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

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1 **1. Extended Data**

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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.: <i>Smith_ED_Fig1.jpg</i>	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 <i>Thermoproteus tenax</i> .	Extended_Data_Figure_2.eps	The error bars are SD from independent growth replicates (n=3).
Extended Data Fig. 3	Growth of <i>Caldivirga maquilensis</i> .	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”, <i>Caldivirga</i> sp. Obs2 and <i>A. fulgidus</i>) and bacteria (<i>D. vulgaris</i> Hildenborough and <i>A. degensii</i>) are shown, as well as from genomes of archaea not capable of sulphate reduction (<i>V. distributa</i> , <i>V. souniana</i> , <i>Caldivirga</i> sp. MU80, <i>Ca. Methanodesulfokores washburnensis</i> 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 “ <i>Candidatus V. moutnovskia</i> ” sequences are marked in red. Expanded insets of the “ <i>Candidatus V. moutnovskia</i> ” QmoA (a) and QmoB (b) clades (marked in blue) are shown in Fig. 5. The scale bar indicates number of substitutions per site.

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2. Supplementary Information:

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A. Flat Files

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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.: <i>Supplementary Figures 1-4, Supplementary Discussion, and Supplementary Tables 1-4.</i>
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

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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.: <i>Smith_SourceData_Fig1.xls</i> , or <i>Smith_Unmodified_Gels_Fig1.pdf</i>	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

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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.

73

74 **Introduction**

75 Microbial dissimilatory sulphate reduction (DSR) is the main driver of the modern sulphur cycle,
 76 and an important actor in the carbon cycle^{1,2}. Although dissimilation of sulphur compounds was
 77 present on the early Earth³, there is dispute regarding the onset of DSR. The Paleoarchean barite
 78 deposits of 3.5, 3.4 and 3.2 Ga in Western Australia, India and South Africa support a well-
 79 mixed, large scale, marine sulphate pool⁴, with low micromolar concentrations^{5,6}. This sulphate
 80 was produced by atmospheric photolysis of volcanic SO₂⁷ and, as observed in modern
 81 environments^{8,9}, the low levels of sulphate did not preclude active DSR. However, this
 82 metabolism did not imprint a strong mass-dependent fractionation on Archean sedimentary
 83 records probably due to a low level of biological activity and an atmospherically-derived
 84 elemental sulphur pool that masked isotopic evidence for DSR¹⁰. Nevertheless, the isotopic and
 85 microfossil evidence for Paleoarchean DSR^{11,12} has been confirmed by recent high-resolution
 86 quadruple sulphur isotope measurements^{4,6,13}, strongly supporting the existence of sulphate-
 87 reducing organisms at 3.55 Ga. However, the evolutionary history of sulphate reducers is poorly
 88 understood.

89 DSR capacity is found in several phyla of Bacteria, including Proteobacteria, Firmicutes,
 90 Thermodesulfobacteria, and Nitrospirae¹, and possibly also in newly identified uncultured
 91 lineages^{14–18}. Among Archaea, DSR has been reported in the hyperthermophilic marine
 92 *Archaeoglobus* genus of the Euryarchaeota, and for some hyperthermophilic terrestrial members
 93 of the Thermoproteaceae family from the Crenarchaeota, including *Caldivirga maquilingensis*¹⁹,
 94 *Vulcanisaeta distributa*²⁰, *Vulcanisaeta souniana*²⁰, *Thermoproteus tenax*²¹, and *Thermoproteus*
 95 *thermophilus*²². However, conclusive experimental proof of DSR, namely quantitative data on
 96 sulphide accumulation from growth with sulphate, has not been reported for the
 97 Thermoproteaceae. DSR is mediated by a specific set of proteins, which include sulphate
 98 adenylyl transferase (Sat), adenosine phosphosulphate reductase (AprAB) and the QmoABC
 99 membrane complex essential for sulphate reduction to sulphite^{23,24}, as well as the dissimilatory
 100 sulphite reductase (DsrAB), its substrate DsrC, and the membrane DsrMK(JOP) complex,
 101 involved in sulphite reduction to sulphide^{24,25}. The presence of DSR in both Archaea and
 102 Bacteria could indicate an ancient origin for this metabolism²⁶. However, phylogenetic analyses
 103 of *Archaeoglobus aprAB* and *dsrAB* genes suggest their acquisition via horizontal gene transfer
 104 (HGT) from Bacteria^{27,28}. In contrast, the Thermoproteaceae *dsrAB* genes represent a deep
 105 archaeal lineage distinct from bacterial sulphate reducers and sulphur oxidisers, supporting the
 106 origin of DsrAB as a reductive enzyme^{14,16,26,27}. Thus, the Thermoproteaceae need to be further
 107 investigated to elucidate the evolutionary history of DSR.

