Carbohydrate-dependent sulfur respiration in halo(alkali)philic archaea

Sorokin, Dimitry Y.; Messina, Enzo; Smedile, Francesco; La Cono, Violetta; Hallsworth, John E.; Yakimov, Michail M.

DOI
10.1111/1462-2920.15421

Publication date
2021

Document Version
Accepted author manuscript

Published in
Environmental Microbiology

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

Dimitry Y. Sorokin[1,2], Enzo Messina[3], Francesco Smedile[3], Violetta La Cono[3], John E. Hallsworth[4] & Michail M. Yakimov[3*]

[1]*Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia;* [2]*Department of Biotechnology, Delft University of Technology, Delft, The Netherlands;* [3]*Institute of Biological Resources and Marine Biotechnology, CNR, Messina, Italy;* [4]*Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, BT9 5DL Northern Ireland, United Kingdom.*

For correspondence: Tel.+39(090)6015437; Email:

Keywords: haloarchaea; hypersaline lakes; soda lakes; sulfur respiration; polysulfide 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 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 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). Natronoarchael 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 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 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_2 as the electron donor and elemental sulfur, thiosulfate or dimethyl sulfoxide (DMSO) as the electron acceptor (Sorokin *et al*., 2017a; 2018a), being a 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_2, C_4-C_9 fatty acids and peptone (Sorokin *et al*., 2018a; 2020b).

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; 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 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 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 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 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 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

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

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 g l^{-1}) as an electron acceptor, the appropriate medium was inoculated with mixed anaerobic sediment/brine slurries (5% v/v). Soluble starch (1 g l^{-1}), glucose or glycerol (2 mM each) were added 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 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 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 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_2 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 *rrn*A and *rrn*B 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/ANIm 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 *rrn*A and *rrn*B 16S rRNA genes (<93% of gene identity). Analysis of these intraspecific polymorphic 16S rRNA gene sequences revealed that the *rrn*A type formed a cluster, mostly related to the corresponding gene of *Halapricum salinum* CBA1105^T (<96.7% of gene identity), while the *rrn*B type was distantly related to *Halosiccatus urmianus* DC8^T (<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 confir 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 isollated 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 riboclones 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 sigle copies, confirming the purity of isolates. Using the Average Nucleotide Identity (ANIb, AAIm) and Amino Acid Identity (AAI) analyses parameters, the genomic similarities between the type strain of the genus *Halapricum salinum* CBA1105^T 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/AAIm 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_2 in the gas phase; (ii) fermentation of several hexoses and glycerol and (iii) anaerobic respiration using elemental sulfur (S_8) 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_2-oxidizing *Halodesulfuriarchaeum* (Sorokin and Yakimov, 2018).

Fermentative growth of all of the neutrophilic isolates was accompanied by production of H_2, 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_2S as the final product instead of H_2. 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_2 as an electron donor at a low level of organic carbon supplied as yeast extract (100 mg l^{-1}), 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_2, while only the most metabolically versatile strain HSR12-2 showed fully positive response to H_2 addition by increase in biomass yield and intensive formation of sulfide in comparison to the incubation without H_2 (Fig. 3). That could be taken as an indication of the respiratory mode of sulfur reduction in these carbohydrate-utilizing haloarchaea, instead of faciltated 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 substrates but with H_2, pointing to an essential role of H_2 as the actual electron donor for sulfur respiration. Moreover, formation of H_2 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 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_2, was provided, thiosulfate reduction by these cells was two- to three-fold less that the corresponding sulfur-reducing activities. The aerobically grown cells did not show any capability of 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. Its inability to respire with DMSO was another phenotypic difference. The total amount of sulfide/sulfane of 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 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 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 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 genes, and two CRISPR repeat regions with one CRISRP-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 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 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

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, 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 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 respirastory type of molybdopterin oxidoreductase Psr/Phs (Sre in other archaea) family within the complex iron-sulfur 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 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 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).

