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Anaerobic carboxydotrophy in sulfur-respiring haloarchaea from hypersaline lakes

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15 Running title: Anaerobic carboxydotrophy in sulfur-reducing haloarchaea

Summary

20 Anaerobic carboxydutrophy is a widespread catabolic trait in bacteria, with two dominant pathways: hydrogenogenic and acetogenic. The marginal mode by direct oxidation to CO₂ using an external *e*-acceptor has only a few examples. Use of sulfidic sediments from two types of hypersaline lakes in anaerobic enrichments with CO as an *e*-donor and elemental sulfur as an *e*-acceptor led to isolation of two pure cultures of anaerobic carboxydutrophs belonging to two genera

25 of sulfur-reducing haloarchaea: *Halanaeroarchaeum* sp. HSR-CO from salt lakes and *Halalkaliarchaeum* sp. AArc-CO from soda lakes. Anaerobic growth of extremely halophilic archaea with CO was obligatory depended on the presence of elemental sulfur as the electron acceptor and yeast extract as the carbon source. CO served as a direct electron donor and H₂ was not generated from CO when cells were incubated with or without sulfur. The genomes of the

30 isolates encode a catalytic Ni,Fe-CODH subunit CooS (distantly related to bacterial homologs) and its Ni-incorporating chaperone CooC (related to methanogenic homologs) within a single genomic locus. Similar loci were also present in a genome of the type species of *Halalkaliarchaeum* closely related to AArc-CO, and the ability for anaerobic sulfur-dependent carboxydutrophy was confirmed for three different strains of this genus. Moreover, similar proteins are encoded in three of the four

35 genomes of recently described carbohydrate-utilizing sulfur-reducing haloarchaea belonging to the genus *Halapricum* and in two yet undescribed haloarchaeal species. Overall, this work demonstrated for the first time the potential for anaerobic sulfur-dependent carboxydutrophy in extremely halophilic archaea.

40 **Key words:** carbon monoxide, anaerobic carboxydutrophy, hypersaline; sulfur-reducing haloarchaea; *Halanaeroarchaeum*; *Halalkaliarchaeum*

Introduction

Prokaryotic carboxydutrophy is based on two different types of unrelated CO-dehydrogenase enzymes (CODH), namely (Cu,Mo,FeS,flavin)-CODH (CoxLMS), which is present in aerobic or denitrifying prokaryotes, and (Ni,FeS)-CODH (CooS) present in anaerobic carboxydutrophs [1-5]. Aerobic carboxydutrophs are predominantly proteobacteria [6], although members of other phyla have recently been found to aerobically oxidize CO, including *Chloroflexi* (growth and oxidation) [7] and the extremely halophilic archaea of the class *Halobacteria* (oxidation without growth) [8]. Anaerobic carboxydutrophy is mainly present in three groups of secondary anaerobic prokaryotes: (i) acetogenic bacteria and archaea via the Wood-Ljungdahl pathway, resulting in the production of CO₂, acetate and/or ethanol; (ii) methanogenic archaea via the archaeal variety of the Wood-Ljungdahl pathway, leading to formation of methane or acetate; and (iii) hydrogenogenic carboxydutrophic bacteria and archaea, producing H₂ and CO₂ [7, 9-10].

The microbial carboxydutrophy is poorly investigated in hypersaline habitats. Until now, halophilic carboxydutrophy has only been observed aerobically by organisms classified within two closely related haloalkaliphilic *Gammaproteobacteria* genera: *Alkalispirillum* and *Alkalilimnicola* [11-12]. The potential for oxidation of CO at low concentrations using O₂, nitrate or perchlorate (albeit without growth) was shown for several members of extremely halophilic archaea belonging to genera *Halorubrum*, *Natronorubrum*, *Haloarcula* and *Halobaculum* [8, 13]. These bacteria and archaea have aerobic type of (Mo,Cu)-CODH. However, the utilization of CO as the energy source for aerobic carboxydutrophic growth was proven only for the *Alkalispirillum*/*Alkalilimnicola* group [12].

Since protons are the final electron acceptors of hydrogenogenic pathway, anaerobic carboxydutrophy would be extremely unlikely in soda lake environment, while both acetogenic and methanogenic CO utilization can be feasible. Indeed, for extremely haloalkaliphilic microorganisms, hydrogenogenic anaerobic carboxydutrophy has never been reported.

Although for some hydrogenogenic carboxydrotrophs the addition of an external electron acceptor such as sulfur, thiosulfate, or sulfate led to a partial redirection of CO-derived electrons from protons to a more favorable acceptor, this did not completely stop the H₂ formation [14-17]. The same seems to be true for those carboxydrotrophic acetogens that can utilize electron acceptors other than CO₂. In this case, some CO-derived electrons is diverted to reduction of the external acceptor, as was shown for the euryarchaeon *Archaeoglobus fulgidus* in the presence of sulfate [18]. However, there is hardly any published evidence for anaerobic microorganisms that could directly oxidize CO to CO₂ in anaerobic respiratory mode as the only energy generation process. One of the possible candidates is the euryarchaeal genus *Thermococcus*, which is apparently capable of both proton-dependent hydrogenogenic carboxydrotrophy and lithoheterotrophic growth by direct oxidation of CO with elemental sulfur as an electron acceptor. The genome of *Thermococcus* sp. AM4 contains two different CODH loci, probably responsible for different modes of CO oxidation: one is associated with membrane-bound hydrogenase (MBH) and the other with a flavin disulfide oxidoreductase, a putative electron acceptor. Both clusters also contain CooF-like ferredoxins, which provide immediate electron transfer during CO oxidation. A close relative of AM4, *T. gammatolerans*, can only grow with CO in the presence of sulfur and has a single *cooS/cooF/cooC* locus in the genome not linked to any recognizable electron transfer complexes [19-21]. Further electron transfer from CODH to sulfur in *Thermococcus* species is still unresolved, although the genomes of both seem to encode the membrane-bound MBX complexes (not associated with CODH loci) with a proven potential for ferredoxin-dependent polysulfide reductase activity in thermophilic archaea [22]. Interestingly, two out of five different CooS-coding loci present in the CO-utilizing sulfate-reducing genus *Desulfofundulus* are structurally very similar to the hydrogenase-independent *Thermococcus* CODH locus and are likely responsible for the oxidation of CO with sulfate as an electron acceptor in these bacteria [17].

So far there is no evidence of anaerobic carboxydrotrophy (based on the Ni,Fe-CODH) for extreme halophiles living in hypersaline pH neutral habitats. There are only two examples of

anaerobic haloalkaliphilic members of *Firmicutes* utilizing CO during acetogenic growth: two
 95 species of the genus *Alkalibaculum* (the low salt and alkalitolerant *A. bachii* and the moderately
 haloalkaliphilic *A. sporogenes*) - a member of the family *Eubacteriaceae* [23-25], and
Natranaerofaba carboxydovora, - an extremely haloalkaliphilic member of the class
Natranaerobiia, which grows optimally in hypersaline soda brines at pH 10 and 50°C [26]. It was
 enriched in a consortium with a triple extremophilic methyl-reducing methanogen of the genus
 100 *Methanonatronarchaeum*, previously found in hypersaline soda lakes [27]. In fact, methane was
 formed in this enrichment from formate, released as one of the products of CO conversion by
 acetogens.