108 Here, we studied terrestrial thermal acidic environments in which DSR was previously
109 detected²⁹. Using community profiling, culture studies and radioisotope measurements, coupled
110 with comparative genomics, proteomics, and phylogenetics, we conclude that among the
111 reported archaeal sulphate reducers with sequenced genomes, only *Archaeoglobus* spp. and
112 “*Candidatus* *Vulcanisaeta moutnovskia*” are capable of DSR. In “*Candidatus* *V. moutnovskia*”
113 (as in *Archaeoglobus* spp.) this activity correlates with the presence of functional (full-length)
114 *qmoABC* genes, which are absent or truncated in the genomes of other Thermoproteaceae.
115 Furthermore, the *qmoABC* genes of “*Candidatus* *V. moutnovskia*” and *Archaeoglobus* spp. are
116 of different evolutionary origin (albeit in both cases bacterial). These observations suggest that
117 DSR arose twice in archaea, both times by acquisition of bacterial genes, and seems to be
118 dependent on functional *qmoABC* genes needed for energy conservation. In contrast,
119 dissimilatory sulphite reduction appears to be more ancient and may have arisen early within the
120 Thermoproteaceae.

121

122 **Community profiling of Oreshek Spring**

123 The microbial community of Oreshek Spring (Moutnovsky Volcano, Kamchatka Peninsula,
124 Russia, temperature of 91°C, pH 3.5, sulphate concentration of 538 mg/l), which displays
125 significant rates of sulphate reduction (1.094 nmol H₂S/cm³·day)²⁹, was analysed by sequencing
126 16S rRNA gene fragments. After quality filtering, we obtained 28152716S rRNA gene
127 fragments corresponding to 434 operational taxonomic units (OTUs) defined with 0.98 similarity
128 threshold. This allowed a coverage of 0.825 for the microbial community, estimated by
129 determining Chao1 richness (Chao1=526). Such coverage does not permit a complete description
130 of phylogenetic diversity, but allows identification of all major groups. The prokaryotic diversity
131 in Oreshek Spring was estimated by the Shannon index, which amounted to 3.37. Archaea
132 dominated the microbial community, accounting for 85.0% of 16S rRNA genes obtained.
133 Bacterial sequences accounted for 13.0%, and 2.0% of sequences could not be affiliated. The
134 archaeal sequences represented three phyla: Crenarchaeota (75% of all library sequences),
135 Thaumarchaeota (2.0%) and Euryarchaeota (8.0%). Among the archaeal representatives, three
136 genera of putative sulphate reducers were detected: *Vulcanisaeta*, *Thermoproteus* and *Caldivirga*
137 (20.0%, 9.0% and 0.9% of library sequences). Among bacterial sequences, no taxa representing
138 known sulphate reducers were detected. Thus, sulphate reduction at Oreshek Spring seems to be
139 performed mainly by archaea, among which *Vulcanisaeta* spp. predominate (**Extended Data**
140 **Figure 1**).

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

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

153

154 **Sulphate reduction by Crenarchaeota**

155 For studies of DSR we used the binary culture 768-28³⁰ (hereafter referred to as the binary
156 culture), which contains “*Candidatus V. moutnovskia*” and *Thermoproteus uzoniensis* (all
157 attempts to isolate the pure cultures were unsuccessful). Using liquid medium with sulphate as
158 electron acceptor and yeast extract as energy source, we tested the DSR capacity of the binary
159 culture and of the pure cultures of putative Thermoproteaceae sulphate reducers *T. tenax*, *V.*
160 *distributa*, *V. souniana*, and *C. maquilingensis*. The binary culture was able to grow by DSR
161 with yeast extract, showing sulphate consumption and sulphide accumulation (**Fig. 1**). Among
162 tested electron donors, only peptone and yeast extract supported growth with sulphate
163 (**Supplementary Table 2**). In sulphate-reducing conditions “*Candidatus V. moutnovskia*”
164 predominated in the binary culture, while *T. uzoniensis* was present only as a minor component.
165 qPCR with 16S rRNA gene-specific primers showed that in DSR conditions the share of *T.*
166 *uzoniensis* was 0.01 to 0.1%, while in sulphur-reducing conditions it increased to 50%. This
167 suggests that DSR is a capacity of “*Candidatus V. moutnovskia*”, while *T. uzoniensis* is likely
168 incapable of sulphate reduction, as supported by genomic and proteomic analyses (see below).