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 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 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 duplication found (as for glycerol-3-phosphate dehydrogenase [G3PDH] and dihydroxyacetone kinase). In bacteria, G3PDH, which oxidizies 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 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_2. 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^+ 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^+-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 α-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).

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 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 gtenome analysis, in our strains, this module likely consists of flavin-containing ferredoxin-NAD(P)H reductase and cytosolic [NiFe]-hydrogenase (H_2ase). 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_2S (acting as sulfur reductase/hydrogenase) to facilitate the fermentation by redirecting electrons from H_2 to a formation of H_2S, 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 the H_2ase-maturation protease, all genes are collocated in a singular operon. A BLAST search with the [NiFe]-H_2ase 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 similar operons harbouring such cytoplasmic heterotetrameric [Ni-Fe] hydrogenase were found in the genome of the hydrogenotrophic sulfur-reducing *Halalkaliarchaeum desulfuricum* AArc-Sl (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

(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_2ase-maturation protease and heteropentameric [NiFe]-hydrogenase of the Group 3d (Fig. 7). Structurally similar pentameric hydrogenases have previously been found in some oxygenic cyanobacteria, anoxigenic 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_2ases made of the dimeric H_2-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, 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 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 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, metanogenic archaea, and acetogenic bacteria (Odom and Peck, 1981a,b; Kulkarni *et al*., 2018; Wiechmann *et al*., 2020), but never previously seen in 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 $2H^+$ 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 H_2-producing 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]-H_2ase of the Group 1a (Fig. 7). The subunits include the small FeS subunit 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 H_2-uptake and O_2-sensitive (NiFe variants) or O_2-tolerant (NiFeSe variants) hydrogenases (Søndergaard *et al*., 2016; 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 Fe^{3+}-reducing *Archaeoglobi*, in methanogens and in 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_3 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 suldide+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 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 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^T genome (CP031310) demonstrated the presence of a single set of polysulfide reductase genes 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_2S) was detected during the one-month-long anaerobic cultivation of *Halapricum salinum* CBA1105^T with glucose as 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; 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:

- 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
- 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.

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 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 neutraphilic haloarchaea are fermentative, producing H_2, while a natronoarchaeon is a nonfermentative saccharolytic.All of them are capable of the 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 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_2-fixing (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.

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 2011-2015. 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 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^{o}C until processing for several days.

To check for possible carbohydrate-dependent sulfur-reducing potential, mixed 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 of three antibiotics (streptomycin, kanomycin and vancomycin, 200 mg l^{-1} 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^{o}C for 3-20 days. During this time there was regular monitoring of sulfide formation as it described below.

Enrichment and cultivation conditions

Two mineral basic media (4 M total Na^+) were used for enrichments and cultivation; an NaCl-base medium and an Na_2CO_3-base medium. The NaCl-base medium, with a final pH 7.0 contained (g l^{-1}): NaCl, 240; KCl, 5; K_2HPO_4, 2.5; NH_4Cl, 0.5; HEPES, 4, was used for neutrophilic haloarchaea. The sodium cabonate-base medium, with final pH 10.0 contained (g l^{-1}): Na_2CO_3, 190; $NaHCO_3$, 30; NaCl,16; KCl, 5.0; and K_2HPO_4, 1.0. After autoclave sterilization, both base media were supplemented with 1 mM $MgCl_2$, 1 ml l^{-1} of acidic trace metal solution and vitamin mix (Pfennig and Lippert, 1966), 1 ml l^{-1} of alkaline Se/W solution (Plugge, 2005) and 20 mg l^{-1} of yeast extract. NH_4Cl (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^+ 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^{-1}. 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 $NaHCO_3$ 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_2. 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_2S from a filter-sterilized 1.0 M stock. H_2 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^{-1}) was added in addition to carbon and energy growth substrate. The final concentration of O_2 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 formadehyde to bind sulfite) with 0.01 N J_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™ Trace GC Ultra, Interscience, Breda, Netherlands), equipped with flame photometric detector (FPD) (150°C) and TCD (160°C) detectors and column Restek column (RT®-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^{-1}) 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_{600}). 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™ 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 Geneiuos 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). 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 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 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 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 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 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 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.