The present work targeted a possibility of elemental sulfur-dependent anaerobic
 carboxydutrophy in two types of hypersaline lakes, with neutral and alkaline pH, with a special
 105 attention to haloarchaea. Extremely halophilic archaea belonging to the class *Halobacteria*
 dominate prokaryotic communities in salt-saturated brines of various hypersaline habitats of marine
 and terrestrial origin. Most of the known species are aerobic organoheterotrophs. Recently,
 however, this point of view has changed. Using elemental sulfur as the terminal electron acceptor,
 we discovered a novel ecotype of haloarchaea living in anoxic sediments by anaerobic dissimilatory
 110 sulfur respiration. To date, four functional varieties of sulfur-reducing haloarchaea have been
 isolated and physiologically characterized from hypersaline habitats. The two groups isolated from
 salt lakes are obligate anaerobes and classified within closely related genera, which include acetate-
 and pyruvate oxidizing *Halanaeroarchaeum* [28-30] and lithoheterotrophic formate/H₂-oxidizing
Halodesulfurarchaeum [31-32]. The third group was found in hypersaline soda lakes. It consists of
 115 facultatively anaerobic haloalkaliphilic archaea, belonging to two new genera *Halalkaliarchaeum*
 and *Natrarchaeobaculum*, with a more versatile range of the electron donors for anaerobic sulfur
 respiration, including formate/H₂, pyruvate, C₄-C₉ fatty acids and peptone [33-36]. The last group
 includes facultatively aerobic haloarchaea, both neutrophilic and alkaliphilic, utilizing
 carbohydrates as an *e*-donor and carbon source for anaerobic sulfur respiration. Neutrophilic

120 members form a new species in the genus *Halapricum*. They can grow either by fermentation with the formation of H₂ or by sulfur respiration, while the single alkaliphilic strain belonging to a novel genus "*Natranaerarchaeum*" possesses a strictly respiratory metabolism growing by either O₂ or sulfur/thiosulfate-dependent respiration [37-38].

In this paper, we describe the isolation, physiological and genomic properties of a fifth
 125 group of sulfur-respiring haloarchaea enriched from hypersaline lake sediments with CO as an electron donor. These lithoheterotrophic carboxydrotrophs directly oxidize CO to CO₂ with elemental sulfur as an electron acceptor, a type of metabolism that has not been known in haloarchaea and is a very rare catabolic trait in the whole archaeal domain.

130 **Experimental procedures**

Sample collection and initial treatments

Samples of the upper 15 cm sediment and near-bottom brine samples were taken from three pH-neutral hypersaline chloride-sulfate lakes (total salinity 220-340 g/l; pH 7.2-7.8) and three
 135 hypersaline soda lakes (total salinity from 150 to 320 g/l, total soluble carbonate alkalinity 1.5-3.8 M, pH 10.2-11.0) in the southern Kulunda Steppe (Altai region, Russia) in July 2015-2017. Samples were collected into a stratometric corer with an inner diameter of 25 mm, extruded into a sterile 100 ml Schott flask, sealed without air bubbles, transported to the laboratory in a insulated box with cooling elements within 3 days after sampling and stored at 10°C until use. For initial
 140 enrichments, the sediment slurries from 3 different salt lakes and soda lakes were mixed in equal proportions to produce two "master mixes" that were used at 5% (v/v) as a combined inoculum.

Enrichment and cultivation conditions

Two mineral basic media (4 M total Na⁺) were used for enrichments and cultivation: a NaCl-base
 145 medium with a final pH 7.0 and a Na₂CO₃-base medium with a final pH 10.0. The former was used

as such for the enrichment and cultivation of neutrophilic extreme halophiles, while for the soda lake cultivation was performed using a 1:1 mix of the NaCl- and Na₂CO₃-base media (final pH 9.7). The NaCl-base contained (g l⁻¹): NaCl, 240; KCl, 5; K₂HPO₄, 2.5; NH₄Cl, 0.5. The sodium carbonate-base medium contained (g l⁻¹): Na₂CO₃ 190; NaHCO₃ 30; NaCl 16; KCl, 5.0; and
 150 K₂HPO₄ 1.0. After autoclave sterilization, both base media were supplemented with 1 mM MgCl₂, 1 ml l⁻¹ of acidic trace metal solution, 1 ml l⁻¹ vitamin mix [39], 1 ml l⁻¹ of alkaline Se/W solution [40] and 100 mg l⁻¹ of yeast extract. Elemental sulfur flour (J.T. Baker, Netherlands) wet paste was sterilized at 110 °C for 30 min and after cooling the clean water phase was removed. Sulfur was used at approximately 2 g l⁻¹. Cultivation was performed at 30°C in 115 ml serum vials with butyl
 155 rubber stoppers filled with 30 ml medium in case of CO or H₂ as the electron donors or in 30 ml bottles with 25 ml media in case of soluble electron donors. Vials 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 Na₂S from a 1.0 M filter sterilized stock solution. CO (20-50%) or H₂ (0.2 bar overpressure) were added through a sterile gas filter over an argon atmosphere. The final
 160 media reduction was done by adding about 20 µl of 10% dithionite in 1 M NaHCO₃ with a syringe. The cultures were incubated statically with occasional shaking of the flasks.

The strategy of isolation of pure cultures consisted of several rounds of decimal transfers to obtain sediment-free enrichments, followed by serial dilution-to-extinction. The maximum positive dilutions were determined by the formation of sulfides. Further purification was only possible using
 165 soft agar approach using maximum positive dilution cultures as starting material. The cultures were serially diluted in anoxic medium heated to 55 °C, mixed 4:1 with 4% washed agar, also kept at 55°C, and the resulted mix was poured into plates (15 ml total). Plates were incubated in 3.5 L anaerobic jars (Oxoid) under argon at 30°C with an O₂-removing catalyzer (Oxoid). The colonies were picked into liquid medium with CO and sulfur. Finally, the purity of isolates was checked by
 170 16S rRNA sequencing.

Potential catabolic activity experiments with washed (resting) cells were carried out in the mineral media (see above) at pH 7 or 9.7 as buffers lacking the N-source and containing 0.1 mM sulfide. 2 ml of the concentrated cell suspension was placed into 7 ml serum bottles containing 10 mg sulfur, made anoxic as described above, reduced by adding 20 μ l of 1% dithionite in 1 M NaHCO₃ and incubated at 30°C with various electron donors. Several 50 μ l liquid samples were periodically taken during 5 days of incubation for sulfide determination and 0.5 ml gas samples for CO/CO₂ analyses as described below.