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

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

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

188

189 Sulphate reduction gene distribution in archaea

190 The previously sequenced “*Candidatus V. moutnovskia*” genome³¹ contains the minimal set of
191 genes necessary for DSR, namely genes for sulphate transporters and pyrophosphatase, *sat*,
192 *aprAB*, *qmoABC*, *dsrAB*, *dsrC* and *dsrMK*. In addition, we found no evidence for possible
193 alternative genes that could be involved in this process, such as coding for membrane complexes
194 or HdrA-related proteins (with one exception, see below). This analysis was extended to a large
195 (meta)genomic dataset which showed that, apart from *Archaeoglobus* spp. and “*Candidatus V.*
196 *moutnovskia*” no other archaeon possesses a complete set of full-length genes necessary for
197 DSR (**Fig. 2 and Extended Data Figure 5**). Among the Thermoproteaceae cultures claimed to
198 be capable of DSR, *T. tenax*, *V. distributa* and *C. maquilingensis* lack the *qmoABC* genes, and *T.*
199 *tenax* and *C. maquilingensis* also contain no *hdrA*-like genes. In the *V. souniana* low quality
200 draft genome, all necessary genes for DSR could be found, but several of them appear to be
201 impaired (with an apparent abundance of sequencing artefacts masking possible natural
202 frameshifts). In the genome of *T. uzoniensis*, the second component of the binary culture, the
203 *dsrAB*, *dsrMK*, and *qmoABC* genes are absent, while *sat*, *aprAB* and *dsrC* are present.

204 Recently, the presence of *dsrABC* genes has been reported in several uncultivated
205 organisms belonging to the Aigarchaeota^{17,34}, Korarchaeota¹⁶ and Hydrothermarchaeota¹⁵
206 candidate phyla. We found that in *Candidatus* Hydrothermarchaeota genomes, no *qmoABC* or
207 bona-fide *aprAB* could be identified, although bona fide *sat*, *dsrABC* and *dsrMK* are present.
208 Interestingly, in both *Ca. Methanodesulfokores washburnensis* MDKW and uncultivated
209 Aigarchaeota JZ bin 15 genomes, in which the presence of *dsrAB*, *dsrC* and *dsrMK* has been
210 reported¹⁷, *qmoABC* gene candidates were also found, albeit no *aprAB* genes were present (**Fig.**
211 **2**). Both metagenomic assemblies have a high level of completeness (above 95%), but it cannot
212 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-reductase³⁵, 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.

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

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

269 The full-length *qmoABC* genes are absent in the Crenarchaeota, with the exception of
 270 “*Candidatus V. moutnovskia*” and the *Caldivirga* sp. Obs2 metagenomic assembly (**Figs. 2 and**
 271 **4**), suggesting a critical role of these genes in DSR. The three genes code for a membrane
 272 complex responsible for electron transfer from the menaquinol pool to APS reductase^{23,39}
 273 (**Supplementary Figure 1**). QmoC oxidizes menaquinol, presumably with proton release to the
 274 periplasm, and electrons are transferred through the QmoC hemes *b* and Fe-S centers to QmoA
 275 and QmoB, which transfers them to APS reductase³⁹. In bacteria, the QmoABC complex was
 276 shown to be essential for sulphate, but not sulphite, reduction⁴⁰, and the corresponding genes are
 277 strictly conserved in sulphate reducers^{24,28}, with the exception of *qmoC* in some clostridial
 278 organisms. In these organisms two *hdrBC* genes (in some *Desulfotomaculum* spp.) or *hdrD*
 279 genes (in some *Desulfosporosinus* spp.) are found instead, but these genes cannot functionally
 280 replace *qmoC* in energy conservation⁴⁰, and their function is unknown. The QmoA and QmoB
 281 proteins are both related to the methanogenic HdrA, a subunit of heterodisulfide reductases^{41,42}.
 282 However, QmoA and QmoB have very distinct physiological functions from HdrA proteins.

283 QmoA is smaller than HdrA and lacks some of its domains and [4Fe-4S] clusters, whereas
284 QmoB has a similar size as HdrA, but also lacks some of its [4Fe-4S] clusters, having an
285 additional C-terminal domain related to the electron transfer protein MvhD²³ (**Fig. 4**). The QmoC
286 protein is distinct and has a cytochrome *b* membrane domain related to HdrE, and an electron-
287 transfer iron-sulphur domain related to HdrC.

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

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

318 from metagenomic Deltaproteobacteria, which is an indication of interdomain HGT. Although
 319 having a higher statistical support, the exact position of these sequences cannot be fully resolved.
 320 The phylogenetic reconstruction of HdrA-like proteins (**Fig. 5 and Extended Data**
 321 **Figure 10**) points to the acquisition of the “*Candidatus V. moutnovskia*” *qmoAB* genes from a
 322 bacterial donor. The nature of the primordial organisms in which the transition from an HdrA to
 323 a QmoA/B protein occurred is not clear. Until some of these organisms are cultivated, Occam’s
 324 razor suggests the bacterial affiliation of this transition since in both *Vulcanisaeta* QmoA/B
 325 clades, the most basal organism known to perform sulphate reduction is *A. degensii*, and in many
 326 of the archaeal lineages basal to the clade there is no evidence for the ability for sulphate or
 327 sulphite reduction. Overall, this data suggests that “*Candidatus V. moutnovskia*” “re-invented”
 328 DSR by acquiring QmoAB proteins from a bacterial organism, where the separate evolution of
 329 Qmo proteins from a typical HdrA took place, and, thus, extant DSR in Thermoproteaceae has
 330 an independent evolutionary history from that in Bacteria and *Archaeoglobus* species.