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 handling and input into writing of the manuscript.

Conflicts of interest

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

Acknowledgements

DYS was supported by research funds from Russian Foundation for Basic Research (RFBR grant 19-04-00401), Russian Ministry of Science and Higher Education and

by the Gravitation-SIAM Program of the Dutch Ministry of Education and Science (grant 24002002). EM, FS, VLC and MMY were supported by the European Commission's Horizon 2020 Program under INMARE Project (Contract 634486).

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. *et al*. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389−3402.

Antunes, A., Taborda., M, Huber, R., Moissl, C., Nobre, M.F. and da Costa, M.S. (2008). *Halorhabdus tiamatea* sp. nov., a non-pigmented, extremely halophilic archaeon from a deep-sea, hypersaline anoxic basin of the Red Sea, and emended description of the genus *Halorhabdus*. *Int J Syst Evol Microbiol* **58**: 215–220.

Arndt, D., Grant, J.R., Marcu, A., Sajed, T., Pon, A., Liang Y. *et al*. (2016). PHASTER: a better, faster version of the PHAST phage search tool. Web Server issue. *Nucleic Acids Res* **44**: W16–W21.

Aziz, R.K., Bartels, D., Best, A.A., De Jongh, M., Disz, T., Edwards, R.A. *et al*. (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* **9**: 75.

Bertelli, C., Laird, M.R., Williams, K.P., Lau, B.Y., Hoad G, Winsor, G.L. *et al*. (2017). IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets. Web Server issue. *Nucleic Acids Res* **45**: W30–W35.

Delcher, A.L., Bratke, K.A., Powers, E.C. and Salzberg, S.L. (2007). Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics* **23**: 673–679.

Dinsdale, E.A., Edwards, R.A., Hall, D., Angly, F., Breitbart, M., Brulc, J.M., *et al*. (2008). Functional metagenomic profiling of nine biomes. *Nature* **452**: 629–632.

Elevi Bardavid, R., Khristo, P., and Oren, A. (2008). Inter-relationships between *Dunaliella* and halophilic prokaryotes in saltern crystallizer ponds. *Extremophiles* **12**: 5–14.

Gonzalez-Garcia, R.A., McCubbin, T., Wille, A., Plan, M., Nielsen, L. And Marcellin, E. (2017). Awakening sleeping beauty: production of propionic acid in *Escherichia coli* through the sbm operon requires the activity of a methylmalonyl-CoA epimerase. *Microb Cell Factor* **16**: 121.

Grissa, I., Vergnaud, G. and Pourcel, C. (2007). The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. *BMC Bioinformatics* **8**: 172.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* **59**: 307−321.

Hilpert, W. and Dimroth, P. (1991). On the mechanism of sodium ion translocation by methylmalonyl-CoA decarboxylase from *Veillonella alcalescens*. *Eur J Biochem* **195**: 79–86.

Iuchi, S., Cameron, D.C. and Lin, E.C. (1989). A second global regulator gene (arcB) mediating repression of enzymes in aerobic pathways of *Escherichia coli*. *J Bacteriol* **171**: 868–873.

Johnson, K., Jiang, Y., Kleerebezem, R., Muyzer, G., van Loosdrecht, M.C. (2009). Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity. *Biomacromolecules* **10**: 670−676.

Kanehisa, M., Sato, Y. and Morishima, K. (2016). BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J Mol Biol* **428**: 726–731.

Kulkarni, G., Mand, T.D., and Metcalf, W.W. (2018). Energy conservation via hydrogen cycling in the methanogenic archaeon *Methanosarcina barkeri*. *MBio* **9**: 1–10.

Krzywinski, M.I., Schein, J.E., Birol, I., Connors, J., Gascoyne, R., Horsman, D., *et al*. (2009). Circos: An information aesthetic for comparative genomics. *Genome Res* **19**: 1639-1645.

