

## Discovery of anaerobic lithoheterotrophic haloarchaea, ubiquitous in hypersaline habitats

Sorokin, Dimitry Y.; Messina, Enzo; Smedile, Francesco ; Roman, Pawel ; Sinninghe Damste, Jaap S ; Ciordia, Sergio; Mena, Maria Carmen; Ferrer, Manuel ; Golyshin, Peter; Kublanov, Ilya V.; Samarov, Nazar I.; Toshchakov, Stepan V.; La Cono, Violetta ; Yakimov, Michail M.

### ISME Journal

DOI:  
[10.1038/ismej.2016.203](https://doi.org/10.1038/ismej.2016.203)

Published: 20/01/2017

Peer reviewed version

[Cyswllt i'r cyhoeddiad / Link to publication](#)

#### *Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):*

Sorokin, D. Y., Messina, E., Smedile, F., Roman, P., Sinninghe Damste, J. S., Ciordia, S., Mena, M. C., Ferrer, M., Golyshin, P., Kublanov, I. V., Samarov, N. I., Toshchakov, S. V., La Cono, V., & Yakimov, M. M. (2017). Discovery of anaerobic lithoheterotrophic haloarchaea, ubiquitous in hypersaline habitats. *ISME Journal*, 11, 1245-1260. <https://doi.org/10.1038/ismej.2016.203>

#### **Hawliau Cyffredinol / General rights**

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

#### **Take down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

## Discovery of anaerobic lithoheterotrophic haloarchaea, ubiquitous in hypersaline habitats.

Dimitry Y. Sorokin<sup>1,2</sup>, Enzo Messina<sup>3</sup>, Francesco Smedile<sup>3</sup>, Pawel Roman<sup>4,5</sup>, Jaap S. Sinninghe Damste<sup>6</sup>, Sergio Ciordia<sup>7</sup>, Maria del Carmen Mena<sup>7</sup>; Manuel Ferrer<sup>8</sup>, Peter N. Golyshin<sup>9,10</sup>, Ilya V. Kublanov<sup>1</sup>, Nazar I. Samarov<sup>10</sup>, Stepan V. Toshchakov<sup>10</sup>, Violetta La Cono<sup>3</sup> and Michail M. Yakimov<sup>3,10</sup>.

<sup>1</sup>Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia; <sup>2</sup>Department of Biotechnology, Delft University of Technology, Delft, The Netherlands; <sup>3</sup>Institute for Coastal Marine Environment, CNR, Messina, Italy <sup>4</sup>Sub-department of Environmental Technology, Wageningen University, Wageningen, The Netherlands; <sup>5</sup>Wetsus, Centre of Excellence for Sustainable Water Technology, Leeuwarden, The Netherlands; <sup>6</sup>Department of Marine Organic Biogeochemistry, NIOZ Royal Netherlands Institute for Sea Research, Den Burg, The Netherlands; <sup>7</sup>Proteomics Unit, National Center for Biotechnology, CSIC, Madrid, Spain; <sup>8</sup>Institute of Catalysis, CSIC, Madrid, Spain; <sup>9</sup>School of Biological Sciences, Bangor University Gwynedd, UK; <sup>10</sup>Immanuel Kant Baltic Federal University, Kaliningrad, Russia.

Correspondence: M. M. Yakimov, Institute for Coastal Marine Environment, IAMC-CNR, Spianata S. Raineri 86, 98122 Messina, Italy. Email:

**Subject Category:** Microbial ecology and functional diversity of natural habitats

**Topic:** Microbial processes and interactions in extreme environments

**Keywords:** haloarchaea; sulfur cycle; hypersaline anoxic habitats; anaerobic hydrogen and formate oxidation; proteomics

**Running Title:** Lithoheterotrophic formate- and H<sub>2</sub>-oxidising sulfidogenic haloarchaea

28 **Abstract**

Hypersaline anoxic habitats harbour numerous novel uncultured archaea whose metabolic  
30 and ecological roles remain to be elucidated. Until recently, it was believed that energy  
generation via dissimilatory reduction of sulfur compounds is not functional at salt  
32 saturation conditions. Recent discovery of the strictly anaerobic acetotrophic  
*Halanaeroarchaeum* compels to change both this assumption and the traditional view on  
34 haloarchaea as aerobic heterotrophs. Here we report on isolation and characterization of a  
novel group of strictly anaerobic lithoheterotrophic haloarchaea, which we propose to  
36 classify as a new genus *Halodesulfurarchaeum*. Members of this previously unknown  
physiological group are capable of utilising formate or hydrogen as electron donors and  
38 elemental sulfur, thiosulfate or dimethylsulfoxide as electron acceptors. Using genome-  
wide proteomic analysis we have detected the full set of enzymes required for anaerobic  
40 respiration and analysed their substrate-specific expression. Such advanced metabolic  
plasticity and type of respiration, never seen before in haloarchaea, empower the wide  
42 distribution of *Halodesulfurarchaeum* in hypersaline inland lakes, solar salterns, lagoons and  
deep submarine anoxic brines. The discovery of this novel functional group of sulfur-  
44 respiring haloarchaea strengthens the evidence of their possible role in biogeochemical  
sulfur cycling linked to the terminal anaerobic carbon mineralisation in so far overlooked  
46 hypersaline anoxic habitats.

## 48 **Introduction**

Extremely halophilic archaea of the class *Halobacteria* represent a unique branch of  
50 *Euryarchaeota* thriving in salt-saturating brines (Andrei *et al.*, 2012) thanks to an  
energetically favourable “salt-in” osmoprotection strategy (Becker *et al.*, 2014). The  
52 emergence of the dominant aerobic heterotrophic haloarchaeal lifestyle is likely the result  
of a large influx of genes from aerobic bacterium to the common halophile ancestor, which  
54 transformed an ancient methanogen into an oxygen-respiring heterotroph (Rhodes *et al.*,  
2011; Nelson-Sathi *et al.*, 2012; 2015; Wolf and Koonin, 2013; Sousa *et al.*, 2016). Corroborating  
56 with this hypothesis, most of the cultivated haloarchaea are aerobic heterotrophs with the  
exception of few examples of facultative anaerobes (Oren and Trüper, 1990; Oren, 1991;  
58 Antunes *et al.*, 2008; Bonete *et al.*, 2008; Andrei *et al.*, 2012; Werner *et al.*, 2014). At the same  
time, the molecular ecology studies based on SSU rRNA phylogeny demonstrated that  
60 highly reduced hypersaline environments are inhabited by a variety of unknown  
haloarchaea with no cultured representatives (Walsh *et al.*, 2005; Youssef *et al.*, 2011;  
62 Lamarche-Gagnon *et al.*, 2015), which could be involved in anaerobic sulfur and carbon  
cycling, as it was proposed in the past (Grant and Ross, 1986; Tindall and Trüper, 1986;  
64 Elshahed *et al.*, 2004a, 2004b). However, until recently, no conclusive evidence for that has  
been found, thus leaving unknown their metabolic capabilities and hence ecological roles.  
66 This has changed with the latest discovery of a strictly anaerobic acetate-oxidizing and S<sup>0</sup>-  
reducing haloarchaeon *Halanaeroarchaeum sulfurireducens* (HAA; Sorokin *et al.* 2016a). The in-  
68 depth characterisation of cultivated representatives demonstrated that aerobic respiration  
is not any longer a universal feature in the haloarchaea (Sorokin *et al.*, 2016a,b; Messina *et*  
70 *al.*, 2016). Moreover, this previously overlooked metabolic type underscores the ongoing  
metabolic diversification within haloarchaea (Sousa *et al.*, 2016) and strengthens the  
72 evidence for involvement of this euryarchaeal branch in biogeochemical sulfur cycling

linked to terminal anaerobic carbon mineralisation in hypersaline anoxic habitats. Further  
74 research into this direction yielded another ecotype of obligate anaerobic haloarchaea,  
which can be considered as lithoheterotrophic. Organisms grew with formate or hydrogen  
76 as the electron donors and sulfur compounds (elemental sulfur, thiosulfate and  
dimethylsulfoxide [DMSO]) as the electron acceptors, while yeast extract served as the  
78 carbon source. We propose to classify this novel group as a new genus and species  
*Halodesulfurarchaeum formicicum* (HDA). Noteworthy, this novel ecotype of haloarchaea was  
80 found in the same hypersaline ecosystems, where HAA was detected, suggesting the  
apparent functional niche diversification and eventual sympatric speciation. In the present  
82 study, we performed in-depth physiological and genomic characterisation of two HDA  
strains and assessed their functional respiratory properties through genome-wide  
84 proteomic studies of cultures grown on different electron acceptors and donors. A focus  
was put on the elucidation of features in HDA that promote its metabolic versatility.

86

## **Materials and Methods**

### *88 Sampling and establishment of enrichment cultures*

Anoxic sediments were obtained from the hypersaline lakes of Kulunda Steppe, lakes Elton  
90 and Baskunchak and solar salterns of Eupatoria (Russia) and Bari (Italy). Additionally,  
anoxic brine was collected in the deep-sea hypersaline lake Medee from approx. 3,100m  
92 depth (Yakimov *et al.*, 2013). Enrichment cultures were initiated by inoculating 1-10 ml of  
collected material into 90ml of the mineral medium after Sorokin *et al.* (2016a). Elemental  
94 sulfur was added directly into each flasks as a wet paste sterilised at 110°C for 30 min at  
final concentrations of ~ 50 mM. 2M sodium thiosulfate (Sigma) and 1M DMSO stock  
96 solutions were filter-sterilized and added at 20 and 10 mM final concentrations,  
respectively. Other tested electron donors/acceptors were added with a syringe from sterile

98 anaerobic 1M stock solutions by syringe at final concentrations 5-10 mM. Formate was  
supplied at the final concentration 30 mM. Routine cultivation was performed at 37° C in 120  
100 ml serum bottles with butyl rubber stoppers filled with the medium to 90% in case of  
formate and 50% in case of H<sub>2</sub>. Hydrogen was added through sterile gas filters at 0.5 bar  
102 overpressure on the top of argon atmosphere. The cultures were incubated at 37 °C with  
periodic shaking of the flasks. Growth in enrichments was monitored by measuring of HS<sup>-</sup>  
104 formation. Since growth in the solid medium was not achieved, pure cultures were obtained  
by serial dilutions of subcultures to the extinction (up to 10<sup>-10</sup>) in 4-6 consecutive series and  
106 the final purity was verified microscopically and by 16S rRNA gene sequencing. Phase  
contrast microphotographs were obtained with a Zeiss Axioplan Imaging 2 microscope  
108 (Jena, Germany). For electron microscopy (JEOL-100, Japan), the cells were fixed with  
paraformaldehyde (3% w/v final) and stained with 1% (w/v) uranyl acetate.

110

#### *Chemical analyses*

112 Sulfide formation was measured by using standard methylene blue method (Trüper and  
Schlegel, 1964) after fixing 10 µl culture supernatant in 0.5 ml 10% Zn acetate. Thiosulfate  
114 and sulfite were determined by iodimetry after removal of sulfide as ZnS. Sulfite was  
blocked by formaldehyde (3% final). Formate consumption was analyzed by HPLC (BioRad  
116 HPX-87H column at 60°C; eluent 1.5mM H<sub>3</sub>PO<sub>4</sub>, 0.6 ml min<sup>-1</sup>; UV/RI detector Waters 2489)  
after cell removal and fivefold diluting of samples with distilled water. The cell protein was  
118 determined by the Lowry method in 1-4ml culture samples after centrifugation 13,000 rpm  
for 20 min. The cell pellets were washed with 4M NaCl solution at pH 5 to remove the cell-  
120 bound FeS. Polysulfides were analyzed after methylation, in the form of dimethyl  
polysulfides as described previously (Roman *et al.*, 2014). Volatile sulfur compounds in the  
122 gas phase were analysed by GC (Thermo Scientific TM Trace GC Ultra with Trace GC Ultra

valve oven, Interscience, Breda, the Netherlands) equipped with FPD (150 °C), Restek  
124 column (RT<sup>®</sup>-U-Bond, 30 m x 0.53 mm di x 20 µm df) as described previously (Roman *et al.*,  
2015). Core membrane lipids and polar phospholipids were analyzed according to Weijers *et*  
126 *al.* (2009) and Sinninghe Damsté *et al.* (2011), respectively (see Supplementary Methods for  
details). Cytochrome oxidase activity was measured spectrophotometrically in sonicated  
128 cells in 4M NaCl buffered with 0.05 M K-P buffer using 1 mM reduced TMPD (tetramethyl-*p*-  
phenyldiamine hydrochloride) as substrate. Cytochrome spectra were recorded on the  
130 UV-Visible diode-array HP 8453 spectrophotometer (Hewlett Packard, Amsterdam, The  
Netherlands) with sodium ascorbate and sodium dithionite as reductants.