331

332 **DISCUSSION**

333 The evolutionary history of DSR is poorly understood, particularly among the archaea. Sulphate is
 334 present in high-temperature submarine, deep subsurface and terrestrial environments where
 335 archaeal organisms are abundant. Several independent studies have reported high-temperature
 336 sulphate reduction in terrestrial neutral or acidic hot springs of Yellowstone National Park⁴³ and
 337 Kamchatka Peninsula^{29,44,45}, but the species responsible for high-temperature sulphate reduction
 338 had not been identified. We report that hyperthermophilic archaea of the genus *Vulcanisaeta* are
 339 one of the dominant groups in the acidic and hot Oreshek Spring. Using growth, radioisotopic,
 340 and proteomic experiments with the binary culture 768-28, we show that “*Candidatus V.*
 341 *moutnovskia*” is responsible for sulphate reduction, while *T. uzoniensis* is only a minor
 342 component in DSR conditions, with its share growing only upon growth with elemental sulphur.
 343 Furthermore, the DSR capacity briefly reported for other four Thermoproteaceae species (*T.*
 344 *tenax*, *C. maquilingensis*, *V. distributa* and *V. souniana*) could not be confirmed in detailed
 345 growth and radioisotopic studies. No quantitative data was provided in any of these previous
 346 studies and the weak growth observed with sulphate was probably not statistically different from
 347 the weak growth without any electron acceptor, a possibility that we confirmed (**Supplementary**
 348 **Table 2, and Extended Data Figure 2 and Extended Data Figure 3**).

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

353 Thermoproteaceae investigated had the *sat*, *aprAB* and *dsrABCMK* genes, but lacked the
354 *qmoABC* genes. The QmoABC respiratory complex²³ was shown to be essential for growth of a
355 sulphate reducing bacterium on sulphate, but not on sulphite⁴⁰, and seems to allow energy
356 conservation during the step of APS reduction. The alternative *aprM* present in sulphur oxidisers
357 (or the *hdrBC* genes in clostridial sulphate reducers), are also absent in Thermoproteaceae. These
358 results suggest that DSR by “*Candidatus V. moutnovskia*” is associated with the acquisition of
359 the *qmoABC* genes by HGT, whereas other Thermoproteaceae may only reduce sulphate for
360 assimilatory purposes, as they lack the QmoABC complex that would enable them to conserve
361 energy from that reduction.

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

374 These results have important implications for understanding the evolution of
375 dissimilatory sulphur metabolism, and three evolutionary scenarios can be considered. The first
376 is that assimilatory or dissimilatory sulphite reduction was already present before the archaeal-
377 bacterial divide. This agrees with the proposed existence of an assimilatory or dissimilatory
378 enzyme for sulphite reduction in ancestral organisms, either prior to the divergence of the
379 bacterial and archaeal domains⁴⁶ or early after the archaeal-bacterial division followed by an
380 interdomain transfer. In this scenario, dissimilatory sulphite reduction was subsequently lost in
381 most archaeal lineages, being only retained in a single Crenarchaeota family. Later interdomain
382 HGTs events would be responsible for the presence of dissimilatory sulphite reduction in other
383 archaeal lineages^{16,17}. The second scenario is that dissimilatory sulphite reduction originated
384 within Archaea (Thermoproteaceae) and was acquired by Bacteria due to an early HGT event.
385 Subsequently, several intradomain HGTs occurred expanding this trait across systematic
386 boundaries. The presence of the *dsr* genes within several bacterial phyla would suggest a high
387 number of such events. The third scenario is that dissimilatory sulphite reduction is of early

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

391 In contrast, DSR has apparently a separate evolutionary history. It may have been
392 preceded by assimilatory sulphate reduction, which could have been the initial physiological
393 function of the AprAB enzyme, namely in the Thermoproteaceae, given the absence of AprM or
394 QmoABC. In this scenario DSR evolved within the bacterial domain, presumably originating
395 through the development of QmoABC proteins, and was only later transferred to a limited
396 number of organisms in the Archaea, namely *Archaeoglobus* spp., “*Candidatus* V.
397 moutnovskia” and *Caldivirga* sp. Obs2, of which at least the first two acquired QmoABC as
398 independent evolutionary events. An alternative, less likely, hypothesis is that ancestors of the
399 Thermoproteaceae were early archaeal dissimilatory sulphate reducers, which subsequently lost
400 this ability, presumably by loss of the *qmoABC* (or *aprM* genes), that were re-acquired by
401 “*Candidatus* V. moutnovskia” and *Caldivirga* sp. Obs2. The ancestry of dissimilatory sulphite
402 over sulphate reduction makes sense from a bioenergetic point of view as ATP is required for
403 sulphate, but not for sulphite, reduction, and also in terms of the different availability of the two
404 compounds on the early Earth. In the anoxic, reduced Earth, sulphate was present at low
405 concentration, produced from SO₂ photolysis⁷, or biologically from sulphide anoxygenic
406 photosynthesis⁴⁷ or disproportionation of sulphur compounds. In contrast, sulphite produced
407 from hydrolysis of volcanic and hydrothermal SO₂ was probably abundant. The evolutionary
408 history of siroheme sulphite reductases also supports the ancestry of sulphite reduction⁴⁶. Recent
409 phylogenomic analysis of novel Diaforarchaea also endorse the view that most organisms with
410 early-branching DsrAB are capable of sulphite, but not sulphate, reduction³⁶.

