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# Carbohydrate-dependent sulfur respiration in halo(alkali)philic euryarchaea

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15 reductase

*Originality significance statement:* Here, we revealed the potential for carbohydrate-dependent sulfur-respiration in novel members of the class *Halobacteria*. This finding underlines the importance of archaea in biogeochemical sulfur cycling linked to the terminal anaerobic carbon mineralization in anoxic sediments of hypersaline habitats  
20 worldwide. It also has astrobiological applications in relation to the habitability of sulfur-containing anoxic brines.

*Authors' contributions:* DYS carried out the hands-on experimental work and physiological analyses. Bioinformatics analyses carried out by EM, FS and VLC. The data were interpreted and manuscript was written by DYS, JEH and MMY. EM, FS  
25 and VLC had advisory roles in the aspects of isolates handling and input into writing of the manuscript.

## Summary

Archaea are environmentally ubiquitous on Earth, and their extremophilic and metabolically versatile phenotypes make them useful as model systems for astrobiology. Here, we reveal a new functional group of halo(natrono)archaea able to utilize alpha-D-glucans (amylopectin, amylose and glycogen), sugars, and glycerol as electron donors and carbon sources for sulfur respiration. They are facultative anaerobes enriched from sediments of hypersaline lakes with either amylopectin, glucose or glycerol as electron/carbon sources and elemental sulfur as the terminal electron acceptor. They include ten strains of neutrophilic haloarchaea enriched either with glucose, starch or glycerol from circum pH-neutral lakes and a natronoarchaeon enriched from soda-lake sediments with glucose. The neutrophilic isolates can grow by fermentation, although addition of  $S^0$  or dimethyl sulfoxide increased growth rate and biomass yield (with a concomitant decrease in  $H_2$ ). Natronoarchaeal isolate AArc-S grew only by respiration, either anaerobically with  $S^0$  or thiosulfate or aerobically with  $O_2$  as the terminal electron acceptors. Via genome analysis of five representative isolates, we detected the full set of enzymes required for the observed catabolic and respiratory phenotypes. These findings provide evidence that sulfur-respiring haloarchaea partake in biogeochemical sulfur cycling, linked to terminal anaerobic carbon mineralization in hypersaline anoxic habitats. We discuss the implications for life detection in analogue environments such as the polar subglacial brine-lakes of Mars.

## Introduction

Extremely halophilic euryarchaea belonging to the class *Halobacteria* (recently suggested to be reclassified into a separate phylum, the *Halobacteriota* on the basis of phylogenomic approach, <https://gtdb.ecogenomic.org>) are a dominant group of

prokaryotes in salt-saturated aerobic habitats worldwide. Their potential absence/presence or ecophysiology in anoxic brines and sediments is as-yet  
55 unresolved. Using elemental sulfur as the terminal electron acceptor we recently described a novel group of haloarchaea able to perform an anaerobic dissimilatory sulfur respiration. Thus far, three different functional groups of such haloarchaea have been characterized:

- the obligate anaerobic archaeon *Halanaeroarchaeum sulfurireducens*, capable of  
60 acetate and pyruvate oxidation during sulfur-dependent respiration - a catabolic route unique in the whole archaeal Domain (Sorokin *et al.*, 2016 a,b; Messina *et al.*, 2016);
- the obligate anaerobic archaeon *Halodesulfurarchaeum formicicum*, that uses formate or H<sub>2</sub> as the electron donor and elemental sulfur, thiosulfate or dimethyl sulfoxide (DMSO) as the electron acceptor (Sorokin *et al.*, 2017a; 2018a), being a  
65 first example of lithoheterotrophs among the haloarchaea;
- the facultatively anaerobic natronoarchaea, belonging to two new genera *Halalkaliarchaeum* and *Natrarchaeobaculum*, which are more versatile in their electron donors repertoire for anaerobic sulfur respiration, including formate/H<sub>2</sub>, C<sub>4</sub>-C<sub>9</sub> fatty acids and peptone (Sorokin *et al.*, 2018a; 2020b).

70 While the first two obligate anaerobic groups listed are neutrophilic halophiles found in various hypersaline chloride-sulfate habitats, the third group includes alkaliphilic haloarchaea living in hypersaline soda lakes, whereby sulfur reduction is a highly active process because of the chemical stability of polysulfide - the actual electron acceptor in sulfur-respiring prokaryotes at high pH (Sorokin *et al.*, 2010;  
75 2011). Most of the soda-lake isolates belong to the novel genus and species *Natrarchaeobaculum sulfurireducens* (Sorokin *et al.*, 2020a), while a single strain from a less-alkaline hypersaline lake (Soap Lake, California) was classified as a novel genus and species, *Halalkaliarchaeum desulfuricum* (Sorokin *et al.*, 2019).

The discovery of these anaerobic sulfur-respiring halo(natrono)archaea living  
80 in anoxic sediments of hypersaline lakes, together with finding in the same habitats of  
extremely halo(alkali)philic and thermophilic methyl-reducing methanogens belonging  
to a novel class *Methanonatronarchaeia* (Sorokin *et al.*, 2017b; 2018b), creates a  
paradigm shift in knowledge that revise what we know about the physiology of  
haloarchaea and their ecological role in hypersaline habitats as aerobic  
85 organoheterotrophs. Apparently, they may also play an important role in  
biogeochemical sulfur cycling linked to the terminal anaerobic carbon mineralization  
in hypersaline anoxic habitats.

To deepen our knowledge in this field, we formulated the following questions.  
Given the existence of haloarchaea that utilize fermentation products as electron  
90 donors for anaerobic sulfur respiration (the microbes in this way act as ‘secondary’  
anaerobes), do haloarchaeal analogues of ‘primary’ anaerobes exist that utilize  
carbohydrates and produce these fermentation products? If so, does the sulfur  
respiration play a role in their catabolism?

Here, we report the enrichment and isolation in pure cultures of ten strains of  
95 neutrophilic haloarchaea from hypersaline salt lakes and a single natronoarchaeon  
from soda lakes able to grow anaerobically with alpha-glucans (glycogen,  
amylopectin and amylose), oligo- and monomeric sugars, and/or glycerol as the  
alternative electron donors either by fermentation or by anaerobic respiration with  
sulfur, thiosulfate or DMSO as the terminal electron acceptors. Collectively, these  
100 isolates form a fourth functional group of sulfur-reducing, carbohydrate-utilizing  
haloarchaea. We employed a holistic approach: knowledge of genomes was  
combined with situational-functional culture-based experiments (where microbes  
were challenged with diverse electron donors with and without electron acceptors to  
determine phenotypic traits). The physiological investigation and analysis of the five

105 finished ungapped genome sequences enabled to dissect and elucidate key aspects  
of their catabolic potential with special attention to anaerobic metabolism.

## Results and Discussion

### *Enrichment, isolation and cultivation*

110 As described in *Experimental procedures*, two different media were used (depending  
on sample origin) to select for and enrich of potential sulfur-reducing  
halo(natrono)archaea. After adding powdered sulfur ( $1 \text{ g l}^{-1}$ ) as an electron acceptor,  
the appropriate medium was inoculated with mixed anaerobic sediment/brine slurries  
(5% v/v). Soluble starch ( $1 \text{ g l}^{-1}$ ), glucose or glycerol (2 mM each) were added  
115 separately as the electron donors and the sulfur-reducing activity (SRAs) was  
measured after one month of incubation in each of settled primary enrichments  
(Supplementary Fig. S1). Generally, using these compounds as sole carbon source  
and energy, the SRA rates were greatest of glucose. All soda-lake enrichments were  
120 characterized by SRA values that were much higher than those of the pH-neutral salt  
lakes. These high SRAs were substantially inhibited by addition of bacteria-specific  
antibiotics, indicating a significant input of bacterial fraction in carbohydrate-  
dependent sulfur reduction in soda lakes. This was not the case in primary  
enrichments derived from pH-neutral salt lakes, where the sulfur reduction was  
mostly insensitive to bacteria-specific antibiotics, strongly suggesting that SRA was  
125 performed by haloarchaea. As shown in Table 1 and Supplementary Table S1,  
besides the Kulunda Steppe hypersaline pH-neutral salt lakes and soda lakes,  
samples from three other sites were used to obtain additional enrichments of  
carbohydrate-utilizing, sulfur-reducing haloarchaea. For the single positive soda-lake  
enrichment, the antibiotic mix was kept during all stages of the enrichment- and  
130 isolation procedure. For enrichments from pH-neutral salt lakes, antibiotics were not

added to the cultivation medium. After three consecutive 1:100 transfers, the grown cultures were subjected to serial dilution-to-extinction steps. It should be noted that, in all cases, obtaining axenic cultures of sulfur-reducing halo(natrono) archaea was not an easy task. In the enrichments of neutrophiles there was a persistent population of minor satellite haloarchaea, identified as hydrogenotrophic *Halodesulfurarchaeum formicicum*. The reason of such coexistence turned out to be the formation of H<sub>2</sub> as a product of sugar fermentation, as explained below. In the enrichment of soda-lake samples, only a single strain (AArc-S) was obtained on media supplemented with glucose and sulfur. From pH-neutral salt lakes, 10 pure cultures were isolated: three isolates using soluble starch, five isolates using glucose and two isolates using glycerol as the sole energy and carbon source. The sulfur-reducing haloarchaeal isolates were mostly large coccoids, often containing refractive inclusions stained positive with Nile-Blue for polyhydroxyalkanoate (further confirmed by genome analysis), while the natronarchaeon AArc-S has small flat polymorphic cells, varying in shape from rods to cocci and without visible refractive inclusions (Supplementary Fig S2). The purity of 5 representative isolates were confirmed by the whole genome analysis.