Lagesen, K., Hallin, P., Rødland, E.A., Staerfeldt, H.H., Rognes, T. and Ussery, D.W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* **35**: 3100−3108.

Lowe, T.M., and Chan, P.P. (2016). tRNAscan-SE on-line: integrating search and context for analysis of transfer RNA genes. *Nucleic Acids Res.* 44: W54−W57.

Ludwig, W., Strunk, O., Westram, R., Richter, L, Meier H, Yadhukumar, B.A., *et a*l. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**:1363–1371.

Ma, K., Schicho, R.N., Kelly, R.M. and Adams, M.W. (1993). Hydrogenase of the hyperthermophile Pyrococcus furiosus is an elemental sulfur reductase or sulfhydrogenase: evidence for a sulfur-reducing hydrogenase ancestor. *Proc Natl Acad Sci U S A* **90**: 5341–5344.

Ma, K., Weiss, R. and Adams, M. (2000). Characterization of hydrogenase II from the hyperthermophilic archaeon *Pyrococcus furiosus* and assessment of its role in sulfur reduction. *J Bacter* **182**: 1864–1871.

Makarova, K.S., Wolf, Y.I. and Koonin, E.V. (2015). Archaeal clusters of orthologous genes (arCOGs): an update and application for analysis of shared features between *Thermococcales*, *Methanococcales*, and *Methanobacteriales*. *Life* (Basel) **5**: 818–840.

Messina, E., Sorokin, D.Y., Kublanov, I.V., Toshchakov, S., Lopatina, A., Arcadi, E. *et al.* (2016). Complete genome sequence of '*Halanaeroarchaeum sulfurireducens*' M27-SA2, a sulfur-reducing and acetate-oxidizing haloarchaeon from the deep-sea hypersaline anoxic lake Medee. *Stand Genomic Sci* **11**: 35.

Mwirichia, R., Alam, I., Rashid, M., Vinu, M., Ba-Alawi, W., Anthony Kamau, A. *et al.* (2016). Metabolic traits of an uncultured archaeal lineage MSBL1 from brine pools of the Red Sea. *Sci Rep* **6**: 19181.

Nelson-Sathi, S., Dagan, T., Landan, G., Janssen, A., Steel, M., McInerney, J.O. *et al.* (2012). Acquisition of 1,000 eubacterial genes physiologically transformed a methanogen at the origin of Haloarchaea. *Proc Natl Acad Sci U S A* **109**: 20537–20542.

Odom, J.M. and Peck, H.D. (1981a). Localization of dehydrogenases, reductases, and electron transfer components in the sulfate-reducing bacterium *Desulfovibrio gigas*. *J Bacteriol* **147**: 161–169.

Odom, J.M. and Peck, H.D. (1981b). Hydrogen cycling as a general mechanism for energy coupling in the sulfate-reducing bacteria, *Desulfovibrio* sp. *FEMS Microbiol Lett* **12**: 47–50.

Oren, A., Bardavid, R.E. and Mana, L. (2014). Perchlorate and halophilic prokaryotes: Implications for possible halophilic life on Mars. *Extremophiles* **18**: 75–80.

Oren A. (2017). Glycerol metabolism in hypersaline environments. *Environ Microbiol* **19**: 851–863.

Orosei, R., Lauro, S.E., Pettinelli, E., Cicchetti, A., Coradini, M., Cosciotti, B. *et al*. (2018). Radar evidence of subglacial liquid water on Mars. *Science* **361**: 490–493.

Lauro, S.E., Pettinelli, E., Caprarelli, G., Guallini, L., Rossi, A.P., Mattei, E. *et al*. (2020). Multiple subglacial water bodies below the south pole of Mars unveiled by new MARSIS data. *Nat Astron*. DOI: 10.1038/s41550-020-1200-6.

