate reducers (or in *T. tenax*, *V. souniana* and *C. maquilingensis*, while it is present in *V. distributa*, which is also not a sulphate reducer), so it is unlikely to be involved in DSR. There are no other HdrA-related proteins encoded in the genome of "*Candidatus* V. moutnovskia". In contrast, no sulphate reduction proteins were detected for cells grown with sulphur.

A second proteomic analysis of the sulphate-grown culture was performed to try to increase the detection of membrane proteins, by extracting proteins with a denaturing detergent (**Extended Data Figure 7, Supplementary Table S3**). This second analysis allowed the detection of "*Candidatus* V. moutnovskia" DSR-related membrane proteins (QmoABC, DsrMK, proton-pumping pyrophosphatase and a sulphate transporter). We found no evidence of other up-regulated proteins in "*Candidatus* V. moutnovskia" whose involvement in sulphate reduction would be conceivable. Thus, the proteomic results confirmed that DSR by the binary culture was performed by "*Candidatus* V. moutnovskia", and showed that several of the proteins involved are among the most highly expressed proteins in cells grown by this mode.

Phylogenetic analysis of archaeal sulphate reduction

We performed a phylogenetic analysis of key genes specific for sulphate reduction, *sat*, *aprAB* and *qmoAB(C)*, to investigate the evolutionary history of DSR. Detailed phylogenetic studies were already reported for *dsrAB*[14,16,26,27,36], indicating the ancient presence or origin of these genes within the Thermoproteaceae, associated with dissimilatory sulphite reduction.

In the *aprA* single gene phylogenies (**Fig. 3 and Extended Data Figure 8**), two highly supported clades are observed, one containing bona-fide *aprA* genes, and another composed of *aprA*-like homologues belonging to different lineages. The *aprA*-like sequences belong to organisms that are not dissimilatory sulphate reducers/sulphur oxidisers or that have a bona-fide *aprA* gene (**Fig. 3 and Extended Data Figure 8a**). These genes probably correspond to proteins with a different and unknown function. Within the AprA clade, the separation of Crenarchaeota and sulphur oxidisers proteobacterial group II from Bacteria/*Archaeoglobus* and sulphur oxidisers proteobacterial group I is highly supported, as previously reported[28,37,38]. Group I sulphur oxidisers, including purple sulphur bacteria, have AprM as physiological partner of AprAB and show an *aprAB* phylogeny mostly congruent with *dsrAB*, whereas Group II sulphur oxidisers and green sulphur bacteria, which have QmoABC as physiological partner of AprAB, apparently acquired the *aprAB* genes from sulphate reducers. Due to the small length of the alignment (163 positions after trimming), the *aprB* phylogeny has many unresolved branches (**Extended Data Figure 8b**), but the general trends observed in the *aprA* phylogeny are retained. The phylogenetic position of the Thermoproteaceae *aprAB* may indicate the ancient origin of these genes in this family, or their acquisition/replacement by an HGT event between this lineage and an ancestor of the group I sulphur oxidisers.

The *sat* gene presents an even more complex evolutionary history (**Extended Data Figure 9**), probably due to its possible role in either assimilatory or dissimilatory sulphate reduction. Indeed, in the *sat* single gene tree Bacteria are not monophyletic and evidence for several recent interdomain HGT events is present.

The full-length *qmoABC* genes are absent in the Crenarchaeota, with the exception of "*Candidatus* V. moutnovskia" and the *Caldivirga* sp. Obs2 metagenomic assembly (**Figs. 2 and 4**), suggesting a critical role of these genes in DSR. The three genes code for a membrane complex responsible for electron transfer from the menaquinol pool to APS reductase[23,39] (**Supplementary Figure 1**). QmoC oxidizes menaquinol, presumably with proton release to the periplasm, and electrons are transferred through the QmoC hemes *b* and Fe-S centers to QmoA and QmoB, which transfers them to APS reductase[39]. In bacteria, the QmoABC complex was shown to be essential for sulphate, but not sulphite, reduction[40], and the corresponding genes are strictly conserved in sulphate reducers[24,28], with the exception of *qmoC* in some clostridial organisms. In these organisms two *hdrBC* genes (in some *Desulfotomaculum* spp.) or *hdrD* genes (in some *Desulfosporosinus* spp.) are found instead, but these genes cannot functionally replace *qmoC* in energy conservation[40], and their function is unknown. The QmoA and QmoB proteins are both related to the methanogenic HdrA, a subunit of heterodisulfide reductases[41,42]. However, QmoA and QmoB have very distinct physiological functions from HdrA proteins.

QmoA is smaller than HdrA and lacks some of its domains and [4Fe-4S] clusters, whereas QmoB has a similar size as HdrA, but also lacks some of its [4Fe-4S] clusters, having an additional C-terminal domain related to the electron transfer protein MvhD[23] (**Fig. 4**). The QmoC protein is distinct and has a cytochrome *b* membrane domain related to HdrE, and an electron-transfer iron-sulphur domain related to HdrC.

To understand the evolutionary relationship between *hdrA*, *qmoA* and *qmoB* genes (**Fig. 5 and Extended Data Figure 10**), a large taxonomic sampling was performed. In this reconstruction "*Candidatus* V. moutnovskia" and *Caldivirga* sp. Obs2 QmoA are inside a bacterial clade, whose most basal sequence is the *Ammonifex degensii* QmoA2. This highly supported clade (**Fig. 5a**) is dominated by metagenomic sequences of uncharacterized HdrA-like proteins affiliated with Bacteroidetes (sister clade of "*Candidatus* V. moutnovskia" QmoA), Chloroflexi, Elusimicrobia and Deltaproteobacteria. The "*Candidatus* V. moutnovskia" QmoAB proteins show a close relationship to QmoAB2 from *A. degensii,* a bacterium that has two *qmoABC* gene clusters. Surprisingly, the "*Candidatus* V. moutnovskia" QmoAB and *Ammonifex* QmoAB2 are more closely related to bacterial HdrA proteins than to canonical QmoAB proteins from Bacteria/*Archaeoglobus* (which include the QmoAB1 from *A. degensii*) (**Extended Data Figure 10)**. The closer proximity between "*Candidatus* V. moutnovskia" branches and *A. degensii* is associated with (or reflects) the presence of two additional [4Fe-4S] clusters in both QmoA and QmoB from *Vulcanisaeta* and *Ammonifex*, as well as the presence of an additional domain (the "inserted ferredoxin domain"[42], absent in bacterial QmoB) and the absence of the C-terminal MvhD domain in *Vulcanisaeta* and *Ammonifex* QmoB, which is encoded in a separate gene upstream of *Vulcanisaeta qmoA* (**Fig. 4**). The "*Candidatus* V. moutnovskia" QmoC protein is more similar to *A. degensii* QmoC1 than to QmoC2, contrary to what might be expected since its QmoAB are more closely related to *Ammonifex* QmoAB2, suggesting a *qmoC* acquisition event separate from the acquisition of *qmoAB* (**Extended Data Figure 5**). Twelve archaeal sequences from mainly uncultivated organisms are basal to the *Vulcanisaeta* and *Ammonifex* QmoA clade that has bona-fide methanogenic HdrA as sister (**Extended Data Figure 10**). However, the low support of these twelve archaeal sequences does not allow to resolve with confidence their exact position within the phylogenetic reconstruction.

The "*Candidatus* V. moutnovskia" QmoB clade is very similar to the QmoA clade (in terms of topology and taxonomic affiliations of their OTUs), wherein "*Candidatus* V. moutnovskia" and *Caldivirga* sp. Obs2 QmoB are also inside a highly supported and resolved bacterial clade with *A. degensii* QmoB2 sequence being basal (**Fig. 5b**). Similarly to QmoA, an HdrA-like archaeal clade with very similar taxonomic distribution of uncultivated organisms is at the base of the "*Candidatus* V. moutnovskia" QmoB bacterial clade but, this time, descending

from metagenomic Deltaproteobacteria, which is an indication of interdomain HGT. Although having a higher statistical support, the exact position of these sequences cannot be fully resolved.

The phylogenetic reconstruction of HdrA-like proteins (**Fig. 5 and Extended Data Figure 10**) points to the acquisition of the "*Candidatus* V. moutnovskia" *qmoAB* genes from a bacterial donor. The nature of the primordial organisms in which the transition from an HdrA to a QmoA/B protein occurred is not clear. Until some of these organisms are cultivated, Occam's razor suggests the bacterial affiliation of this transition since in both Vulcanisaeta QmoA/B clades, the most basal organism known to perform sulphate reduction is *A. degensii*, and in many of the archaeal lineages basal to the clade there is no evidence for the ability for sulphate or sulphite reduction. Overall, this data suggests that "*Candidatus* V. moutnovskia" "re-invented" DSR by acquiring QmoAB proteins from a bacterial organism, where the separate evolution of Qmo proteins from a typical HdrA took place, and, thus, extant DSR in Thermoproteaceae has an independent evolutionary history from that in Bacteria and *Archaeoglobus* species.

DISCUSSION

The evolutionary history of DSR is poorly understod, particularly among the archaea. Sulphate is present in high-temperature submarine, deep subsurface and terrestrial environments where archaeal organisms are abundant. Several independent studies have reported high-temperature sulphate reduction in terrestrial neutral or acidic hot springs of Yellowstone National Park[43] and Kamchatka Peninsula[29,44,45], but the species responsible for high-temperature sulphate reduction had not been identified. We report that hyperthermophilic archaea of the genus *Vulcanisaeta* are one of the dominant groups in the acidic and hot Oreshek Spring. Using growth, radioisotopic, and proteomic experiments with the binary culture 768-28, we show that "*Candidatus* V. moutnovskia" is responsible for sulphate reduction, while *T. uzoniensis* is only a minor component in DSR conditions, with its share growing only upon growth with elemental sulphur. Furthermore, the DSR capacity briefly reported for other four Thermoproteaceae species (*T. tenax, C. maquilingensis, V. distributa* and *V. souniana*) could not be confirmed in detailed growth and radioisotopic studies. No quantitative data was provided in any of these previous studies and the weak growth observed with sulphate was probably not statistically different from the weak growth without any electron acceptor, a possibility that we confirmed (**Supplementary Table 2, and Extended Data Figure 2 and Extended Data Figure 3**).

Our observations on DSR ability by the Thermoproteaceae may be explained through genomic analyses of available complete archaeal genomes: the minimal set of genes shown to be required for DSR in bacteria[24,40] was only found in the genomes of *Archaeoglobus* spp. and "*Candidatus* V. moutnovskia". Notably, with the exception of *Caldivirga* sp. Obs2, the other

Thermoproteaceae investigated had the *sat*, *aprAB* and *dsrABCMK* genes, but lacked the *qmoABC* genes. The QmoABC respiratory complex[23] was shown to be essential for growth of a sulphate reducing bacterium on sulphate, but not on sulphite[40], and seems to allow energy conservation during the step of APS reduction. The alternative *aprM* present in sulphur oxidisers (or the *hdrBC* genes in clostridial sulphate reducers), are also absent in Thermoproteaceae. These results suggest that DSR by "*Candidatus* V. moutnovskia" is associated with the acquisition of the *qmoABC* genes by HGT, whereas other Thermoproteaceae may only reduce sulphate for assimilatory purposes, as they lack the QmoABC complex that would enable them to conserve energy from that reduction.

Phylogenetic analyses show that the *"Candidatus* V. moutnovskia*" dsr* genes, like those of other Thermoproteaceae, are rooted in the Archaea domain, contrary to *dsr* genes of *Archaeoglobus* spp., which were acquired from bacterial lineages[14,16,26,27]. In contrast, the *"Candidatus* V. moutnovskia*" qmoABC* genes have a distinct evolutionary history. The *qmoAB* genes, together with the bacterial *A. degensii qmoAB2* genes and genes of other bacterial organisms from the same clade, are more closely related to typical *hdrA* genes, than to bacterial *qmoAB* genes. Thus, the "*Candidatus* V. moutnovskia" *qmo* genes are not derived from canonical *qmo* genes from Bacteria, but are apparently the result of a separate functional evolution directly from bacterial *hdrA* genes. This indicates that the acquisition of the *qmoAB(C)* genes by "*Candidatus* V. moutnovskia", apparently conferring DSR capacity to this organism, may have been a more recent event than their ability to perform dissimilatory sulphite and/or assimilatory sulphate reduction.