411 In conclusion, assimilatory/dissimilatory sulphite reduction may have been present before
412 the Bacteria/Archaea divide or shortly after, and likely preceded dissimilatory sulphate
413 reduction. Nevertheless, DSR is an ancient process dating back at least to 3.5 Ga, which was
414 likely restricted to Bacteria that were limited in biological activity by the low levels of sulphate.
415 In the Thermoproteaceae, assimilatory sulphate reduction may have been an ancient trait and our
416 results suggest that a later acquisition of the *qmoABC* genes allowed “*Candidatus* V.
417 moutnovskia” to grow by reduction of sulphate. Nevertheless, further experimental studies of
418 archaeal sulphate reduction are required to confirm this idea and firmly clarify the role of the
419 *qmo* and *hdr* genes in archaea, as additional or different genes may be involved. At about 2.4 Ga,
420 the rise in O₂ levels started the slow oxygenation of the oceans and atmosphere, with the
421 associated increase in marine sulphate concentration. This process then led to the strong
422 diversification of bacterial lineages capable of sulphate reduction, as observed today.

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547

548 **ONLINE METHODS**

549 **Environmental sampling**

550 The sample used in this work was collected from Oreshek Spring (N52° 31.818 E158° 11.499, T
551 91°C, pH 3.5) at the foot of Moutnovsky volcano (Kamchatka Peninsula, Russia). The sample of
552 thermal water and sediment was placed into 50-mL glass vial with gas-tight butyl rubber stopper
553 and aluminium screw cap, which was filled to capacity, hermetically sealed, and transported to
554 the laboratory at ambient temperature. For DNA isolation, a mixed sample of sediment and water
555 was used. Prior to arrival in the laboratory, the fixed sample was stored at ambient temperature
556 for several days and then at –20°C until evaluation.

557

558 **Microbial cultures**

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

563 The binary culture 768-28 (referred to as “the binary culture”) containing “*Candidatus V.*
564 *moutnovskia*” was initially regarded as a pure isolate³⁰. The culture was obtained on medium
565 with ethanol, yeast extract, and sulphate, but was then routinely maintained on medium with
566 yeast extract and elemental sulphur, which provided for better growth. Later it was found that
567 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 culture³⁰ 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): NH₄Cl, 0.33; KCl, 0.33; MgCl₂·2H₂O, 0.33; CaCl₂·6H₂O, 0.33; KH₂PO₄, 0.33; Na₂S·9H₂O, 0.5; yeast extract, 1.0; trace element solution⁵⁰, 1 ml; vitamin solution⁵¹. 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. Na₂SO₄ (1.0 g L⁻¹) and S⁰ (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 CO₂. 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 (N₂/CO = 1:1, 4:1, or 20:1, v/v), and H₂ (H₂/CO₂ = 80:20, v/v) were tested as alternative electron donors for the binary culture.

589

590 **Radioisotopic tracing of sulphate reduction**

Sulphate reduction rates were measured by means of the radioisotope technique using ³⁵S-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% CO₂, 10% inoculum was introduced, and 0.2 mL of ³⁵SO₄²⁻ (37 × 10⁴ Bq) was injected into each tube with a syringe through the stopper. The tubes were incubated in thermostats at a temperature of 85 °C for 4 days; after incubation, the samples were fixed with 1 mL of 2 M NaOH. The subsequent treatment was performed as described previously⁴⁴.

598

599 **DNA isolation**

Sediment samples (2-4 ml) were placed in centrifuge tubes and precipitated for 10 min at 12,000×g and 4 °C. 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 37°C.

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

615

616 **PCR, DGGE, and qPCR analyses**

617 The following primers were used in this work for amplification of 16S rRNA genes of Bacteria
618 and Archaea: Bact338F- ACTCCTACGGGAGGCAGCAG⁵² and Bact907R-
619 CCGTCAATTCMTTGTGAGTTT⁵³ and A338F- GGCCCTAYGGGGYGCASCAGGC⁵⁴ and
620 A915R- GTGCTCCCCGCCAATTCCT⁵⁵. 20 μl of the PCR mixture contained 2 μl of 10x
621 buffer (Eurogen buffer with 1.5 mM MgCl_2); deoxynucleoside triphosphates (Eurogen, Russia),
622 200 μM each; 20 pmol of each of the Bact338F and Bact907R or A338F and A915R primers; 1.2
623 U of *Taq* polymerase (Eurogen); and approximately 10 ng of DNA. The PCR mixture was
624 incubated for 5 min at 94°C, followed by 33 cycles of 30 s at 93°C, 30 s at 52°C (bacterial
625 primer system) or 68°C (archaeal primer system), and 60 sec at 72°C, with final extension at
626 72°C for 10 min.