132

#### *Sequencing, assembly and annotation of genomes of strains HSR6 and HTSR1*

134 We succeeded with isolation of 8 different strains, and 2 of these strains, HTSR1 and HSR6,  
were sequenced. The HTSR1 genome was sequenced with MiSeq<sup>™</sup> Personal Sequencing  
136 System technology of Illumina Inc. (San Diego, CA, USA) using paired-end 250-bp reads. For  
sequencing of HSR6 genome, both paired-end and mate-paired DNA libraries were used.  
138 Detailed descriptions of all methodological procedures used in this study can be found in  
the Supplementary Methods. Obtained reads were assembled with both ALLPATHS-LG  
140 (Butler *et al.*, 2008) and SPADES 3.7.0 (Nurk *et al.*, 2013) assemblers and refined by Geneious  
7.1 software (Biomatters Ltd, New Zealand), resulting in a fully closed circular  
142 chromosomes. Genes were predicted by Glimmer 3.02 (Delcher *et al.*, 2007), rRNA genes was  
predicted by RNAmmer 1.2 Server online tool (Lagesen *et al.*, 2007), while tRNA-coding  
144 sequences were predicted by tRNAscan-SE 1.21 online tool (Lowe *et al.*, 1997). Operon  
prediction was performed by using the FgenesB online tool  
146 (<http://linux1.softberry.com/berry.phtml?topic=fgenesb&group=programs&subgroup=gfin>  
[db](#)). For each predicted gene, the similarity search was performed by Geneious 7.1 BLAST

148 embedded tool against public amino acid sequence databases (nr) and conserved domains  
families databases (COG, KEGG). Finally, annotations were manually curated using the  
150 Artemis 16.0 program (Rutherford *et al.*, 2001), and refined for each gene with NCBI blastx  
against nr and KEGG database (Altschul *et al.*, 1997, <http://www.genome.jp/tools/blast/>).  
152 The Average Nucleotide Identity (ANI) index was used to estimate the average nucleotide  
identity between HTSR1 and HSR6, as calculated by Goris *et al.*, 2007. 16S rRNA gene  
154 phylogeny of the HDA strains was inferred from a 16S rRNA gene sequence alignment with  
PAUP\*4.b10 using a LogDet/paralinear distance method as it described elsewhere (Sorokin  
156 *et al.*, 2016a).

#### 158 *Proteomic analyses*

Shotgun proteomic analyses were conducted using the HTSR1 cells grown on formate/S<sup>0</sup>,  
160 formate/thiosulfate and formate/DMSO couples. Detailed descriptions of all methodological  
procedures used in this study (protein extraction, protein concentration, in-gel trypsin  
162 digestion and nano-liquid chromatography tandem mass spectrometry) can be found in the  
Supplementary Methods. Abundance of each detected protein was treated separately with a  
164 custom C++ Linux-Shell program in order to be accepted as User graph from the DNAPlotter  
tool inside Artemis 16.0 program (Rutherford *et al.*, 2001). Data normalisation was  
166 performed automatically by the program.

#### 168 *Data and strains deposition*

16S rRNA gene sequences were deposited in the GenBank database (accession no. from  
170 KX664089 to KX664094). The genome sequences of strains HSR6<sup>T</sup> and HTSR1 have been  
submitted to the GenBank with accession numbers CP016804 and CP016070. All HSR  
172 formate-oxidizing isolates have been deposited in the UNIQEM culture collection

(Collection of Unique Extremophilic Microorganisms, Russian Academy of Sciences,  
174 Moscow, Russia). The type strain HSR6<sup>T</sup> (UNIQEM U983<sup>T</sup>) was additionally deposited in Japan  
Collection of Microorganisms under the number JCM 30662<sup>T</sup>.

176

## Results and Discussion

178 *Enrichment and isolation of lithoheterotrophic sulfur-respiring haloarchaea*

The eight novel sulfidogenic haloarchaeal isolates described in this study were obtained  
180 from anoxic sediment/brine samples taken from hypersaline circumneutral habitats at  
different geographical locations (Table 1). Most of the strains were enriched with a  
182 combination of formate as electron donor and elemental sulfur as electron acceptor, with a  
supplementation of yeast extract (10 mg l<sup>-1</sup>). Sulfide formation was registered after 2-4  
184 weeks of incubation at 37°C with maximal accumulation up to 5 mM within 2 months in  
samples from Kulunda Steppe. Addition of a mixture of streptomycin, ampicillin and  
186 vancomycin (100 mg l<sup>-1</sup> each) did not affect the sulfidogenesis but shortened the isolation  
procedure. The active sediment slurry incubations were further used as an inoculum (1%  
188 vol/vol) in artificial medium containing 4 M NaCl. However, the cell growth was extremely  
weak and after the third transfer the growth of cultures ceased. Increasing the  
190 concentration of yeast extract to 100 mg l<sup>-1</sup> allowed the full recovery of the cultures.  
Apparently these haloarchaea were heterotrophic and required yeast extract as the carbon  
192 source. Consecutive dilutions to extinction series produced six pure cultures of formate-  
dependent sulfur reducers designated as HSR6, HSR8, HSR9, HSR15, Bari-SA6 and Medee-  
194 SA6. Using thiosulfate as an electron acceptor instead of elemental sulfur, resulted in  
isolation from the Kulunda Steppe salt lake sediments of an additional formate-oxidising  
196 sulfidogenic strain HTSR1. A combination of hydrogen as an electron donor and elemental  
sulfur as an acceptor resulted in isolation of a strain HSR14 from the Kulunda Steppe salt

198 lake sediments. The characteristic property of this hydrogenotrophic isolate was a higher  
sulfide production at slower growth and lower biomass yield in comparison with the  
200 formate-oxidising cultures (Table 1 and Supplementary Figure S1). The tolerance to oxygen  
was checked by exposing the HDA cultures (10% [vol / vol] liquid to gas ratio) to the gas  
202 phase containing from 0.1 to 5% O<sub>2</sub>. No growth was observed in any trials. The tests for  
cytochrome oxidase were also negative.

204 Phylogenetic analysis revealed that all 8 isolates are closely related one to another (98.4-  
100% 16S rRNA gene identity) and form a novel genus-level branch within the order  
206 *Halobacteriales* (Gupta *et al.*, 2016). We propose to classify this novel group as a new genus  
and species *Halodesulfurarchaeum formicum* (HDA). The members of this genus clustered  
208 with the acetate-oxidising *Halanaeroarchaeum* strains (HAA) (95.3-95.5% identity), forming a  
separate clade of obligate anaerobic sulfur-respiring haloarchaea (Figure 1). This novel  
210 clade seems to be widely distributed across the globe and can be found in numerous  
microbially-explored anoxic hypersaline environments: solar salterns, high-altitude and  
212 flatland salt lakes of America, Central Asia and Europe and in the deep-sea anoxic brine  
lakes of Mediterranean Sea and Gulf of Mexico (Figure S2). Noteworthy, sulfur-respiring  
214 haloarchaea are likely of a significant ecological importance since they represent a  
predominant group (up to 20% of the total population) in a variety of hypersaline  
216 ecosystems worldwide (Supplementary Table S1).

#### 218 *Cell morphology and physiological characterisation*

Cells of the HDA strains isolated on formate and elemental sulfur had very similar cell  
220 morphologies, predominantly long flattened rods, which were actively motile with  
peritrichous archaella. Two strains, HTSR1 and HSR14, while growing on formate +

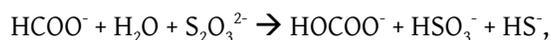
222 thiosulfate and hydrogen + S<sup>0</sup>, respectively, had much smaller cells (0.6 x 1.0 μm) than the  
cells grown on formate + S<sup>0</sup> (1.0-1.3 x 1.5-5.0 μm) (Figure 2).

224 Growth tests including other electron donors (acetate, ethanol, pyruvate, lactate,  
propionate, butyrate, butanol, succinate, glucose, fructose, ribose, glutamate and yeast  
226 extract) with sulfur as electron acceptor were negative for all isolates. No growth was  
obtained for all cultures while using arsenate, ferrihydrite, nitrate, nitrite, manganese  
228 dioxide, selenate, selenite, sulfate, sulfite and tetrathionate as alternative electron  
acceptors with formate as electron donor. Disproportionation of sulfur, thiosulfate and  
230 sulfite was negative. All strains were able to use DMSO as the electron acceptor with  
formate as the electron donor reducing it to DMS. DMSO was toxic to all strains at  
232 concentrations above 10 mM. A cross check of all isolates for the capability of  
hydrogenotrophy demonstrated that only HSR14 and HTSR1 strains were able to use H<sub>2</sub>  
234 with sulfur as the electron acceptor. Only HTSR1, HSR8 and HSR9 strains could use  
thiosulfate as electron acceptor with formate as electron donor. Therefore, it might be  
236 concluded, that despite their close phylogenetic relation, the obtained isolates possess  
slightly different (but functionally important) phenotypes and are likely adapted to specific  
238 catabolic conversions.

We performed the further in-depth characterisation of the HSR6 and HTSR1 isolates,  
240 which, within the group of lithoheterotrophic haloarchaea, represent two metabolic  
extremes, the narrowest and broadest, correspondingly. The NaCl range for growth and  
242 sulfidogenic activity was characteristic for extreme halophiles with the growth range from  
2.5 to 5.0 M (optimum at 3.5-4.0 M) and sulfidogenic activity range from 1.5 to 5.0 M  
244 (optimum at 3.5-4.5 M). Both strains grew with the couple formate/S<sup>0</sup>, but there was a  
substantial difference in growth dynamics and maximum sulfide formation between them  
246 (Figure S3). Maximum amount of sulfide recovered in HSR6 culture was 14 mM with a

concomitant consumption of 15 mM formate from 30 mM supplied, well corresponding to  
248 the 2 electron reduction of zero-valent sulfur. No further growth of HSR6 was observed  
likely due to the inhibition by accumulating sulfide. Apart from sulfide, a trace presence of  
250 volatile organic sulfur compounds, methanethiol (CH<sub>3</sub>SH) and carbon disulfide (CS<sub>2</sub>), at  
concentrations of 81 and 11.5 ppmv, respectively, were detected in the gas phase of the  
252 HSR6 culture at the stationary growth phase. In a control medium with 10 mM sulfide added  
initially, CH<sub>3</sub>SH and CS<sub>2</sub> concentrations were also detected but at significantly lower  
254 concentrations (13 and 2 ppmv, respectively). This allows to conclude on the biological  
origin of, at least, CH<sub>3</sub>SH.

256 The anaerobic growth rate and biomass yield of HTSR1 with thiosulfate and formate, a  
type of respiration never seen before in haloarchaea, was comparable to that of HSR6 on  
258 elemental sulfur (Figure S3). Although, the sulfide production by HTSR1 was significantly  
lower during growth on thiosulfate. Judging from a nearly 1:1 stoichiometry between the  
260 consumed formate and thiosulfate and produced sulfide,



262 equimolar amount of thiosulfate was reduced to sulfide and sulfite. Sulfite formation was  
monitored analytically. The experiments with washed cells (Supplementary Table S2)  
264 demonstrated that the cells of HSR6 and HTSR1 grown with S<sup>0</sup>, were active only with sulfur  
as electron acceptor, while the HTSR1 cells grown on thiosulfate were equally active both  
266 with thiosulfate and sulfur as electron acceptors. The cells of both strains grown with DMSO  
were active with DMSO and elemental sulfur, while no respiration with thiosulfate was  
268 observed.

270 *General genomic features of HSR6 and HTSR1 strains*

We determined complete genome sequences of the HSR6 and HTSR1 strains (most of the  
272 data are present in Supplementary Tables S3-S9 and Supplementary Figures S4-S5). Both  
genomes were single circular replicons of 1,972,283 bp (HTSR1) and 2,085,946 bp (HSR6),  
274 with GC molar content of 63.76% and 63.62%, respectively. Both genomes harbour a single  
rRNA operon and 45 tRNA genes. Genomes exhibited the average nucleotide identity (ANI)  
276 98.58%, while containing several strain-specific genomic regions: one in HTSR1 and four in  
HSR6. The unique island “D” in HSR6 genome encodes CRISPR and CRISPR-associated  
278 proteins (Deveau *et al.*, 2010). Following the current classification, HSR6 CRISPR-Cas was  
affiliated to I-B (*E. coli*) or CASS7 (Makarova *et al.*, 2011). Using the ACLAME database (Leplae  
280 *et al.*, 2010), spacer sequences were blasted against Plasmid, Virus and Prophages databases  
using ACLAME web site tool (<http://aclame.ulb.ac.be/>) with default parameters. Only spacer  
282 #38 was found distantly related (1e-03) to phage-related DNA polymerase (NC\_004556).  
Remarkably, HSR6 and HSR2 showed no homology between the spacer sequences, likely  
284 implying a different history of phage interaction for the strains, regardless of their isolation  
from the same environments.