These results have important implications for understanding the evolution of dissimilatory sulphur metabolism, and three evolutionary scenarios can be considered. The first is that assimilatory or dissimilatory sulphite reduction was already present before the archaeal-bacterial divide. This agrees with the proposed existence of an assimilatory or dissimilatory enzyme for sulphite reduction in ancestral organisms, either prior to the divergence of the bacterial and archaeal domains[46] or early after the archaeal-bacterial division followed by an interdomain transfer. In this scenario, dissimilatory sulphite reduction was subsequently lost in most archaeal lineages, being only retained in a single Crenarchaeota family. Later interdomain HGTs events would be responsible for the presence of dissimilatory sulphite reduction in other archaeal lineages[16,17]. The second scenario is that dissimilatory sulphite reduction originated within Archaea (Thermoproteaceae) and was acquired by Bacteria due to an early HGT event. Subsequently, several intradomain HGTs occurred expanding this trait across systematic boundaries. The presence of the *dsr* genes within several bacterial phyla would suggest a high number of such events. The third scenario is that dissimilatory sulphite reduction is of early

bacterial origin and Crenarchaeota acquired the *dsr* genes by an early HGT to the Thermoproteaceae ancestor. This scenario agrees with recent studies[16,17,36], which indicate several independent archaeal acquisitions of dissimilatory sulphite reduction genes from bacteria.

In contrast, DSR has apparently a separate evolutionary history. It may have been preceded by assimilatory sulphate reduction, which could have been the initial physiological function of the AprAB enzyme, namely in the Thermoproteaceae, given the absence of AprM or QmoABC. In this scenario DSR evolved within the bacterial domain, presumably originating through the development of QmoABC proteins, and was only later transferred to a limited number of organisms in the Archaea, namely *Archaeoglobus* spp., *"Candidatus* V. moutnovskia*"* and *Caldivirga* sp. Obs2, of which at least the first two acquired QmoABC as independent evolutionary events. An alternative, less likely, hypothesis is that ancestors of the Thermoproteaceae were early archaeal dissimilatory sulphate reducers, which subsequently lost this ability, presumably by loss of the *qmoABC* (or *aprM* genes), that were re-acquired by *"Candidatus* V. moutnovskia*"* and *Caldivirga* sp. Obs2. The ancestry of dissimilatory sulphite over sulphate reduction makes sense from a bioenergetic point of view as ATP is required for sulphate, but not for sulphite, reduction, and also in terms of the different availability of the two compounds on the early Earth. In the anoxic, reduced Earth, sulphate was present at low concentration, produced from SO_2 photolysis[7], or biologically from sulphide anoxygenic photosynthesis[47] or disproportionation of sulphur compounds. In contrast, sulphite produced from hydrolysis of volcanic and hydrothermal SO_2 was probably abundant. The evolutionary history of siroheme sulphite reductases also supports the ancestry of sulphite reduction[46]. Recent phylogenomic analysis of novel Diaforarchaea also endorse the view that most organisms with early-branching DsrAB are capable of sulphite, but not sulphate, reduction[36].

In conclusion, assimilatory/dissimilatory sulphite reduction may have been present before the Bacteria/Archaea divide or shortly after, and likely preceded dissimilatory sulphate reduction. Nevertheless, DSR is an ancient process dating back at least to 3.5 Ga, which was likely restricted to Bacteria that were limited in biological activity by the low levels of sulphate. In the Thermoproteaceae, assimilatory sulphate reduction may have been an ancient trait and our results suggest that a later acquisition of the *qmoABC* genes allowed *"Candidatus* V. moutnovskia*"* to grow by reduction of sulphate. Nevertheless, further experimental studies of archaeal sulphate reduction are required to confirm this idea and firmly clarify the role of the *qmo* and *hdr* genes in archaea, as additional or different genes may be involved. At about 2.4 Ga, the rise in O_2 levels started the slow oxygenation of the oceans and atmosphere, with the associated increase in marine sulphate concentration. This process then led to the strong diversification of bacterial lineages capable of sulphate reduction, as observed today.

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ONLINE METHODS

Environmental sampling

The sample used in this work was collected from Oreshek Spring (N52° 31.818 E158° 11.499, T 91°C, pH 3.5) at the foot of Moutnovsky volcano (Kamchatka Peninsula, Russia). The sample of thermal water and sediment was placed into 50-mL glass vial with gas-tight butyl rubber stopper and aluminium screw cap, which was filled to capacity, hermetically sealed, and transported to the laboratory at ambient temperature. For DNA isolation, a mixed sample of sediment and water was used. Prior to arrival in the laboratory, the fixed sample was stored at ambient temperature for several days and then at –20°C until evaluation.

Microbial cultures

The following cultures were obtained from DSMZ: *Thermoproteus tenax* DSM2078^T, *Vulcanisaeta souniana* DSM 14430^T**,** *Vulcanisaeta distributa* DSM 14429^T, and *Caldivirga maquilingensis* DSM 13496^T. The purity and identity of the cultures were checked by 16S rRNA gene amplification and sequencing.

The binary culture 768-28 (referred to as "the binary culture") containing "*Candidatus* V. moutnovskia" was initially regarded as a pure isolate[30]. The culture was obtained on medium with ethanol, yeast extract, and sulphate, but was then routinely maintained on medium with yeast extract and elemental sulphur, which provided for better growth. Later it was found that this culture contained an additional organism, besides the *Vulcanisaeta* sp., belonging to the

genus *Thermoproteus.* The genomes of both components were previously sequenced, closed and annotated[31,48]. The *Vulcanisaeta* component was found to represent a novel species named "*Candidatus* Vulcanisaeta moutnovskia" with 98% 16S rRNA gene similarity to *Vulcanisaeta distributa*[20], while the *Thermoproteus* component had 100% 16S rRNA gene similarity with *Thermoproteus uzoniensis*[49]. For the present studies the binary culture[30] was obtained from M.I. Prokofeva (Federal Research Center of Biotechnology, RAS). The culture grows robustly on medium with yeast extract and sulphur or sulphate, and is maintained in sulphate conditions.

Liquid medium of the following composition was used (g L^{-1}): NH_4Cl, 0.33; KCl, 0.33; $MgCl_2 \cdot 2H_2O$, 0.33; $CaCl_2 \cdot 6H_2O$, 0.33; KH_2PO_4, 0.33; $Na_2S \cdot 9H_2O$, 0.5; yeast extract, 1.0; trace element solution[50], 1 ml; vitamin solution[51]. Sodium sulphide (0.5 g L^{-1}) was used as a reducing agent. Resazurin (1.0 mg $L^{-}1$) was added as a redox indicator. Na_2SO_4 (1.0 g L^{-1}) and S^0 (10.0 g L^{-1}) were used as electron acceptors. To adjust the pH of the medium to 5.5 (for *T. tenax*), 4.8 (for *V. souniana, V. distributa* and the binary culture) and 4.0 (for *C. maquilingensis*), 3N HCl was added. The medium was dispensed in 5-ml portions into 17-ml Hungate tubes; the headspace was filled with CO_2. The incubation temperature was 85°C.

Peptone (1 g L^{-1}), glucose, lactose, maltose, sucrose (5 mM each), acetate, lactate, pyruvate, malate, propionate, butyrate, fumarate, succinate, citrate, ethanol, propanol, glycerol, methanol (20 mM each), glycine, leucine, tyrosine, serine, glutamate, glutamine, aspartate, asparagine, phenylalanine, alanine, lysine, arginine, histidine, cysteine, valine, proline, methionine (20 mM each), CO (N_2/CO = 1:1, 4:1, or 20:1, v/v), and H_2 (H_2/CO_2 = 80:20, v/v) were tested as alternative electron donors for the binary culture.

Radioisotopic tracing of sulphate reduction

Sulphate reduction rates were measured by means of the radioisotope technique using ^{35}S-sulphate. Radioisotope experiments were performed in the laboratory using 15-mL Hungate tubes with screw caps. Each tube contained 5 mL of the medium. The headspace was filled with 100% CO_2, 10% inoculum was introduced, and 0.2 mL of $^{35}SO_4^{2-}$ (37×10^4 Bq) was injected into each tube with a syringe through the stopper. The tubes were incubated in thermostats at a temperature of 85 ºC for 4 days; after incubation, the samples were fixed with 1 mL of 2 M NaOH. The subsequent treatment was performed as described previously[44].

DNA isolation

Sediment samples (2-4 ml) were placed in centrifuge tubes and precipitated for 10 min at 12,000×g and 4 ºC. Sediment samples were resuspended in TNE buffer, pH 7.4 (20 mM Tris, 15 mM NaCl, 20 mM EDTA), frozen in liquid nitrogen, ground, and melted in a water bath at 37ºC.

The freezing and melting cycle was repeated twice. Lysozyme (200 μg ml^{-1}) and RNase (DNase-free, 5 μg ml^{-1}) were added, and the mixture was incubated for 30 min at room temperature. Proteinase K (5-10 μg ml^{-1}) and SDS (0.5%) were added, and the mixture was incubated for 30 min at 54ºC. After mixing, and cooling, an equal volume of a cooled phenol - chloroform - isoamyl alcohol (50:50:1) mixture was added, followed by agitation for 10 min and centrifugation for 10 min at 12,000×g. The water phase was collected, supplemented with an equal volume of chloroform, agitated for 5 min, centrifuged, the water phase was collected, and chloroform extraction was repeated. 0.1 volume of 3 M sodium citrate (pH 5.2) and two volumes of cooled 96% ethanol were added to the water phase. For DNA precipitation, the mixture was allowed to stay at -20 ºC for 60 min. DNA was gathered by centrifugation for 5 min at 12,000×g, washed with 70 and 96% ethanol, dried, and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). 5 μl of the preparation was applied to 1% agarose gel for the DNA visualisation.

PCR, DGGE, and qPCR analyses

The following primers were used in this work for amplification of 16S rRNA genes of Bacteria and Archaea: Bact338F- ACTCCTACGGGAGGCAGCAG[52] and Bact907R- CCGTCAATTCMTTTGAGTTT[53] and A338F- GGCCCTAYGGGGYGCASCAGGC[54] and A915R- GTGCTCCCCCGCCAATTCCT[55]. 20 μl of the PCR mixture contained 2 μl of 10x buffer (Eurogen buffer with 1.5 mM $MgCl_2$); deoxynucleoside triphosphates (Eurogen, Russia), 200 μM each; 20 pmol of each of the Bact338F and Bact907R or A338F and A915R primers; 1.2 U of *Taq* polymerase (Eurogen); and approximately 10 ng of DNA. The PCR mixture was incubated for 5 min at 94°C, followed by 33 cycles of 30 s at 93°C, 30 s at 52°C (bacterial primer system) or 68°C (archaeal primer system), and 60 sec at 72°C, with final extension at 72°C for 10 min.

The following primers were used for amplification of *dsrAB* genes of "*Candidatus* V. moutnovskia": N1 Vmut592F 5'AAAATAAAGTTCAAGTTTAGTGGTTGTCCAATGG and N2 Vmut 2021R 5'- TATGCACTTCCTCTCATCAACCTCAATACC. The PCR mixture was incubated for 5 min at 94°C, followed by 33 cycles of 30 s at 93°C, 30 s at 69°C, and 120 sec at 72°C, with final extension at 72°C for 20 min.

High-throughput sequencing of 16S rRNA gene fragments

The V4 region of 16S rRNA genes was amplified, and libraries were prepared as described by Caporaso et al.[56], with some modification. The following primer set was used for amplification: the forward primer was 5'-AATGATACGGCGACCACCGAGATCTACAC TATGGTAATT

GTGTGBCAGCMGCCGCGGTAA-3', consisting of 5′ Illumina adapter, forward primer pad, forward primer linker and 515f primer[57] and the reverse primer was 5'-CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGTCAG CC GGACTACHVGGGTATCTAAT-3', consisting of reverse complement of 3′ Illumina adapter, Golay barcode, reverse primer pad, reverse primer linker and 806R primer[56]. Each sample was amplified in two replicates, which were then pooled into a single volume and run on a standard agarose gel for visualisation and size selection purposes. Prior to sequencing, equal amounts of amplicon DNA from each sample were combined to create a single library pool, which was then diluted to a concentration of 4 nM according to the Illumina Sample Preparation Guide. Library denaturation and sample loading were performed according to the Illumina Sample Preparation Guide using a MiSeq Reagent Kit v3 (600 cycles) (Illumina). The sequencing of the library was performed with the Illumina MiSeq platform. Most of the data analysis, including quality trimming, demultiplexing, taxonomic assignments, and core diversity analyses were performed with QIIME (version 1.9.1[62]) and SILVA online data analysis service[58].The coverage of the microbial community was estimated by determining Chao1 richness[59].

Analytical methods

Sulphide formation was determined using the colourimetric method with N,N-dimethyl-para-phenylenediamine[60]; the developing blue colouration was quantified by spectrophotometry at λ = 670 nm. Sulphate ion concentrations were determined on a Stayer ion chromatograph (Russia).