627 The following primers were used for amplification of *dsrAB* genes of “*Candidatus V.*
628 *moutnovskia*”: N1 Vmut592F 5’AAAATAAAGTTCAAGTTTAGTGGTTGTCCAATGG and
629 N2 Vmut 2021R 5’- TATGCACTTCCTCTCATCAACCTCAATACC. The PCR mixture was
630 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
631 72°C, with final extension at 72°C for 20 min.

632

633

634 **High-throughput sequencing of 16S rRNA gene fragments**

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

638 GTGTGBCAGCMGCCGCGGTAA-3', consisting of 5' Illumina adapter, forward primer pad,
639 forward primer linker and 515f primer⁵⁷ and the reverse primer was 5'-
640 CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXXX AGTCAGTCAG CC
641 GGACTACHVGGGTATCTAAT-3', consisting of reverse complement of 3' Illumina adapter,
642 Golay barcode, reverse primer pad, reverse primer linker and 806R primer⁵⁶. Each sample was
643 amplified in two replicates, which were then pooled into a single volume and run on a standard
644 agarose gel for visualisation and size selection purposes. Prior to sequencing, equal amounts of
645 amplicon DNA from each sample were combined to create a single library pool, which was then
646 diluted to a concentration of 4 nM according to the Illumina Sample Preparation Guide. Library
647 denaturation and sample loading were performed according to the Illumina Sample Preparation
648 Guide using a MiSeq Reagent Kit v3 (600 cycles) (Illumina). The sequencing of the library was
649 performed with the Illumina MiSeq platform. Most of the data analysis, including quality
650 trimming, demultiplexing, taxonomic assignments, and core diversity analyses were performed
651 with QIIME (version 1.9.1⁶²) and SILVA online data analysis service⁵⁸. The coverage of the
652 microbial community was estimated by determining Chao1 richness⁵⁹.

653

654 **Analytical methods**

655 Sulphide formation was determined using the colourimetric method with N,N-dimethyl-para-
656 phenylenediamine⁶⁰; the developing blue colouration was quantified by spectrophotometry at λ =
657 670 nm. Sulphate ion concentrations were determined on a Stayer ion chromatograph (Russia).

658

659 **Proteomics**

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

672 and liquid chromatography and mass spectrometry analysis are provided was performed as
673 described previously⁶¹.

674

675 *Proteomic analyses based on denaturing detergent (Method II)*

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

686

687 *S-TrapTM Digestion.* Protein digestion in the S-Trap filter (Protifi, Huntington, NY, USA) was
688 performed following the manufacturer's procedure with slight modifications. Briefly, 20 µg of
689 protein of each sample was diluted to 40 µL with 5% SDS. Afterwards, 12% phosphoric acid and
690 then seven volumes of binding buffer (90% methanol; 100 mM TEAB) were added to the sample
691 (final phosphoric acid concentration: 1.2%). After mixing, the protein solution was loaded to an
692 S-Trap filter in two consecutive steps, separated by a 2 min centrifugation at 3,000×g. Then the
693 filter was washed 3 times with 150 µL of binding buffer. Finally, 1 µg of Pierce MS-grade
694 trypsin (Thermo-Fisher Scientific) in 20 µL of a 100 mM TEAB solution was added to each
695 sample in a ratio 1:20 (w/w) and spun through the S-Trap prior to digestion. Flow-through was
696 then reloaded to the top of the S-Trap column and allowed to digest in a wet chamber at 37°C
697 overnight. To avoid liquid leakage from the S-Trap column, a customized yellow tip with 9
698 Empore 3M C18 disks (Sigma-Aldrich) was placed at the bottom tip of the S-Trap column
699 during digestion. To elute peptides, two step-wise buffers were applied (1) 40 µL of 25 mM
700 TEAB and 2) 40 µL of 80% acetonitrile and 0.2% formic acid in H₂O), separated by a 2 min
701 centrifugation at 3,000×g in each case. Eluted peptides were pooled and vacuum centrifuged to
702 dryness.