Analyses

Cumulative free sulfide + sulfane from polysulfide formed during elemental sulfur reduction were measured by the methylene blue method [41] after fixing the supernatant in 10% (w/v) zinc acetate. Gases were analyzed using a GC (Chromateck Crystall 5000 [Russia], Hayesep column 80–100 mesh, 2 m x 3 mm, 40°C) equipped with a methanizer and thermal conductivity detector for H₂ and flame ionization detector (for CO and CO₂) (200°C; carrier gas argon; flow rate 25 ml min⁻¹); the injection volume was 250 μ l. Cellular 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 iron (II) sulfide bound to the cells. Microbial growth was monitored by the increase in optical density at 600 nm (OD₆₀₀). Phase contrast microphotographs were obtained with a Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany).

Genome sequencing and phylogenetic analysis

The complete genomes of two CO-utilizing 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). Both genomes were assembled as a single circular chromosome and a small plasmid and analyzed as described previously [37]. The genome statistics are given in the Supplementary **Table S1**.

For the identification of the isolates phylogenetic reconstructions based on sequences of 122 archaeal single copy conservative marker genes [42] as well as of 16S rRNA gene sequences were used. For phylogenetic reconstruction of *CooS*, database consisting of 1942 non-redundant protein sequences described in Inoue et al., 2019 was used. In all cases phylogenetic trees were built with IQ-TREE 2 [43] and PhyML 3.0 [44] software with the approximate likelihood-ratio test for branches[45]. MAFFT version 7.487 was used for a multiple alignment [46]. Models for phylogenetic reconstructions were selected using ModelFinder software [47]. 5 automated alignment trimming strategies were used in trimAl 1.2rev59 software [48]: 1) strictplus mode; 2) automated1 mode; 3) strictplus mode plus deletion of columns that contain more than 10%; 4) automated1 mode plus deletion of columns that contain more than 5%; 5) deletion of columns that contain more than 5%. After trimming all alignments were manually reviewed.

Whole cell lysate shotgun proteomic analysis

Cell lysis, protein extraction and proteolytic digestion was performed as described previously [26]. Briefly, approx. 10-20 mg of each cell pellet (biomass wet weight) were lysed using Complete Bacterial Protein Extraction Reagent (B-PER; Thermo-Fisher)/TEAB buffer and bead beating followed by centrifugation at 14,000 g under cooling, using a bench top centrifuge. TCA was added to the protein supernatant (20% TCA, v/v), incubated at 4°C and centrifuged to collect the protein precipitate. The pellet was washed with ice cold acetone and dissolved in 200 mM ammonium bicarbonate containing 6 M urea. The disulfides were reduced with dithiothreitol (10 mM) and sulfhydryl groups were blocked with iodoacetamide (20 mM). The solution was diluted to below 1 M urea and digested using sequencing grade Trypsin (Promega). The proteolytic peptide digest was desalted using an Oasis HLB solid phase extraction well plate (Waters) according to the manufacturers protocol. An aliquot corresponding to approximately 500 ng of protein digest was analysed with a shotgun proteomics approach. The peptides were analysed using a nano-liquid-

chromatography system consisting of an EASY nano LC 1200, equipped with an Acclaim PepMap RSLC RP C18 separation column (50 $\mu\text{m} \times 150 \text{ mm}$, 2 μm , 100Å, Thermo Scientific) and a QE plus Orbitrap mass spectrometer (Thermo Fisher, Germany). The flow rate was maintained at 350 nL over a linear gradient from 5% to 30% solvent B over 38 minutes and finally to 60% B over 15 min. Solvent A was H_2O containing 0.1% formic acid, and solvent B consisted of 80% acetonitrile in H_2O and 0.1% formic acid. The Orbitrap was operated in data-dependent acquisition (DDA) mode acquiring peptide signals from 385–1250 m/z . The top 10 signals were isolated at a window of 2.0 m/z and fragmented at a NCE of 28. The collected mass spectrometric raw data were analysed against the constructed genome sequence database (described above), using PEAKS Studio 10 (Bioinformatics Solutions, Canada) allowing 20 ppm parent ion and 0.02 Da fragment ion mass error tolerance, 2 missed cleavages, carbamidomethylation as fixed and methionine oxidation and N/Q deamidation as variable modifications. Peptide spectrum matches were filtered for 1% false discovery rate (FDR) and protein identifications with at least 2 unique peptides were considered as significant.

Data deposition

16S rRNA gene sequences of strains HSR-CO and AArc-CO were deposited in the NCBI GenBank database with accession no. MZ466393-MZ466394 and their complete genomes were deposited in the GenBank database by the NCBI Genome submission portal with the accession numbers CP081498-CP081499 (chromosome-plasmid) for AArc-CO and CP081500-CP081501 (chromosome-plasmid) for HSR-CO. The proteomic data were deposited at the proteomics identification database (PRIDE Archive) and are available through the proteome exchange server (<http://www.proteomexchange.org/>, <http://www.ebi.ac.uk/pride>, (project code PXD028241); reviewer account details: reviewer_pxd028241@ebi.ac.uk).

Results and Discussion

Enrichment and isolation of pure cultures

250 No CO consumption and H₂ formation was observed in primary anaerobic enrichments without electron acceptors and in presence of thiosulfate, nitrate/nitrite and ferrihydride as the electron acceptors. CO consumption was only obtained when elemental sulfur served in this capacity. The salt-lake enrichment formed 2.5 mM sulfide and the soda-lake enrichment formed 6.3 mM sulfane (in polysulfides) after 2 months incubation (with endogenous controls without CO subtracted).

255 After several stabilizing 1:100 transfers, sediment-free cultures were obtained in which the rates of CO-dependent sulfur reduction increased approximately two times for the salt-lake and four times for the-soda lake cultivations. The two cultures were different in appearance. The salt-lake culture was only slightly turbid and colourless (Supplementary **Fig. S1a**). The soda-lake enrichment showed denser growth and was orange due to accumulation of polysulfide (S_n²⁻), spontaneously

260 formed from biologically produced sulfide and the remaining sulfur, since S_n²⁻ is chemically stable at highly alkaline anoxic conditions (Supplementary **Fig. S1b**). Several rounds of dilution-to-extinction did not lead to the isolation of pure cultures: 16S rRNA gene sequencing determined the microdiversity of archaea in the salt-lake enrichment, while the bacterial component was present along with the archaea in the soda-lake enrichment. Therefore, for the final purification, an attempt

265 was made using soft agar plating under anaerobic conditions, although elemental sulfur could not be evenly distributed in the agar even after sonication of the suspended sulfur in liquid medium before mixing with the agar. Indeed, plating of the neutrophilic enrichment was difficult, since the culture did not form distinct colonies and grew as a diffuse cloud on the surface of sulfur conglomerates. However, the target organism was easy to differentiate from contamination by blackening of the

270 sulfur particles with the *de novo* formed FeS. Microscopy showed that such changed conglomerates indeed contain a dense population of flattened cocci typical for haloarchaea, and their incubation in liquid medium with CO resulted in a pure carboxydotrophic haloarchaeal culture designated as

strain HSR-CO. Plating under microaerobic conditions (1% O₂ in the gas phase) on the medium with yeast extract and pyruvate did not yield any colonies for this culture after 2 months incubation.