Proteomics

Proteomic analysis of the binary sulphate-reducing archaeal culture 768-28, grown with sulphate and with sulphur as electron acceptors (see above), was performed by liquid chromatography and mass spectrometry analysis. In order to guarantee access to the highest diversity and amount of proteins two different methods for protein extraction were used. First, one (Method I)[61] involving extraction with a multi-chaotropic lysis buffer containing a non-denaturing zwitterionic detergent, most efficient for extracting cytosolic proteins. Second, one (Method II) involving a denaturing anionic detergent allowing total disruption of the membranes and capable of extracting both membrane (hydrophobic) and non-membrane (water-soluble, hydrophilic) proteins. Following extraction, 1D-nano Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC ESI-MSMS) analysis with Triple TOF 5600 and Orbitrap technologies were used for protein identification. Extensive description of methods for protein extraction based on a non-denaturing detergent and on a denaturing detergent, protein digestion,

and liquid chromatography and mass spectrometry analysis are provided was performed as described previously[61].

Proteomic analyses based on denaturing detergent (Method II)

Lysis and protein extraction. Cell pellets were dissolved with multi-chaotropic lysis buffer containing 7 M urea (USB Corporation, Cleveland, OH), 2 M thiourea (Sigma-Aldrich), 5 % SDS (Sigma-Aldrich), 100 mM triethylammonium bicarbonate (TEAB) (Thermo Fisher Scientific) and a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific). Samples were reduced and alkylated by adding 5 mM tris(2-carboxyethyl)phosphine and 10 mM chloroacetamide for 1 hour at 37ºC and homogenized by micro tip probe ultrasonication for 1 min on UP50H ultrasonic lab homogenizer (Hielscher Ultrasonics). The homogenate was centrifuged at 16,000 × g for 15 min at 4°C, and the supernatant containing the solubilized proteins was used for further analysis. Protein concentration was estimated by Pierce 660nm protein assay (Thermo Fisher Scientific).

S-Trap™ Digestion. Protein digestion in the S-Trap filter (Protifi, Huntington, NY, USA) was performed following the manufacturer's procedure with slight modifications. Briefly, 20 µg of protein of each sample was diluted to 40 µL with 5% SDS. Afterwards, 12% phosphoric acid and then seven volumes of binding buffer (90% methanol; 100 mM TEAB) were added to the sample (final phosphoric acid concentration: 1.2%). After mixing, the protein solution was loaded to an S-Trap filter in two consecutive steps, separated by a 2 min centrifugation at 3,000×g. Then the filter was washed 3 times with 150 µL of binding buffer. Finally, 1 µg of Pierce MS-grade trypsin (Thermo-Fisher Scientific) in 20 µL of a 100 mM TEAB solution was added to each sample in a ratio 1:20 (w/w) and spun through the S-Trap prior to digestion. Flow-through was then reloaded to the top of the S-Trap column and allowed to digest in a wet chamber at 37°C overnight. To avoid liquid leakage from the S-Trap column, a customized yellow tip with 9 Empore 3M C18 disks (Sigma-Aldrich) was placed at the bottom tip of the S-Trap column during digestion. To elute peptides, two step-wise buffers were applied (1) 40 µL of 25 mM TEAB and 2) 40 µL of 80% acetonitrile and 0.2% formic acid in H_2O), separated by a 2 min centrifugation at 3,000×g in each case. Eluted peptides were pooled and vacuum centrifuged to dryness.

MS/MS data obtained for individual samples were processed using Analyst® TF 1.7 Software (SCIEX) (for Method I) and Proteome Discoverer v2.4 (Thermo Fisher Scientific) (for Method II). Raw data file conversion tools generated mgf files which were also searched against protein

database that included "*Candidatus* V. moutnovskia" and *Thermoproteus uzoniensis* protein sequences from Uniprot/Swissprot Knowledgebase (last update: 2020/03/04, 4.573 sequences) using the Mascot Server v. 2.5.1 (Matrix Science, London, UK) (for Method I) or Mascot Server v2.7.0.1 (Matrix Science, London, UK) (for Method II). Search parameters were set as follows: enzyme, trypsin; allowed missed cleavages, 2; carbamidomethyl (C) as fixed modification and acetyl (Protein N-term), Gln to pyro-Glu (N-term Q), Glu to pyro-Glu (N-term E) and Oxidation (M) as variable modifications. Peptide mass tolerance was set to 25 ppm and 0.05 Da for fragment masses derived from Method I and ± 10 ppm for precursors and 0.02 Da for fragment masses from Method II. The confidence interval for protein identification was set to ≥ 95% (p<0.05) and only peptides with an individual ion score above the 1% False Discovery Rates (FDR) at spectra level were considered correctly identified. The threshold of only one identified peptide per protein identification was used because FDR controlled experiments counter-intuitively suffer from the two-peptide rule[61].

To rank the protein abundance in each sample, the Exponentially Modified Protein Abundance Index (emPAI) was used in the present study as a relative quantitation score of the proteins in a complex mixture based on protein coverage by the peptide matches in a database search result[62].

Genomic analysis

The genome analyses of "*Candidatus* V. moutnovskia" and other archaea were performed at the site https://blast.ncbi.nlm.nih.gov/Blast.cgi. Protein sequences of known sulphate reducing organisms were used as queries for blastp: *Desulfovibrio vulgaris ssp. vulgaris* str. Hildenborough (Sat – AAS95773, AprA – AAS95326, AprB – AAS95327, DsrA – AAS94885, DsrB – AAS94886, DsrC – AAS97248, DsrJ – AAS95766, DsrK – AAS95767, DsrM – AAS95768, DsrO – AAS95765 , DsrP – AAS95764, HdrA – AAS96875, HdrB – AAS96876, HdrC – AAS96877, PpaC – AAS96114, QmoA – AAS95328, QmoB – AAS95329, QmoC – AAS95330), and *Archaeoglobus fulgidus* DSM 4304 (Sat – AAB89581, AprA – AAB89579, AprB – AAB89580, DsrA – AAB90812, DsrB – AAB90811, DsrC – AAB89026, DsrJ – AAB90740, DsrK – AAB90735 , DsrM – AAB90736, DsrO – AAB90738, DsrP – AAB90737, HdrA – AAB89867, HdrB – AAB89869, HdrC – AAB89868, PpaC – AAB90480, QmoA – AAB90578, QmoB – AAB90579, QmoC – AAB90580). In addition, *Caldivirga maquilingensis* IC-167 (HppA – ABW01781), *Syntrophobacter fumaroxidans* MPOB (HppA – ABK18711), *Allochromatium vinosum* DSM 180 (AprM – ADC62058) and *Hyphomicrobium denitrificans* (HdrA –WP_013214728.1) were used as queries. The locus tags of the "*Candidatus* V.

741 moutnovskia" sulphate reduction genes and their function are described in Supplementary Figure
742 1.
743
744 **Phylogenetic analysis**
745 A dataset consisting of 289 archaeal genomes (253 complete and 36 assembled as contigs or
746 scaffolds) and 4,856 complete bacterial genomes were downloaded from NCBI and JGI
747 (Supplementary Table S4) and mapped to NCBI taxonomy. The completeness and contamination
748 of metagenomic records were retrieved from the original publication. Proteins involved in
749 sulphate reduction were identified using the reciprocal best BLAST hit (rBBH) approach
750 (version 2.4.0+)[63] (*E*-value $\leq 10^{-10}$ and identity cut-off of 25%) using as queries proteins from
751 characterised bacterial and archaeal sulphate reducers (see Genomic analysis). Pairwise-global
752 identities were calculated considering local identity, sequence and alignment length. Recent
753 duplications, defined as sequences with identity ≥ 70% to identified rBBHs, were kept. Hits were
754 mapped using NCBI feature tables and gene clusters were plotted using in-house Perl scripts, R-
755 Studio (version 1.0.153) and R-package genoPloR[64] (version 0.1). The syntheny allowed the
756 identification of canonical sequences of *hdrA*s from several methanogens and sulphate reducers
757 organised in an *hdrABC* cluster and the identification of (frameshifted) genes filtered out by
758 coverage. Transmembrane (TM) helices prediction was performed with TMHMM (TMHMM
759 Server v. 2.0)[65] using default parameters.
760 For phylogenetic analysis, the HdrA-like sequences from the metagenomic dataset were
761 combined with the ones retrieved from the NCBI nr search. For the small *sat*, *aprA* and *aprB*
762 phylogenies, 10 selected bacterial genomes of representative sulphate reducers and/or sulphur
763 oxidisers from different phylogenetic lineages and 26 archaeal genomes from genera in which at
764 least one genome contains *dsrAB* genes were considered. All alignments were performed with
765 clustalw2[66] (version 2.1) and trimmed in TrimAl[67]. Furthermore, in the HdrA-like recontruction,
766 only the "HdrA domain" common to QmoA, QmoB and HdrA families and defined by the
767 Conserved Domain Database at NCBI (CDD version 3.16 or higher) was considered. Maximum
768 likelihood phylogenies were reconstructed using IQ-TREE[68] (version 1.5.2 or higher) with best
769 model selection using 100 non-parametric bootstraps for the small reconstructions and 1,000
770 ultrafast bootstraps for the large HdrA-like and AprA families. Phylogenies were rooted with the
771 Minimal Ancestor Deviation method[69] and/or using succinate dehydrogenase/fumarate
772 reductases as outgroup (*aprA*) and visualised in FigTree (version 1.4.3)
773 (http://tree.bio.ed.ac.uk/publications/). Both *aprA* rooting methods selected the same root.
774 A combined analysis of sequence similarity, cofactor composition and syntheny, was
775 used for the distinction between bona-fide *qmoA/B* genes and closely-related heterodisulphide

reductase genes (*hdr*), usually present in several copies with varied domain composition and functional role[24,70] in sulphur metabolising organisms.

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Reporting summary

Further information on research design is available in Reporting Summary linked to this article.

Code availability

The small scripts used in this paper are available at https://figshare.com/s/fb5a1541be68639f4026

Data availability statement

Candidatus Vulcanisaeta mountnovskia 768-28 has been deposited into the All-Russian Collection of Microorganisms (VKM) under the number VKM B-3479. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE[71] partner repository with the dataset identifier PXD012750 and 10.6019/PXD012750. Pyrosequencing read data obtained for the 16S rRNA gene fragments were deposited in Sequence Read Archive (SRA) under the accession numbers PRJNA642261. To reproduce the results reported, the raw data are to be downloaded from SRA and submitted to the SILVAngs analysis platform (https://ngs.arb-silva.de/silvangs/). We did not obtain any new genomic assemblies, as the genomes used for computational analyses have already been reconstructed in previous and published work and are publicly available. The accession codes and taxonomic information of the genomes from the dataset are given in Supplementaty Table S4. The binary culture genomes used here have been previously sequenced and published[31,48] and have the accession codes (GenBank CP002529, CP002590). The alignments and phylogenies at the basis of Figures 3, and 5 and Extended Data Figures 8, 9 and 10 are available in Figshare (https://figshare.com/s/fb5a1541be68639f4026). The source data for Figures 1, and 2 and Extended Data Figures 2, 3, 4, 5, 6, and 7 are given in this paper and in its supplementary information.

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Author contributions

N.A.C. designed the research. N.A.C. and E.N.F. performed the fieldwork and sediment activity determinations. E.N.F. and M.L.M. enriched and isolated pure cultures and performed their microbiological studies. S.N., F.L.S., N.A.C. and A.V.L. performed genomic analysis and evolutionary reconstructions. A.Y.M. analysed 16S rRNA genes in sediments and carried out

qPCR. N.V.P. performed radioisotope research. S.C., M.C.M., M.F. and P.N.G. performed proteomic analysis. N.A.C., A.V.L., I.A.C.P., F.L.S, S.N., E.N.F., D.Y.S. and E.A.B-O analysed the data and wrote the paper. All authors have seen and approved the final version submitted.

Competing interests

All local, national and international regulations and conventions, and normal scientific ethical practices have been respected. The authors state no conflicts of interest.