Pavankumar, T.L., Mittal, P. and Hallsworth, J.E. Molecular insights into the ecology of a psychrotolerant *Pseudomonas syringae*. *Environ Microbiol* doi.org/10.1111/1462-2920.15304.

Peters, J.W., Schut, G.J., Boyd, E.S., Mulder, D.W., Shepard EM, Broderick JB *et al*. 2015. [FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation. *Biochim Biophys Acta* **1853**: 1350–1369.

Pfennig, N. and Lippert K.D. (1966). Über das Vitamin B12-Bedürfnis phototropher Schwefelbakterien. *Arch Mikrobiol* **55**: 245–256.

Plugge, C.M. (2005). Anoxic media design, preparation, and considerations. *Methods Enzymol* **397**: 3–16.

Rawls, K.S., Martin, J.H. and Maupin-Furlow, J.A. (2011). Activity and transcriptional regulation of bacterial protein-like glycerol-3-phosphate dehydrogenase of the haloarchaea in *Haloferax volcanii*. *J Bacteriol* **193**: 4469-4476.

Roh, S.W., Nam, Y.-D., Chang, H.-W., Sung, Y., Kim, K.-H., Oh, H.-M. et al. (2007). *Halalkalicoccus jeotgali* sp. nov., a halophilic archaeon from shrimp jeotgal, a traditional Korean fermented seafood. *Int J Syst Evol Microbiol* **57**: 2296–2298.

Rummel, J.D., Beaty, D.W., Jones, M.A., Bakermans, C., Barlow, N.G., Boston, P.J. *et al*. (2014). A new analysis of Mars “special regions”: findings of the second MEPAG Special Regions Science Analysis Group (SR-SAG2). *Astrobiol* **14**: 887–968.

Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice P, Rajandream, M.A. *et al*. (2000). Artemis: sequence visualization and annotation. *Bioinformatics* **16**: 944–945.

Sato, T., Atomi, H. and Imanaka, T. (2007). Archaeal type IIIRuBisCOs function in a pathway for AMP metabolism. *Science* **315**: 1003–1006.

Sherwood, K.E., Cano, D.J. and Maupin-Furlow, J.A. (2009). Glycerol-mediated repression of glucose metabolism and glycerol kinase as the sole route of glycerol catabolism in the haloarchaeon *Haloferax volcanii*. *J Bacteriol* **191**: 4307–4315.

Sievers, F. and Higgins, D.G. (2014). Clustal Omega, accurate alignment of very large numbers of sequences. *Methods Mol Biol* **1079**: 105–116.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W. *et al*. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* **7**: 539.

Solovyev, V., and Salamov, A. (2011). Automatic annotation of microbial genomes and metagenomic sequences. In: Li RW (eds.), *Metagenomics and its Applications in Agriculture, Biomedicine and Environmental Studies*, p. 61−78. Nova Science Publishers.

Søndergaard, D. and Pedersen, C.N.S. (2016). HydDB: a web tool for hydrogenase classification and analysis. *Sci Rep* **6**: 34212.

Sorokin, D.Y., Rusanov, I.I., Pimenov, N.V., Tourova, T.P., Abbas, B. and Muyzer, G. (2010). Sulfidogenesis under extremely haloalkaline conditions in soda lakes of Kulunda Steppe (Altai, Russia). *FEMS Microbiol Ecol* 73: 278−290.

Sorokin, D.Y., Kuenen, J.G. and Muyzer, G. (2011). The microbial sulfur cycle at extremely haloalkaline conditions of soda lakes. *Front Microbiol* **2**: 44.

Sorokin, D.Y., Kublanov, I.V., Gavrilov, S.N., Rojo, D., Roman, P., Golyshin, P.N. *et al*. (2016a). Elemental sulfur and acetate can support life of a novel strictly anaerobic haloarchaeon. *ISME J.* **10**: 240−252.