275 In contrast, plating of the soda lake highest positive dilution culture produced visible coloured colonies both under anoxic condition in a CO-containing atmosphere and under microaerobic conditions with yeast extract and pyruvate. The surface microaerophilic colonies were bright red and had two shapes, but only one of them grew back anaerobically with CO. Anaerobic colonies formed under CO were of only a single type (pale pink lens-shaped). Notably, these colonies
280 created a clearance zone around them due to the dissolution of fine sulfur particles and the conversion of the solubilized sulfur into a yellow polysulfide fringe outside the clearance zone (Supplementary **Fig. S1 c-d**). Inoculation of the anaerobic colonies into liquid medium with CO and sulfur resulted in a positive culture designated AArc-CO. Both HSR-CO and AArc-CO cells were mostly angled flattened coccoids, typical for haloarchaea (Supplementary **Fig. S2**). The purity of
285 the isolates was confirmed by 16S rRNA gene and whole genome sequencing. The strains were deposited in the Japanese Culture Collection of Microorganisms with the accession numbers JCM 34029 (HSR-CO) and JCM 34103 (AArc-CO).

Identification of the isolates

290 The HSR-CO genome contained a single *rrn* operon with its 16S rRNA gene 99.0% identical to halophilic *Halanaeroarchaeum sulfurireducens* HSR2^T, while the AArc-CO genome has two nearly identical *rrn* operons with 99.9% identity of 16S rRNA genes to each other and 98.4% identity to the haloalkaliphilic *Halalkaliarchaeum desulfuricum* AArc-SI^T 16S rRNA gene. Despite the high similarity of 16S rRNA genes of both these carboxydotrophic strains to their nearest relatives
295 (Supplementary **Fig. S3**), they represent new species in the respective genera of sulfur-respiring haloarchaea according to ANI, AAI and DDH analysis (Supplementary **Table S2**) and according to phylogenomic reconstruction based on 122 archaeal single copy conserved protein marker genes (**Fig. 1**).

300 **Carboxydotrophic growth and CO-dependent sulfidogenic activity**

Already at the state of highly enriched cultures, it became clear that anaerobic utilization of CO is obligatory associated with the respiration on elemental sulfur, rather than more common pathways of anaerobic acetogenic or hydrogenogenic carboxydotrophy. This was confirmed in growth experiments with pure haloarchaeal cultures, which also revealed another aspect – the obligate
 305 dependence of growth on the presence of yeast extract as a carbon source. Taken together, the carboxydotrophic metabolism of isolated haloarchaea can be characterized as lithoheterotrophy assuming that CO is similar to H₂ in its capacity as an electron donor.

Both cultures grew and reduced sulfur only with yeast extract, but in the presence of CO a concentration-dependent increase in biomass (up to two times) and a significant increase in
 310 sulfidogenic activity (up to twelve times) were observed. There was a clear difference in the rate and intensity of carboxydotrophic growth between the neutro- and alkali-philic isolates, including the following (**Fig. 2**): (i) the maximum cell density and sulfide formation during HSR-CO cultivation were reached after 35-40 days, while in case of AArc-CO it peaked in 20-23 days; (ii) the maximum CO-dependent increase in biomass (compared to the controls without CO) was
 315 similar in the two cultures, while sulfide/sulfane formation was three times higher in AArc-CO; (iii) the growth of neutrophilic culture was suppressed at CO concentrations above 50% in the gas phase, while the alkaliphilic culture was not inhibited at up to 80% CO, although growth and sulfidogenic activity did not increase further compared with the maximum reached at 40% CO; (iv) in abiotic control at neutral pH, there was no spontaneous sulfur reduction in the presence of CO in
 320 contrast to a weak abiotic reaction occurred at a pH above 9.5 at 30°C, becoming significant (up to 10% of living cultures) upon increase of temperature to 37°C and a CO concentration above 50% in the gas phase. Interestingly, no chemical reaction occurred in the activity tests (see below) using the same alkaline buffer applied in the AArc-CO cultivation medium (but without trace metals addition). This observation points to the possibility of an abiotic sulfur/polysulfide reduction by CO

325 at high pH/temperature catalyzed by trace metals. The most likely mechanism here is the initial reaction of CO with polysulfide(s), leading to the formation of carbonyl sulfide (COS), followed by its hydrolysis to sulfide and CO₂. This reaction is favoured by high pH, temperature and CO/polysulfide concentrations [49].

The optimal CO concentrations for growth and sulfidogenic activity in both strains was 30-
 330 40% in the gas phase. Stimulation of biomass yield with CO was clearly less effective than sulfidogenesis, indicating that CO was used only as an electron donor, and growth efficiency was limited by the assimilatory metabolism. The same situation was observed for those sulfur-respiring haloarchaea that utilize H₂ or formate as the electron donors, including the genera *Halodesulfurarchaeum* and *Halalkaliarchaeum*. The stoichiometry of CO consumption in relation
 335 to the production of sulfide/sulfane was close to the theoretical 1:1. The only gaseous product of anaerobic CO oxidation in the neutrophilic culture was CO₂, while in the alkaliphilic culture the accumulation of CO₂ in the gas phase was very low due to its absorption in the alkaline liquid phase (Fig. 2).

Both organisms were also able to grow lithoheterotrophically with two other low-potential
 340 electron donors, including H₂ and formate with sulfur as the acceptor. HSR-CO was significantly more active with H₂ (up to 11 mM HS⁻ and 3 mM on formate over 30 days of incubation), while AArc-CO showed the opposite trend (9 and 23 mM HS⁻ on H₂ and formate after 25 days, respectively).

345 **Activity of CO-dependent sulfur reduction in resting cells**

The cells of both strains, grown anaerobically with only yeast extract and sulfur, did not show any stimulation of sulfur-reducing activity in presence of CO during the first 5 days of incubation, but the activity began to manifest during further incubation. Since *de novo* protein synthesis in such incubations is unlikely, it is possible that the CODH is present but inactive in cells grown without
 350 CO and is activated after prolong exposure to CO. In contrast, in cells grown with CO, the CO-

stimulated sulfidogenesis began immediately, and the maximum CO-specific sulfur reduction in strain AArc-CO was twice that in HSR-CO, in line with what was observed in cultivation experiments (see above) (**Fig. 3**). Another difference between the cells of the two strains was that the CO-dependent sulfur reduction in neutrophilic HSR-CO was only active when dithionite was added as a second reductant to HS⁻ at the beginning of the experiment, while the cells of alkaliphilic AArc-CO were already active in presence of only 0.2 mM HS⁻. This may indicate that under alkaline conditions the low redox potential required to activate (Ni-Fe) CODH can be more easily achieved even at low HS⁻ concentrations. Growth on CO also induced the high H₂-dependent sulfur reducing activity observed in resting cells, while the effect of formate stimulation was much lower (**Fig. 3**). Overall, these experiments clearly demonstrated that CO and H₂ are strong electron donors for two novel isolates of sulfur-reducing haloarchaea.