Figure Legends

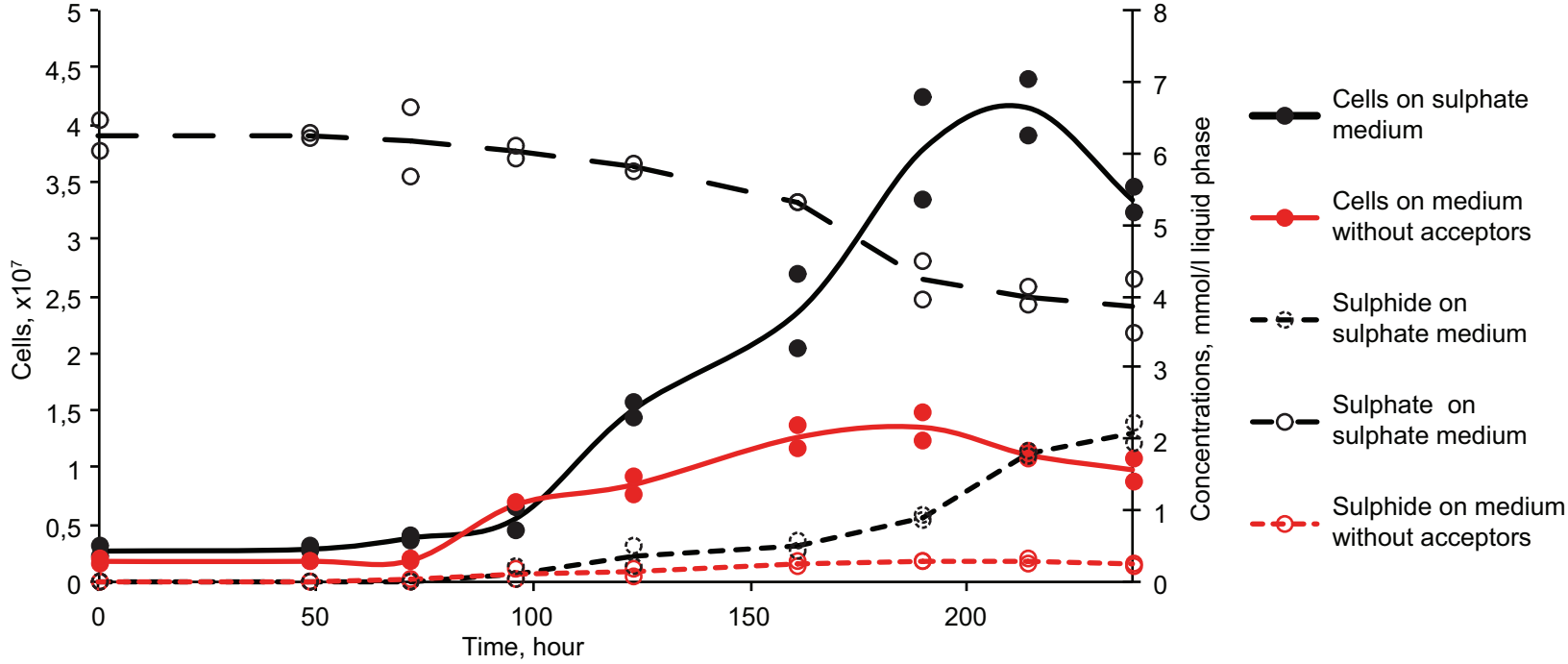
Fig. 1. Growth of the binary culture on sulphate. Data points shown correspond to 2 independent growth experiments. Lines represent the mean of n = 2 independent experiments.

Fig. 2. Distribution of sulphate reduction/sulphur oxidation genes in archaea versus established bacterial groups. Only organisms belonging to genera in which *dsr* genes are found are represented in the figure. When experimentally known, the initial substrate of the enzyme set is indicated in the first column. Abbreviations: HppA/ PpaC, pyrophosphatases, Sat, sulphate adenylyl transferase; AprAB, APS reductase; DsrABCKM, genes involved in dissimilatory sulphite reduction/oxidation; Qmo complex, QmoABC; in some sulphur oxidisers AprM, instead of QmoABC, is the physiological partner of AprAB, providing a link to the membrane.

Fig. 3. Phylogenetic analysis of AprA proteins. Maximum likelihood reconstruction (LG+I+G4) of AprA and AprA-like proteins. The tree was rooted by using as outgroup succinate dehydrogenases. The same root was obtained by the minimal ancestor deviation method[73]. Black circles indicate support values of 100, and all other node support values are omitted for simplicity. The scale bar indicates number of substitutions per site.

Fig. 4. Gene synteny and cofactor composition of the QmoAB/HdrA family.

Fig. 5. Phylogenetic analysis of HdrA-like proteins, including QmoA and QmoB. Maximum-likelihood reconstruction (LG+I+G4) of the HdrA-domain showing the expanded "*Candidatus* V. moutnovskia" QmoA (a) and QmoB (b) clades. The full tree is given in Extended Data Figure 10. The scale bar indicates number of substitutions per site.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Concentrations, mmol/l liquid phase
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1,5
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3,5
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4,5
5
0
1
2
3
4
5
6
7
8
0
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100
150
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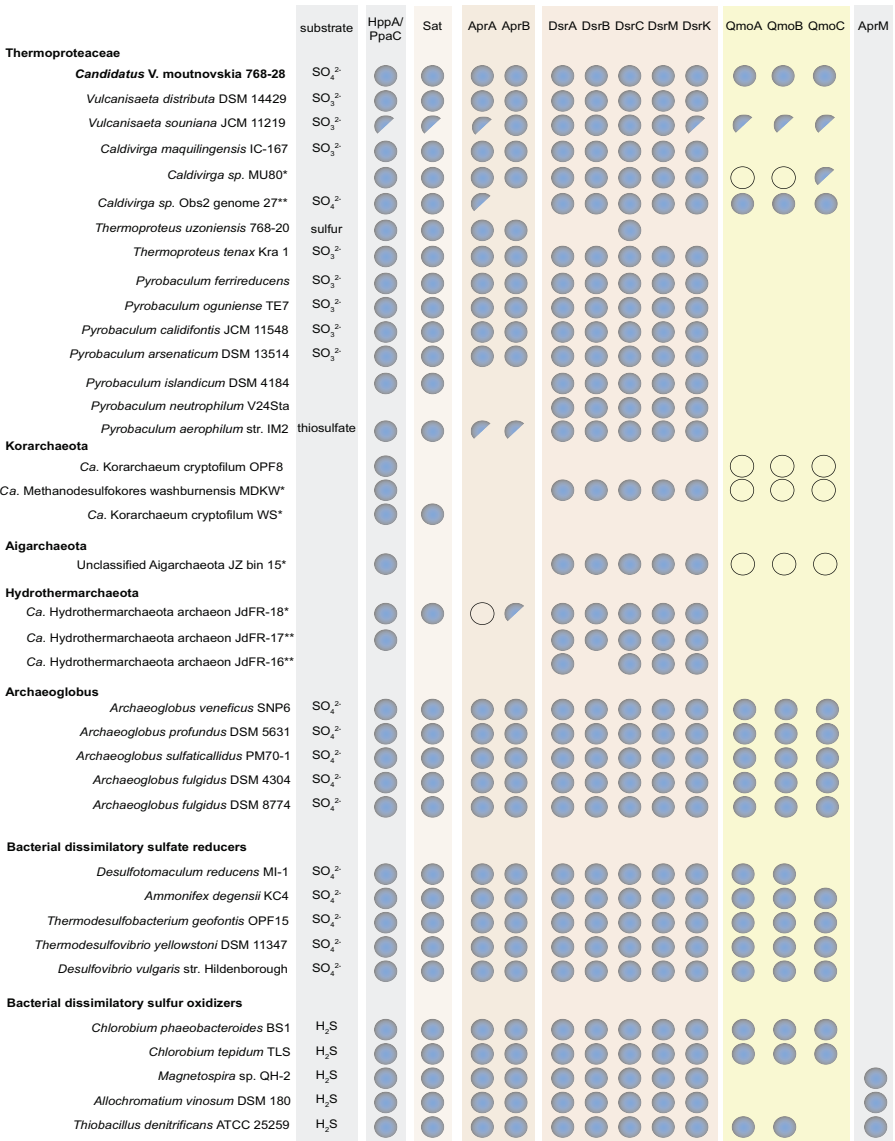
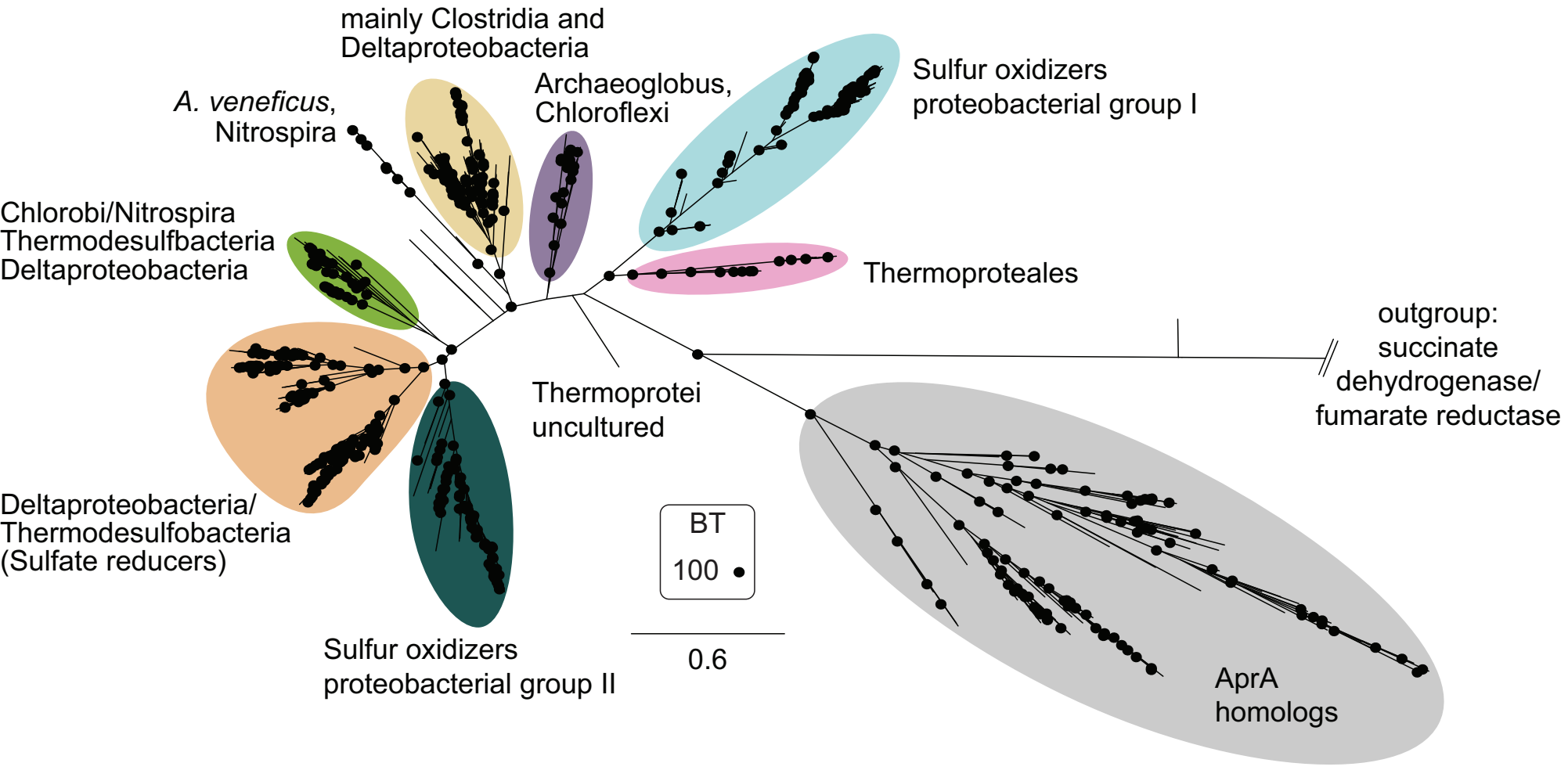
Sulphide on sulphate medium
Sulphate on sulphate medium
Sulphide on medium without acceptors