### *Phylogeny of the isolates*

Together with the single soda-lake isolate AArc-S, strains that represent the starch- (HSR-Est), glucose- (HSR12-1 and HSR12-2) and glycerol-utilising (HSR-Bgl) neutrophilic groups were chosen for further genome sequencing and detailed phylogenomic analyses. The genome of soda-lake isolate AArc-S possesses two rRNA operons with identical *rnaA* and *rnaB* 16S rRNA genes, distantly related to members of the genus *Natronoarchaeum* (94.03-94.57% of 16S-rRNA gene sequence identity) (Supplementary Fig. S3). Both ANIb/ANI<sub>m</sub> and AAI values

obtained from pairwise comparison of the available genomes of the genus *Natronoarchaeum* (Supplementary Table S2) are consistent with phylogenetic placement of the strain AArc-S as a separate genus-level lineage in the family *Halobacteriaceae* for which we gave the provisional name “*Natranaeroarchaeum sulfidigenum*”.

The genome of each neutrophilic isolate harbours two divergent rRNA operons with highly dissimilar *rnnA* and *rnnB* 16S rRNA genes (<93% of gene identity). Analysis of these intraspecific polymorphic 16S rRNA gene sequences revealed that the *rnnA* type formed a cluster, mostly related to the corresponding gene of *Halapricum salinum* CBA1105<sup>T</sup> (<96.7% of gene identity), while the *rnnB* type was distantly related to *Halosiccatus urmianus* DC8<sup>T</sup> (<91.4% of gene identity), with both species occurring within the family *Haloarculaceae*. Therefore, the 16S rRNA gene-based phylogeny for such organism can not resolve the true position on the tree and, furthermore, can not confirm the purity of isolates. To resolve this problem, a phylogenomic analysis of a concatenated alignment of six ribosomal proteins S2, S3 and L1, L2, L3 and L4 (always present in single copies) from the 5 newly isolated strains and extracted from 61 haloarchaeal genomes was performed (Fig 1). Using maximum parsimony criteria within the ARB software (Ludwig *et al.*, 2004), the master alignment in SILVA Release 132SSURef NR99 suggested that the nearest neighbours of the novel physiological group of haloarchaea (~97-98% of sequence identity) were the Chinese solar saltern isolates CK28-2, DL47, DL50 and SY-39, all possessing the intraspecific 16S rRNA gene sequences polymorphism (Supplementary Fig. S3). Together with our isolates and other ribocloned retrieved from around the world, they formed a separate novel genus-level taxon within the family *Haloarculaceae* for which we propose a provisional name “*Halarchaeoglobus desulfuricus*”. The analysis also showed that those genes encoding the conserved



single-copy ribosomal protein markers were present in single copies, confirming the purity of isolates. Using the Average Nucleotide Identity (ANIb, AAI) and Amino Acid Identity (AAI) analyses parameters, the genomic similarities between the type strain of the genus *Halapricum salinum* CBA1105<sup>T</sup> and the “*Halarchaeoglobus desulfuricus*” strains produced the indices in the range of inter-genus level, which supported the conclusion based on phylogenomic analysis (Supplementary Table S2). Although this is beyond the aims of the current study, based on ANIb/AAI and AAI indices, the strain HSR-Est could be qualified as a distinct species, which is consistent with clear-cut phenotypic differences observed between the groups (ability/inability to degrade alpha-D-glucans).

#### *Growth physiology and sulfidogenic activity*

All of the neutrophilic isolates are facultative anaerobes, able to grow in three different modes: (i) microaerobically in the presence of 2-5% (v/v) O<sub>2</sub> in the gas phase; (ii) fermentation of several hexoses and glycerol and (iii) anaerobic respiration using elemental sulfur (S<sub>8</sub>) and DMSO (all strains) or thiosulfate (strain HSR12-2) as alternative terminal electron acceptors. Furthermore, we observed that in addition to DMSO, the HSR isolates can grow with methionine sulfoxide and tetramethylene sulfoxide as alternative electron acceptors. A subgroup, enriched with starch (strains HSR-Bst, HSR-Est and HSR-Kst) can grow anaerobically with starch and other alpha-glucans, including cyclodextrin, dextrin, glycogen and pullulan. The maximum sulfide production by HSR isolates was in the range of 7-10 mM, similar to that of the acetate-oxidizing *Halanaeroarchaeum* (Sorokin *et al.*, 2016 a,b), but significantly lower than that of the formate/H<sub>2</sub>-oxidizing *Halodesulfuriarchaeum* (Sorokin and Yakimov, 2018).

Fermentative growth of all of the neutrophilic isolates was accompanied by production of H<sub>2</sub>, acetate and lactate. In general, this type of catabolism seems to be less efficient, because the addition of terminal acceptors, i.e. creation of conditions for anaerobic respiration, has stimulated the yield of both end metabolites (acetate and lactate) and biomass (Fig. 2). Hydrogen formation was also detected during the anaerobic respiration, although to a much less extent, indicating that part of the reducing power generatedn from sugar fermentation was used for the formation of H<sub>2</sub>S as the final product instead of H<sub>2</sub>. Notably, the sulfidogenic oxidation of glucose (but not glycerol) promoted the production of propionate in significant quantities (up to 8.5 mM), never seen before under any other conditions. We also tested whether three representative strains of "*Halarchaeoglobus desulfuricus*" (HSR12-1, HSR12-2 and HSR-Bgl) could grow lithoheterotrophically with H<sub>2</sub> as an electron donor at a low level of organic carbon supplied as yeast extract (100 mg l<sup>-1</sup>), glucose (0.5 mM) or glycerol (1 mM). Strain HSR12-1 was clearly negative, strain HSR-Bgl did show an increase in sulfide formation in presence of H<sub>2</sub>, while only the most metabolically versatile strain HSR12-2 showed fully positive response to H<sub>2</sub> addition by increase in biomass yield and intensive formation of sulfide in comparison to the incubation without H<sub>2</sub> (Fig. 3). That could be taken as an indication of the respiratory mode of sulfur reduction in these carbohydrate-utilizing haloarchaea, instead of facilitated fermentation more common in fermentative archaea, also further confirmed by genomic analysis (see below).

Washed cells of neutrophilic isolates, cultivated with appropriate electron donors and acceptors, were tested to see whether they retained sulfur- and DMSO-reducing activity. In all cases, the sulfidogenic activities were much higher than the DMSO-reducing activities, even in the cells grown with DMSO as acceptor (Fig. 4). It is notable that resting cells grown with either glucose or starch and sulfur as the

electron acceptor showed the highest sulfur-reducing activities not with respected  
235 substrates but with H<sub>2</sub>, pointing to an essential role of H<sub>2</sub> as the actual electron donor  
for sulfur respiration. Moreover, formation of H<sub>2</sub> by resting HSR12-2 cells from  
glucose was substantially decreased upon addition of an electron acceptor with  
maximum drop caused by sulfur (Supplementary Fig. S4) - same phenomenon as  
observed in the growth experiments. The tendency to obtain an elevated constitutive  
240 sulfur-reducing activity was also true for the washed HSR12-2 cells, grown with  
thiosulfate (HSR12-2 was the only neutrophilic isolate capable of using thiosulfate as  
the terminal electron acceptor). When either glucose or H<sub>2</sub>, was provided, thiosulfate  
reduction by these cells was two- to three-fold less than the corresponding sulfur-  
reducing activities. The aerobically grown cells did not show any capability of  
245 anaerobic respiration, indicating that either expression or activities of constitutively  
expressed anaerobic respiratory reductases was completely inhibited by oxygen.