Genomic analysis and comparative proteomics

Utilization of CO as the electron donor

A search for functional genes specific for anaerobic CODH was carried out in genomes of novel isolates. Both genomes contained a bicistron operon encoding the Ni-FeS catalytic subunit CooS and its Ni-chaperon CooC (CODH maturase). Using the CooS from AArc-CO and HSR-CO for a search of similar sequences within the class *Halobacteria* yielded a very limited number of matches including four closely related hits in the genomes of previously described sulfur-reducing haloarchaea. CooS from AArc-CO had the highest identity with the homologue from *Halalkaliarchaeum desulfuricum* AArc-SI^T [34], while CooS from HSR-CO had a high-level identity with CooS homologues encoded in the genomes of three of the four genome-sequenced strains (HSR-12-1, HSR12-2^T and HSR-Est) of carbohydrate-utilizing *Halapricum desulfuricans* [37-38] (Supplementary **Table S3**). There were two additional hits including undescribed *Halorubrum* sp. CSM-61 (more similar to CooS of *Halalkaliarchaeum*) and *Salinarchaeum* sp.

IM2453 (although it might be wrongly assigned to this genus, judging from the level of its 16S rRNA gene sequence). In addition, a *Halobacteria* metagenome-assembled genome (MAG) recovered from hypersaline industrial tailings in Canada, containing two copies of CooS with low homology to CooS from HSR-CO was identified. This indicates that anaerobic carboxydutrophy is an extremely rare trait in haloarchaea with only a few examples bearing its functional determinates detected so far. Interestingly, in all seven cultivated strains of haloarchaea, CooS was colocalized with CooC in one operon (with the exception of the second copy in the *Halobacteria* MAG), indicating the importance of this small maturase in the functionality of catalytic CooS, which is consistent with the results of biochemical studies [50]. The N-terminus of the CooC proteins of two *Halalkaliarchaeum* strains is particularly interesting in that it has a large locus of histidine-aspartate (HD) repeats, which are known to be present in Ni-chaperones, such as CooJ in *Rhodospirillum rubrum* [51], HypB (Ni,Fe hydrogenase assembly proteins) and in Ni-storage/transport proteins, Hpn and RcnA, present in some pathogens such as *Helicobacter pylori* [52]. Moreover, in AArc-CO, this HD-rich region was double that of the type strain of *Halalkaliarchaeum* (Supplementary **Table S3**). On the other hand, the CooC from the previously isolated neutrophilic HSR strains and the two undescribed haloarchaea lacks this characteristic pattern. It is likely, that the Ni-transport and handling in alkaliphiles is more difficult than under neutral hypersaline conditions.

Due to the presence of CooS/CooC operon in the genomes of three *Halapricum desulfuricans* strains, we tested these haloarchaea (stored and maintained in our personal culture collection) for their ability to grow anaerobically with CO and sulfur. Controls without CO in the presence of yeast extract exhibited no growth and sulfide formation, but in the presence of limited concentrations of glucose addition of 20% CO to the gas phase substantially increased sulfide formation in the type strain of *H. desulfuricans* HSR12-2, while growth stimulation was only marginal. The most pronounced effect of CO addition was observed at the lowest glucose concentration used (Supplementary Fig. S4). Interestingly, among the closely related *Halapricum*

desulfuricum isolates, HSR12-2^T was the only one capable of lithoheterotrophic growth with H₂ as the electron donor and sulfur as the electron acceptor [37].

The carboxydutrophic potential in the alkaliphilic genus *Halalkaliarchaeum*, was more prominent. In addition to the novel strain AArc-CO, enriched with CO, we have also recently
 405 described strain AArc-GB capable of using glycine betaine as a carbon source and electron donor and sulfur as an electron acceptor [53]. The complete genome of the strain was not yet sequenced, but because of its proximity to AArc-CO (99% of the 16S rRNA gene sequence identity), it was also included into the test for carboxydutrophy. The results showed that both the type species *H. desulfuricum* AArc-SI^T and strain AArc-GB, can grow anaerobically with CO as the electron donor,
 410 sulfur as electron acceptor and yeast extract as the carbon source in the following order of sulfidogenic intensity: AArc-CO>AArc-GB>>AArc-SI^T.

Phylogeny of CooS from sulfur-reducing carboxydutrophic haloarchaea

In order to define the phylogenetic position of CooS from anaerobic haloarchaea, a multiple
 415 sequence alignment was applied included 1942 non-redundant protein sequences and the previously established Ni,Fe CODH clade structure [4, 7]. Sequences of CooS from haloarchaea are located in clade F (**Fig. 4**) and formed a separate deep cluster. Based on the resulting reconstruction, we can assume that these haloarchaea have acquired CooS not during a recent horizontal gene transfer event, but evolved with it for a long time. All haloarchaeal CooS in this cluster belong to the
 420 structural group F-1 which mostly include the ECH-associated proteins. The F-1 Ni-CODHs were mainly found in bacteria, with a few exceptions [4]. Taking into account this fact and the fact, that all clades, except clade A, are mainly bacterial we can assume, that haloarchaea have initially acquired Ni-CODH from bacterial anaerobic carboxydutrophs.

In general our phylogenetic reconstructions is consistent with previously published works
 425 including subdivision into clades A-F [4, 7]. However, during our work, we have also noticed an

instability of clade F. Depending on the methods of alignment trimming (see Experimental procedures section), this biggest CooS clade in many cases became paraphyletic. Probably, a larger number of diverse sequences of CooS from various sources would improve our understanding of CooS phylogeny, specifically clade F.

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Possible routes of electron transfer from CooS in sulfur-reducing haloarchaea

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In addition to the functional CODH proteins, a most important question for understanding the anaerobic CO-oxidizing system in sulfur-reducing haloarchaea is the nature of electron transfer from the cytoplasmic CooS to the extracellular polysulfide reductase complex Psr. Since the electron transport system is well established only in hydrogenogenic carboxydrotrophs (via CooF ferredoxin to membrane-bound H₂-forming hydrogenases), it is difficult to predict what acts in the direct anaerobic oxidation of CO to CO₂ with sulfur as an actual electron acceptor. However, one thing is clear – the membrane-associated subunit of Psr (PsrC) can accept as the direct electron donor only menaquinol, a membrane electron shuttle that is ubiquitous in all haloarchaea [54]. Each of the *Halalkaliarchaeum* genomes revealed the presence of at least three electron transfer flavoprotein (Etf) complexes, known to be coupled to several redox reactions in the cytoplasm including, ferredoxin-dependent bifurcation [55-56]. Noteworthy, the four-subunit EtfABCX complex containing the ferredoxin EtfX and the quionone-reductase EtfC (AArcCO_1914-1917) and the three subunits EtfABC (AArcCO_1725-1727) may be involved in electron transfer from the cytoplasmic CODH to the membrane menaquinone pool (**Fig. 5**).