286

#### *Energy generation and proton-translocation machinery*

288 In addition to the use of elemental sulfur, the HDA strains demonstrated their capability to  
utilise DMSO and thiosulfate as terminal electron acceptors, which are the predominant  
290 products of corresponding oxidation of dimethylsulfoniopropionate and sulfide in saline  
environments (Jørgensen, 1990; Matsuzaki *et al.*, 2006). The HTSR1 genome encodes 10  
292 molybdopterin oxidoreductases from CISM superfamily (Duval *et al.*, 2008) that may be  
potentially involved in the central catabolic reactions (Supplementary Table S9-S11). To  
294 facilitate comparison of these enzymes in various strains, we established their sequential  
numeration, based on appearance in the HTSR1 genome. This set exceeds by 2.5-fold the

296 numbers of corresponding enzymes in the acetate-oxidising sulfur-reducing HAA (Sorokin  
et al., 2016a), which coheres with the advanced metabolic versatility of the novel group of  
298 anaerobic haloarchaea. Noteworthy, HSR6 differs from HTSR1 by the absence of only one  
molybdopterin oxidoreductase, HTSR\_0625-0630, which, taking into account the inability of  
300 HSR6 to grow on thiosulfate, seems to play a pivotal role in the utilisation of this compound  
as an electron acceptor.

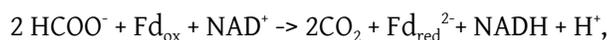
302 The phylogenetic analysis of detected CISM s suggested they can exhibit various activities  
(Figure 3). To improve the inference, we tried to assign the activity of detected  
304 molybdopterin oxidoreductases by using the physiological data. Molecular basis of formate-  
dependent respiration with sulfur compounds likely relies upon three respiratory  
306 dehydrogenases and seven terminal reductases. All strains of sulfur-reducing haloarchaea  
with currently completed genomes (HSR2, M27-SA2, HSR6 and HTSR1) possess four  
308 common CISM oxidoreductases. One of them belongs to the haloarchaeal branch of  
tetrathionate (Ttr) reductase family (Duval et al., 2008), while three others are the members  
310 of polysulfide/thiosulfate (Psr/Phs) reductases (Figure 3). HAA strains do not grow on  
DMSO as electron acceptor, obviously due to lack of two enzymatic complexes present in  
312 both HDA genomes, which belong to DMSO/trimethylamine-N-oxide (TMAO)/nitrate  
reductases (NAR) family. The nearest characterised enzyme, (Q9HR74), is a DMSO/TMAO  
314 reductase from *Halobacterium salinarum* (Müller and DasSarma, 2005), which makes the  
assignment of their metabolic function more reliable. Noteworthy, all these DMSO  
316 reductases form a deep branch within nitrate reductases cluster Nar and likely represent an  
ancient form of DMSO-reducing enzymes. HTSR1 has a unique deeply branched CISM  
318 oxidoreductase (HTSR\_0627), missing in all genomes of S<sup>0</sup>-reducing haloarchaea. We could  
not affiliate this enzyme with any known CISM family. However, despite it does not fall into  
320 the Psr/Phs family, its function seems to be related with thiosulfate reduction since this

enzyme was not found in other genomes and the capability to grow on thiosulfate as an  
322 electron acceptor is the key physiological feature of HTSR1.

Inspection of the HDA genomes revealed that formate metabolism in  
324 *Halodesulfurarchaeum* is considerably diversified, pointing at a great importance of formate  
for these organisms in sustaining the life. These haloarchaea are evolved to exploit the low  
326 reduction potential of formate ( $E_0'$  [ $\text{CO}_2/\text{HCOO}^-$ ] = -430 mV (Thauer *et al.*, 1977) to derive the  
energy by coupling its oxidation to the reduction of various electron acceptors. To facilitate  
328 these physiological roles, different types of formate dehydrogenase (Fdh) enzymes, both  
membrane-anchored and cytoplasmic, are present in the HDA genomes. The first type of  
330 Fdh coded by an operon of membrane-bound peripherally oriented formate dehydrogenase  
(HTSR\_1573-1576), resembled that of a thoroughly investigated bacterial analogue *Wolinella*  
332 *succinogenes* (Figure 3). In *W. succinogenes*, membrane-bound Fdh is suggested to be involved  
in the periplasmic dissimilatory reduction of nitrite to ammonia and elemental sulfur to  
334 sulfide, catalysed by corresponding reductases Nrf and Psr, respectively (Simon, 2002;  
Simon and Klotz, 2013). By analogy, the electron transfer chain of the HDA strains has to  
336 possess membrane menaquinone pool that mediate transfer of electrons between  
membrane-bound respiratory Fdh dehydrogenase and terminal reductases. It is very likely  
338 that this transfer is coupled to the consumption of protons from the inside and to proton  
release on the outside of the membrane, thus contributing to the proton motive force  
340 generation. The genomes of HDA strains contain 10 (methyl)menaquinone biosynthetic  
genes located in four loci: two separate *ubiE* genes (HTSR\_1082 and 1583), the *menA-ubiE*  
342 (HTSR\_1105-1106) and the *menFDBACE* (HTSR\_1290-1295) clusters. Such gene array  
unambiguously points at the occurrence of the classical menaquinone biosynthetic pathway  
344 via isochorismate, o-succinylbenzoate and 1,4-dihydroxy-2-naphthoate (Chen *et al.*, 2013;  
Zhi *et al.*, 2014). All predicted HDA menaquinone biosynthetic proteins were remarkably

346 similar (42-75% of identity) with the corresponding proteins of *Natronobacterium gregoryi*,  
whose menaquinone composition is known. This haloarchaeon possesses four major  
348 respiratory quinones corresponding to unsaturated and VIII-dihydrogenated menaquinones  
and methylmenaquinones with 8 isoprene units [MK-8, MK-8(VIII-H<sub>2</sub>), MMK-8 and MK-  
350 8(VIII-H<sub>2</sub>)] (Collins and Tindall, 1987).

Cytoplasmic type of Fdh is coded by two copies of genes for FdhA catalytic subunits  
352 (HTSR\_1736 and 1740), that are located in close vicinity. As it is typical for cytoplasmic  
Fdh (Maia *et al.*, 2015), we did not find genes, encoding other subunits present in  
354 membrane-bound CISM oxidoreductases complexes. Phylogenetic analysis revealed their  
similarity to cytoplasmic catalytic subunits FdhA of archaeal (*Methanococcus*) and bacterial  
356 (*Clostridium acidurici*) lineages (Figure 3). As it was proposed for some formate-utilising  
methanogens (Wood *et al.*, 2003), cytoplasmic FdhA is required to oxidise formic acid to CO<sub>2</sub>  
358 and to generate reduced electron carriers for energy conservation. To interact with the  
electron acceptors, cytoplasmic FdhAs of HDA need an appropriate “interface” to use  
360 ferredoxins and NAD. The genes located next to the cytoplasmic *fdhA* operon could encode  
this putative “interface”, namely, the electron transfer flavoprotein EtfAB (HTSR\_1748-49).  
362 Apparently, this flavin-containing module can be combined with the FdhA to perform  
energy conservation by coupled reduction of ferredoxin and NAD<sup>+</sup> via mechanism of flavin-  
364 based electron bifurcation (Buckel and Thauer, 2013):



366 just as it was documented for the first bifurcating formate dehydrogenase of *Clostridium*  
*acidurici* (Wang *et al.*, 2013).

368 Aforementioned physiological studies revealed, that besides formate HTSR1 can use  
hydrogen, a well-known electron donor utilised for microbial litho(auto)trophic growth.  
370 Before our findings, utilisation of formate and H<sub>2</sub> as electron donors was not observed in

any known species of the class *Halobacteria*. Strain HTSR1 has a gene cluster HTSR\_0658-  
372 0657 encoding [NiFe]-hydrogenase, consisting of three subunits, HydA (39.3 KDa), HydB  
(55.3 KDa) and HydC (37.4 KDa). Noteworthy, the HSR6 strain possesses identical [NiFe]-  
374 hydrogenase gene cluster, but fails to use hydrogen as the electron donor. Phylogenetic  
analysis of full-length subunits HydA and HydB of HDA revealed that they belong to Group 1  
376 of the [NiFe]-hydrogenases (Figure 4). The hydrogenases in Group 1 are known as  
membrane-bound, respiratory uptake hydrogenases capable of supporting growth with H<sub>2</sub>  
378 as an energy source (Vignais *et al.* 2001; Vignais and Billoud, 2007). Although the exact  
mechanism for the generation of the electrochemical proton gradient with formate or H<sub>2</sub> as  
380 electron donors is yet to be elucidated in HDA, both membrane-bound Fdh and Hyd could  
reduce menaquinone with formate/H<sub>2</sub> with concomitant transfer of electrons to terminal  
382 reductases and outward proton pumping (Figure 5).

Besides aforementioned enzymes, the Complex 1-like oxidoreductase (HTSR\_1171-1181)  
384 is the supplementary component of proton-translocation machinery in HDA. Similar to HAA  
and other obligate anaerobes (Castelle *et al.*, 2013; Probst *et al.*, 2014; Sorokin *et al.*, 2016a),  
386 this complex lacks the NADH-binding module and is hypothesized to use reduced  
ferredoxin as the electron donor for the generation of the proton gradient (Battchikova *et*  
388 *al.*, 2011). We propose, that one of the sources of reduced ferredoxins is the cytoplasmic  
oxidation of formate by the monomeric FdhA. The proton gradient can be further utilised  
390 by the V-type archaeal H<sup>+</sup>-ATP synthase complex (HTSR\_1802-1811) for the generation of  
ATP, thus providing an attractive mechanism for efficient energy conservation in  
392 *Halodesulfurarchaeum* (Figure 5).

394 *One-carbon metabolism*

The extra- and intracellular oxidation of formate is the key catabolic property of novel  
396 haloarchaea and of utmost interest since it had never been observed in any known  
haloarchaeal species. Apart from three CISM formate dehydrogenases, both HDA genomes  
398 harbour the full set of genes encoding the tetrahydrofolate (THF)-dependent enzymes  
involved in the reversible conversion of formate to methyl-THF (Figure 6). The  
400 corresponding enzymes likely provide C<sub>1</sub>-units for purine and thymidylate synthesis,  
similarly to the euryarchaeon SM1 and *Methanosarcina barkeri* (Buchenau and Thauer, 2004;  
402 Probst *et al.*, 2014). The other major requirement for C<sub>1</sub>-units comes from the provision of  
methyl groups for multiple biosynthetic methylation reactions (Brosnan *et al.*, 2015). The  
404 HDA genomes encode the full set of enzymes, needed to catabolise methionine via its  
conversion to S-adenosylmethionine (SAM) with the transfer of SAM methyl group to a  
406 substrate for methylation, producing S-adenosylhomocysteine (SAH) and the methylated  
substrate (Figure 6). In accordance with this route, we found more than 35 different  
408 methyltransferases including two SAM-dependent methyltransferases (HTSR\_1450, 1509). It  
seems that beside these two canonical biosynthetic functions of one-carbon metabolism  
410 (methylation reactions and purine/thymidylate synthesis), the third metabolic function is  
also operative in HDA cells: serine and glycine metabolism via glycine cleavage system  
412 (Figure 6). In case these substrates can provide HDA cells with more one-carbon groups  
than they need, the oxidative conversion of methylene-THF back to formate and ultimately  
414 to carbon dioxide might be a mechanism for their disposal. As proposed by Brosnan *et al.*  
(2015), this process in addition to oxidizing the excess of C<sub>1</sub>-units can reduce substantial  
416 quantities of NADP<sup>+</sup> to NADPH and produce ATP.

Noteworthy, before entering in SAM-cycle, methylene-THF is reduced to methyl-THF,  
418 which is highly exergonic reaction using NADH. This reductant produced by cytoplasmic  
oxidation of formate can be used by methylenetetrahydrofolate reductase, which was

420 suggested to be involved in energy conservation by reducing ferredoxin via electron  
bifurcation (Hess *et al.*, 2013). Thus, the one-carbon metabolism in HDA likely has the fourth  
422 metabolic function - to fuel the bioenergetic coupling site via NADH-dependent methylene-  
THF reduction (Figure 6).