Unlike neutrophilic strains, the facultatively anaerobic soda lake isolate AArc-S  
was unable to grow by fermentation and exhibited an obligate requirement during  
anaerobic growth for either sulfur or thiosulfate as an alternative electron acceptor.  
250 Its inability to respire with DMSO was another phenotypic difference. The total  
amount of sulfide/sulfane or polysulfide produced by this organism was considerably  
higher than that produced by neutrophilic strains, especially when sulfur served as  
the terminal acceptor of electrons (Fig. 5). This phenomenon/fact seems to be a  
common trait for sulfur-reducing natronoarchaea, which is most likely mediated by  
255 the superior chemical stability of polysulfide under highly alkaline anoxic conditions  
(Sorokin *et al.*, 2018a; 2019). Interestingly, the aerobically grown AArc-S cells still  
exhibited sulfur/thiosulfate-reducing activity. Also, thiosulfate-reducing activity was  
detected in cells grown with sulfur as the terminal electron acceptor. Both findings

suggest the basic constitutive expression level of the corresponding anaerobic  
260 terminal reductases, independently of the type of respiration.

### *Characterization of fully assembled complete genomes*

To help to explain and confirm the metabolic properties of the carbohydrate-oxidizing  
and sulfur-reducing halo(natrono)archaea observed growth and resting cells  
265 experiments, we determined complete genome sequences for strains AArc-S,  
HSR12-1, HSR12-2, HSR-Bgl and HSR-Est. Their general features are presented in  
Supplementary Table S3. The genome of alkaliphilic “*Natranaeroarchaeum*  
*sulfidigenum*” AArc-S consists of a circular chromosome of 3,041,127 bp and  
contains 3,120 predicted protein-coding genes, two identical rRNA operons, 50 tRNA  
270 genes, and two CRISPR repeat regions with one CRISPR-Cas system of the type I-  
D. The genomes of four neutrophilic “*Halarchaeoglobus desulfuricus*” strains  
comprise the circular chromosome of 2.70–2.94 Mb. A single plasmid pHSR-Bgl-01  
(137.7 kbp) and a single plasmid pHSR-Est-01 (180.7 kbp) were detected in strains  
HSR-Bgl and pHSR-Est, respectively. Similarly to AArc-S isolate, two CRISPR repeat  
275 regions with one CRISPR-Cas system of type I-D were found in the genomes of  
HSR12-2 and HSR-Bgl, whereas two different CRISPR-Cas systems of type I-B were  
found in the HSR12-1 genome. No CRISPR systems were detected in the genome of  
HSR-Est (Supplementary Figure S5). Analysis of the detected CRISPR-Cas systems  
showed no homology between both repeater and spacer sequences. Among 433  
280 spacers detected in all CRISPRs, only 16 resulted similar to hits obtained from viral  
fraction obtained from the metagenomic investigation in saltern San Diego, CA  
(Dinsdale *et al.*, 2008). The absence of similarity between spacer sequences derived  
from different CRISPRs systems likely implies a different history of phage/mobile  
elements interaction for the strains, which was anticipated based on their isolation

285 from geographically distant locations. Circos ribbon plots (Krzywinski *et al.*, 2019)  
were used to compare synteny between the genomes and to find the clusters of  
orthologous proteins. High overall collinearity and high similarity in terms of gene  
context (> 70% of amino-acid identity) was found between all them along with the  
presence of few organism-specific genome rearrangements, genomic islands,  
290 CRISPR and CRISPR-associated elements (Supplementary Figure S6, Extended  
Data 1). This finding strongly suggests the common origin of all studied neutrophilic  
members of this novel ecotype of carbohydrate-oxidizing and sulfur-reducing  
halo(natrono)archaea.

Besides the incapability to grow anaerobically either with DMSO or by  
295 fermentation and to produce hydrogen, the alkaliphilic AArc-S strain is a phenotypic  
counterpart of the HSR strains. Hence its genomic features were considered only for  
the phylogenetic and evolutionary comparisons of the terminal polysulfide- and  
thiosulfate reductases, both belonging to the respiratory type of molybdopterin  
oxidoreductase Psr/Phs (Sre in other archaea) family within the complex iron-sulfur  
300 molybdoenzyme (CISM) superfamily.

#### *Genomic reconstruction of the catabolic features*

Phenotypic traits consistent with carbohydrate-based heterotrophy, fermentation and  
types of respiration were confirmed using analysis of all five genomes sequenced. In  
305 concordance with the observed usage of a broad spectrum of sugars as the sole  
carbon source, these organisms possess various genes involved in the transportation  
and metabolism of sugars and their N-glycan and amino-, phospho- and nucleotide  
derivates. Like many other halophilic archaea, the oxidative pentose phosphate  
pathway was not present in our strains and sources of ribulose phosphates likely are  
310 ribose sugars, produced via the archaeal nucleotide salvage pathway (Sato *et al.*,

2007; Mwirichia *et al.*, 2016). In line with the cultivation results, we predicted the key metabolic pathways and found the full sets of genes encoding for the glycolysis / gluconeogenesis, citrate cycle (TCA cycle), pyruvate and glycerol metabolism (Figure 6, Extended Data 2).

315 Glycerol is an important intermediate in organic carbon mineralization in hypersaline habitats, being a major compatible solute of extremely halophilic algae of the genus *Dunaliella* (Elevi *et al.*, 2008) and halophilic fungi (Stevenson *et al.*, 2017). Many pure cultures of haloarchaea are capable of aerobic growth on glycerol as the sole source of carbon and energy (Williams *et al.*, 2017; Oren, 2017). They possess  
320 two pathways (referred to the *sn*-glycerol-3-phosphate [G3P] and dihydroxyacetone [DHA] pathways) of its catabolism, likely acquired from bacteria as part of their evolutionary transformation to heterotrophic nutrition (Nelson-Sathi *et al.*, 2012). There is circumstantial evidence that some haloarchaea may use glycerol as a stress metabolite (Pavankumar *et al.*, in press). Prior to the present study, however, there  
325 was no direct evidence of anaerobic growth of haloarchaea on glycerol, and a potential for this kind of metabolism was only suggested for *Halalkalicoccus jeotgali*, isolated from fermented food (Roh *et al.*, 2007; Williams *et al.*, 2017). Our isolates can grow on glycerol both aerobically and anaerobically and their genomes are fully equipped by the genes encoding for both catabolic pathways, even with some  
330 duplication found (as for glycerol-3-phosphate dehydrogenase [G3PDH] and dihydroxyacetone kinase). In bacteria, G3PDH, which oxidizes G3P to dihydroxyacetone phosphate (DHAP), exists in two forms: the homodimeric 'aerobic' GlpD, and the heterotrimeric 'anaerobic' GlpABC dehydrogenase. The differentiation between these isozymes came from the highest activity levels observed in bacteria  
335 after aerobic or anaerobic growth, respectively (Iuchi *et al.*, 1989). The heterotrimeric G3P dehydrogenase (GlpABC) seems the only isoform acquired by haloarchaea and

its function during aerobic growth on glycerol was documented for *Haloferax volcanii* (Sherwood *et al.*, 2009; Rawls *et al.*, 2011). Thus, more work is needed to understand the functional activities of GlpABC in glycerol metabolism in sulfur-reducing haloarchaea during aerobic or anaerobic growth.

Both the G3P- and DHA-pathways of the glycerol catabolism end with the production of DHAP, an important metabolite that can be directed via glycolysis (Embden-Meyerhof pathway) to formation of pyruvate (Fig. 6). The four-subunit pyruvate:ferredoxin oxidoreductase complex (PFOR) catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA and CO<sub>2</sub>. ADP-forming acetyl-CoA synthetase likely terminates the oxidative pathway of pyruvate metabolism with generation of ATP and formation of acetate, one of the major soluble end fermentation products in our isolates. Oxidation of glucose to pyruvate involves the reduction of NAD<sup>+</sup> to NADH and thus, to avoid stopping glycolysis, the cells may have to re-oxidize the metabolically unused excess of this reduced electron/energy shuttle. In the line with detection of lactate among the fermentation products, there are indications from the genomes that pyruvate could be used in the reductive pathway as an electron acceptor via NADH-dependent reduction by lactate dehydrogenase acting in reverse and resulting in lactate formation.