703

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

707 database that included “*Candidatus V. moutnovskia*” and *Thermoproteus uzoniensis* protein
708 sequences from Uniprot/Swissprot Knowledgebase (last update: 2020/03/04, 4.573 sequences)
709 using the Mascot Server v. 2.5.1 (Matrix Science, London, UK) (for Method I) or Mascot Server
710 v2.7.0.1 (Matrix Science, London, UK) (for Method II). Search parameters were set as follows:
711 enzyme, trypsin; allowed missed cleavages, 2; carbamidomethyl (C) as fixed modification and
712 acetyl (Protein N-term), Gln to pyro-Glu (N-term Q), Glu to pyro-Glu (N-term E) and Oxidation
713 (M) as variable modifications. Peptide mass tolerance was set to 25 ppm and 0.05 Da for
714 fragment masses derived from Method I and ± 10 ppm for precursors and 0.02 Da for fragment
715 masses from Method II. The confidence interval for protein identification was set to $\geq 95\%$
716 ($p < 0.05$) and only peptides with an individual ion score above the 1% False Discovery Rates
717 (FDR) at spectra level were considered correctly identified. The threshold of only one identified
718 peptide per protein identification was used because FDR controlled experiments counter-
719 intuitively suffer from the two-peptide rule⁶¹.

720 To rank the protein abundance in each sample, the Exponentially Modified Protein
721 Abundance Index (emPAI) was used in the present study as a relative quantitation score of the
722 proteins in a complex mixture based on protein coverage by the peptide matches in a database
723 search result⁶².

724

725 **Genomic analysis**

726 The genome analyses of “*Candidatus V. moutnovskia*” and other archaea were performed at the
727 site <https://blast.ncbi.nlm.nih.gov/Blast.cgi>. Protein sequences of known sulphate reducing
728 organisms were used as queries for blastp: *Desulfovibrio vulgaris ssp. vulgaris* str.
729 Hildenborough (Sat – AAS95773, AprA – AAS95326, AprB – AAS95327, DsrA – AAS94885,
730 DsrB – AAS94886, DsrC – AAS97248, DsrJ – AAS95766, DsrK – AAS95767, DsrM –
731 AAS95768, DsrO – AAS95765, DsrP – AAS95764, HdrA – AAS96875, HdrB – AAS96876,
732 HdrC – AAS96877, PpaC – AAS96114, QmoA – AAS95328, QmoB – AAS95329, QmoC –
733 AAS95330), and *Archaeoglobus fulgidus* DSM 4304 (Sat – AAB89581, AprA – AAB89579,
734 AprB – AAB89580, DsrA – AAB90812, DsrB – AAB90811, DsrC – AAB89026, DsrJ –
735 AAB90740, DsrK – AAB90735, DsrM – AAB90736, DsrO – AAB90738, DsrP – AAB90737,
736 HdrA – AAB89867, HdrB – AAB89869, HdrC – AAB89868, PpaC – AAB90480, QmoA –
737 AAB90578, QmoB – AAB90579, QmoC – AAB90580). In addition, *Caldivirga maquilingensis*
738 IC-167 (HppA – ABW01781), *Syntrophobacter fumaroxidans* MPOB (HppA – ABK18711),
739 *Allochromatium vinosum* DSM 180 (AprM – ADC62058) and *Hyphomicrobium denitrificans*
740 (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 $\geq 70\%$ 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 genoPloR⁶⁴ (version 0.1). The syntheny allowed the
756 identification of canonical sequences of *hdrAs* 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 clustalw2⁶⁶ (version 2.1) and trimmed in TrimAl⁶⁷. Furthermore, in the HdrA-like reconstruction,
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-TREE⁶⁸ (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 method⁶⁹ 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

776 reductase genes (*hdr*), usually present in several copies with varied domain composition and
777 functional role^{24,70} in sulphur metabolising organisms.

778

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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 PRIDE⁷¹ 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](https://www.ncbi.nlm.nih.gov/sra/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 Supplementary 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.

867

868

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870

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896

897 **Author contributions**

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

902 qPCR. N.V.P. performed radioisotope research. S.C., M.C.M., M.F. and P.N.G. performed
903 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
904 the data and wrote the paper. All authors have seen and approved the final version submitted.

905

906 **Competing interests**

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

909

910

911

912 **Figure Legends**

913

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

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

923 **Fig. 3. Phylogenetic analysis of AprA proteins.** Maximum likelihood reconstruction
924 (LG+I+G4) of AprA and AprA-like proteins. The tree was rooted by using as outgroup succinate
925 dehydrogenases. The same root was obtained by the minimal ancestor deviation method⁷³. Black
926 circles indicate support values of 100, and all other node support values are omitted for
927 simplicity. The scale bar indicates number of substitutions per site.