424

#### *Heterotrophy*

426 Anoxic sediments of hypersaline lakes and salterns receive a variety of forms of detrital  
organic matter from the overlying compartments, which provide carbon and nitrogen to  
428 anaerobic microbial communities. Consistently with current insight (Oren, 2011; Yakimov *et al.*,  
*et al.*, 2013), the anaerobic metabolic diversity at the highest salinities is poor due to energetic  
430 constraints and is restricted primarily to fermentation, methylotrophic methano- and  
acetogenesis and recently discovered acetoclastic sulfur reduction (Sorokin *et al.*, 2016a).  
432 HDA strains represent a novel type of haloarchaeal anaerobic metabolism, which is  
operative at the highest salinities, i.e. hydrogen/formate-dependent lithoheterotrophy. As  
434 mentioned above, the presence of yeast extract is essential for growth of all HDA strains.  
According to this, two oligopeptide/dipeptide ABC transporters and 9 transporters for  
436 amino acids were found along with 22 cytoplasmic and membrane-associated proteases and  
peptidases (Supplementary Table S9). The genome inspection of HDA strains revealed the  
438 synthesis pathway for lysine was incomplete, pointing at the dependence of HDA on  
external sources of this amino acid. In accordance with the cultivation tests, sugars cannot  
440 be used by HDA, likely due to the lack in the genomes of hexokinase and  
phosphofructokinase, the enzymes initiating the glycolysis. The presence of an  
442 unidirectional fructose-1,6-biphosphate aldolase/phosphatase suggests that the metabolic  
fluxes are oriented in gluconeogenic direction (Say and Fuchs, 2010) from pyruvate to  
444 phosphoenolpyruvate (via phosphoenolpyruvate synthase) and further to fructose-6-

phosphate. At the same time, the well-developed routes for amino acids degradation are  
446 encoded by both HDA genomes confirming their capacity for using these compounds as the  
sole carbon sources, ultimately catabolising those via the tricarboxylic acid (TCA) cycle.  
448 Most proteins involved in the canonical oxidative TCA cycle are encoded in HDA genomes,  
except for 2-oxoglutarate dehydrogenase, which is, as in case with HAA, replaced by 2-  
450 oxoglutarate:ferredoxin oxidoreductase.

Each of the HDA genomes possessed genes for two ADP-forming acetyl-CoA synthetases that  
452 have been proven to catalyse the acetate production in various archaea (Glasemacher *et al.*,  
1997; Musfeld *et al.*, 1999). The presence of this enzyme suggests that acetyl-CoA, produced  
454 via deamination and subsequent oxidation of amino acids, aside from entering in TCA cycle,  
could be converted to acetate, thus generating one ATP by substrate level phosphorylation.  
456 We are aware of this assumption and despite the apparent advantage of this reaction, the  
production of acetate in traceable amount was not detected in formate-growing HDA  
458 cultures (data not shown). Unlike the acetoclastic HAA, none of the genes encoding for  
enzymes of the glyoxylate shunt, which allows acetate to be used as the sole carbon source,  
460 were identified in HDA genomes. Additionally, none of the genes associated with the  
methyaspartate cycle, an alternative pathway of acetate assimilation in certain haloarchaea  
462 (Khomyakova *et al.*, 2011), were found. Therefore, we believe that if acetate is produced, it is  
likely excreted as an end-metabolite by formate/oxalate antiporter, which is  
464 simultaneously involved in uptake of formate (Probst *et al.*, 2014). As far as acetate is the key  
substrate for acetoclastic *Haloanaeroarchaeum* (Sorokin *et al.*, 2016a), it can be an important  
466 link between these haloarchaea. Hence the potential ability of HDA to generate acetate  
could greatly influence the terminal anaerobic degradation cascade of organic matter in  
468 hypersaline ecosystems.

470 *Energy metabolism confirmed by comparative proteome analysis*

In the present work, the proposed metabolic pathways during respiration with different  
472 terminal acceptors, were analysed through the proteome assays (Figure 7). Although we did  
not aim here to perform the detailed comparative analysis of HTSR1 proteomes, the  
474 description of the peptide-level scoring metrics is provided in Supplementary Table S11 and  
in Supplementary Discussion. It must be specified that inspection of the proteome revealed  
476 that the proposed C<sub>1</sub> metabolism in HDA is active, since eleven enzymes of the pathways  
depicted on Figure 6 were among the most abundant proteins.

478 The experiments with washed cells demonstrated that the formate/S<sup>0</sup> grown HTSR1 cells  
were active only with sulfur, while no reduction of either thiosulfate or DMSO was detected  
480 (Supplementary Table S2). Consistently with this observation, the analysis of expressed  
CISM complexes revealed that neither DMSO reductases (HTSR\_0423 and 0517), nor the  
482 putative thiosulfate reductase (HTSR\_1522) were induced during the growth on elemental  
sulfur (Figure 7B). In contrary, all three polysulfide reductases, together with the ‘Deep’  
484 CISM were induced. This observation confirms that they are essential components of the  
energy production machinery during sulfur respiration with formate. Among the last group  
486 of enzymes, the PsrA HTSR\_1347, co-transcribed together with periplasmatic  
sulfurtransferase/rhodanese-like protein HTSR\_1348, was the most abundant, i.e. was 3-4  
488 times higher than the other Psr reductases. This finding corroborates with the significance  
of sulfurtransferase in transformation of practically insoluble S<sup>0</sup> into soluble polysulfide,  
490 thus functioning as the sulfur supplier for the catalytic subunit of Psr reductases (Klimmek  
*et al.*, 1998; Hedderich *et al.*, 1999; Campbell *et al.*, 2009; Aussignargues *et al.*, 2012), which was  
492 also the case in HAA (Sorokin *et al.*, 2016a).

Washed cells of HTSR1 grown with formate/thiosulfate were equally active with both  
494 thiosulfate and sulfur as terminal electron acceptors, but not with DMSO (Supplementary

Table S2). Correspondingly, the total protein expression profile of thiosulfate-grown cells  
496 was very similar to that of sulfur-respiring cells, with only few differences observed. First of  
all, thiosulfate induced the expression of HTSR\_1522 reductase of the Ttr family, repressed  
498 in other HTSR1 proteomes, and significantly increased (68%) the abundance of unaffiliated  
CISM 'Deep' reductase (HTSR\_0627). Another remarkable difference with S<sup>0</sup>-grown cells is  
500 that the utilisation of thiosulfate as terminal electron acceptor was accompanied by a 2.5-  
fold decrease in the abundance of membrane-bound formate dehydrogenase HTSR\_1576  
502 with a simultaneous increase in the abundance of cytoplasmic FdhA dehydrogenases  
HTSR\_1736 and HTSR\_1740. A notable aspect of the 2-electron reduction of thiosulfate is  
504 that under standard conditions, the reduction potential (E°') of the S<sub>2</sub>O<sub>3</sub><sup>2-</sup>/(HS<sup>-</sup> + SO<sub>3</sub><sup>2-</sup>)  
electron acceptor couple is -402 mV, which is considerably lower than that of the MK/MKH<sub>2</sub>  
506 electron donor couple (-74 mV), resulting in an unfavourable ΔE°= of -328 mV for the  
reaction. The principle, that the reduction potentials of cleavage reaction are considerably  
508 higher under physiological conditions (Stoffels *et al.*, 2012), can diminish unfavorable ΔE°,  
but anyhow the two-electron reduction catalysed by thiosulfate reductase has to be linked  
510 to an exergonic process in order to operate in the endergonic direction. Therefore, we  
assumed that, similarly to formate-dependent thiosulfate reduction in *Salmonella enterica*  
512 (Stoffels *et al.*, 2012), the proton motive force (PMF) is only just sufficient to drive the  
thiosulfate reductase reaction. Above we suggested a mechanism in which formate is  
514 oxidised in the cytoplasm to produce the additional Fd<sub>red</sub>. This reductant is further used by  
the ferredoxin:menaquinone Complex 1-like oxidoreductase (HTSR\_1171-1181) to generate  
516 the extra PMF, which is likely necessary to overcome the unfavourable red-ox conditions of  
respiration with thiosulfate (Figure 5).

518 When the HTSR1 cells were grown with DMSO as the electron acceptor, they were active  
also with elemental sulfur, while thiosulfate was not used as the sulfidogenic substrate

520 (Supplementary Table S2). This may indicate that DMSO, as in the case of thiosulfate, the  
sulfur reduction is rather a constitutive phenotype of the HTSR1 strain. Proteomic data  
522 corroborates with physiological testing, whereby the strong induction of DMSO reductase  
HTSR\_0517 was observed (Figure 7B). Noteworthy, the second DMSO reductase (HTSR\_0423)  
524 was not found among the expressed proteins, indicating that this reductase did not  
contribute to the respiration activity of the HTSR1 strain. During the growth with DMSO,  
526 the proton-translocation machinery in HDA does not seem to require a significant amount  
of the membrane-bound Fdh reductase, judging from its decreased expression (6.5-fold  
528 lower than in sulfur-respiring cells).

Taken together, the results of differential proteome analyses and respiration  
530 experiments revealed that polysulfide reductases are constitutively expressed in HDA cells,  
indicating that elemental sulfur, despite being one of the energetically least favourable,  
532 serves as the preferential electron acceptor. The growth with other electron acceptors  
requires the induction of corresponding oxidoreductases. Thus, *Halodesulfurarchaeum*  
534 strains possess a remarkable adaptation machinery to thrive in hypersaline anoxic habitats,  
while exhibiting capacity of utilising low-potential electron acceptors at the  
536 thermodynamic edge of life.

### 538 *Classification*

On the basis of phylogenetic and phenotypic properties we propose to classify the novel  
540 group of anaerobic haloarchaea described above as a novel genus and species  
*Halodesulfurarchaeum formicicum* gen. nov., sp. nov. within the family *Halobacteriaceae*.

542

### **Description of *Halodesulfurarchaeum* gen. nov.**

544 [hal.o.de.sul'fu.ri. Gr.n. *hals*, *halos* salt of the sea; L. pref. *de-*, from; L.n. *sulfur*, sulfur; N.L.  
neut. n. *archaeum* archaeon from Gr. adj. *archaios*-ê-on ancient; N.L. neut. n.  
546 *Halodesulfurarchaeum* sulfur-reducing haloarchaeon].

548 Extremely halophilic, neutrophilic, obligately anaerobic euryarchaea growing by sulfur-  
dependent respiration with formate or hydrogen as electron donor, thereby representing a  
550 first example of haloarchaea with the lithotrophic metabolism. A member of the family  
*Halobacteriaceae*. Found in hypersaline chloride brines of terrestrial and marine origin.  
552 Recommended three-letter abbreviation: *Hda*. The type species is *Halodesulfurarchaeum*  
*formicum*.

554

**Description of *Halodesulfurarchaeum formicum* sp. nov.**

556 [for.mi'ci.cum. N.L. neut. n. *acidum formicum*, formic acid; L. neut. *suffixicum*, suffix used  
with the sense of belonging to, pertaining to; N.L. neut. adj. *formicum*, pertaining to formic  
558 acid].

560 Cells are variable in shape and size at different growth conditions: from flattened motile  
rods 1.0-5.0 x 0.6-0.8 µm (growth on sulfur and DMSO) to small nonmotile cocci 0.6-0.8 x 1.0  
562 µm (with thiosulfate). The cell wall consists of a thin proteinaceous layer. The cells lyse at  
salt concentration below 2 M. Carotenoids are absent. The core membrane lipid analysis  
564 demonstrated a presence of two dominant components: archaeol (C<sub>20</sub>-C<sub>20</sub> diglycerol ether  
[DGE], 40% of the total) and extended archaeol (C<sub>20</sub>-C<sub>25</sub> DGE, 59% of the total). Trace presence  
566 (1.2% in total) of the monoglycerol ether (MGE) lipids (2-C<sub>20</sub> MGE, 1-C<sub>20</sub> MGE and 2-C<sub>25</sub> MGE)  
was also detected. The phospholipids were dominated by phosphatidylglycerophosphate  
568 methylester, while phosphatidylglycerol, phosphatidylethanolamine and two unidentified  
C<sub>45</sub>/C<sub>40</sub> lipid species were less abundant. Obligately anaerobic, growing by elemental sulfur  
570 and DMSO (all strains) or thiosulfate (some strains) respiration with either formate (all  
strains) or hydrogen (some strains) as electron donor. Sulfur is reduced to sulfide with

572 intermediate formation of polysulfides and traces of organic sulfides, such as methanethiol  
and CS<sub>2</sub>. Some strains are capable of incomplete thiosulfate reduction to sulfide and sulfite,  
574 while DMSO is reduced to DMS. Yeast extract can serve as carbon source, but not as energy  
source. Ammonium and is utilised as N-source. Optimum growth temperature is 37°C  
576 (maximum at 50°C). Extremely halophilic, with the range of NaCl for growth from 2.5 to 5 M  
(optimum at 3.5-4 M), and neutrophilic, with the pH range for from 6.5 to 8 (optimum at 7.0-  
578 7.2). The G+C content of genomic DNA in the type strain HSR6 is 63.62 mol%. Habitats:  
hypersaline lakes and solar salterns. The type strain (HSR6<sup>T</sup>=JCM 30662<sup>T</sup>=UNIQEM U983<sup>T</sup>)  
580 was isolated from mixed anaerobic sediments of hypersaline chloride-sulfate lakes in  
Kulunda Steppe (Altai, Russia).