In addition to acetate and lactate, propionate was also found as a product of in anaerobic metabolism in sugar-utilizing strains (Fig. 2). We checked all genomes for the presence of any of metabolic pathways known to allow the formation of propionate, but the enzymes likely to be responsible for this process could not be clearly annotated. In contrast, propionate production by the alkaliphilic strain likely occurs via the succinate pathway (Gonzalez-Garcia *et al.*, 2017). Both key enzymes of this pathway, methylmalonyl-CoA mutase (AArcS\_0912, \_0922 and \_1052) and methylmalonyl-CoA decarboxylase (AArcS\_0961), were found in the genome of

“*Natranaeroarchaeum sulfidigenum*”. In *Propionigenium modestum* and some other fermenting bacteria, the catabolism of pyruvate or phosphoenolpyruvate to succinate (via the dicarboxylic branch of the TCA cycle) is employed as an electron sink, an alternative to formation of ethanol. While less energy is gained via production of succinate and acetate rather than via the dissimilation of glucose to acetate and ethanol, this bacterium has evolved the mixed acid fermentation strategy of energy conservation (Hilpert and Dimroth, 1991; Gonzalez-Garcia *et al.*, 2017). This pathway in *Propionigenium modestum* couples the decarboxylation of methylmalonyl-CoA, derived from succinyl-CoA, to propionyl-CoA with the pumping of two sodium ions across the cell membrane. Although it is premature to assert the existence of similar machinery in AArc-S, it should be emphasized that two secondary Na<sup>+</sup>-translocating pumps (sodium:calcium antiporter, AArcS\_0954 and solute sodium symporter, AArcS\_0962) are collocated in the same operon with methylmalonyl-CoA decarboxylase.

As stated above, the only two whole genome-sequenced strains, HSR-Est and AArc-S were able to directly utilize complex polysaccharides such as glycogen, starch and other alpha-D-glucans. These data were confirmed by genome annotations. In fact, despite the presence of an impressive amount of CAZymes genes found in all sequenced genomes (Table 2), only these two strains harbour multiple extracellular  $\alpha$ -amylases of GH13 family. Joint action of these hydrolases outside the cells can ensure the cleavage of alpha-glucosidic linkages, present in exogenous glucans, and production of malto-dextrins and, finally, glucose (Fig. 6).

385

#### *Hydrogen production and hydrogen uptake*

The conversion of pyruvate by PFOR is accompanied by the formation of reduced ferredoxin, which may participate in the removal of excess electrons, generated by



the oxidative branch of glycolysis, through the production of hydrogen. This was  
390 detected during the anaerobic cultivation of all our neutrophilic isolates. To convert  
this reduced electron carrier to hydrogen, an appropriate 'interface' for energy  
conservation should be implemented, resembling the redox balance module in  
acetogen *Acetobacterium woodii* by Wiechmann *et al.* (2020). According to the  
genome analysis, in our strains, this module likely consists of flavin-containing  
395 ferredoxin-NAD(P)H reductase and cytosolic [NiFe]-hydrogenase (H<sub>2</sub>ase). The latter  
enzyme is a heterotetramer, structurally resembling the [NiFe]-hydrogenases of the  
Group 3b, found primarily in thermophilic archaea (Vignais *et al.*, 2001; Peters *et al.*,  
2015). Some of tetrameric hydrogenases of this group are bidirectional and might be  
able to reduce sulfur to H<sub>2</sub>S (acting as sulfur reductase/hydrogenase) to facilitate the  
400 fermentation by redirecting electrons from H<sub>2</sub> to a formation of H<sub>2</sub>S, as reported in  
*Pyrococcus furiosus* (Ma *et al.*, 1993; 2000). Like these complexes, the haloarchaeal  
large (HydA) and small (HydB) subunits represent the minimal [NiFe]-hydrogenase  
structure, with the two other subunits (HydC and HydE) containing FAD/NAD(P)-  
binding domain and iron-sulfur clusters, respectively. Along with the *hydD* gene for  
405 the H<sub>2</sub>ase-maturation protease, all genes are collocated in a singular operon. A  
BLAST search with the [NiFe]-H<sub>2</sub>ase subunits revealed that structurally identical  
operons occurred only in the genomes of *Halorhabdus* species that are facultatively  
fermenting haloarchaea with proven activities to produce gas from sugars (Antunes  
*et al.*, 2008; Werner *et al.*, 2014). Besides bacterial counterparts, the only other  
410 similar operons harbouring such cytoplasmic heterotetrameric [Ni-Fe] hydrogenase  
were found in the genome of the hydrogenotrophic sulfur-reducing *Halalkaliarchaeum*  
*desulfuricum* AArc-SI (Sorokin *et al.*, 2018a) and in the metagenome-assembled  
genomes of various euryarchaea, such as extremely halophilic MSBL1 candidate  
division (Mwirichia *et al.*, 2016), *Thermoplasmata* and *Candidatus* Bathyarchaeota

415 (Fig. 7). This observation suggests that the acquisition of the Group 3b [NiFe]-hydrogenase by the sulfur-respiring and *Halorhabdus*-related fermentative haloarchaea occurred relatively recently.

Remarkably, the adjacent downstream operon harbours a set of genes encoding the additional H<sub>2</sub>ase-maturation protease and heteropentameric [NiFe]-hydrogenase of the Group 3d (Fig. 7). Structurally similar pentameric hydrogenases  
420 have previously been found in some oxygenic cyanobacteria, anoxygenic photosynthetic purple sulfur gammaproteobacteria of the genera *Allochromatium* and *Thiocaspa*, but have never been reported in archaea (Vignais and Billoud, 2007 for further references). These latter bidirectional [NiFe]-H<sub>2</sub>ases made of the dimeric H<sub>2</sub>-  
425 activating hydrogenase moiety HoxYH and trimeric NAD-activating diaphorase moiety HoxFUE (Vignais and Billoud, 2007; Søndergaard and Pedersen, 2016). The subunits of diaphorase moiety contain NAD-, FMN- and Fe-S-binding sites and are highly homologous to NuoF, NuoG and NuoE subunits of peripheral arm of NADH:ubiquinone oxidoreductase (Complex I). In addition to this structural similarity,  
430 there is another remarkable feature inherent in genomes of cyanobacteria and '*Halarchaeoglobus*' isolates, namely the absence of the NADH:ubiquinone oxidoreductase subunits. Although a hypothesis proposing a common use of the diaphorase subunits by the bidirectional Group 3d [NiFe]-hydrogenase and the respiratory Complex I in cyanobacteria was formulated (Vignais and Billoud, 2007 for  
435 further references), without additional experiments we can neither discount nor verify the possibility that heteropentameric [NiFe]-hydrogenase of the Group 3d could be linked to Complex I in our isolates.

Apart from the production of hydrogen, our physiological experiments showed that at least one '*Halarchaeoglobus desulfuricus*' isolate (HSR12-2) could gain  
440 energy from an anaerobic hydrogen oxidation with elemental sulfur as the terminal

electron acceptor (Fig. 3). This finding alludes to their capacity for intracellular hydrogen cycling; a rather rare metabolic ability, previously described in some sulfate reducing bacteria, methanogenic archaea, and acetogenic bacteria (Odom and Peck, 1981a,b; Kulkarni *et al.*, 2018; Wiechmann *et al.*, 2020), but never previously seen in  
445 haloarchaea. According to the model of Odom and Peck (1981b), hydrogen that is produced inside the cell diffuses across the membrane and is oxidised outside the cell, producing  $2\text{H}^+$  and electrons. Thus, production of scalar electrons lead to creation of a proton motive force across the cytoplasmic membrane that drives ATP synthesis. This mechanism requires both (a) soluble, cytoplasmic  $\text{H}_2$ -producing  
450 hydrogenase(s) and an extracellular, membrane-bound uptake hydrogenase in a single organism.