Sorokin, D.Y., Kublanov, I.V., Yakimov, M.M., Rijpstra, W.I., Sinninghe Damsté, J.S. (2016b). *Halanaeroarchaeum sulfurireducens* gen. nov., sp. nov., the first obligately anaerobic sulfur-respiring haloarchaeon, isolated from a hypersaline lake. *Int J Syst Evol Microbiol* **66**: 2377−2381.

Sorokin, D.Y., Messina, E., Smedile, F., Roman, P., Sinninghe Damsté, J.S., Ciordia, S. *et al.* (2017a). Discovery of anaerobic lithoheterotrophic haloarchaea, ubiquitous in hypersaline habitats. *ISME J* 11: 1245−1260.

Sorokin, D.Y., Makarova, K.S., Abbas, B., Ferrer, M., Golyshin, P.N., Galinski, E.A. *et al.* (2017b). Discovery of extremely halophilic, methyl-reducing euryarchaea provides insights into the evolutionary origin of methanogenesis. *Nat Microbiol* **2**: 17081.

Sorokin, D.Y., Messina, E., La Cono, V., Ferrer, M., Ciordia, S., del Carmen Mena, M., Toshchakov, S.V., *et al*. (2018a). Sulfur respiration in a group of facultatively anaerobic natronoarchaea ubiquitous in hypersaline soda lakes. *Front Microbiol* **9**: article 2359.

Sorokin, D.Y., Merkel, A.Y., Abbas, B., Makarova, K., Wolf, Y., Rijpstra, W.I.C., *et al*. (2018b). *Methanonatronarchaeum thermophilum* gen. nov., sp. nov, and '*Candidatus* Methanohalarchaeum thermophilum' - extremely halo(natrono)philic methyl-reducing methanogens from hypersaline lakes representing a novel euryarchaeal class *Methanonatronarchaeia* classis nov. *Int J Syst Evol Microbiol* **68**: 2199-2208.

Sorokin D.Y. and Yakimov M.M. (2018). Genus *Halodesulfuriarchaeum*. In: *Bergey's Manual of Systematic of Bacteria and Archaea*. Whitman WB (ed.), John Wiley & Sons, Ltd: Chichester. Published on line: 12 December 2018. DOI: 10.1002/9781118960608.gbm01528.

Sorokin, D.Y., Yakimov, M.M., Messina, E., Merkel, A.Y., Bale N.J. and Sinninghe Damsté J.S. (2019). *Natronolimnobius sulfurireducens* sp. nov., and *Halalkaliarchaeum desulfuricum* gen. nov., sp. nov., the first sulfur-respiring alkaliphilic haloarchaea from hypersaline alkaline lakes. *Int J Syst Evol Microbiol* **69**: 2662-2673.

Sorokin, D.Y., Merkel, A.Y., Messina, E., Yakimov, M.M., Itoh T, Mesbah NM *et al*. (2020a). Reclassification of the genus *Natronolimnobius*: proposal of two new genera, *Natronolimnohabitans* gen. nov. to accommodate *Natronolimnobius innermongolicus* and *Natrarchaeobaculum* gen. nov. to accommodate *Natronolimnobius aegyptiacus* and *Natronolimnobius sulfurireducens*. *Int J Syst Evol Microbiol* **70**: 3399–3405.

Sorokin, D.Y., Merkel, A.Y., Yakimov, M.M. and Oren, A. (2020b). *Halalkaliarchaeum*, p. 1–8. *In* Bergey's Manual of Systematics of Archaea and Bacteria. American Cancer Society. Published online : 19 June 2020. DOI: 10.1002/9781118960608.gbm01943.

Stevenson, A., Hamill, P.G., O'Kane, C.J., Kminek, G., Rummel, J.D., Voytek, M.A. *et al*. (2017). *Aspergillus penicillioides* differentiation and cell division at 0.585 water activity: Fungal cell division at 0.585 water activity. *Environ Microbiol* **19**: 687–697.

Trüper, H.G., and Schlegel, H.G. (1964). Sulfur metabolism in *Thiorhodaceae*. 1. Quantitative measurements on growing cells of *Chromatium okenii*. *Ant. van Leeuwenhoek* 30, 225–238.