582

## Conclusions

584 This study has demonstrated that even the well-studied microbial habitats could reveal a  
significant new knowledge on novel microbial taxa and their metabolism, by applying a  
586 hypothesis-driven combination of cultivation, physiological and in-depth 'omic' analyses.  
We have discovered and characterised a novel lifestyle of haloarchaea, prevalent in anoxic  
588 hypersaline systems worldwide, yet very different from that of all previously described  
members of the class *Halobacteria*. We proposed the new genus, *Halodesulfurarchaeum*, within  
590 family *Halobacteriaceae* to accommodate this new lineage. We further propose that, along  
with recently described genus *Halanaeroarchaeum*, this new genus partitions the class  
592 *Halobacteria* into distinct phenotypes, consisting of aerobic (with the exception of few  
facultative anaerobes) and obligate anaerobic organisms. Evidence supporting last proposal  
594 includes: (i) lineage-specific features, such as acetotrophy and lithoheterotrophy, coupled  
with the previously overlooked type of sulfur-dependent respiration and (ii) significant  
596 intra-lineage diversity and abundance within geographically distinct hypersaline habitats

worldwide. The sister grouping of ‘anaerobic’ and ‘aerobic’ haloarchaea reflects their  
598 plausible derivation from an ancient common aerobic halophilic ancestor. The ongoing  
metabolic diversification than resulted in subsequent divergence along separate  
600 evolutionary paths. A second possible scenario implies a consideration, that obligate  
anaerobic sulphur-reducing haloarchaea have a different evolutionary history than the  
602 aerobic counterparts and their ancestors avoided the massive lateral gene transfer event  
from aerobic bacteria.

604 In addition to the metabolic peculiarities, lineage-specific characteristics of ‘anaerobic’  
haloarchaea, attributed to adaptation to anoxic habitats at the thermodynamic edge of life,  
606 include their compact genome and single-copy rRNA operon, rarely seen among  
haloarchaea. These features have been proposed to minimize metabolic costs in energy-  
608 limited habitats where neither broad metabolic repertoire nor high numbers of paralogous  
proteins are needed. The sporadic identification of sulfur-respiring ‘anaerobic’ haloarchaea  
610 (up to 20% of the total archaeal communities in Lake Tirez) in microbial surveys of  
hypersaline communities worldwide (Supplementary Figure S2 and Table S1) suggests that  
612 they represent so far overlooked but significant fraction of the biomass and diversity in  
these habitats. The inability of earlier studies to recognise their significant contribution to  
614 anaerobic part of sulfur and carbon cycling in hypersaline habitats is likely due to  
limitations in cultivation methods routinely used to assess the diversity of extreme  
616 halophiles. It is therefore not surprising, that before our studies the list of hypersaline  
archaeal isolates described to date did not include any obligate anaerobes.

618

### **Conflict of interest**

620 The authors declare no conflict of interest.

## 622 **Acknowledgements**

This work was supported by research funds from European Commission's Horizon 2020  
624 Program under INMARE Project (Contract 634486), the Italian National Flagship Project  
RITMARE funded the Italian Ministry of Education, University and Research, the Russian  
626 Foundation of Basic Research (16-04-00035, 13-04-40205-N), the Gravitation SIAM grant  
24002002 (NWO, The Netherlands) and the Russian Academy of Science Program "Molecular  
628 and Cellular Biology". This work was further funded by grant BIO2014-54494-R from the  
Spanish Ministry of Economy and Competitiveness. We sincerely thank Erika Arcadi and  
630 Gina La Spada for technical assistance.

## 632 **References**

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W et al. (1997). Gapped BLAST  
634 and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids  
Res* **25**: 3389–3402.
- 636 Andrei AS, Banciu HL, Oren A. (2012). Living with salt: metabolic and phylogenetic diversity  
of archaea inhabiting saline ecosystems. *FEMS Microbiol Lett* **330**: 1–9.
- 638 Antunes A, Taborda M, Huber R, Moissl C, Nobre MF, da Costa MS. (2008). *Halorhabdus  
tiamatea* sp. nov., a non-pigmented extremely halophilic archaeon from a deep-sea,  
640 hypersaline anoxic basin of the Red Sea, and emended description of the genus  
*Halorhabdus*. *Int J Syst Evol Microbiol* **58**: 215–220.
- 642 Aussignargues C, Giuliani MC, Infossi P, Lojou E, Guiral M, Giudici-Orticoni MT et al. (2012).  
Rhodanese functions as sulfur supplier for key enzymes in sulfur energy metabolism. *J  
644 Biol Chem* **287**: 19936–19948.
- Battchikova N, Eisenhut M, Aro EM. (2011). Cyanobacterial NDH-1 complexes: novel insights  
646 and remaining puzzles. *Biochim Biophys Acta* **1807**: 935–944.
- Becker EA, Seitzer PM, Tritt A, Larsen D, Krusor M, Yao AI et al. (2014). Phylogenetically  
648 driven sequencing of extremely halophilic archaea reveals strategies for static and  
dynamic osmo-response. *PLoS Genet* **10**: e1004784.

- 650 Bonete MJ, Martínez-Espinosa RM, Pire C, Zafrilla B, Richardson DJ. (2008). Nitrogen  
metabolism in haloarchaea. *Saline Systems* **4**: 9.
- 652 Braakman R, Smith E. (2012). The emergence and early evolution of biological carbon-  
fixation. *PLoS Comput Biol* **8**: e1002455.
- 654 Brosnan ME, MacMillan L, Stevens JR, Brosnan JT. (2015). Division of labour: how does folate  
metabolism partition between one-carbon metabolism and amino acid oxidation?  
656 *Biochem J* **472**: 135–146.
- Buchenau B, Thauer RK. (2004). Tetrahydrofolate-specific enzymes in *Methanosarcina barkeri*  
658 and growth dependence of this methanogenic archaeon on folic acid or p-  
aminobenzoic acid. *Arch Microbiol* **182**: 313–325.
- 660 Buckel W, Thauer RK. (2013). Energy conservation via electron bifurcating ferredoxin  
reduction and proton/Na(+) translocating ferredoxin oxidation. *Biochim Biophys Acta*  
662 **1827**: 94–113.
- Butler J, MacCallum I, Kleber M, Shlyakhter IA, Belmonte MK, Lander ES *et al.* (2008).  
664 ALLPATHS: de novo assembly of whole-genome shotgun microreads. *Genome Res* **18**:  
810-820.
- 666 Campbell BJ, Smith JL, Hanson TE, Klotz MG, Stein LY, Lee CK *et al.* (2009). Adaptations to  
submarine hydrothermal environments exemplified by the genome of *Nautilia*  
668 *profundicola*. *PLoS Genet* **5**: e1000362.
- Castelle CJ, Hug LA, Wrighton KC, Thomas BC, Williams KH, Wu D *et al.* (2013). Extraordinary  
670 phylogenetic diversity and metabolic versatility in aquifer sediment. *Nat Commun* **4**:  
2120.
- 672 Chen M, Ma X, Chen X, Jiang M, Song H, Guo Z. (2013). Identification of a hotdog fold  
thioesterase involved in the biosynthesis of menaquinone in *Escherichia coli*. *J Bacteriol*  
674 **195**: 2768–2775.
- Collins MD, Tindall BJ. (1987). Occurrence of menaquinones and some novel methylated  
676 menaquinones in the alkaliphilic, extremely halophilic archaeobacterium  
*Natronobacterium gregoryi*. *FEMS Microbiol Lett* **43**: 307–312.
- 678 Delcher AL, Bratke KA, Powers EC, Salzberg SL. (2007). Identifying bacterial genes and  
endosymbiont DNA with Glimmer. *Bioinformatics* **23**: 673–679.
- 680 Deveau H, Garneau JE, Moineau S. (2010). CRISPR/Cas system and its role in phage-bacteria  
interactions. *Annu Rev Microbiol* **64**: 475–493.

- 682 Duval S, Ducluzeau AL, Nitschke W, Schoepp-Cothenet B. (2008). Enzyme phylogenies as  
markers for the oxidation state of the environment: the case of respiratory arsenate  
684 reductase and related enzymes. *BMC Evol Biol* **8**: 206.
- Elshahed MS, Najjar FZ, Roe BA, Oren A, Dewers TA, Krumholz LR *et al.* (2004a). Survey of  
686 archaeal diversity reveals an abundance of halophilic Archaea in a low-salt, sulfide-  
and sulfur-rich spring. *Appl Environ Microbiol* **70**: 2230–2239.
- 688 Elshahed MS, Savage KN, Oren A, Gutierrez MC, Ventosa A, Krumholz LR *et al.* (2004b).  
*Haloferax sulfurifontis* sp. nov., a halophilic archaeon isolated from a sulfide- and sulfur-  
690 rich spring. *Int J Syst Evol Microbiol* **54**: 2275–2279.
- Glasemacher J, Bock AK, Schmid R, Schönheit P. (1997). Purification and properties of  
692 acetyl-CoA synthetase (ADP-forming), an archaeal enzyme of acetate formation and  
ATP synthesis, from the hyperthermophile *Pyrococcus furiosus*. *Eur J Biochem* **244**: 561–  
694 567.
- Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM. (2007). DNA-  
696 DNA hybridization values and their relationship to whole-genome sequence  
similarities. *Int J Syst Evol Microbiol* **57**: 81–91.
- 698 Grant WD, Ross HNM. (1986). The ecology and taxonomy of *Halobacteria*. *FEMS Microbiol Rev*  
**39**: 9–15.
- 700 Gupta N, Pevzner PA. (2009) False discovery rates of protein identifications: a strike against  
the two-peptide rule. *J Proteome Res* **8**: 4173–4181.
- 702 Gupta RS, Naushad S, Fabros R, Adeolu M. (2016). A phylogenomic reappraisal of family-  
level divisions within the class *Halobacteria*: proposal to divide the order  
704 Halobacteriales into the families *Halobacteriaceae*, *Haloarculaceae* fam. nov., and  
*Halococcaceae* fam. nov., and the order *Haloferacales* into the families, *Haloferacaceae* and  
706 *Halorubraceae* fam nov. *Antonie Van Leeuwenhoek* **109**: 565–587.
- Hedderich R, Klimmek O, Kröger A, Dirmeier R, Keller M, Stetter KO *et al.* (1999). Anaerobic  
708 respiration with elemental sulfur and with disulfides. *FEMS Microbiol Rev* **22**: 353–381.
- Hess V, Schuchmann K, Müller V. (2013). The ferredoxin:NAD<sup>+</sup> oxidoreductase (Rnf) from  
710 the acetogen *Acetobacterium woodii* requires Na<sup>+</sup> and is reversibly coupled to the  
membrane potential. *J Biol Chem* **288**: 31496–31502.
- 712 Jørgensen BB. (1990). A thiosulfate shunt in the sulfur cycle of marine sediments. *Science*  
**249**: 152–154.