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

929 **Fig. 5. Phylogenetic analysis of HdrA-like proteins, including QmoA and QmoB.** Maximum-
930 likelihood reconstruction (LG+I+G4) of the HdrA-domain showing the expanded “*Candidatus*
931 *V. moutnovskia*” QmoA (a) and QmoB (b) clades. The full tree is given in Extended Data Figure
932 10. The scale bar indicates number of substitutions per site.

	substrate	HppA/ PpaC	Sat	AprA	AprB	DsrA	DsrB	DsrC	DsrM	DsrK	QmoA	QmoB	QmoC	AprM
Thermoproteaceae														
<i>Candidatus V. moutnovskia</i> 768-28	SO ₃ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Vulcanisaeta distributa</i> DSM 14429	SO ₃ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Vulcanisaeta souniana</i> JCM 11219	SO ₃ ²⁻	◐	◐	◐		●	●	●	●	●	◐	◐	◐	
<i>Caldivirga maquilingensis</i> IC-167	SO ₃ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Caldivirga</i> sp. MU80*	SO ₃ ²⁻	●	●	●	●	●	●	●	●	●	○	○	◐	
<i>Caldivirga</i> sp. Obs2 genome 27**	SO ₄ ²⁻	●	●	◐		●	●	●	●	●	●	●	●	
<i>Thermoproteus uzoniensis</i> 768-20	sulfur	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Thermoproteus tenax</i> Kra 1	SO ₃ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Pyrobaculum ferrireducens</i>	SO ₃ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Pyrobaculum oguniense</i> TE7	SO ₃ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Pyrobaculum caldifontis</i> JCM 11548	SO ₃ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Pyrobaculum arsenaticum</i> DSM 13514	SO ₃ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Pyrobaculum islandicum</i> DSM 4184	SO ₃ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Pyrobaculum neutrophilum</i> V24Sta	SO ₃ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Pyrobaculum aerophilum</i> str. IM2	thiosulfate	●	●	◐	◐	●	●	●	●	●	●	●	●	
Korarchaeota														
<i>Ca. Korarchaeum cryptofilum</i> OPF8	SO ₃ ²⁻	●									○	○	○	
<i>Ca. Methanodesulfokores washburnensis</i> MDKW*	SO ₃ ²⁻	●				●	●	●	●	●	○	○	○	
<i>Ca. Korarchaeum cryptofilum</i> WS*	SO ₃ ²⁻	●	●											
Aigarchaeota														
Unclassified Aigarchaeota JZ bin 15*	SO ₃ ²⁻	●				●	●	●	●	●	○	○	○	
Hydrothermarchaeota														
<i>Ca. Hydrothermarchaeota archaeon</i> JdFR-18*	SO ₃ ²⁻	●	●	○	◐	●	●	●	●	●	●	●	●	
<i>Ca. Hydrothermarchaeota archaeon</i> JdFR-17**	SO ₃ ²⁻	●				●	●	●	●	●	●	●	●	
<i>Ca. Hydrothermarchaeota archaeon</i> JdFR-16**	SO ₃ ²⁻	●				●	●	●	●	●	●	●	●	
Archaeoglobus														
<i>Archaeoglobus veneficus</i> SNP6	SO ₂ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Archaeoglobus profundus</i> DSM 5631	SO ₄ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Archaeoglobus sulfatocaldus</i> PM70-1	SO ₄ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Archaeoglobus fulgidus</i> DSM 4304	SO ₂ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Archaeoglobus fulgidus</i> DSM 8774	SO ₄ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
Bacterial dissimilatory sulfate reducers														
<i>Desulfotomaculum reducens</i> MI-1	SO ₂ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Ammonifex degensii</i> KC4	SO ₂ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Thermodesulfobacterium geofontis</i> OPF15	SO ₄ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Thermodesulfovibrio yellowstoni</i> DSM 11347	SO ₂ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Desulfovibrio vulgaris</i> str. Hildenborough	SO ₄ ²⁻	●	●	●	●	●	●	●	●	●	●	●	●	
Bacterial dissimilatory sulfur oxidizers														
<i>Chlorobium phaeobacteroides</i> BS1	H ₂ S	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Chlorobium tepidum</i> TLS	H ₂ S	●	●	●	●	●	●	●	●	●	●	●	●	
<i>Magnetospira</i> sp. QH-2	H ₂ S	●	●	●	●	●	●	●	●	●				●
<i>Allochrocatium vinosum</i> DSM 180	H ₂ S	●	●	●	●	●	●	●	●	●				●
<i>Thiobacillus denitrificans</i> ATCC 25259	H ₂ S	●	●	●	●	●	●	●	●	●	●	●		●

** high quality metagenome (>90% completeness, <5% contamination)
 * medium to low quality metagenome

● gene present ○ homologue ◐ frameshifted gene

Canonical HdrABC (from *M. thermolithotrophicus*)

Canonical HdrED

4Fe-4S cluster

3Fe-4S cluster

2C redox active Cys

FAD Flavin

mvhD domain

Transmembranar helix
(with conserved His)

CCG

Non-cubane
cluster

a **b**

Sulfate reducers

Vulcanisaeta moutnovskia 768-28

Caldivirga sp. Obs2 genome 027

Desulfovibrio vulgaris str. Hildenborough

Archaeoglobus fulgidus DSM 4304

Ammonifex degensii KC4

Sulfite reducers

Vulcanisaeta distributa DSM 14429

Vulcanisaeta souniana JCM 11219

Caldivirga sp. MU80

Ca. Methanodesulfokores washburnensis MDKW

Unclassified Aigarchaeota JZ bin 15

a **b**

a

b

■ bacteria
■ archaea