Inspection of annotated '*Halarchaeoglobus desulfuricus*' genomes revealed a set of genes, collocated in a single operon, encoding the classical membrane-bound [NiFe]- $\text{H}_2$ ase of the Group 1a (Fig. 7). The subunits include the small FeS subunit  
455 HyaA (39.2 KDa), the large NiFe subunit HyaB (55.1 KDa) and membrane-anchored diheme cytochrome *b* subunit HyaC (35.9 KDa). Following the current hydrogenase classifier HydDB, all known members of the Group 1a are the heterotrimeric respiratory  $\text{H}_2$ -uptake and  $\text{O}_2$ -sensitive (NiFe variants) or  $\text{O}_2$ -tolerant (NiFeSe variants) hydrogenases (Søndergaard *et al.*, 2016;  
460 <https://services.birc.au.dk/hyddb/>). Phylogenetic analysis of the full-length HyaA subunit revealed that together with homologous subunits of sulfur-respiring lithoheterotrophic haloarchaea they are organized in a deeply branched and topologically robust cluster, distantly related to the Group 1a [NiFe] hydrogenases found in hydrogen-oxidizing  $\text{Fe}^{3+}$ -reducing *Archaeoglobi*, in methanogens and in  
465 some unclassified archaea (Figure 7).

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

In our cultivation experiments, both neutrophilic and alkaliphilic isolates grew under microoxic conditions. Genomic analysis indicated that their aerobic respiration likely proceeds via a membrane-bound electron transport chain (Complex I, NADH-quinone oxidoreductase and Complex II, succinate: quinone oxidoreductase) that is terminated in the heme-copper containing terminal oxidases (Complex IV). All genomes lack genes encoding the Complex III (quinol: cytochrome *c* oxidoreductase) and genes encoding for the *c*-type cytochromes. All these findings indicated that their Complex IV should belong to the quinol-type of terminal oxidases, which are capable of using menaquinols as the electron donors. Most likely, the quinol oxidase belongs to the cytochrome *bo*<sub>3</sub> family (Figure 6; Extended Data 2).

Apart from the capability for fermentation, all novel '*Halarchaeoglobus*' isolates and AArc-S exhibit the type of anaerobic respiration very similar to those of previously described sulfidogenic haloarchaea (Sorokin *et al.*, 2016a, 2017a, 2018a). Taken together all of them are able to grow aerobically, to use elemental sulfur and DMSO for anaerobic respiration and two out of 10 isolates can also respire thiosulfate (2-electron reduction to sulfide+sulfite). In correspondence to observed phenotypes, the '*Halarchaeoglobus*' genomes encode an analogous set of molybdopterin oxidoreductases (Psr/Phs, DMSOr and Ttr/Arr) from the CISM superfamily and lack the formate dehydrogenases (Fdh) (Figure 8). Remarkably, the AArc-S genome consists of only two Psr/Phs reductases (AArcS\_0638-41 and AArcS\_0990-2), thus representing a 'minimal' respiratory suite, needed for polysulfide and thiosulfate respiration. This irreducible genetic potential allows AArc-S to be used as a model for unambiguously assigning these terminal respiratory reductases to the observed respiration types. Among them, the terminal respiratory reductase AArcS\_0990-2 appears to be the polysulfide reductase, since its subunits

are transcribed together with extracellular sulfurtransferase/rhodanese-like protein AArcS\_0988. Previously, we pointed out the significance of sulfurtransferase in  
495 respiration with sulfur, acting as a polysulfide-binding carrier and sulfur supplier for the catalytic subunit PsrA of polysulfide reductases (Sorokin *et al.*, 2016a, 2017a; 2018a).

Phylogenetic analysis of the catalytic subunits of CISM enzymes, detected in the '*Halarchaeoglobus*' isolates and AArc-S, revealed that together with few other  
500 haloarchaeal counterparts, their Psr/Phs, DMSOr and Ttr/Arr enzymes form deep branches within the corresponding CISM families and likely represent ancient forms of molybdopterin oxidoreductases acquired from bacteria (Figure 8). Analysis of the currently available *Halapricum salinum* CBA1105<sup>T</sup> genome (CP031310) demonstrated the presence of a single set of polysulfide reductase genes  
505 ACP98\_RS15450-95, which resembles the genuine rhodanese-containing Psr operon (Sorokin *et al.*, 2018a), suggesting that this organism might be capable of anaerobic sulfur respiration. Indeed, using the elemental sulfur as terminal electron acceptor, a slight sulfidogenic activity (3 mM H<sub>2</sub>S) was detected during the one-month-long anaerobic cultivation of *Halapricum salinum* CBA1105<sup>T</sup> with glucose as  
510 an electron donor.

## Conclusion

Interest in, and missions/planned mission for, life detection on Mars, Enceladus, and Europa – and the recent detection of subglacial brines on Mars (Orosei *et al.*, 2018; 515 Lauro *et al.*, 2020) – make it imperative to understand the types of metabolism that terrestrial microbes are capable of, especially in saline and briny environments. The extremely halophilic archaea (particularly those that are lithoheterotrophic) are

especially useful as models for studying the metabolic and biophysical adaptability or terrestrial life in the context of astrobiology (Rummel *et al.*, 2014). Many of them are:

- 520 - physiologically versatile,
- polyextremophilic,
- extremely xerophilic (Stevenson *et al.*, 2015),
- either psychrotolerant/-philic or thermotolerant/-philic,
- tolerant to radiation, perchlorate and magnesium (Oren *et al.*, 2014), and
- 525 - can potentially survive in many kinds of planetary bodies (at low temperature, extremes of pressure, salt saturation, anoxia, high concentration of magnesium sulfate and perchlorate). It is halophilic archaea, for example, that are the most likely candidates, capable of inhabiting the Martian subglacial polar lakes or the icy moons Enceladus and Europa.

530           The current study acted to conceptually define a minimal, independent, and self-sufficient hypersaline anoxic ecosystem, based on (or including) haloarchaea. Initially, we isolated and characterised the obligate anaerobic haloarchaea that act as 'secondary' (litho)heterotrophic anaerobes, living by utilization of hydrogen, formate and acetate (fermentation products) as electron donors for anaerobic sulfur

535           respiration (Sorokin *et al.*, 2016a; 2017a; 2018a). Here, we report the enrichment and isolation in pure culture of two variety of haloarchaeal analogues of 'primary' anaerobes that utilize alpha-glucans, simple sugars and glycerol as carbon and energy source. The neutrophilic haloarchaea are fermentative, producing H<sub>2</sub>, while a natronoarchaeon is a nonfermentative saccharolytic. All of them are capable of the

540           dissimilatory sulfur respiration; and sulfur is known to be ubiquitous in our Solar System.

          Currently, the only component missing for a complete haloarchaeal sulfur-reducing ecosystem to function in a self-sufficient manner is the presence of a certain

type of organisms (phototrophs or chemolithotrophs), which are responsible for the  
545 primary production of organic material. In case of terrestrial hypersaline anoxic  
ecosystems, they are fed by the constant input of organic material settled from the  
superior aerobic compartments. Yet, to the best of our knowledge, the nature, origin  
and type of metabolism of eventual primary producers is hardly discussed in the  
context of astrobiology. If we could manage to find and cultivate a CO<sub>2</sub>-fixing  
550 (autotrophic) member of the haloarchaeal sulfur-reducing ecosystem, this would be a  
major breakthrough in understanding of metabolic adaptation and would lead to new  
discoveries in astrobiology and the possible distribution of life elsewhere in the  
universe.

## 555 **Experimental procedures**

### *Samples and sediment activity incubation*

The upper 10 cm of sediment and near-bottom brine samples (2-5 cm above the  
sediment surface) were obtained from three pH-neutral hypersaline chloride-sulfate  
lakes and three hypersaline soda lakes in Kulunda Steppe (Altai, Russia) in 2011-  
560 2015. Additional pH-neutral samples were obtained from two large hypersaline lakes  
Elton and Baskunchak in the south Russia (2012-2013) and from a sea solar saltern  
end evaporation pond in Eupatoria (Crimea) (Supplementary Table S1). Sediment  
samples and overlaid brines were collected into a corer with an internal diameter of  
25 mm, extruded into a sterile 200 ml Schott flask, closed without air bubbles and  
565 transported into the laboratory in insulated box within 3 days after sampling. After  
arrival at the laboratory, the samples were transferred immediately into glass bottles  
closed with rubber stoppers and kept under argon at 8°C until processing for several  
days.