- 714 Klappenbach JA, Dunbar JM, Schmidt TM. (2000). rRNA operon copy number reflects  
ecological strategies of bacteria. *Appl Environ Microbiol* **66**: 1328–1333.
- 716 Klimmek O, Kreis V, Klein C, Simon J, Wittershagen A, Kröger A *et al.* (1998). The function of  
the periplasmic Sud protein in polysulfide respiration of *Wolinella succinogenes*. *Eur J*  
718 *Biochem* **253**: 263–269.
- Lagesen K, Hallin PF, Rødland E, Stærfeldt HH, Rognes T, Ussery DW. (2007). RNAmmer:  
720 consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res* **35**: 3100–  
3108.
- 722 Lamarche-Gagnon G, Comery R, Greer CW, Whyte LG. (2015). Evidence of in situ microbial  
activity and sulphidogenesis in perennially sub-0 °C and hypersaline sediments of a  
724 high Arctic permafrost spring. *Extremophiles* **19**: 1–15.
- Lepplae R, Lima-Mendez G, Toussaint A. (2010). ACLAME: a CLAssification of Mobile genetic  
726 Elements, update 2010. *Nucleic Acids Res* **38**: D57–D61.
- Lowe TM, Eddy SR. (1997). tRNAscan-SE: a program for improved detection of transfer RNA  
728 genes in genomic sequence. *Nucleic Acids Res* **25**: 955–964.
- Lu P, Vogel C, Wang R, Yao X, Marcotte EM. (2007). Absolute protein expression profiling  
730 estimates the relative contributions of transcriptional and translational regulation.  
*Nat Biotechnol* **25**: 117–124.
- 732 Maia LB, Moura JJ, Moura I. (2015). Molybdenum and tungsten-dependent formate  
dehydrogenases. *J Biol Inorg Chem* **20**: 287–309.
- 734 Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P *et al.* (2011).  
Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol* **9**: 467–477.
- 736 Matsuzaki M, Kubota K, Satoh T, Kunugi M, Ban S, Imura, S. (2006). Dimethyl sulfoxide-  
respiring bacteria in Suribati Ike, a hypersaline lake, in Antarctica and the marine  
738 environment. *Polar bioscience* **20**: 73–81.
- Messina E, Sorokin DY, Kublanov IV, Toshchakov S, Lopatina A, Arcadi E *et al.* (2016).  
740 Complete genome sequence of 'Halanaeroarchaeum sulfurireducens' M27-SA2, a  
sulfur-reducing and acetate-oxidizing haloarchaeon from the deep-sea hypersaline  
742 anoxic lake Medee. *Stand Genomic Sci* **11**: 35.
- Musfeldt M, Selig M, Schönheit P (1999). Acetyl coenzyme A synthetase (ADP forming) from  
744 the hyperthermophilic archaeon *Pyrococcus furiosus*: identification, cloning, separate

- expression of the encoding genes, *acdAI* and *acdBI*, in *Escherichia coli*, and in vitro  
746 reconstitution of the active heterotetrameric enzyme from its recombinant subunits. *J*  
*Bacteriol* **181**: 5885-5888.
- 748 Müller JA, DasSarma S. (2005). Genomic analysis of anaerobic respiration in the archaeon  
*Halobacterium* sp. strain NRC-1: dimethyl sulfoxide and trimethylamine N-oxide as  
750 terminal electron acceptors. *J Bacteriol* **187**: 1659–1667.
- Nelson-Sathi S, Dagan T, Landan G, Janssen A, Steel M, McInerney JO *et al.* (2012). Acquisition  
752 of 1,000 eubacterial genes physiologically transformed a methanogen at the origin of  
Haloarchaea. *Proc Natl Acad Sci USA* **109**: 20537–20542.
- 754 Nelson-Sathi S, Sousa FL, Roettger M, Lozada-Chávez N, Thiergart T, Janssen A, Bryant D *et*  
*al.* (2015). Origins of major archaeal clades correspond to gene acquisitions from  
756 bacteria. *Nature* **517**: 77–80.
- Nurk S, Bankevich A, Antipov D, Gurevich AA, Korobeynikov A, Lapidus A *et al.* (2013).  
758 Assembling single-cell genomes and mini-metagenomes from chimeric MDA products.  
*J Comput Biol* **20**: 714-737.
- 760 Oren A, Trüper HG. (1990). Anaerobic growth of halophilic archaeobacteria by reduction of  
dimethylsulfoxide and trimethylamine N-oxide. *FEMS Microbiol Lett* **70**: 33–36.
- 762 Oren A. (1991). Anaerobic growth of archaeobacteria by reduction of fumarate. *J Gen*  
*Microbiol* **137**: 1387–1390.
- 764 Probst AJ, Weinmaier T, Raymann K, Perras A, Emerson JB, Rattei T *et al.* (2014). Biology of a  
widespread uncultivated archaeon that contributes to carbon fixation in the  
766 subsurface. *Nat Commun* **5**: 5497.
- Rhodes ME, Spear JR, Oren A, House CH. (2011). Differences in lateral gene transfer in  
768 hypersaline versus thermal environments. *BMC Evol Biol* **11**: 199.
- Roman P, Bijmans MF, Janssen AJ. (2014). Quantification of individual polysulfides in lab-  
770 scale and full-scale desulfurisation bioreactors. *Environ Chem* **11**: 702–708.
- Roman P, Veltman R, Bijmans MFM, Keesman K, Janssen AJ. (2015). Effect of methanethiol  
772 concentration on sulfur production in biological desulfurization systems under  
haloalkaline conditions. *Environ Sc. Technol* **49**: 9212–9221.
- 774 Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA *et al.* (2001). Artemis:  
sequence visualization and annotation. *Bioinformatics* **16**: 944–945.

- 776 Simon J. (2002). Enzymology and bioenergetics of respiratory nitrite ammonification. *FEMS Microbiol Rev* **26**: 285–309.
- 778 Simon J, Klotz MG. (2013). Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. *Biochim Biophys Acta* **1827**: 114–135.
- 780 Sinninghe Damsté JS, Rijpstra WIC, Hopmans EC, Weijers JWH, Foesel BU, Overmann J., Dedysh S.N. (2011). 13,16-Dimethyl octacosanedioic acid (iso-diabolic acid): A common  
782 membrane-spanning lipid of *Acidobacteria* subdivisions 1 and 3. *Appl Env Microbiol* **77**: 4147–4154.
- 784 Sorokin DY, Kublanov IV, Gavrillov SN, Rojo D, Roman P, Golyshin PN *et al.* (2016a). Elemental sulfur and acetate can support life of a novel strictly anaerobic haloarchaeon. *ISME J*  
786 **10**: 240–252.
- Sorokin DY, Kublanov IV, Yakimov MM, Rijpstra WI, Sinninghe Damsté JS. (2016b).  
788 *Halanaeroarchaeum sulfurireducens* gen. nov., sp. nov., the first obligately anaerobic sulfur-respiring haloarchaeon, isolated from a hypersaline lake. *Int J Syst Evol Microbiol*  
790 **66**: 2377–2381.
- Sousa FL, Nelson-Sathi S, Martin WF. (2016). One step beyond a ribosome: The ancient  
792 anaerobic core. *Biochim Biophys Acta* **1857**: 1027–1038.
- Stoffels L, Krehenbrink M, Berks BC, Uden G. (2012). Thiosulfate reduction in *Salmonella enterica* is driven by the proton motive force. *J Bacteriol* **194**: 475–485.
- 794 Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* **30**: 2725–2729.
- 796 Thauer RK, Jungermann K, Decker K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* **41**: 100–180.
- 798 Tindall BJ, Trüper HG. (1986). Ecophysiology of the aerobic halophilic archaeobacteria. *Syst Appl Microbiol* **7**: 202–212.
- 800 Trüper HG, Schlegel HG. (1964). Sulphur metabolism in *Thiorhodaceae* I. Quantitative measurements on growing cells of *Chromatium okenii*. *Antonie van Leeuwenhoek* **30**: 225–238.
- 802 Vignais PM, Billoud B, J. Meyer J. (2001) Classification and phylogeny of hydrogenases. *FEMS Microbiol Rev* **25**: 455–501.
- 804 Vignais PM, Billoud B. (2007). Occurrence, classification, and biological function of hydrogenases: an overview. *Chem Rev* **107**: 4206–4272.
- 806

- 808 Walsh DA, Papke RT, Doolittle WF. (2005). Archaeal diversity along a soil salinity gradient  
prone to disturbance. *Environ Microbiol* **7**: 1655–1666.
- 810 Wang S, Huang H, Kahnt J, Thauer RK. (2013). *Clostridium acidurici* electron-bifurcating  
formate dehydrogenase. *Appl Environ Microbiol* **79**: 6176–6179.
- 812 Weijers JWH, Panoto E, van Bleijswijk J, Schouten S, Balk M, Stams AJM *et al.* (2009).  
Constraints on the biological source(s) of the orphan branched tetraether membrane  
814 lipids. *Geomicrobiol J* **26**: 402–414.
- Werner J, Ferrer M, Michel G, Mann AJ, Huang S, Juarez S *et al.* (2014). *Halorhabdus tiamatea*:  
816 proteogenomics and glycosidase activity measurements identify the first cultivated  
euryarchaeon from a deep-sea anoxic brine lake as potential polysaccharide degrader.  
818 *Environ Microbiol* **16**: 2525–2537.
- Wolf YI, Koonin EV. (2013). Genome reduction as the dominant mode of evolution. *BioEssays*  
820 **35**: 829–837.
- Wood GE, Haydock AK, Leigh JA. (2003). Function and regulation of the formate  
822 dehydrogenase genes of the methanogenic archaeon *Methanococcus maripaludis*. *J*  
*Bacteriol* **185**: 2548–2554.
- 824 Yakimov MM, La Cono V, Slepak VZ, La Spada G, Arcadi E, Messina E *et al.* (2013) Microbial  
life in the Lake *Medee*, the largest deep-sea salt-saturated formation. *Sci Report* **3**: 3554.
- 826 Youssef NH, Ashlock-Savage KN, Elshahed MS. (2011). Phylogenetic diversities and  
community structure of members of the extremely halophilic Archaea (order  
828 *Halobacteriales*) in multiple saline sediment habitats. *Appl Environ Microbiol* **78**: 1332–  
1344.
- 830 Zhi XY, Yao JC, Tang SK, Huang Y, Li HW, Li WJ. (2014). The futasine pathway played an  
important role in menaquinone biosynthesis during early prokaryote evolution.  
832 *Genome Biol Evol* **6**: 149–160.

## Table and Figure Legends

836 **Table 1.** Growth characteristics of isolated lithoheterotrophic sulfur-respiring haloarchaea

838 **Figure 1** Phylogenetic position of the proposed genus *Halodesulfurarchaeum* within the order  
 840 *Halobacteriales* inferred from a 16S rRNA gene sequence alignment with PAUP\*4.b10 using a  
 842 LogDet/paralinear distance method as it described elsewhere (Sorokin *et al.*, 2016a). A  
 844 phylogenetic tree based on 16S rRNA gene sequences from members of the class *Halobacteria*  
 846 covering all known genera (Gupta *et al.*, 2016). The members of orders *Natrialbales*, and  
 848 *Haloferacales* are collapsed. The members of sulfur-respiring genera *Halanaeroarchaeum* and  
*Halodesulfurarchaeum* are highlighted in yellow and orange, respectively. 16S rRNA gene  
 phylogeny of the HDA strains was Support for nodes in this tree corresponds to bootstrap  
 values for 1000 pseudo-replicates. Only bootstrap values at nodes greater than 75% are  
 displayed as solid circles. The tree has been arbitrarily rooted with the sequences from  
*Methanohalophilus halophilus* (FN870068) used for out-grouping.

850 **Figure 2** Cell morphology of four different *Halodesulfurarchaeum* isolates. Phase contrast  
 microphotographs: (a) strain HSR6 (formate + S<sup>0</sup>); (c) strain HTSR1 (formate + thiosulfate);  
 852 (d) strain HTSR14 (hydrogen + S<sup>0</sup>). Transmission electron microscopy (b) shows flagellation  
 of cells of the strain HSR6.

854

**Figure 3** Maximum Likelihood phylogenetic tree of CISM catalytic subunits A. Totally 168  
 856 sequences were taken for the analysis. The tree with the highest log likelihood (-  
 132625.7976) is shown. The bootstrap values (100 replicates) are shown next to the  
 858 branches. All positions with less than 95% site coverage were eliminated. There were a total  
 of 580 positions in the final dataset. The tree was constructed in MEGA6 (Tamura *et al.*,  
 860 2013). CISM proteins of three sulfur-reducing haloarchaea *Halanaeroarchaeum sulfurireducens*  
 HSR2<sup>T</sup> (HLASF) and *Halodesulfurarchaeum formicicum* strains HTSR1 and HSR6 are highlighted  
 862 in bold (locus tag prefixes are HLASF, HTSR and HSR6, respectively). Sequential numeration  
 of all HDA CISM is used as in Table S10. Abbreviations used: Aro, arsenite oxidases family;  
 864 Arr, arsenate reductase family; Nar/DMSO, nitrate / DMSO reductase family; Nas/Nap/Fdh,  
 assimilatory (periplasmic) nitrate reductase / formate dehydrogenase family; Ttr,

866 tetrathionate reductase family; Psr/Phs, polysulfide/thiosulfate reductase family; Unk,  
unknown family. Bar is 0.3 aminoacid substitutions per site.

868

**Figure 4** Phylogenetic tree of [NiFe]-hydrogenases constructed with full-length enzymes  
870 from small HydA (A) and large HydB (B) subunits of subgroup representatives. Based on the  
report Vignais and Billoud (2007), the alignment was made with Clustal W584 and  
872 MacVector 11.1.2. Trees were computed with PhyML586 using the bootstrap procedure with  
1000 replicates and bootstrap values of more than 700 (70%) are displayed as percentages  
874 close to the corresponding nodes. The nodes are displayed so that the corresponding small  
and large subunits can be read in the same top-down order. Branch lengths along the  
876 horizontal axis reflect the degree of relatedness of the sequences (20%).