** high quality metagenome (>90% completeness, <5% contamination)
* medium to low quality metagenome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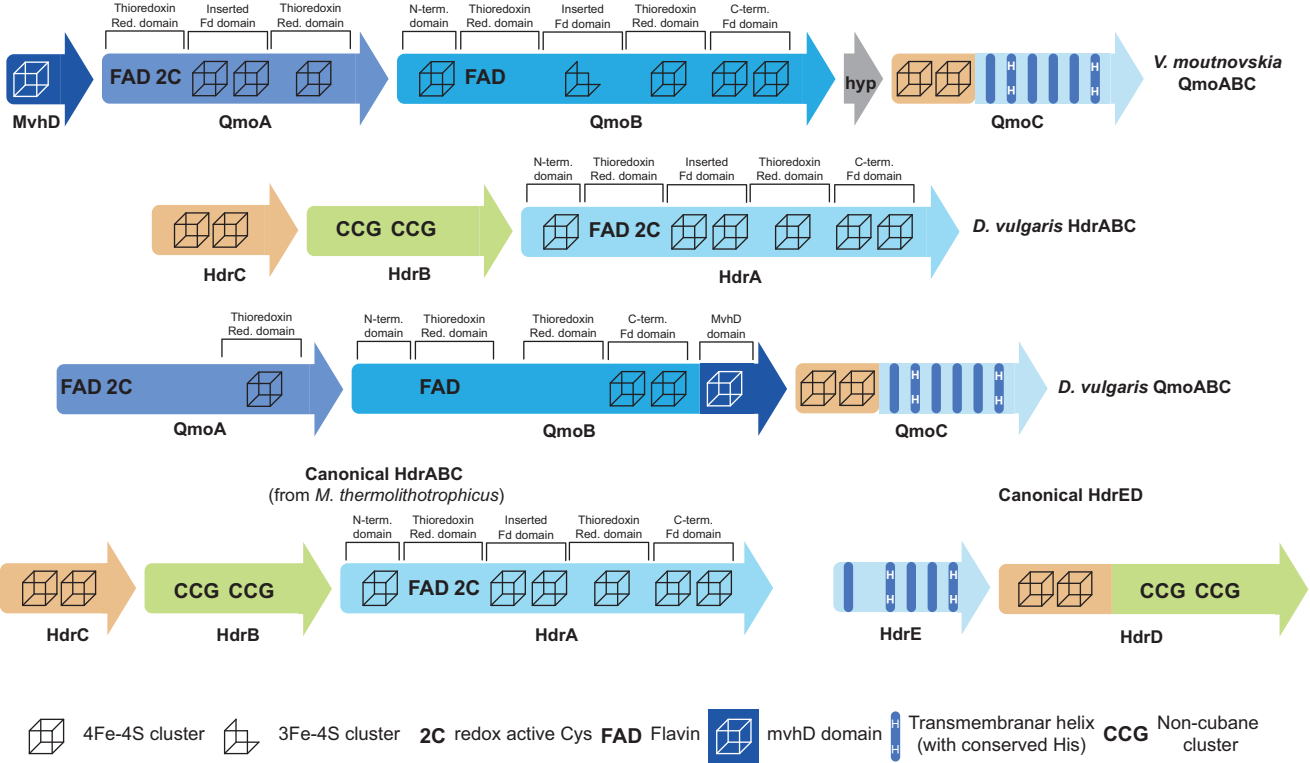
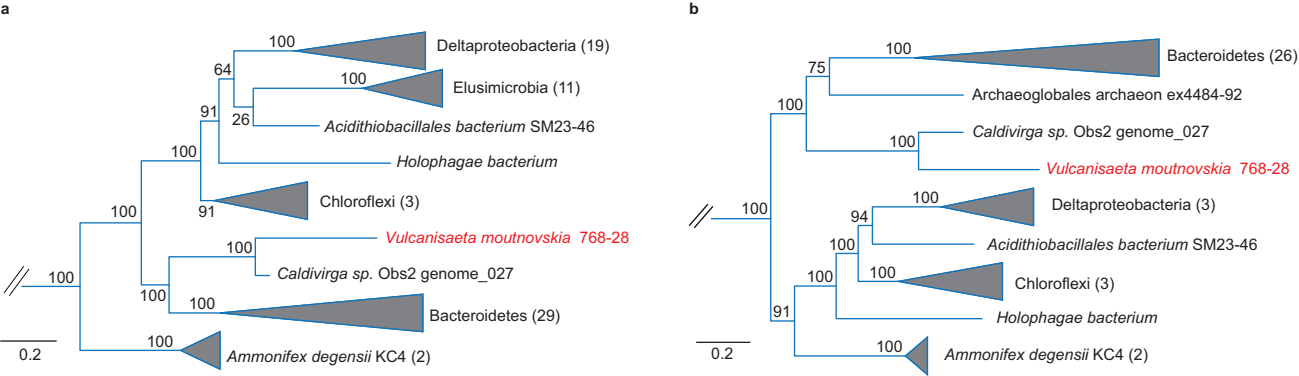
Deltaproteobacteria
A. veneficus,
Nitrospira
Archaeoglobus,
Chloroflexi
Sulfur oxidizers
proteobacterial group I
Chlorobi/Nitrospira
Thermodesulfbacteria
Deltaproteobacteria
Thermoproteales
outgroup:
succinate
dehydrogenase/
fumarate reductase
Thermoprotei
uncultured
Deltaproteobacteria/
Thermodesulfobacteria
(Sulfate reducers)
BT
100
0.6
Sulfur oxidizers
proteobacterial group II
AprA
homologs


Thioredoxin Red. domain
Inserted Fd domain
Thioredoxin Red. domain
N-term. domain
Thioredoxin Red. domain
Inserted Fd domain
Thioredoxin Red. domain
C-term. Fd domain
MvhD
FAD 2C
QmoA
FAD
QmoB
hyp
QmoC
V. moutnovskia QmoABC
N-term. domain
Thioredoxin Red. domain
Inserted Fd domain
Thioredoxin Red. domain
C-term. Fd domain
HdrC
CCG CCG
HdrB
FAD 2C
HdrA
D. vulgaris HdrABC
Thioredoxin Red. domain
N-term. domain
Thioredoxin Red. domain
Thioredoxin Red. domain
C-term. Fd domain
MvhD domain
FAD 2C
QmoA
FAD
QmoB
QmoC
D. vulgaris QmoABC
Canonical HdrABC
(from M. thermolithotrophicus)
Canonical HdrED
N-term. domain
Thioredoxin Red. domain
Inserted Fd domain
Thioredoxin Red. domain
C-term. Fd domain
HdrC
CCG CCG
HdrB
FAD 2C
HdrA
HdrE
CCG CCG
HdrD
4Fe-4S cluster
3Fe-4S cluster
2C redox active Cys
FAD Flavin
mvhD domain
Transmembranar helix (with conserved His)
CCG Non-cubane cluster


a
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Elusimicrobia (11)
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Acidithiobacillales bacterium SM23-46
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Holophagae bacterium
91
Chloroflexi (3)
100
100
Vulcanisaeta moutnovskia 768-28
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Caldivirga sp. Obs2 genome_027
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100
Bacteroidetes (29)
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Ammonifex degensii KC4 (2)
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b
100
Bacteroidetes (26)
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Archaeoglobales archaeon ex4484-92
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Caldivirga sp. Obs2 genome_027
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Vulcanisaeta moutnovskia 768-28
100
100
Deltaproteobacteria (3)
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Acidithiobacillales bacterium SM23-46
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Holophagae bacterium
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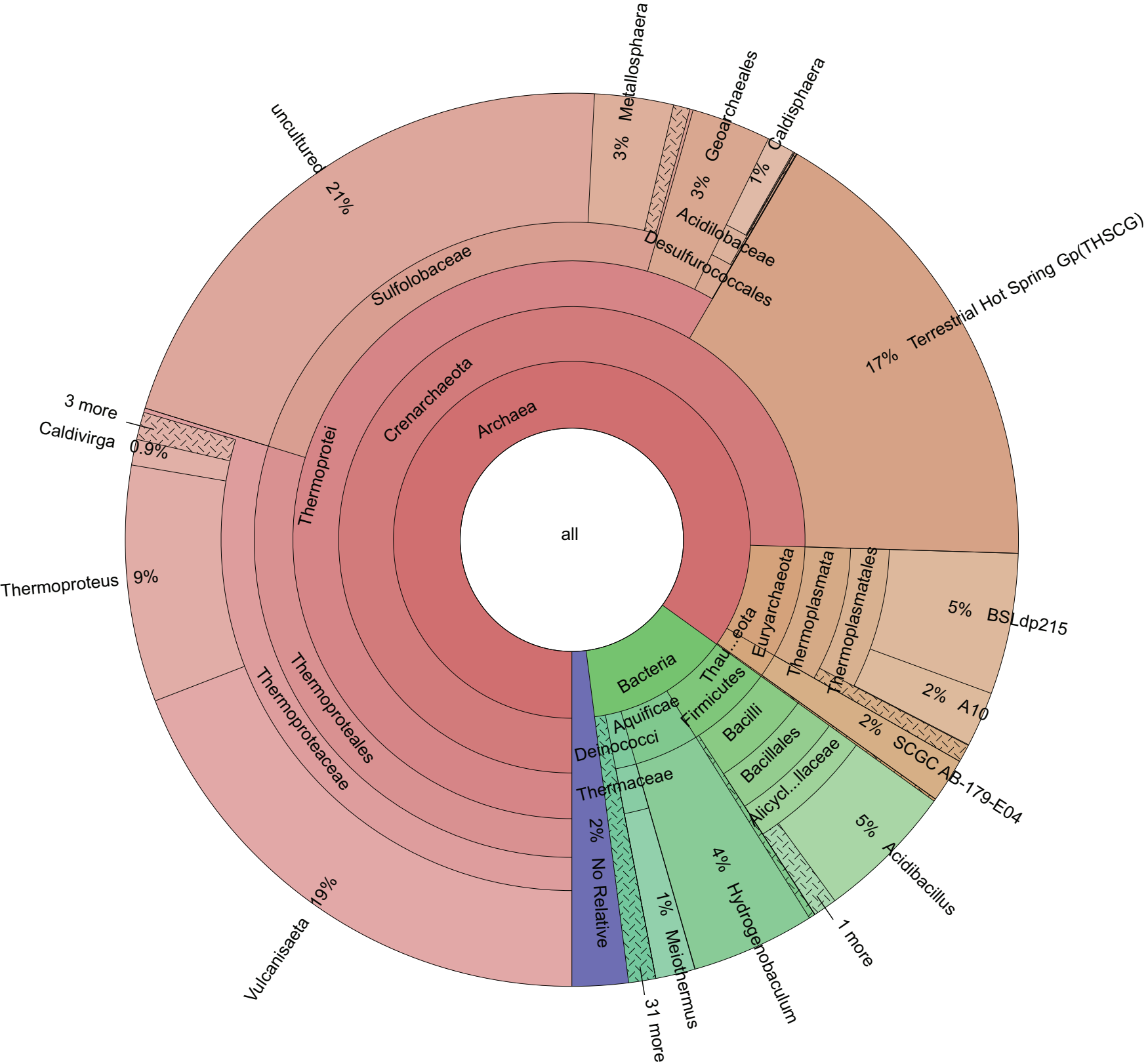
all
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Crenarchaeota
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Metallosphaera
3%
Geoarchaeales
3%
Acidilobaceae
Caldisphaera
1%
Terrestrial Hot Spring Gp(THSCG)
17%
Euryarchaeota
Thermoplasmata
Thermoplasmatales
BSLdp215
5%
A10
2%
SCGC AB-179-E04
2%
Bacteria
Thau...eota
Aquificae
Firmicutes
Deinococci
Bacilli
Thermaceae
Bacillales
Alicycl...llaceae
Acidibacillus
5%
1 more
Hydrogenobaculum
4%
Meiothermus
1%
31 more
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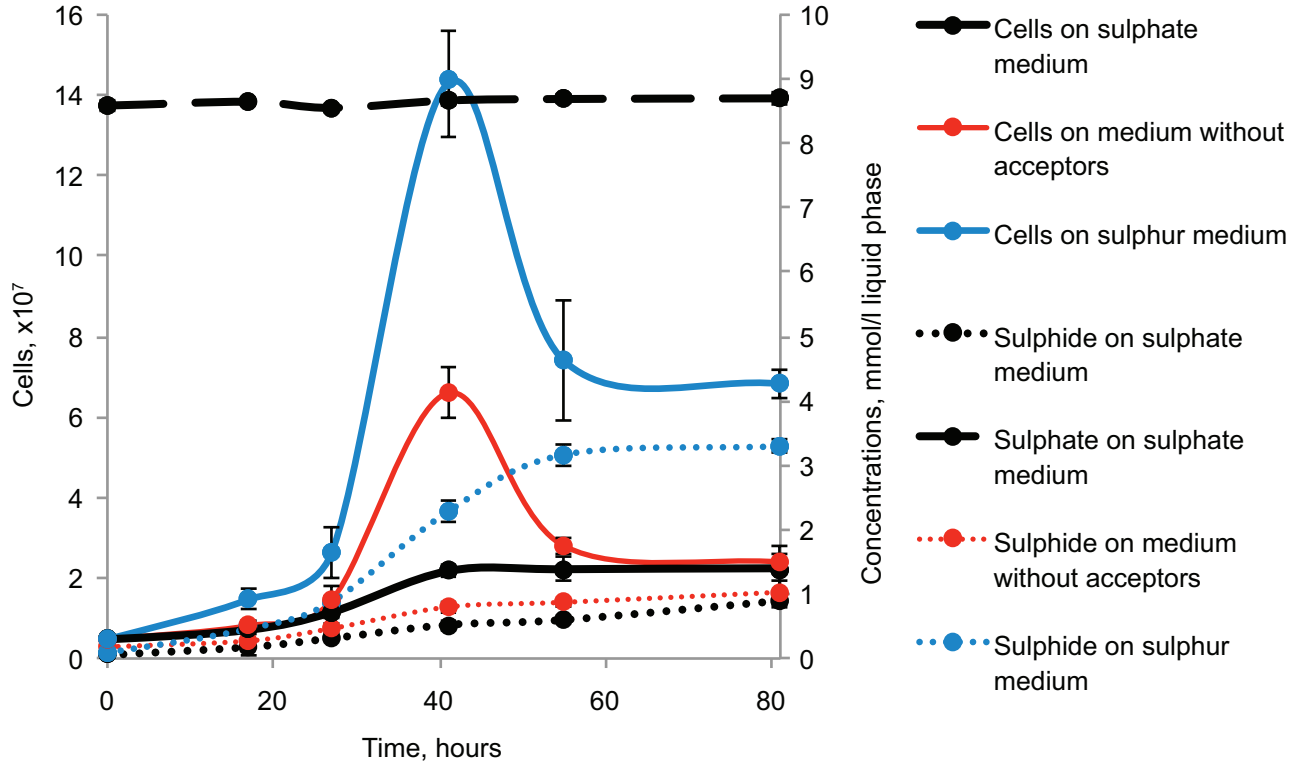
Vulcanisaeta
19%
Thermoproteales
Thermoproteaceae
Thermoproteus
9%
Caldivirga
0.9%
3 more