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In the genome of neutrophilic HSR-CO, the CODH operon is immediately preceded by a membrane-bound Ni,Fe uptake hydrogenase HyaABC accompanied with its full set of maturation factors HypABCDE/HyaD. It would be tempting to suggest that both [Ni,Fe] catalytic subunits HyaB (located outside the CPM) and the cytoplasmic Ni,Fe CooS can use the common membrane menaquinol-reductase subunit HyaC to donate electrons to the terminal electron-accepting PsrABC complex (**Fig. 5**). Such a connection would mimic, at least superficially, the hydrogenogenic system

with the CooS linked to membrane-bound H₂-forming hydrogenases. The high H₂-dependent rates of sulfur reduction, observed in cells of HSR-CO grown with CO supports the proposed electron transfer scheme. However, both schemes (for AArc-CO and HSR-CO) are purely hypothetical, and
 455 deep biochemical and genetic studies will be required to understand the real electron transport route from CO to sulfur Psr in carboxydutrophic haloarchaea.

Another possibility of converting low-potential electrons from CO oxidation into a cytoplasmic redox active donor could be via ferredoxin-NAD⁺ oxidoreductase (Fnor) [57] with further menaquinone reduction through the NADH dehydrogenase (Nuo complex I)
 460 (CO→Fnor→NADH→Nuo→menaquinol→Psr). The genomes of both HSR-CO and AArc-CO do indeed contain homologues of both the Fnor and Nuo complexes (NuoAB-C/D-HIJ1J2KLMN). Notably, in both of them, the Nuo complexes lack three subunits of the NADH dehydrogenase N-module (NuoEFG), indicating that these carboxydutrophic haloarchaea use other low-potential electron donors rather than NADH, such as F₄₂₀-H₂ or ferredoxin, potentially formed by oxidation
 465 of CO [58].

H₂ and formate as the electron donors

Apart from the CODH and consistent with the growth and cell activity tests, the genomes of HSR-CO and AArc-CO encode the type 1a membrane-bound Ni,Fe uptake hydrogenase Hya/HydABC
 470 and its maturation factors and two types of formate dehydrogenases: a membrane-bound FdoGHI and a cytoplasmic monosubunit FdhH. While both of those have previously been found in the genome of *Halkaliarchaeum desulfuricum* closely related to AArc-CO, neither are present in the type species of *Halanaeroarchaeum sulfurireducens* which utilize only acetate or pyruvate as the electron donors. Therefore, despite close phylogenetic relation, HSR-CO has a very different key
 475 catabolic properties from the type species, more reminiscent of the lithoheterotrophic *Halodesulfurarchaeum*, a sister genus to *Halanaeroarchaeum*.

Dissimilatory sulfur reduction

Apart of revealed capability for anaerobic CO oxidation, AArc-CO and HSR-CO exhibit anaerobic
 480 respiration patterns very similar to those of previously described sulfidogenic halo(natrono)archaea
 mentioned in the Introduction. In addition to the aforementioned formate dehydrogenases, both
 genomes encode an analogous set of DMSO reductase DmsABC, polysulfide reductase PsrABC
 and tetrathionate reductase TtrABC molybdopterin oxidoreductases from the CISM superfamily
 [59], acting as potential terminal electron acceptors (**Figure 6**). Interestingly, while HSR-CO has
 485 two polysulfide- and four DMSO-reductases, the AArc-CO set of CISM enzymes contains only
 single Psr and DMSOr. Such a “minimal” respiratory suit, which determines CO- or formate-
 dependent respiration with elemental sulfur and DMSO, makes this organism an ideal model for
 unambiguously identifying the main mechanisms of the observed types of respiration. A single set
 of AArc-CO Psr polysulfide reductase genes is located in one polycistronic operon, which includes
 490 genes encoding MoPterin (*psrA*), FeS subunit (*psrB*), membrane quinol-interacting anchor protein
 (*psrC*) and system-specific chaperone (*psrD*). In addition to the *psrA-D* genes (AArcCO_1246-8),
 the AArc_1245 gene, which encodes rhodanese-like sulfurtransferase belonging to the SseA family,
 is located upstream. We recently hypothesized that this sulfurtransferase acts as a potential
 polysulfide-binding carrier that is responsible for the mobilization of sulfur during sulfur respiration
 495 in sulfur-respiring halo(natrono)archaea [28, 31, 33]. It is noteworthy that such a rhodanese-
 containing Psr operon was also found as a single set of polysulfide reductase genes in another
 sulfidogenic natronoarchaea, *Natranaerobaculum sulfurireducens* [33]. This finding strongly
 suggests that the products of this operon are sufficient to determine the type of respiration based on
 the use of elemental sulfur as a terminal electron acceptor. The catalytic subunits of CISM enzymes
 500 found in the carboxydophilic isolates AArc-CO and HSR-CO clustered together with the
 homologues from the previously described sulfur reducing halo(natrono)archaea (**Figure 6**)
 forming deep branches within the respective CISM families and likely representing ancient forms
 of molybdopterin oxidoreductases acquired in the past from bacteria.

The genome of AArc-CO also contains three homologous gene clusters with a similar structure: (1) ferredoxin>NrfC1D1>membrane protein>NrfC2fD2; (2) ferredoxin>membrane protein>NrfC3D3; (3) ferredoxin>NrfC4D4>membrane protein; the membrane protein in all three loci is conserved but its function cannot be predicted, while the ferredoxin seems to belong to the family of ferredoxin-NAD(P) oxidoreductase family. The NrfC and D are homologues of PsrB and C, respectively, and recently this membrane associated quinol-dehydrogenase module was found to be widely present in various anaerobic quinol-interacting respiratory complexes, for example in the Qrc (as QrcCD) and Dsr (as DsrOP) complexes of sulfate-reducing bacteria [60]. The presence of four such modules in AArc-CO genome strongly suggest that they are an important part of the anaerobic respiration in this organism, but, once more, since they are not in a direct association with the loci encoding either CO, H₂ or formate-oxidizing enzymes, their true function can not be predicted for now.