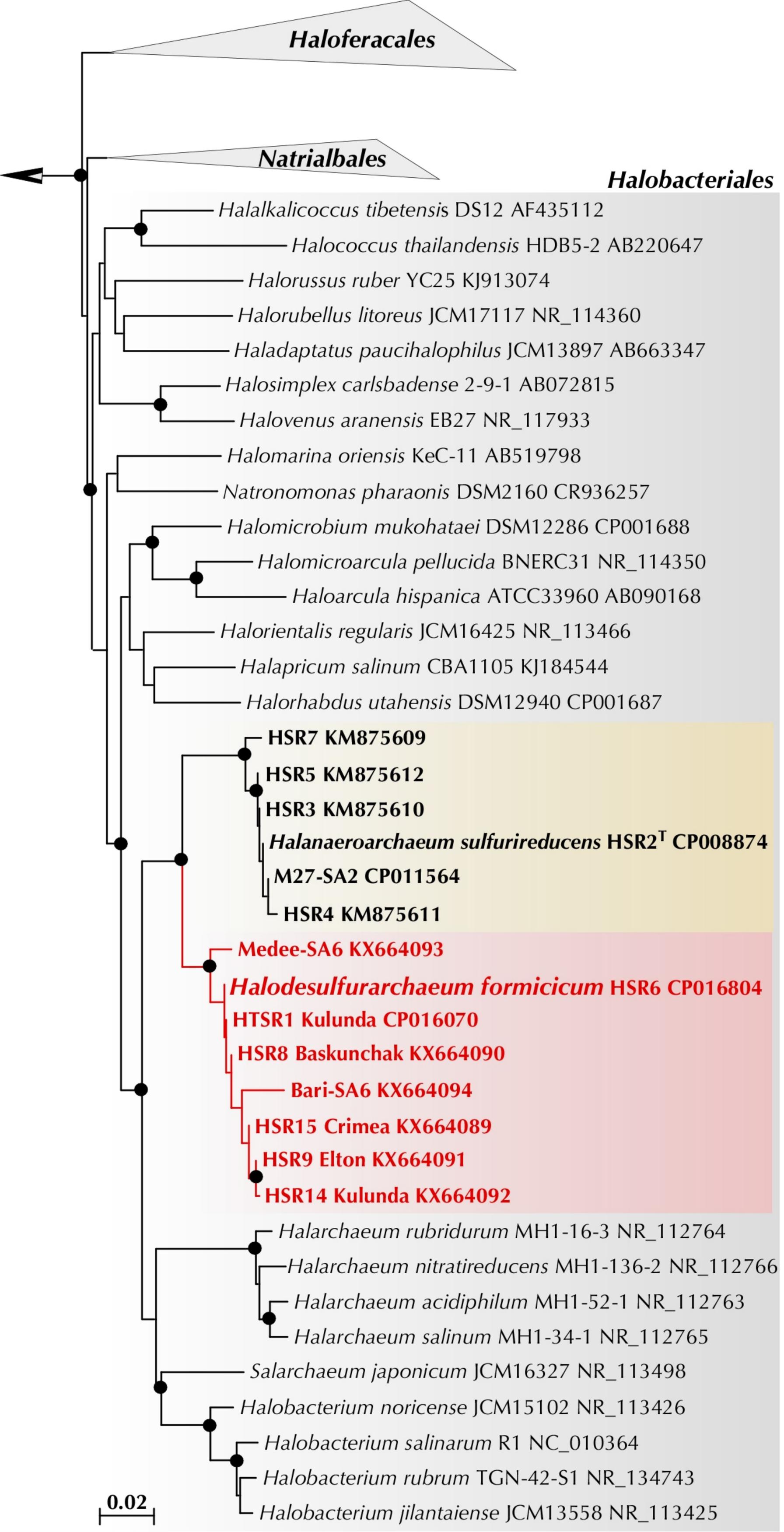
878 **Figure 5** Proposed pathways for energy generation and proton-translocation machinery in  
*Halodesulfurarchaeum*. Molybdopterin- and [Ni-Fe]-containing catalytic subunits of  
880 respiratory complexes are shown in blue and green, correspondingly. Subunits, that  
transfer electrons and predicted to possess four iron-sulfur centers, are shown in yellow,  
882 while integral membrane subunits, that anchor the other two subunits to the membrane  
and predicted to contain the site for  $MH_2$  oxidation and two heme cofactors, are shown in  
884 red. Abbreviations: A-ATPase, archaeal ATP synthase;  $CH_2$ -THF, methylene-tetrahydrofolate;  
 $CH_3$ -THF, methyl-tetrahydrofolate; DMSOR, DMSO reductase; Etf, electron transfer  
886 flavoprotein; FDH, formate dehydrogenase; FT, formate transporter; HYD, uptake  
hydrogenase; (M)MK, oxidised (methyl)menaquinone; (M)MKH<sub>2</sub>, reduced  
888 (methyl)menaquinone; MTHFR, 5,10-methylene-tetrahydrofolate reductase; PSR,  
polysulfide reductase; ST, sulfurtransferase; TSR, thiosulfate reductase.

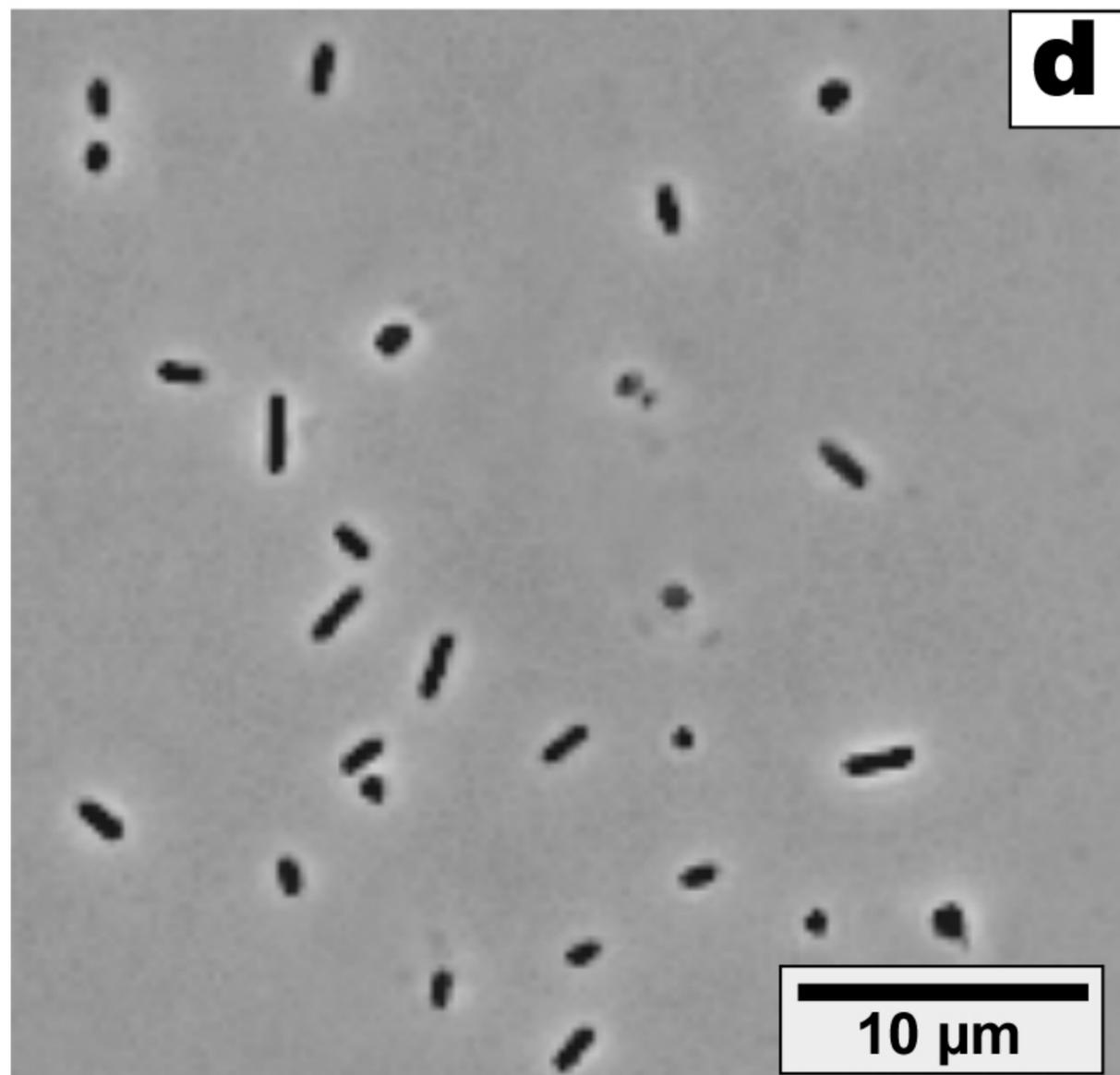
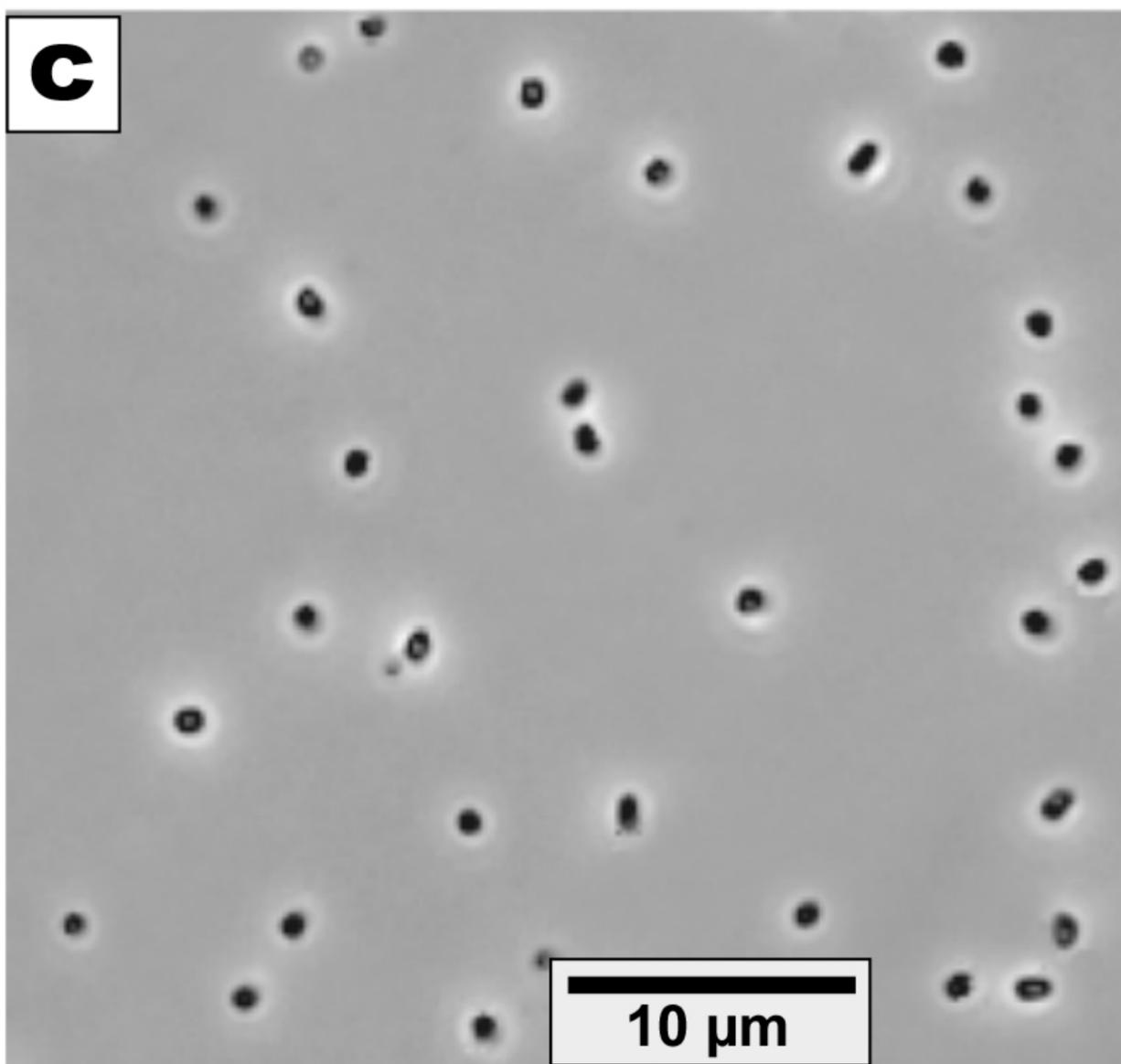
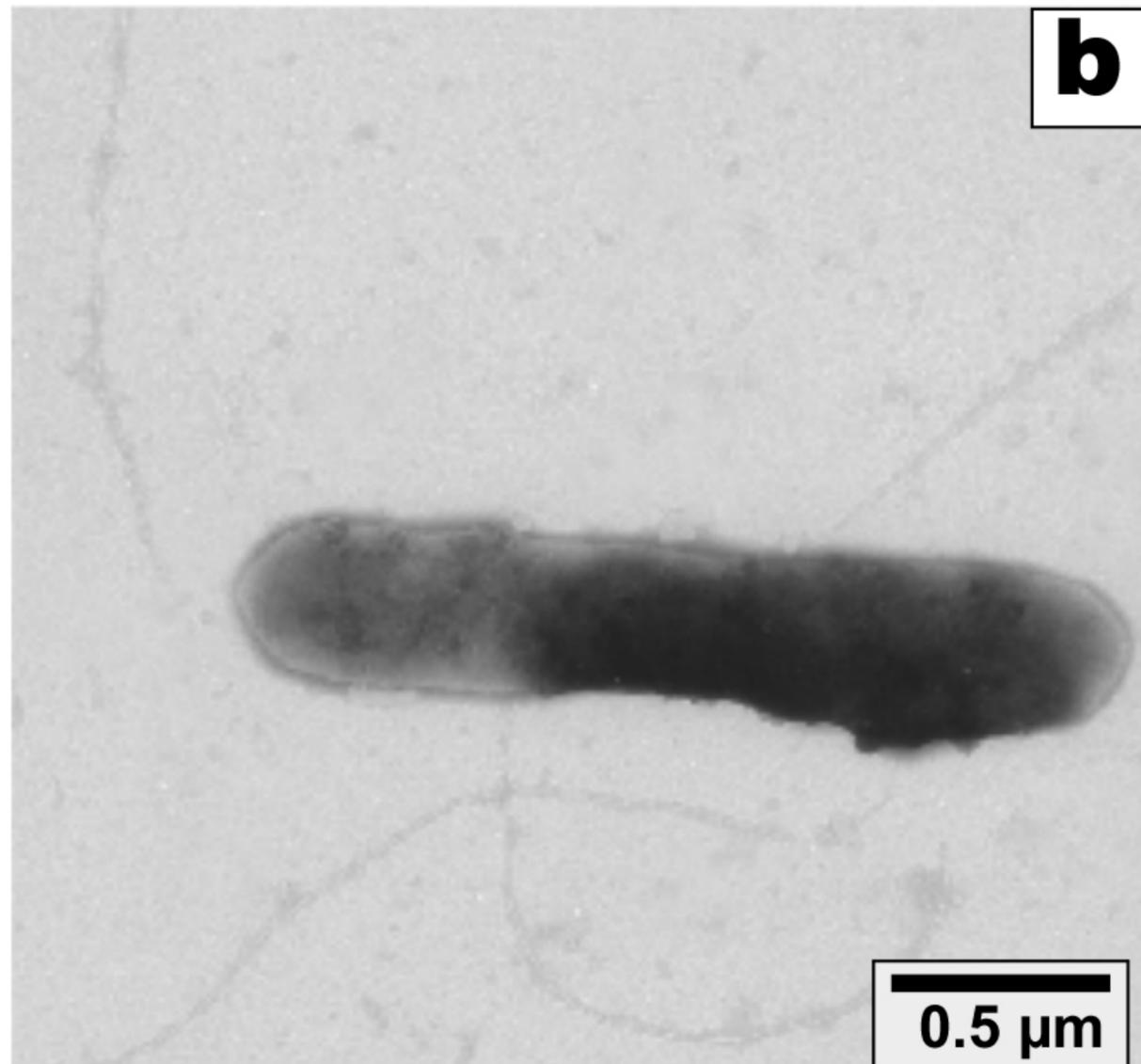
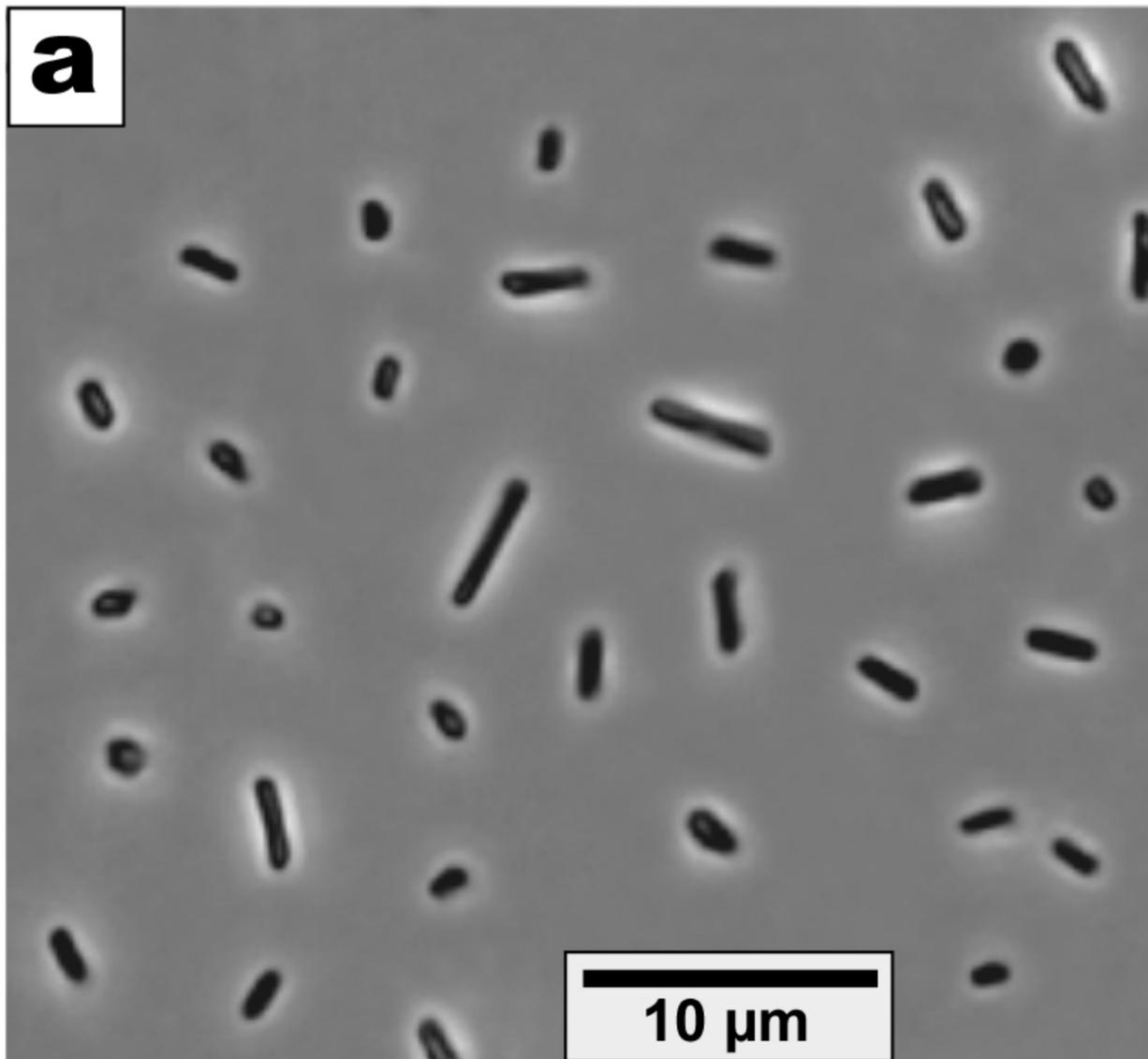
890