Cells, x10^7
Concentrations, mmol/l liquid phase
Time, hours
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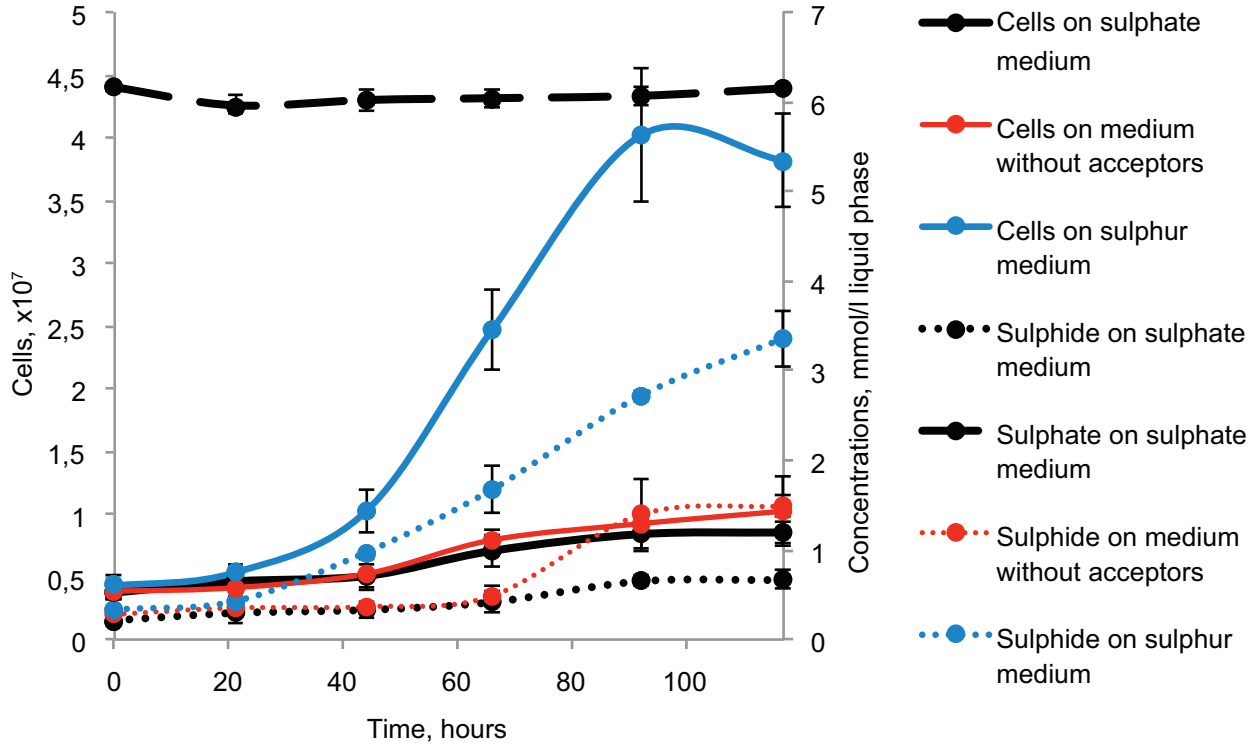
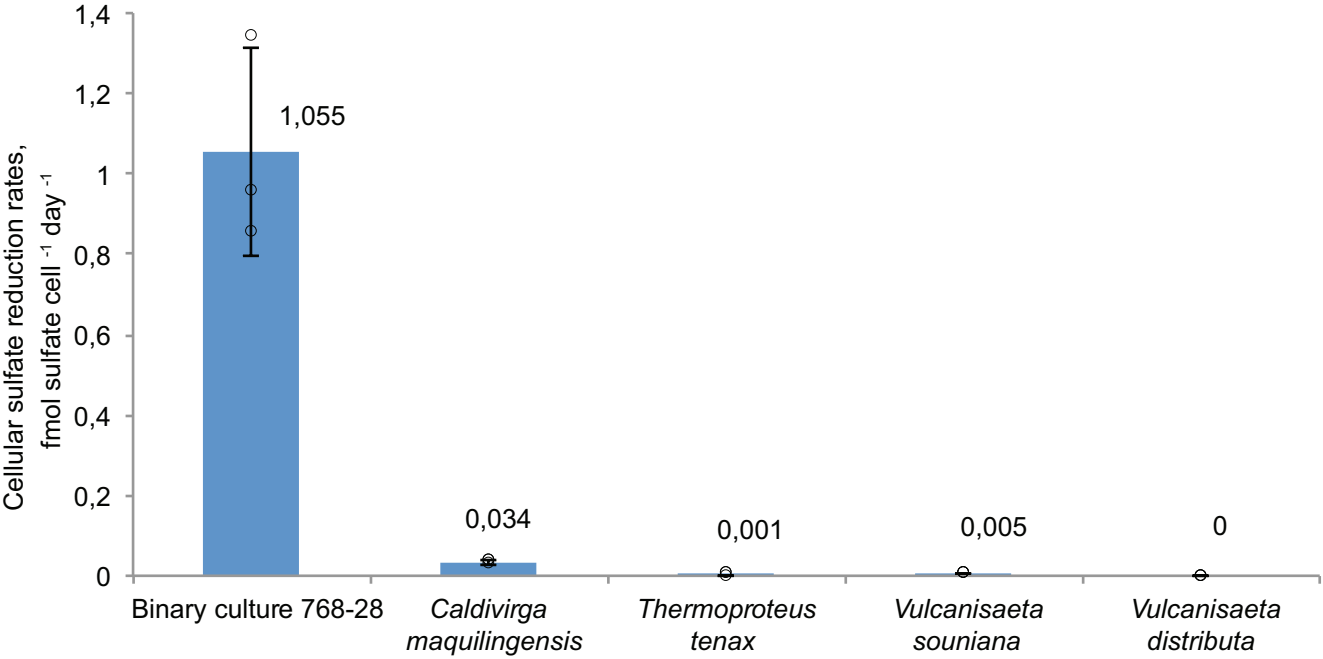
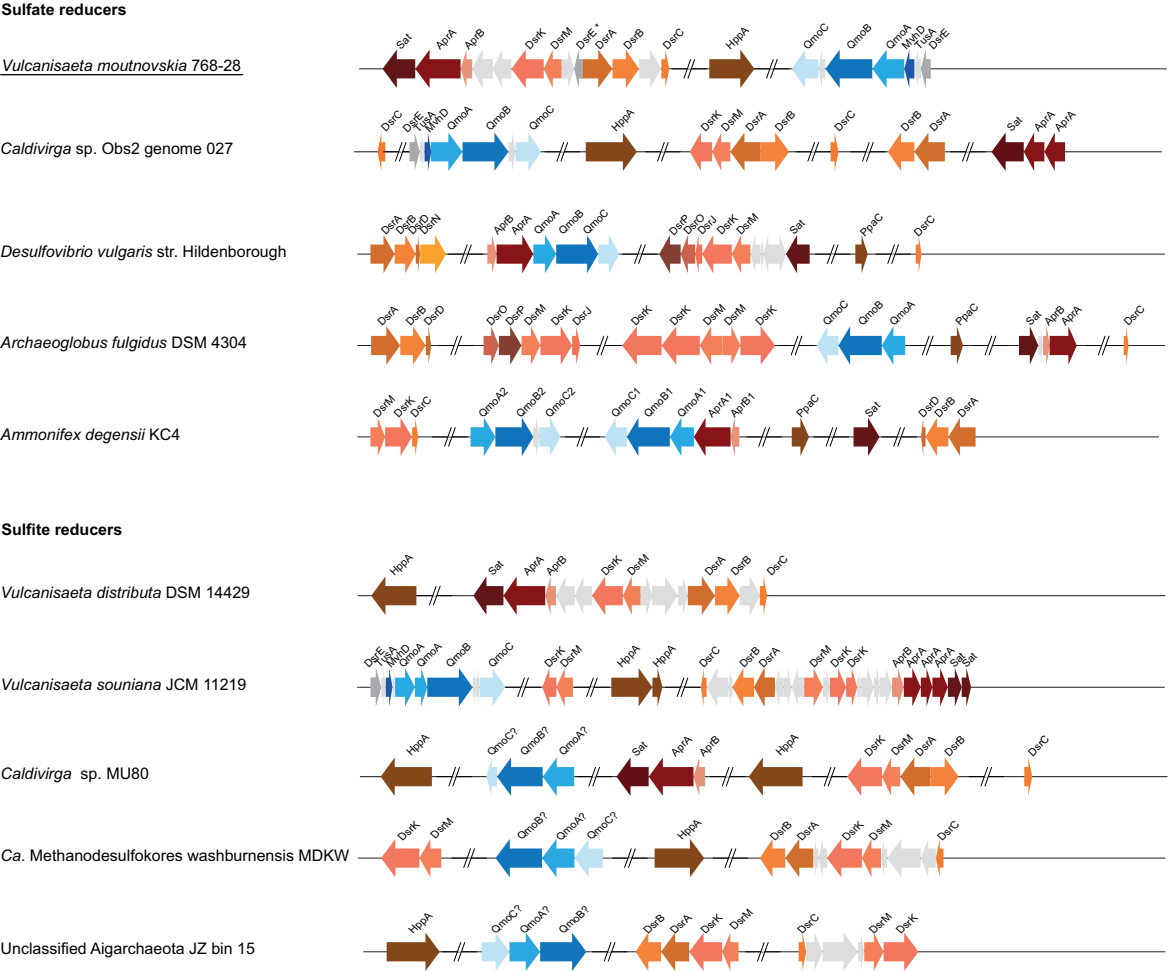
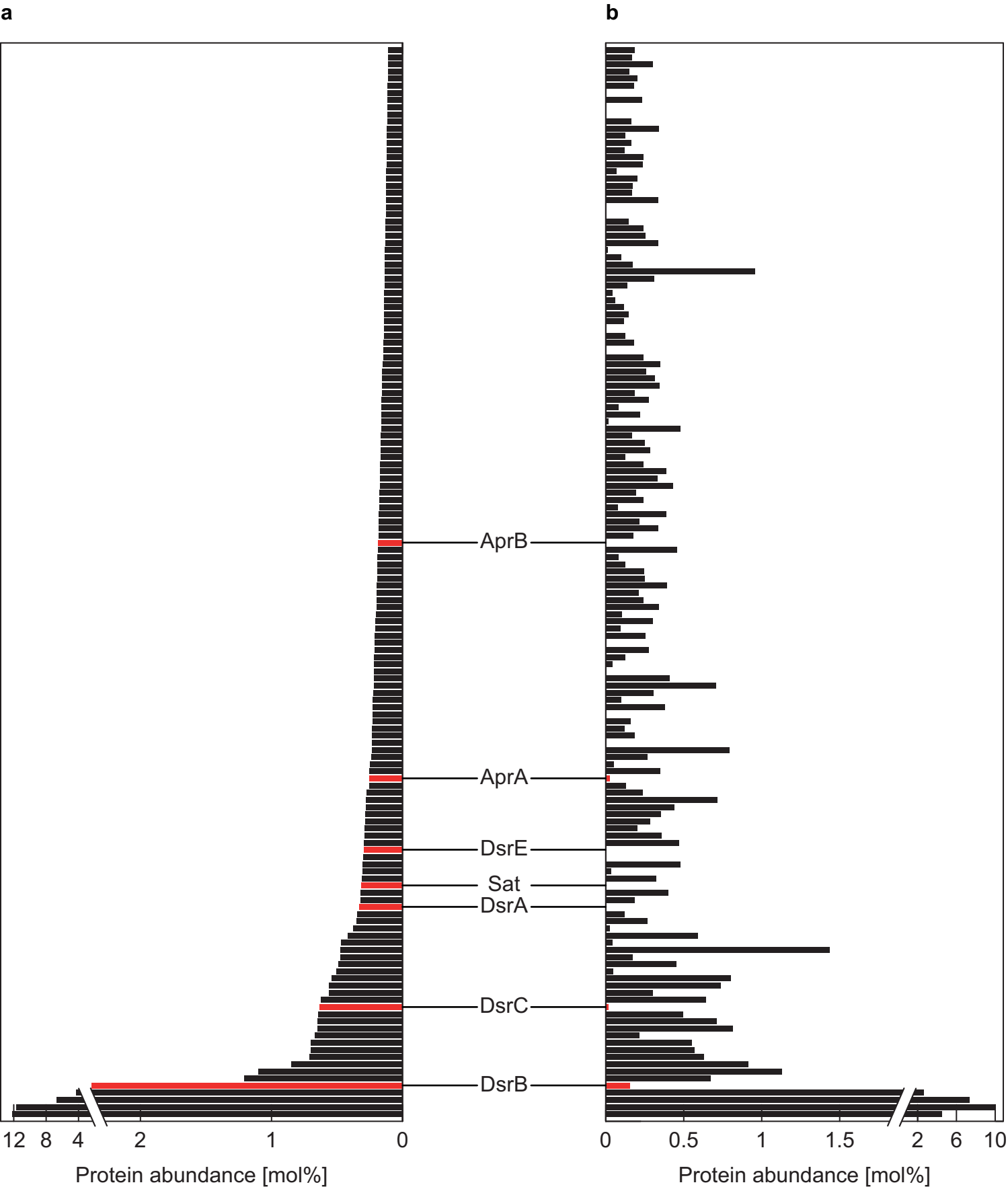
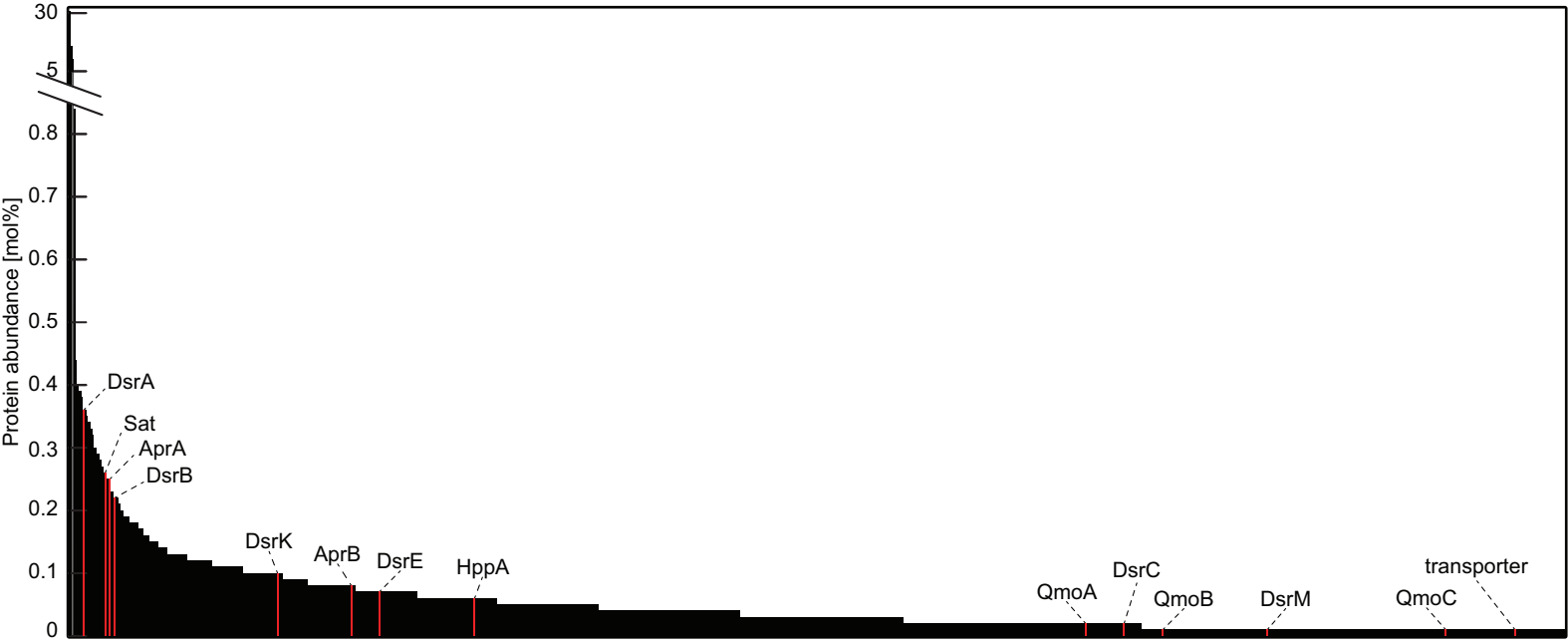
40
60
80
2
4
6
8
10
12
14
16
1
3
5
7
9
Cells on sulphate medium
Cells on medium without acceptors
Cells on sulphur medium
Sulphide on sulphate medium
Sulphate on sulphate medium
Sulphide on medium without acceptors
Sulphide on sulphur medium