To check for possible carbohydrate-dependent sulfur-reducing potential, mixed  
570 sediment slurries (10 ml) were prepared from the sediment-surface layer (upper 5  
cm) and bottom brines (1:1). 10-ml slurries were dispensed into 30-ml serum bottles  
and powdered sulfur (10 mg) was added as the electron acceptor. Soluble starch (10  
mg), glucose (2 mM), or glycerol (2 mM) were added as electron donors. One set of  
incubations was done for determinations of total activity and to another one a mixture  
575 of three antibiotics (streptomycin, kanomycin and vancomycin, 200 mg l<sup>-1</sup> each) was  
added to determine any contribution of haloarchaea to the sulfur reduction process.  
The bottles, closed with butyl rubber stoppers, were made anoxic by three cycles of  
evacuation-flushing with argon gas and incubated statically, with periodic hand-  
mixing, at 25°C for 3-20 days. During this time there was regular monitoring of sulfide  
580 formation as it described below.

#### *Enrichment and cultivation conditions*

Two mineral basic media (4 M total Na<sup>+</sup>) were used for enrichments and cultivation;  
an NaCl-base medium and an Na<sub>2</sub>CO<sub>3</sub>-base medium. The NaCl-base medium, with  
585 a final pH 7.0 contained (g l<sup>-1</sup>): NaCl, 240; KCl, 5; K<sub>2</sub>HPO<sub>4</sub>, 2.5; NH<sub>4</sub>Cl, 0.5; HEPES,  
4, was used for neutrophilic haloarchaea. The sodium carbonate-base medium, with  
final pH 10.0 contained (g l<sup>-1</sup>): Na<sub>2</sub>CO<sub>3</sub>, 190; NaHCO<sub>3</sub>, 30; NaCl, 16; KCl, 5.0; and  
K<sub>2</sub>HPO<sub>4</sub>, 1.0. After autoclave sterilization, both base media were supplemented with  
1 mM MgCl<sub>2</sub>, 1 ml l<sup>-1</sup> of acidic trace metal solution and vitamin mix (Pfennig and  
590 Lippert, 1966), 1 ml l<sup>-1</sup> of alkaline Se/W solution (Plugge, 2005) and 20 mg l<sup>-1</sup> of yeast  
extract. NH<sub>4</sub>Cl (4 mM) was also added to the sodium carbonate basic medium. A 1:1  
mix of these two media with a final pH 9.6 was used for enrichment and cultivation of  
natronoarchaea from soda lakes. For the pH range in activity tests of  
natronoarchaea, a range of buffers containing 4M total Na<sup>+</sup> was employed: 50 mM



595 HEPES (pH from 6 to 8); combination of two basic media in different proportions (pH  
8.5-9.5) and titrating by 4M NaOH of the two media buffers mix (1:1) (pH from 9.5 to  
11). Elemental sulfur flour (J.T. Backer, Netherlands) was made as a paste by adding  
Milli-Q water (10% of sulfur, vol/vol) and sterilized at 110 °C for 30 min and after  
cooling the clean water phase was decanted. Sulfur was added to enrichment at  
600 approximately 2 g l<sup>-1</sup>. Both DMSO and sodium thiosulfate stock solutions (2 M in each  
case; both from Sigma-Aldrich) were filter-sterilized and added to enrichments at 10  
and 20 mM, respectively. For the neutrophilic haloarchaea 40 mM of filter-sterilized  
NaHCO<sub>3</sub> was added to prevent acidification due to formation of organic acids during  
carbohydrate fermentation. Cultivation was performed at 30°C (natronoarchaea) and  
605 at 37°C - 48°C (haloarchaea) in serum bottles with butyl rubber stoppers filled with  
liquid to 90% of capacity in case of soluble electron donors and 30% of capacity in  
case of using H<sub>2</sub>. The bottles with sterile medium were subjected to 3 cycles of  
evacuation/flushing with sterile argon. Anaerobic conditions were achieved by final  
addition of 0.2 mM Na<sub>2</sub>S from a filter-sterilized 1.0 M stock. H<sub>2</sub> was added through  
610 sterile gas filter at 0.5 bar overpressure on the top of argon atmosphere. The cultures  
were incubated statically with periodic shaking of the flasks. The ability for aerobic  
growth was tested in the absence of sulfur and sulfide, and with liquid-to-gas ratio of  
1:10. Yeast extract (100 mg l<sup>-1</sup>) was added in addition to carbon and energy growth  
substrate. The final concentration of O<sub>2</sub> in the gas phase varied from 2 to 5% (v/v).  
615 Pure cultures of neutrophilic haloarchaea were obtained by multiple rounds of  
decimal serial dilution-to-extinction, which were performed under anaerobic  
conditions in 20 ml serum bottles filled with 10 ml media described above with either  
sulfur as electron acceptor, since colony formation was not observed in soft shake-  
agar cultures. The maximum positive dilutions were determined by sulfide formation.  
620 The single natronarchaeal isolate was purified from colonies obtained in soft agar.

For this, maximal positive serial dilution culture was serially diluted in anoxic medium heated up to 55°C, mixed 3:2 with 4% washed agar also kept at 55°C and the resulted mix pored into plates (total 15 ml). The plates were incubated in 3.5 L anaerostat jars (Oxoid) under argon at 30°C. Single colonies forming decolorized halo around (by reduction of polysulfide) were picked into liquid medium with glucose and sulfur. Finally, the purity of isolates was checked both microscopically and by 16S rRNA sequencing.

### *Analyses*

Formation of sulfide (in case of cultivation of haloarchaea) or sulfane of polysulfide (in case of cultivation of natronoarchaea) was measured using the methylene blue method (Trüper and Schlegel, 1964) after fixing supernatant in 10% (w/v) zinc acetate. Thiosulfate and sulfite were titrated (as a sum and thiosulfate separately in presence of 5% v/v formaldehyde to bind sulfite) with 0.01 N  $I_2$  solution (prepared from a commercial 0.5 M stock, Sigma-Aldrich) in 5% v/v acetic acid after removal of sulfide/polysulfide as zinc sulfide. Fermentation products were analysed by HPLC (BioRad HPX-87H column at 60°C; mobile phase 1.5 mM  $H_3PO_4$ , flow rate 0.6 ml  $min^{-1}$ ; UV/Refraction Index Detector, Waters 2489) after cell removal and 5x dilution of the supernatant to reduce the salt concentration.  $H_2$  and  $CO_2$  were measured by the gas chromatograph (GC) equipped with the methanator catalyst (Chromateck Crystall 5000 [Russia], column Hayesep 80–100 mesh, 2 m x 3 mm, 40°C; Thermal Conductivity Detector [TCD] [for  $H_2$ ] and Flame Ionization Detector [FID] [for  $CO_2$ ], 200°C; carrier gas argon; flow rate 25 ml  $min^{-1}$ ). Dimethyl sulfide (DMS) was analysed by GC (Thermo Scientific<sup>TM</sup> Trace GC Ultra, Interscience, Breda, Netherlands), equipped with flame photometric detector (FPD) (150°C) and TCD (160°C) detectors and column Restek column (RT<sup>®</sup>-U-Bond, 30 m x 0.53 mm di x 20

µm df). The inlet temperature was 190°C. Oven temperature was 70°C first 2 min, and then increased up to 190°C for 5 min. Helium (10 ml min<sup>-1</sup>) was the carrier gas and the injection volume was 250 µl. The cell protein was determined by the Lowry method in 1-2 ml culture samples after centrifugation 13,000 x g for 20 min. The cell pellets were washed with 4 M NaCl at pH 5.0 to remove the cell-bound iron(II) sulfide. Microbial growth was monitored according to increase in optical density at 600 nm (OD<sub>600</sub>). Phase contrast microphotographs were obtained with a Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany) and intracellular polyhydroxyalkanoate inclusions were identified by Nile-Blue staining (Johnson *et al.*, 2009).