Vignais, P.M., Billoud, B. and Meyer, J. (2001). Classification and phylogeny of hydrogenases. *FEMS Microbiol Rev* **25**:455–501.

Vignais, P.M. and Billoud, B. (2007). Occurrence, classification, and biological function of hydrogenases: an overview. *Chem Rev* **107**: 4206–4272.

Werner, J., Ferrer, M., Michel, G., Mann, A.J., Huang, S., Juarez, S. *et al.* (2014). *Halorhabdus tiamatea*: proteogenomics and glycosidase activity measurements identify the first cultivated euryarchaeon from a deep-sea anoxic brine lake as potential polysaccharide degrader. *Environ Microbiol* **16**: 2525–2537.

Wick, R.R., Judd, L.M., Gorrie, C.L. and Holt, K.E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol* **13**: e1005595.

Wiechmann, A., Ciurus, S., Oswald, F., Seiler, V. N., and Müller, V. (2020). It does not always take two to tango: “Syntrophy” via hydrogen cycling in one bacterial cell. *ISME J* **14**: 1561-1570.

Williams, T., Allen, M., Tschitschko, B. and Cavicchioli R. (2017). Glycerol metabolism of haloarchaea. *Environ Microb* **19**: 864-877.

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[a]	*e*-acceptors
AArc-S[a] (JCM 34033)	Hypersaline Kulunda soda lakes brine/sediment	Glucose + S_8	9.8	30	Starch, glycogen, cyclodextrin, fructose, glucose, maltose, mannose, raffinose, trehalose, glycerol	S_8, $S_2O_3^{2-}$, O_2[b]
HSR12-1 (JCM 34031)	pH-neutral hypersaline Kulunda lakes brine/sediment (Altai, Russia)	Glucose + S_8	7.5	30	Fructose, glucose, maltose, melibiose, raffinose, sucrose, trehalose, glycerol	S_8, DMSO[c], O_2[b]
HSR12-2[a] (JCM 34032)	pH-neutral hypersaline Kulunda lakes brine/sediment (Altai, Russia)	Glucose + S_8	7.5	30	Fructose, galactose, glucose,maltose, melibiose, raffinose, sucrose, trehalose, glycerol	S_8, DMSO[c], O_2[b], $S_2O_3^{2-}$
HSR-T1	pH-neutral hypersaline Kulunda lakes brine/sediment (Altai, Russia)	Glucose + S_8	7.5	46	Fructose, glucose, maltose, melibiose, raffinose, sucrose, trehalose, glycerol	S_8, DMSO, O_2[b]
HSR-T2	pH-neutral hypersaline Kulunda lakes sediment (Altai, Russia)	Glucose + S_8	7.5	46	Fructose, glucose, maltose, melibiose, raffinose, sucrose, trehalose, glycerol	S_8, DMSO, O_2[b]
HSR-T3	pH-neutral solar saltern brine/sediment (Crimea, Russia)	Glucose + S_8	7.0	48	Fructose, glucose, maltose, mannose, melezitose, raffinose, sucrose, trehalose, glycerol	S_8, DMSO, O_2[b]
HSR-Kgl	pH-neutral hypersaline Kulunda lakes brine/sediment (Altai, Russia)	Glycerol + S_8	7.5	37	Fructose, glucose, maltose, raffinose, sucrose, trehalose, glycerol	S_8, DMSO, O_2[c]
HSR-Bgl	pH-neutral hypersaline Lake Baskunchak brine/sediment (South Russia)	Glycerol + S_8	6.8	37	Fructose, trehalose, glycerol	S_8, DMSO[c], O_2[b]
HSR-Kst	pH-neutral hypersaline Kulunda lakes brine/sediment (Altai, Russia)	Starch + S_8	7.5	37	Starch, pullulan, dextrin, glycogen, cyclodextrin, glucose, maltose, sucrose, glycerol	S_8, DMSO, O_2[b]
HSR-Bst	pH-neutral hypersaline Lake Baskunchak brine/sediment (South Russia)	Starch + S_8	6.6	37	Starch, pullulan, dextrin, glycogen, cyclodextrin, glucose, maltose, sucrose, glycerol	S_8, DMSO, O_2[b]
HSR-Est	pH-neutral hypersaline Lake Elton brine/sediment (South Russia)	Starch + S_8	6.6	37	Starch, pullulan, dextrin, glycogen, cyclodextrin, glucose, maltose, sucrose, glycerol	S_8, DMSO[c], O_2[b]