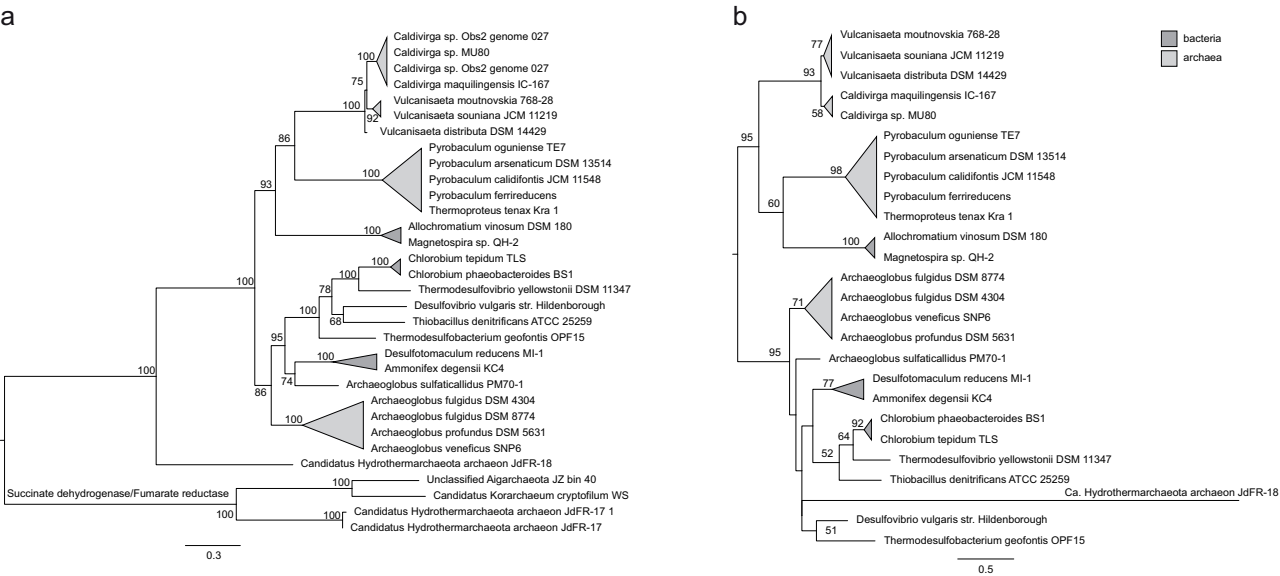

Cells, x10^7
Concentrations, mmol/l liquid phase
Time, hours
0
0,5
1
1,5
2
2,5
3
3,5
4
4,5
5
0
1
2
3
4
5
6
7
0
20
40
60
80
100
Cells on sulphate medium
Cells on medium without acceptors
Cells on sulphur medium
Sulphide on sulphate medium
Sulphate on sulphate medium
Sulphide on medium without acceptors
Sulphide on sulphur medium

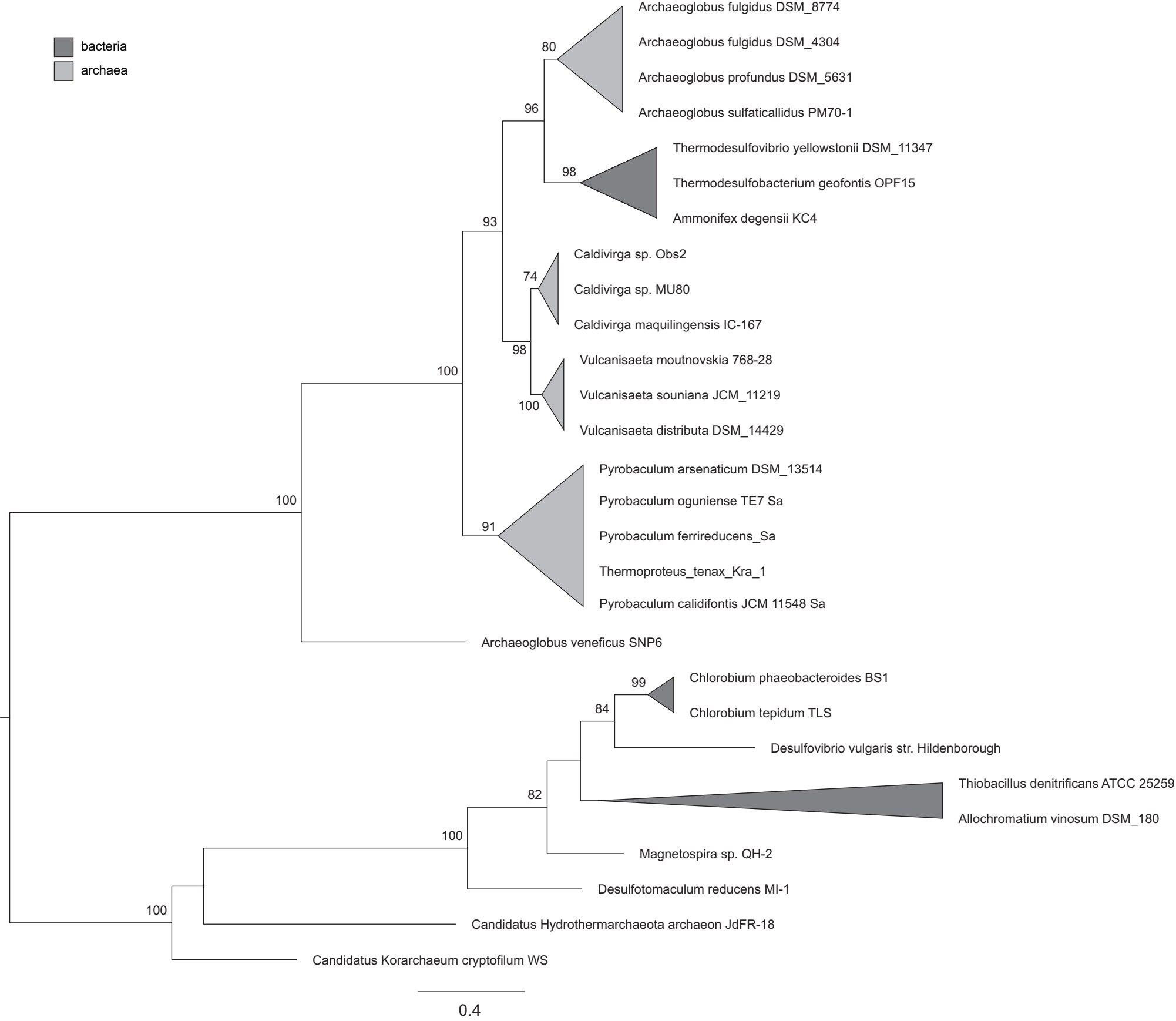

Cellular sulfate reduction rates, fmol sulfate cell -1 day -1
1,4
1,2
1
0,8
0,6
0,4
0,2
0
1,055
0,034
0,001
0,005
0
Binary culture 768-28
Caldivirga maquilingensis
Thermoproteus tenax
Vulcanisaeta souniana
Vulcanisaeta distributa