Other electron transfer complexes

Both genomes contain four-gene operons encoding a full quinone-reducing electron transfer flavoprotein complex EtfABCX (archael homologue of the bacterial FixBACX [61]), but since they are not located in proximity to the CooS/CooC operons, their possible link to CO oxidation can not be predicted from only genomic content. In contrast to the classical FixC of bacteria, which is the integral membrane quinone-reductase, in the studied archaea this protein is predicted to be cytoplasmic having only a small hydrophobic patch at the N-terminus which would allow it to attach to the inner face of the CPM similar to the characterized EtfABCX from a hyperthermophilic crenarchaeon *Pyrobaculum aerophilum* [62].

Both AArc-CO and HSR-CO genomes encode a non-proton pumping quinol oxidase *bd*, frequently present in obligate anaerobes, including members of two obligate anaerobic sulfur-reducing haloarchaeal genera *Halanaeroarchaeum* and *Halodesulfurarchaeum*. Unexpectedly, in addition to this oxidase, the HSR-CO genome contains a cluster of 5 genes (HSRCO_1948-52), apparently encoding a full set of the heme-copper superfamily cytochrome *c*-oxidase *caa*₃ (COX I-

530 III and COX cluster proteins 1 and 2), which theoretically should allow this organism to grow by aerobic respiration. Despite this, all attempts to grow HSR-CO under microaerophilic conditions with variety of electron donors, including CO, H₂, formate, organic acids and pepton/yeast extract, have been unsuccessful. In contrast, strain AArc-CO can be easily transferred from the sulfur-reducing cultures to full aerobiosis through intermediate microaerophily (2% of O₂ in gaseous
535 phase) using a heterotrophic medium with pyruvate+yeast extract, similar to the other two strains of *Halakaliarchaeum* (AArc-SI^T and AArc-GB) [34, 53]. In agreement with this result, AArc-CO genome contains gene clusters encoding for the proton-pumping quinol oxidases *bo₃* (CyoAB) and *ba₃* (CbaABDE) also present the genome of AArc-SI^T.

Another unexpected feature of the HSR-CO genome is the presence of an operon encoding
540 an archaeal type of extracellularly located membrane-bound nitrate reductase, which includes NarGHI/BC [63]. Moreover, downstream of the *nar* cluster, there is a locus encoding an enigmatic complex reminiscent of the ammonifying nitrite reductase NrfABCD of the enteric gammaproteobacteria [64]. This complex includes an octaheme cytochrome *c* (annotated as the hydroxylamine oxidoreductase), a tetraheme *c* of the NrfB/DmsE family, a membrane-bound quinol
545 dehydrogenase module NrfCD (ferredoxine/membrane diheme cytochrome *b*) and eight cytochrome *c* biogenesis proteins. As far as we know, the presence of such a complex has never been observed in haloarchaeal genomes. Among other archaea, the octaheme *c* protein of HSR-CO has only a low identity (26%) to octaheme *c* hypothetical proteins from *Ca. Methanoperedens nitroreducens*. Also, there is an apparent resemblance of the complex to a part of the hypothetical scheme of
550 extracellular electron transport from a quinol pool to MnO₂ in recently discovered Mn-reducing anaerobic methane oxidizing *Ca. Methanoperedens manganicus* and *Ca. Methanoperedens manganireducens* (Fig.5 in [65]). This includes a membrane-bound quinol-dehydrogenase NrfCD-like module and several multiheme *c* cytochromes conducting extracellular electron transport to MnO₂ or Fe(III) oxides. Attempts to grow HSR-CO anaerobically at hetero- and lithoheterotrophic
555 conditions with nitrate as the electron acceptor showed positive results when H₂ served as the

electron donor and yeast extract as the C-source, but not with CO or formate/yeast extract or at heterotrophic conditions with pyruvate or lactate as substrates. But even with H₂, nitrate (5 mM) was only reduced to nitrite (Supplementary **Fig. S5**) and there was no growth with nitrite even at minimal tested concentration of 1 mM. Nevertheless, these are remarkable results, since, to our knowledge, even incomplete nitrate respiration to nitrite with H₂ as the electron donor has never been demonstrated before in haloarchaea [66].

Anaerobic methylotrophy in the genus Halalkaliarchaeum

As previously mentioned, a new member of the genus *Halalkaliarchaeum*, strain AArc-GB, was enriched and isolated in pure culture from hypersaline soda lakes using glycine betaine (GB, trimethylglycine) as energy and carbon source with sulfur as an electron acceptor, being the first haloarchaeon with such anaerobic methylotrophic metabolism [53]. Since this strain was closely related to AArc-CO, as well as to the type strain of *Halalkaliarchaeum*, *H. desulfuricum* AArc-SI^T, the latter two were also checked for their ability for anaerobic methylotrophy with GB in presence of sulfur as the electron acceptor. The results were positive with the following order of sulfidogenic activity: AArc-GB>AArc-CO>AArc-SI^T. Previous genome analysis of the type strain AArc-SI^T identified a substantial number of genomic loci which, taken together, suggest a hypothetical scheme of the sequential demethylation of GB to glycine with a concomitant methyl group oxidation via the methylotrophic C₁ pathway. The first demethylation step driven by the GB-specific methyltransferases MtgABC is unique for anaerobes, while the second and the third steps catalyzed by the dimethylglycine dehydrogenase Dmg monomeric sarcosine oxidase SoxB, respectively, are described for aerobic methylotrophs utilizing GB as the carbon and energy source. Furthermore, a full complement of the tetrahydrofolate-dependent methyl group oxidative pathway is also present in the same genomic island [53 and the references therein]. The AArc-CO genome seems to encode similar proteins with the exception of a few variations in the number of the homologs (Supplementary **Table S4**) suggesting that both the anaerobic sulfur-dependent C₁

catabolism (carboxydo- and methylo- trophy) are inherent property in the members of genus *Halalkaliarchaerum* isolated in pure culture so far.

585 *Whole cell lysate shotgun proteomic analysis*

The whole cell lysates of the AArc-Co and HSR-CO cells grown at sulfur-reducing conditions either heterotrophically with yeast extract only or lithoheterotrophically with CO+yeast extract were analysed using a shotgun proteomics approach which confirmed the expression of the presumed key enzymes/pathways and moreover provided the following observations (summary of selected
590 enzymes/pathways can be found on **Supplementary Table S5**):

i) Catalytic subunits of the key catabolic enzymes - CooS of the CODH and PsrA of the polysulfide reductase were equally present among the most highly abundant proteins in cells grown with or without CO in both strains. While for the latter it is reasonable, since in both growth conditions sulfur was used as the electron acceptor, the high expression level of the CooS in cells grown
595 without CO would signify the constitutive nature of this enzyme. This contradicts to what was observed in the resting cell experiments (see above). Presumably the observed activation following incubation with CO for several days might indicate the necessity for maturation of constitutively expressed CooS by CooC. The apparently low expression level of the CooC protein in AArc-CO might be (artefactually) connected to interference of the large locus of multiple HD repeats (see
600 above) with the standard sample preparation protocol.