#### *Genome sequencing and phylogenomic analysis*

The complete genomes of four neutrophilic and one alkaliphilic sulfur-reducing haloarchaea were sequenced with the MiSeq System of Illumina Inc. (San Diego, CA, USA) using short insert paired-end library (2x250 bp, MiSeq Reagent Kit v2). These genomes were also sequenced with PacBio<sup>TM</sup> technology of Pacific Biosciences of California Inc. (Menlo Park, CA, USA) and, only for strain HSR-Bgl, with MinION system of Oxford Nanopore Technologies (Oxford Science Park, UK) in order to provide the necessary long reads to perform a hybrid assembly. The obtained reads, equivalent to genome coverage of about 322× for HSR12-1, 198× for HSR12-2, 250× for HSR-Bgl, 223× for HSR-Est, and 191× for AArc-S, were respectively assembled by the Unicycler 0.4.6 program (Wick *et al.*, 2017), and then refined with the map to reference function tool provided by Geneious 7.1.9 software (Biomatters Ltd, New Zealand). Contigs assembled with Geneious 7.1.9 software were also used for control. Protein genes, rRNA operons, tRNAs and CRISPR regions were respectively predicted by Glimmer 3.02 (Delcher *et al.*, 2007),

RNAmmer 1.2 online server (Lagesen *et al.*, 2007), tRNAscan-SE 2.0 online tool (Lowe and Chan, 2016), and CRISPRFinder online program (Grissa *et al.*, 2007).  
675 The presence of prophages was investigated by the online tool PHASTER (<https://phaster.ca/>) (Arndt *et al.*, 2016). Genomics islands prediction was obtained by IslandViewer 4 (Bertelli *et al.*, 2017). Further checks for annotation consistency were performed using the FgenesB online tool for operon prediction (Solovyev and Salamov, 2011), PATRIC/RAST server (Aziz *et al.*, 2008), and NCBI blastx against  
680 nr, arCOG and KEGG database (Altschul *et al.*, 1997; Makarova *et al.*, 2015; <http://www.genome.jp/tools/blast/>) were used for protein annotation and EC number control. Metabolic pathways reconstruction was refined using BlastKOALA online tool (<https://www.kegg.jp/blastkoala/>) (Kanehisa *et al.*, 2016). Genomics comparisons were visualized using Circos (Krzywinski *et al.*, 2009). The annotation data and  
685 bidirectional blastp comparison of amino-acid identity between different genomes were obtained by RAST server. Artemis 16.0 software (Rutherford *et al.*, 2000) was used for final manual check before submission on NCBI GenBank. Maximum Likelihood tree of 61 haloarchaeal genomes was constructed in the same manner described in Sorokin *et al.* (2016a), by selecting six ribosomal conserved proteins  
690 concatenated to form a sequence ready to be aligned with Clustal Omega 1.2.3 (Sievers *et al.*, 2011, 2018) and then constructed using PhyML 3.3 (Guindon *et al.*, 2010) with Blosum62 substitution model and 100 bootstrap resampling. 16S rRNA gene phylogeny of the strains was computed from a 16S rRNA gene sequence alignment with PAUP\*4.b10 using a LogDet/paralinear distance method as it  
695 described elsewhere (Sorokin *et al.*, 2016a).

#### *Data deposition*

16S rRNA gene sequences were deposited on NCBI GenBank database (accession no. MW183131 and MW183132). The complete genomes of five sulfur-reducing  
700 strains were deposited on GenBank database by the NCBI Genome submission portal. BioProject PRJNA670096 has been processed as *Halobacteriaceae* archaeon AArc-S. BioProjects PRJNA670127, PRJNA670151, and PRJNA670153 were merged into PRJNA670125, and all four of the Biosamples are linked to this BioProject. The organism name for the BioProject is *Haloarculaceae* archaeon and  
705 for the Biosamples the organism names are: SAMN16484195 *Haloarculaceae* archaeon HSR-Bgl; SAMN16484257 *Haloarculaceae* archaeon HSR-Est; SAMN16484274 *Haloarculaceae* archaeon HSR12-1 and SAMN16484277 *Haloarculaceae* archaeon HSR12-2.

#### 710 **Author contribution**

DS performed sampling and field measurements, sediment incubations and isolation and physiological characterisation of pure cultures. Bioinformatics analyses carried out by EM, FS and VLC. The data were interpreted and manuscript was written by DYS, JEH and MMY. EM, FS and VLC had advisory roles in the aspects of isolates  
715 handling and input into writing of the manuscript.

#### **Conflicts of interest**

The authors declare that they have no current or potential competing financial interests.

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**Table 1.** Sulfur-respiring carbohydrate-oxidizing halo(natrono)archaea from hypersaline lakes.

Isolate	Environmental origin	Conditions for enrichment and isolation			Growth physiology	
		Substrates	pH	T, °C	e-donors <sup>a</sup>	e-acceptors
<b>AArc-S<sup>a</sup></b> (JCM 34033)	Hypersaline Kulunda soda lakes brine/sediment	Glucose + S <sub>8</sub>	9.8	30	Starch, glycogen, cyclodextrin, fructose, glucose, maltose, mannose, raffinose, trehalose, glycerol	S <sub>8</sub> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup> , O <sub>2</sub> <sup>b</sup>
<b>HSR12-1</b> (JCM 34031)	pH-neutral hypersaline Kulunda lakes brine/sediment (Altai, Russia)	Glucose + S <sub>8</sub>	7.5	30	Fructose, glucose, maltose, melibiose, raffinose, sucrose, trehalose, glycerol	S <sub>8</sub> , DMSO <sup>c</sup> , O <sub>2</sub> <sup>b</sup>
<b>HSR12-2<sup>a</sup></b> (JCM 34032)	pH-neutral hypersaline Kulunda lakes brine/sediment (Altai, Russia)	Glucose + S <sub>8</sub>	7.5	30	Fructose, galactose, glucose, maltose, melibiose, raffinose, sucrose, trehalose, glycerol	S <sub>8</sub> , DMSO <sup>c</sup> , O <sub>2</sub> <sup>b</sup> , S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>
<b>HSR-T1</b>	pH-neutral hypersaline Kulunda lakes brine/sediment (Altai, Russia)	Glucose + S <sub>8</sub>	7.5	46	Fructose, glucose, maltose, melibiose, raffinose, sucrose, trehalose, glycerol	S <sub>8</sub> , DMSO, O <sub>2</sub> <sup>b</sup>
<b>HSR-T2</b>	pH-neutral hypersaline Kulunda lakes sediment (Altai, Russia)	Glucose + S <sub>8</sub>	7.5	46	Fructose, glucose, maltose, melibiose, raffinose, sucrose, trehalose, glycerol	S <sub>8</sub> , DMSO, O <sub>2</sub> <sup>b</sup>
<b>HSR-T3</b>	pH-neutral solar saltern brine/sediment (Crimea, Russia)	Glucose + S <sub>8</sub>	7.0	48	Fructose, glucose, maltose, mannose, melezitose, raffinose, sucrose, trehalose, glycerol	S <sub>8</sub> , DMSO, O <sub>2</sub> <sup>b</sup>
<b>HSR-Kgl</b>	pH-neutral hypersaline Kulunda lakes brine/sediment (Altai, Russia)	Glycerol + S <sub>8</sub>	7.5	37	Fructose, glucose, maltose, raffinose, sucrose, trehalose, glycerol	S <sub>8</sub> , DMSO, O <sub>2</sub> <sup>c</sup>
<b>HSR-Bgl</b>	pH-neutral hypersaline Lake Baskunchak brine/sediment (South Russia)	Glycerol + S <sub>8</sub>	6.8	37	Fructose, trehalose, glycerol	S <sub>8</sub> , DMSO <sup>c</sup> , O <sub>2</sub> <sup>b</sup>
<b>HSR-Kst</b>	pH-neutral hypersaline Kulunda lakes brine/sediment (Altai, Russia)	Starch + S <sub>8</sub>	7.5	37	Starch, pullulan, dextrin, glycogen, cyclodextrin, glucose, maltose, sucrose, glycerol	S <sub>8</sub> , DMSO, O <sub>2</sub> <sup>b</sup>
<b>HSR-Bst</b>	pH-neutral hypersaline Lake Baskunchak brine/sediment (South Russia)	Starch + S <sub>8</sub>	6.6	37	Starch, pullulan, dextrin, glycogen, cyclodextrin, glucose, maltose, sucrose, glycerol	S <sub>8</sub> , DMSO, O <sub>2</sub> <sup>b</sup>
<b>HSR-Est</b>	pH-neutral hypersaline Lake Elton brine/sediment (South Russia)	Starch + S <sub>8</sub>	6.6	37	Starch, pullulan, dextrin, glycogen, cyclodextrin, glucose, maltose, sucrose, glycerol	S <sub>8</sub> , DMSO <sup>c</sup> , O <sub>2</sub> <sup>b</sup>

<sup>a</sup> Thiosulfate reduction in HSR12-2 and AArc-S (shown by grey shading) resulted in equimolar formation of HS<sup>-</sup> and SO<sub>3</sub><sup>2-</sup> (two-electron reduction).

925 <sup>b</sup> Microoxic conditions of cultivation for all isolates.

<sup>c</sup> In addition to DMSO, the HSR12-1, HSR12-2, HSR-Bgl and HSR-Est isolates were also able to use methionine and tetramethylene sulfoxides as electron acceptors for anaerobic growth with glucose

**Table 2.** Glycosyl hydrolase genes found in the genomes of sulfur-respiring carbohydrate-oxidizing halo(natrono)archaea from hypersaline lakes.