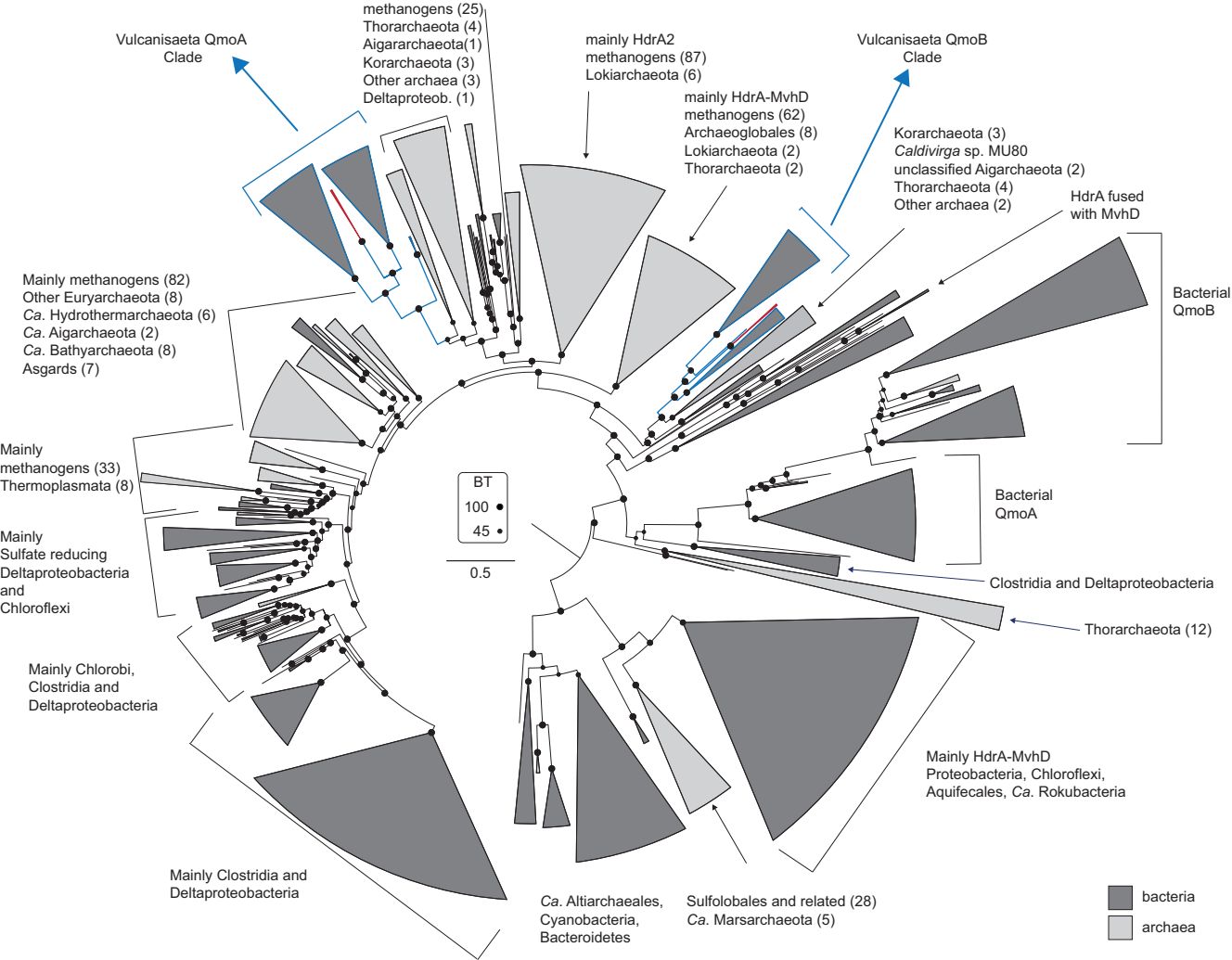

Sulfate reducers
Vulcanisaeta moutnovskia 768-28
Sat
AprA
AprB
DsrK
DsrM
DsrE*
DsrA
DsrB
DsrC
HppA
QmoC
QmoB
QmoA
MvhD
TusA
DsrE
Caldivirga sp. Obs2 genome 027
DsrC
DsrE
TusA
MvhD
QmoA
QmoB
QmoC
HppA
DsrK
DsrM
DsrA
DsrB
DsrC
DsrB
DsrA
Sat
AprA
AprA
Desulfovibrio vulgaris str. Hildenborough
DsrA
DsrB
DsrD
DsrN
AprB
AprA
QmoA
QmoB
QmoC
DsrP
DsrO
DsrJ
DsrK
DsrM
Sat
PpaC
DsrC
Archaeoglobus fulgidus DSM 4304
DsrA
DsrB
DsrD
DsrO
DsrP
DsrM
DsrK
DsrJ
DsrK
DsrK
DsrM
DsrM
DsrK
QmoC
QmoB
QmoA
PpaC
Sat
AprB
AprA
DsrC
Ammonifex degensii KC4
DsrM
DsrK
DsrC
QmoA2
QmoB2
QmoC2
QmoC1
QmoB1
QmoA1
AprA1
AprB1
PpaC
Sat
DsrD
DsrB
DsrA
Sulfite reducers
Vulcanisaeta distributa DSM 14429
HppA
Sat
AprA
AprB
DsrK
DsrM
DsrA
DsrB
DsrC
Vulcanisaeta souniana JCM 11219
DsrE
TusA
MvhD
QmoA
QmoA
QmoB
QmoC
DsrK
DsrM
HppA
HppA
DsrC
DsrB
DsrA
DsrM
DsrK
DsrK
AprB
AprA
AprA
AprA
Sat
Sat
Caldivirga sp. MU80
HppA
QmoC?
QmoB?
QmoA?
Sat
AprA
AprB
HppA
DsrK
DsrM
DsrA
DsrB
DsrC
Ca. Methanodesulfokores washburnensis MDKW
DsrK
DsrM
QmoB?
QmoA?
QmoC?
HppA
DsrB
DsrA
DsrK
DsrM
DsrC
Unclassified Aigarchaeota JZ bin 15
HppA
QmoC?
QmoA?
QmoB?
DsrB
DsrA
DsrK
DsrM
DsrC
DsrM
DsrK


a
b
AprB
AprA
DsrE
Sat
DsrA
DsrC
DsrB
12 8 4 2 1 0
0 0.5 1 1.5 2 6 10
Protein abundance [mol%]
Protein abundance [mol%]


Protein abundance [mol%]
30
5
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DsrA
Sat
AprA
DsrB
DsrK
AprB
DsrE
HppA
QmoA
DsrC
QmoB
DsrM
QmoC
transporter


a
Caldivirga sp. Obs2 genome 027
Caldivirga sp. MU80
Caldivirga sp. Obs2 genome 027
Caldivirga maquilingensis IC-167
Vulcanisaeta moutnovskia 768-28
Vulcanisaeta souniana JCM 11219
Vulcanisaeta distributa DSM 14429
Pyrobaculum oguniense TE7
Pyrobaculum arsenaticum DSM 13514
Pyrobaculum calidifontis JCM 11548
Pyrobaculum ferrireducens
Thermoproteus tenax Kra 1
Allochromatium vinosum DSM 180
Magnetospira sp. QH-2
Chlorobium tepidum TLS
Chlorobium phaeobacteroides BS1
Thermodesulfovibrio yellowstonii DSM 11347
Desulfovibrio vulgaris str. Hildenborough
Thiobacillus denitrificans ATCC 25259
Thermodesulfobacterium geofontis OPF15
Desulfotomaculum reducens MI-1
Ammonifex degensii KC4
Archaeoglobus sulfaticallidus PM70-1
Archaeoglobus fulgidus DSM 4304
Archaeoglobus fulgidus DSM 8774
Archaeoglobus profundus DSM 5631
Archaeoglobus veneficus SNP6
Candidatus Hydrothermarchaeota archaeon JdFR-18
Unclassified Aigarchaeota JZ bin 40
Succinate dehydrogenase/Fumarate reductase
Candidatus Korarchaeum cryptofilum WS
Candidatus Hydrothermarchaeota archaeon JdFR-17 1
Candidatus Hydrothermarchaeota archaeon JdFR-17
0.3
b
bacteria
archaea
Vulcanisaeta moutnovskia 768-28
Vulcanisaeta souniana JCM 11219
Vulcanisaeta distributa DSM 14429
Caldivirga maquilingensis IC-167
Caldivirga sp. MU80
Pyrobaculum oguniense TE7
Pyrobaculum arsenaticum DSM 13514
Pyrobaculum calidifontis JCM 11548
Pyrobaculum ferrireducens
Thermoproteus tenax Kra 1
Allochromatium vinosum DSM 180
Magnetospira sp. QH-2
Archaeoglobus fulgidus DSM 8774
Archaeoglobus fulgidus DSM 4304
Archaeoglobus veneficus SNP6
Archaeoglobus profundus DSM 5631
Archaeoglobus sulfaticallidus PM70-1
Desulfotomaculum reducens MI-1
Ammonifex degensii KC4
Chlorobium phaeobacteroides BS1
Chlorobium tepidum TLS
Thermodesulfovibrio yellowstonii DSM 11347
Thiobacillus denitrificans ATCC 25259
Ca. Hydrothermarchaeota archaeon JdFR-18
Desulfovibrio vulgaris str. Hildenborough
Thermodesulfobacterium geofontis OPF15
0.5


bacteria
archaea
Archaeoglobus fulgidus DSM_8774
Archaeoglobus fulgidus DSM_4304
Archaeoglobus profundus DSM_5631
Archaeoglobus sulfaticallidus PM70-1
Thermodesulfovibrio yellowstonii DSM_11347
Thermodesulfobacterium geofontis OPF15
Ammonifex degensii KC4
Caldivirga sp. Obs2
Caldivirga sp. MU80
Caldivirga maquilingensis IC-167
Vulcanisaeta moutnovskia 768-28
Vulcanisaeta souniana JCM_11219
Vulcanisaeta distributa DSM_14429
Pyrobaculum arsenaticum DSM_13514
Pyrobaculum oguniense TE7 Sa
Pyrobaculum ferrireducens_Sa
Thermoproteus_tenax_Kra_1
Pyrobaculum calidifontis JCM 11548 Sa
Archaeoglobus veneficus SNP6
Chlorobium phaeobacteroides BS1
Chlorobium tepidum TLS
Desulfovibrio vulgaris str. Hildenborough
Thiobacillus denitrificans ATCC 25259
Allochromatium vinosum DSM_180
Magnetospira sp. QH-2
Desulfotomaculum reducens MI-1
Candidatus Hydrothermarchaeota archaeon JdFR-18
Candidatus Korarchaeum cryptofilum WS
80
96
98
93
74
98
100
100
100
91
99
84
82
100
100
0.4


Vulcanisaeta QmoA Clade
methanogens (25)
Thorarchaeota (4)
Aigararchaeota(1)
Korarchaeota (3)
Other archaea (3)
Deltaproteob. (1)
mainly HdrA2
methanogens (87)
Lokiarchaeota (6)
mainly HdrA-MvhD
methanogens (62)
Archaeoglobales (8)
Lokiarchaeota (2)
Thorarchaeota (2)
Vulcanisaeta QmoB Clade
Korarchaeota (3)
Caldivirga sp. MU80
unclassified Aigarchaeota (2)
Thorarchaeota (4)
Other archaea (2)
HdrA fused with MvhD
Bacterial QmoB
Mainly methanogens (82)
Other Euryarchaeota (8)
Ca. Hydrothermarchaeota (6)
Ca. Aigarchaeota (2)
Ca. Bathyarchaeota (8)
Asgards (7)
Mainly methanogens (33)
Thermoplasmata (8)
Mainly Sulfate reducing Deltaproteobacteria and Chloroflexi
Mainly Chlorobi, Clostridia and Deltaproteobacteria
BT
100
45
0.5
Bacterial QmoA
Clostridia and Deltaproteobacteria
Thorarchaeota (12)
Mainly HdrA-MvhD Proteobacteria, Chloroflexi, Aquifecales, Ca. Rokubacteria
Mainly Clostridia and Deltaproteobacteria
Ca. Altiarchaeales, Cyanobacteria, Bacteroidetes
Sulfolobales and related (28)
Ca. Marsarchaeota (5)
bacteria
archaea