**Figure 6** Summary of  $C_1$  metabolism in *Halodesulfurarchaeum formicicum* HTSR1 representing  
892 ancestral routes of glycine, serine and methyl group chemistry (Braakman and Smith, 2012).  
Pathways shown in the model were deduced on the basis of genome annotation and  
894 genome-wide proteomic analysis of cells grown on different electron acceptors. Enzymes  
involved: (1) formate transporter (HTSR\_0446) or formate/oxalate antiporter (HTSR\_1713);  
896 (1A) numerous amino acid permeases; (2) formate dehydrogenase subunit alpha  
(HTSR\_1736 [A], 1740 [B]); (3) formate--tetrahydrofolate ligase (HTSR\_1739); (4) 5,10-  
898 methylenetetrahydrofolate reductase (HTSR\_1746); (5) methylenetetrahydrofolate

dehydrogenase (NADP+) (HTSR\_1747); (6) serine hydroxymethyltransferase (HTSR\_0671);  
900 (7) glycine cleavage system proteins H, P and T (HTSR\_0503-0506, 1750); (8)  
methylenetetrahydrofolate reductase (NADPH) (HTSR\_1220); (9) electron transfer  
902 flavoprotein (HTSR\_1748-1749); (10) methionine synthase (HTSR\_1805); (11) S-  
adenosylmethionine hydroxide adenosyltransferase (HTSR\_1447); (12) S-  
904 adenosylmethionine-dependent methyltransferase (HTSR\_1450); (13)  
adenosylhomocysteinase (HTSR\_0160); (14) D-3-phosphoglycerate dehydrogenase  
906 (HTSR\_0539), (15) phosphoserine phosphatase (HTSR\_1388). The data for the 150 most  
abundant proteins from proteomic analysis are outlined in the small nested box. The  
908 proteins are sorted according to their relative abundance in cells grown on formate +  
thiosulfate. Proteins involved in C<sub>1</sub> metabolism are indicated in red. Abbreviations: Fd,  
910 ferredoxin; THF, tetrahydrofolate.

912 **Figure 7** The 1.97-Mbp genome and differential proteome of *Halodesulfurarchaeum*  
*formicum* HTSR1. (a) The outermost ring indicates the position on the genome map of the  
914 10 sequentially numbered CISM enzymatic complexes (as in Figure 3 and Table S10),  
including two DMSO reductases DMSOR (1, 2), one unaffiliated CISM complex 'Deep' (3),  
916 three polysulfide reductases PSR (4, 7, 8), one thiosulfate reductase TSR (5) and three  
formate dehydrogenases FDH (6, 9-10). The second, third and fourth rings (histograms) are  
918 the relative abundances of proteins detected in corresponding proteomes, normalized  
versus the most abundant protein in all three proteomes, glycine cleavage system protein T  
920 (HTSR\_1750, 100%). Two innermost cyan rings indicate predicted ORFs on the plus and  
minus strands, respectively. The Venn diagram in the centre shows the numbers of proteins  
922 detected in sulfur- (red), thiosulfate- (blue) and DMSO-respiring (green) cells. (b) Relative  
abundances of the 10 sequentially numbered CISM enzymatic complexes identified in  
924 corresponding proteomes. Key to protein annotations: 1. DMSOR (catalytic subunit  
HTSR\_0423); 2. DMSOR (catalytic subunit HTSR\_0517); 3. Deeply branched CISM (catalytic  
926 subunit HTSR\_0627); 4. PSR (catalytic subunit HTSR\_1347); 5. TSR (catalytic subunit  
HTSR\_1522); 6. FDH (catalytic subunit HTSR\_1576); 7. PSR (catalytic subunit HTSR\_1661); 8.  
928 PSR (catalytic subunit HTSR\_1699); 9. FDH (catalytic subunit HTSR\_1736); 10. FDH (catalytic  
subunit HTSR\_1740). Relative abundances of all proteins identified in the global proteome is  
930 provided in Supplementary Table S11.





Nas/Nap/Fdh

10

9

HSR6-1806  
 HTSR-1740  
 HSR6-1802  
 HSR6-1736  
 Methanocaldococcus P61159  
 Clostridium acidurici AFS79904  
 Clostridium acidurici AFS79903  
 Escherichia BAE78081  
 Staphylococcus Q49ZN0  
 Bacillus P42434

6

Aro

HSR6\_1645  
 HTSR\_1576  
 Arcobacter OCL86836  
 Wollinella CAA37989

2

Nar/DMSO

1

HSR6\_0501  
 HTSR\_0517  
 Halobacterium Q9FR74  
 HSR6\_0408  
 HTSR\_0423

7

Psr/Phs

8

Desulfocaspa M1P680  
 HLASF\_1287  
 HSR6\_1729  
 Natronobacterium LOAJT9  
 HTSR\_1661  
 HSR6\_1768  
 HTSR\_1699  
 HLASF\_0052  
 Natronobacterium LOAF89  
 Halobiforma M0M8T3  
 Halonotius U1PCL7  
 HLASF\_0694  
 HSR6\_1419  
 HTSR\_1347

4

3

Unk

HTSR\_0627  
 HTSR\_1522

0.3

5

Ttr

Carboxydotherrmus Q3AB12  
 Bacillus D6XY26  
 HSR6\_1593  
 HTSR\_1522  
 HLASF\_0530  
 Natronococcus L9XXG8  
 Halobifoma M0L3B4