Glycosyl hydrolase (GH) CAZy Family	Provisional function	Provisional location	Isolates				
			AArc-S <sup>T</sup>	HSR12-1 <sup>T</sup>	HSR12-2	HSR-Bgl	HSR-Est
GH2	$\beta$ -galactosidase / $\beta$ -glucuronidase	cytoplasmic	0	0	0	0	1
GH2	$\beta$ -galactosidase / $\beta$ -glucuronidase	extracellular	1	0	0	0	0
GH3	$\beta$ -glucosidase	cytoplasmic	1	3	0	1	0
GH3	$\beta$ -glucosidase	extracellular	0	0	0	0	1
GH4	$\alpha$ -glucosidase / $\alpha$ -galactosidase	cytoplasmic	1	1	1	1	1 <sup>a</sup>
GH13	$\alpha$ -amylase	cytoplasmic	7	10	10	10	13
<b>GH13</b>	<b><math>\alpha</math>-amylase</b>	<b>extracellular</b>	<b>5</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>4</b>
GH15	Glucoamylase	cytoplasmic	2	0	0	0	1
GH27	$\alpha$ -galactosidase	cytoplasmic	0	1	1	1	1 <sup>a</sup>
GH31	$\alpha$ -glucosidase	Cytoplasmic	0	2	2	2	2
GH32	$\beta$ -fructosidase	Cytoplasmic	1	0	0	1	0
GH42	$\beta$ -galactosidase	Cytoplasmic	1	1	1	1	1 <sup>a</sup>
GH68	Levansucrase / invertase	Cytoplasmic	1	0	0	0	0
GH77	$\alpha$ -glucanotransferase / Amylomaltase	Cytoplasmic	1	2	2	2	1
GH81+CBM6	Endoglucanase with carbohydrate-binding module 6	Extracellular	0	1	0	1	0
<b>Total amount of GHs</b>			<b>21</b>	<b>21</b>	<b>17</b>	<b>20</b>	<b>26</b>

<sup>a</sup> Plasmid

## Figure legends

**Figure 1.** A phylogenetic species tree for the phylum *Halobacteriota* inferred from concatenated alignments of 6 ribosomal proteins, encoded by 61 selected genomes that were obtained from the Genome Taxonomy Database. The amino acids sequences were aligned by Clustal W 2.1 program with Blosum cost matrix and the phylogeny was inferred by PhyML 3.0 plugin software inside Geneious 7.1 with Blosum62 substitution model and 1,000 bootstrap replicates. Bootstrap support values (if >50) are indicated for selected groups at the nodes. Carbohydrate-utilizing sulfur-respiring halo(natrono)archaeal isolates are highlighted in red. The scale bar represents the average number of substitutions per site.

**Figure 2.** Anaerobic growth kinetics (left panels) and product formation (right panels) obtained for carbohydrate-utilizing sulfur-reducing '*Halarchaeoglobus*' isolates grown at 4.0 M NaCl, pH 7 and 37°C: strain HSR12-1 (A, B); strain HSR12-2 (C, D); strain HSR-Bgl (E, F) and strain HSR-Est (G, H). Strains HSR12-1 and HSR12-2 were grown with 10 mM glucose, strain HSR-Bgl - with 10 mM glycerol and strain HSR-Est - with 1 g l<sup>-1</sup> soluble starch. The data are mean values from 2 parallel incubations.

**Figure 3.** Ability of strain HSR12-2 to utilize H<sub>2</sub> as an electron donor for anaerobic growth and sulfur respiration in organic carbon-limited conditions. Yeast extract (YE) and glucose were added in amounts of 200 mg l<sup>-1</sup> and 500 μM, respectively. The data are mean values from duplicate experiments.

**Figure 4.** Anaerobic respiratory activity measured with washed cells of carbohydrate-utilizing sulfur-reducing '*Halarchaeoglobus*' isolates grown with three different electron

acceptors and incubated at 4 M NaCl, pH 7 and 37°C. Cells for cell-suspension experiments were collected at the end of exponential growth phase, washed in anoxic 4 M NaCl buffered with 4 g l<sup>-1</sup> HEPES at pH 7 and resuspended at final protein concentration of 0.2-0.3 mg ml<sup>-1</sup>. The data are mean values from 3 parallel incubations.

**Figure 5.** Anaerobic growth kinetic (A) and sulfide formation (B) obtained for carbohydrate-utilizing sulfur-reducing '*Halarchaeoglobus*' isolate AArc-S grown at 4.0 M total Na<sup>+</sup>, pH 7 and 30°C. Sulfidogenic activity was measured with resting AArc-S cells pre-grown with different electron acceptors (S<sub>8</sub>, thiosulfate and oxygen). The data are mean values from 2 parallel incubations.

**Figure 6.** Predicted key catabolic pathways, energy generation and proton-translocation machineries shared by novel group of sugar-oxidizing sulfur-reducing halo(natrono)archaea.  $\alpha$ -Glucan degradation to malto-dextrins and glucose is likely performed by multiple extracellular  $\alpha$ -amylases of GH13 family produced by strains HSR-Est and AArc-S. Eventual common use in HSR strains of the [NiFe]H<sub>2</sub>ase's diaphorase moiety as peripheral arm by the respiratory Complex I is highlighted as red circle. Enzymes and compounds abbreviations: ABC, ABC-type transporter; CoA, coenzyme A; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; DMSOR, DMSO reductase; F-6P, fructose-6-phosphate; F1,6P<sub>2</sub>, fructose-1,6-biphosphate; G1,3P<sub>2</sub>, 1,3-biphosphoglycerate; G2P, 2-phosphoglycerate; G3P, 3-phosphoglycerate; GA3P, glyceraldehyde-3-phosphate; Gly3P, glycerol-3-phosphate; HYA, membrane-bound [NiFe]-H<sub>2</sub>ase; MFS, major facilitator superfamily transporter; NNT, nicotinamide nucleotide transhydrogenase; PEP, phosphoenolpyruvate; PHS, thiosulfate reductase; PSR, polysulfide reductase; TAT, twin-arginine translocation system.

**Figure 7.** Organization of gene clusters encoding [NiFe]-hydrogenases HydECBA, HoxEFUYH and HyaABC in the genome of '*Halarchaeoglobus desulfuricus*' HSR12-1 (A) and phylogenetic tree of [NiFe]-hydrogenases of sulfur-reducing halo(natrono)archaea constructed with full-length catalytic subunits (B). Arrows show the direction of transcription. Bars positioning of operons and the genes are drawn to scale. The genes associated with neither [NiFe]-hydrogenase subunit are shown in black colour. Localization and predicted multimeric structure of both soluble (cytosolic) and membrane-bound [NiFe]-hydrogenases is shown as cartoon between the operons. The '*Halarchaeoglobus desulfuricus*' isolates and halo(natrono)archaea with proven capability of sulfur-reducing growth are highlighted in red and black, respectively. The bootstrap values of more than 50%, supporting topological placement of catalytic subunits, are displayed as grey circles to the corresponding nodes. The Group 2a [NiFe]-hydrogenase of *Ca. Syntrophoarchaeum caldarius* (GenBank accession no. OFV67551) was used as the out-group. Branch lengths along the horizontal axis reflect the degree of relatedness of the sequences (50%).

**Figure 8.** Maximum Likelihood phylogenetic tree of catalytic subunits of molybdopterin oxidoreductases Psr/Phs, DMSOr and Ttr/Arr from the CISM superfamily (A) and respiratory chain components in experimentally proven sulfur-reducing haloarchaea (B). Totally 108 sequences were taken for the CISM analysis. The tree with the highest log likelihood is shown. Locus tags of CISM proteins of novel group of sugar-oxidizing sulfur-reducing halo(natrono)archaea and other sulfur-reducing members of the phylum *Halobacteriota* are highlighted in bold red and black, respectively. The polysulfide reductase subunits, transcribed together with sulfurtransferase/rhodanese-like protein, are shown by asterisk. Abbreviations used: DMSO/Nar, DMSO/nitrate reductase family;



Psr/Phs, polysulfide/thiosulfate reductase family; Ttr/Arr, tetrathionate/arsenate reductase family. Scale bar is 0.1 amino acid substitutions per site. Purple boxes (B) highlight the organisms with Psr/Phs reductases representing a “minimal” respiratory suit, needed for observed respiration type.