[a] Thiosulfate reduction in HSR12-2 and AArc-S (shown by grey shading) resulted in equimolar formation of HS^- and SO_3^{2-} (two-electron reduction).

[b] Microoxic conditions of cultivation for all isolates.

[c] 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

930

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^T	HSR12-1^T	HSR12-2	HSR-Bgl	HSR-Est
GH2	β-galactosidase / β-glucuronidase	eytoplasmic	0	0	0	0	1
GH2	β-galactosidase / β-glucuronidase	extracellular	1	0	0	0	0
GH3	β-glucosidase	cytoplasmic	1	3	0	1	0
GH3	β-glucosidase	extracellular	0	0	0	0	1
GH4	α-glucosidase / α-galactosidase	cytoplasmic	1	1	1	1	1[a]
GH13	α-amylase	cytoplasmic	7	10	10	10	13
GH13	**α-amylase**	**extracellular**	**5**	**0**	**0**	**0**	**4**
GH15	Glucoamylase	cytoplasmic	2	0	0	0	1
GH27	α-galactosidase	cytoplasmic	0	1	1	1	1[a]
GH31	α-glucosidase	Cytoplasmic	0	2	2	2	2
GH32	β-fructosidase	Cytoplasmic	1	0	0	1	0
GH42	β-galactosidase	Cytoplasmic	1	1	1	1	1[a]
GH68	Levansucrase / invertase	Cytoplasmic	1	0	0	0	0
GH77	α-glucanotransferase / Amylomaltase	Cytoplasmic	1	2	2	2	1
GH81+CBM6	Endoglucanase with carbohydrate-binding module 6	Extracellular	0	1	0	1	0
	Total amount of GHs		**21**	**21**	**17**	**20**	**26**

935

[a] 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 carbohydarate-utilizing sulfur-reducing '*Halarchaeoglobus*' isolates grown at 4.0 M NaCl, pH 7 and 37^{o}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^{-1} soluble starch. The data are mean values from 2 parallel incubations.

Figure 3. Ability of strain HSR12-2 to utilize H_2 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^{-1} and 500 µM, respectively. The data are mean values from duplicate experiments.

Figure 4. Anaerobic respiratory activity measured with washed cells of carbohydarate-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^{-1} HEPES at pH 7 and resuspended at final protein concentration of 0.2-0.3 mg ml^{-1}. The data are mean values from 3 parallel incubations.

Figure 5. Anaerobic growth kinetic (A) and sulfide formation (B) obtained for carbohydarate-utilizing sulfur-reducing '*Halarchaeoglobus*' isolate AArc-S grown at 4.0 M total Na^+, pH 7 and 30^oC. Sulfidogenic activity was measured with resting AArc-S cells pre-grown with different electron acceptors (S_8, 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. α-Glucan degradation to malto-dextrins and glucose is likely performed by multiple extracellular α-amylases of GH13 family produced by strains HSR-Est and AArc-S. Eventual common use in HSR strains of the [NiFe]H_2ase'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_2, fructose-1,6-biphosphate; G1,3P_2, 1,3-biphosphoglycerate; G2P, 2-phosphoglycerate; G3P, 3-phosphoglycerate; GA3P, glyceraldehyde-3-phosphate; Gly3P, glycerol-3-phosphate; HYA, membrane-bound [NiFe]-H_2ase; 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.