ii) In the working hypothesis on the nature of electron transfer complexes possibly involved in carboxydotrophy, the uptake Ni,Fe hydrogenase is implicated as the CODH partner in HSR-CO (based on the proximity in the genome) and the EtfABC or EtfABCX in case of AArc-CO (on the basis of its quinone-reductase potential and the presence of a ferredoxin subunit). The proteomic
605 data showed that the Ni,Fe hydrogenase catalytic subunits HyaAB are among the highly expressed proteins in HSR-CO cells, although only HyaB had a higher expression level in the presence of CO. In this respect the proteomic results are well in agreement with the high hydrogenase activity

observed in the CO-grown cells of HSR-CO cells (see **Fig. 3**). Among the four Etf complexes in AArc-CO (two EtfAB, EtfABC and EtfABCX), the EtfABC was definitely positively influenced by the presence of CO.

iii) Regarding the other possibilities for channelling electrons from CO oxidation to Psr, it is worth mentioning that cells of AArc-CO grown with CO had a higher level of expression of the ferredoxin-NAD⁺ reductase (Fnr) in comparison with the cells grown heterotrophically only with yeast extract (Supplementary **Table S5**). These cumulatively might be utilized for an alternative rout of the menaquinone reduction with the low-potential electrons derived from CO oxidation. Furthermore, in AArc-CO, only the cells grown with CO expressed one of the four copies of the NrfCD/PsrBC-like membrane-bound complex which is supposed to be able to interact with the quinone pool in various respiratory complexes [60], indicating that it might potentially be involved in the electron transfer from CODH as well.

iv) The overall proteomic results (Supplementary **Table S5**) showed that cells of alkaliphilic AArc-CO responded more actively to the presence of CO in comparison to the cells of neutrophilic HSR-CO.

Conclusions

In this work, we demonstrate for the first time that haloarchaea isolated from hypersaline lakes with neutral and alkaline pH are capable of anaerobic growth with CO as the electron donor and sulfur as the electron acceptor. The direct anaerobic sulfur-dependent carboxydutrophy of the new *Halanaeroarchaem* and *Halalkaliarchaeum* isolates is a catabolic trait which has previously been shown only for a single archaeal genus *Thermococcus*. The exact mechanism of the electron transport from anaerobic CODH to a final electron acceptor (sulfur in this case) remains unclear yet either in bacteria or archaea. Genomic and proteomic analysis of carboxydutrophic haloarchaea suggest several candidates for linking anaerobic CO oxidation to polysulfide reductase via a menaquinone pool, such as the membrane-bound Ni,Fe uptake hydrogenase HyaABCD and the

electron transfer flavoprotein complex EtfAB(CX), but both biochemical characterization and
 635 genetic manipulation analysis are still needed to confirm it. In addition to the anaerobic
 carboxydutrophy, members of the genus *Halalkaliarchaeum* are also capable of anaerobic sulfur-
 dependent methylotrophy with glycine betaine as a substrate, making this genus truly unique among
 haloarchaea.

640 **Author contribution**

DS performed sampling, isolation and physiological characterisation of pure cultures.
 Bioinformatics analyses was carried out by DS, AM, EM and MY. MP and CT performed
 proteomic analysis. PG was responsible for the genome sequencing. The data were interpreted and
 manuscript was written by DS, AM and MY.

645 **Conflicts of interest**

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

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840 **Figure legends**

Figure 1. Phylogenomic placement of carboxydotrophic in sulfur-reducing isolates AArc-CO and HSR-CO among their nearest relatives based on maximum likelihood inference and concatenated partial amino acid sequences of 122 archaeal single copy conserved marker proteins. Bootstrap consensus tree is shown with values above 90% placed at the nodes. Bar, 0.1 changes per position.

845 **Figure 2.** Anaerobic growth kinetics of carboxydotrophic sulfur-reducing haloarchaea grown at 4.0 M total Na^+ /pH 7/37°C (HSR-CO) or pH 9.5/30°C (AArc-CO) with 0.5 g l⁻¹ yeast extract as the carbon source, CO as the electron donor and elemental sulfur as the electron acceptor. (a-b), growth dynamics at 55 mM CO; (c), influence of CO concentration on growth and sulfur-reducing activity. The data are mean values from three parallel incubations.

850 **Figure 3.** Sulfidogenic activity of resting cells of carboxydotrophic haloarchaea grown anaerobically with CO and sulfur. Cells of HSR-CO were incubated in 4 M NaCl containing 50 mM K-P buffer at pH 7. Cells of AArc-CO were incubated in an alkaline buffer containing 3.5 M NaCl and 0.5 M Na^+ as sodium carbonates, pH 9.5. The incubation temperature was 30°C. The anoxic cell suspensions were reduced by 0.2 mM sulfide and 10 µl of 10% dithionite in 1 M NaHCO_3 /2 ml. The chemical controls were buffers without
855 cells and were negative during the whole incubation period (up to 7 days). The data are mean values from duplicate experiments.

Figure 4. Phylogeny of the CooS catalytic subunit from haloarchaeal Ni,Fe-CODH based on maximum likelihood inference. Bootstrap consensus tree is shown with values above 80% placed at the nodes. Bar, 0.1 changes per position. The clades are according to Inoue et al. [4].

860 **Figure 5.** Hypothetical scheme of electron transport from CooS to PsrABC in two anaerobic carboxydotrophic haloarchaea (based on genomic content).

Figure 6. Maximum Likelihood phylogenetic tree of catalytic subunit A of molybdopterin oxidoreductases, members of the Complex iron-sulfur molybdoenzyme (CISM) superfamily according to Duval et al. [67] (A) and respiratory chain components in sulfur-reducing haloarchaea, experimentally proven by us (B). In total, 158 sequences were taken for CISM analysis, including 61 nonhaloarchaeal members (highlighted in gray). The filled circles at the nodes indicate the bootstrap values >70% (1000 bootstrap iterations) in the tree with the highest log likelihood. The loci of the CISM catalytic subunits A of new carboxydotrophic sulfur-reducing halo(natrono)archaea of the genus *Halalkaliarchaeum* (red), and the genus *Halanaeroarchaeum* (blue) are highlighted in bold. The unknown CISM family is shown with an asterisk. Scale bar - 0.06 amino
865 acid substitutions per site. The purple rectangle in (B) highlights the polysulfide reductase PsrABCD, which is transcribed together with sulfurtransferase/rhodanese-like protein. Organisms containing only this PsrABCD polysulfide reductase, which thus represents the ‘minimum’ respiratory outfit required for their detected respiration pattern, are shown in empty black circles. Abbreviations: DMSO/Nar, DMSO/nitrate reductase family; Fdh/Nas/Nap/, assimilatory nitrate reductase/formate dehydrogenase family; [NiFe]-H₂ase, [NiFe] uptake hydrogenase; Psr/Phs, polysulfide/thiosulfate reductase family; Ttr/Arr, tetrathionate/arsenate
870 reductase family.











