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Natranaeroarchaeum sulfidigenes gen. nov., sp. nov., carbohydrate-utilizing sulfur-respiring haloarchaeon from hypersaline soda lakes, a member of a new family *Natronoarchaeaceae* fam. nov. in the order *Halobacteriales*

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ABSTRACT

A pure culture of alkaliphilic haloarchaeon strain AArc-S^T capable of anaerobic growth by carbohydrate-dependent sulfur respiration was obtained from hypersaline lakes in southwestern Siberia. According to phylogenetic analysis, AArc-S^T formed a new genus level branch most related to the genus *Natronoarchaeum* in the order *Halobacteriales*. The strain is facultatively anaerobic with strictly respiratory metabolism growing either by anaerobic respiration with elemental sulfur and thiosulfate as the electron acceptors or by aerobic respiration at microoxic conditions. Thiosulfate is reduced partially to sulfide and sulfite. It is a first sulfur-reducing alkaliphilic haloarchaeon utilizing sugars, starch and glycerol as substrates for anaerobic growth. It is extremely halophilic (optimum at 3.5 M total Na^+) and obligately alkaliphilic (optimum at pH 9.5). The dominant polar lipids include PG and PGP-Me with the archaeol (C_{20}-C_{20}) or extended archaeol (C_{20}-C_{25}) cores. The dominant respiratory lipoquinone is MK-8:8. On the basis of unique physiological properties and results of phylogenetic analysis, the soda lake isolate is suggested to be classified into a novel genus and species *Natranaeroarchaeum sulfidigenes* gen. nov., sp. nov. (=JCM 34033^T = UNIQEM U1000^T). Furthermore, on the bases of phylogenomic reconstruction, a new family *Natronoarchaeaceae* fam. nov. is proposed within the order *Halobacteriales* incorporating *Natranaeroarchaeum* and three related genera: *Natronoarchaeum*, *Salinarchaeum* and *Halostella*.

Introduction

Our previous research into anaerobic haloarchaea growing by sulfur respiration found in anoxic sulfidic sediments of hypersaline soda lakes resulted in the discovery of two new genera of facultatively anaerobic alkaliphilic haloarchaea *Natrarchaeobaculum* and *Halalkaliarchaeum* capable of anaerobic growth with elemental sulfur as the electron acceptor and a broad range of simple electron donors, mostly representing fermentation products, including H_2, formate, CO, pyruvate and C4-C8 fatty acids [28–30,32]. In continuation of elucidation of the role of haloarchaea in anaerobic mineralization of organic matter in hypersaline lakes, a novel functional group which would perform sulfur respiration using more complex substrates, such as carbohydrates, was thought of and indeed found, both in salt and soda lakes. It included multiple closely related isolates from salt lakes with fermentative metabolism recently described as *Halapricum desulfuricans* sp. nov. and a single natronoarchaeal strain AArc-S^T with strictly respiratory metabolism which was most related the genus *Natronoarchaeum* [31,33]. Here we provide a taxonomic description of *Natranaeroarchaeum sulfidigenes* gen. nov., sp. nov., and also propose to form a new family *Natronoarchaeaceae* to accommodate a monophyletic phylogenomic cluster including four genera: *Natronoarchaeum, Salinarchaeum* and *Halostella* and *Natranaeroarchaeum* gen. nov.

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Materials and methods

Enrichment, isolation and cell morphology

The sources of the inocula were anaerobic sulfidic sediments (3–20 cm deep) from 3 hypersaline soda lakes in southwestern Siberia (Kulunda Steppe, Altai region, Russia). The samples were taken by a stratometer corer tube into a sterile 100 ml bottle, filled to the top and closed without air bubbles. The enrichment and isolation procedures, the medium composition, cultivation conditions and analyses of growth and sulfide/polysulfide formation have been described previously [31]. In short, routine cultivation was performed in a mineral medium was prepared by 1:1 mixing of the neutral base (4 M NaCl adjusted to pH 7 by 50 mM K-P buffer) and sodium carbonate-bicarbonate base containing 4 M total Na^+ at pH 10, resulting in the 2 M total carbonate alkalinity and final pH 9.7. After sterilization, the medium was supplemented with 4 mM NH_4Cl, 1 mM $MgSO_4$, 1 ml l^{-1} each of trace metals and vitamins [21], 20 mg l^{-1} of yeast extract and either 5 mM glucose, 5 mM glycerol (from 1 M filter-sterilized stock solutions) or soluble starch (from 10% heat-sterilized stock solution). Furthermore, a mixture of antibiotics (streptomycin, kanamycin and vancomycin, Sigma-Aldrich) at a final concentration 100 mg l^{-1} each were used to suppress growth of bacteria in the soda lake enrichments. The medium was reduced with 0.5 mM sulfide and the cultivation was done in serum bottles varying in volumes from 12 to 115 ml after creating anoxic conditions by evacuation-argon flushing. For colony formation, the grown culture was serially diluted in the above mentioned reduced anoxic liquid medium, mixed at 50 °C with 4% washed and melted agar, poured into Petri plates and incubated in anaerobic jars under argon with O_2-scavenging catalyst (Oxoid). Sulfide formation was measured with methylene blue method after fixation with 10% Zn acetate [34].

Cell morphology was examined by using phase contrast microscopy (Zeiss Axioplan Imaging 2, Germany) and electron microscopy. For the flagella detection, the paraformaldehyde-fixed cells in 4 M NaCl were positively stained with 1% uranyl acetate for 1 min and the salts were removed with a brief emersion into demineralized water. The preparations were examined with the JEOL-100 model transmission electron microscope (Japan).

Chemotaxonomy

The intact polar lipids (IPLs) and respiratory quinones were extracted from freeze-dried biomass using a modified Bligh-Dyer procedure [2]. Briefly, the biomass was treated ultrasonically twice for 10 min with a solvent mixture of methanol, dichloromethane (DCM) and phosphate buffer (2:1:0.8, v:v:v). After sonication, the combined supernatants were phase-separated by adding additional dichloromethane and buffer to a final solvent ratio of 1:1:0.9 (v:v:v). The organic phase containing the IPLs was collected and the aqueous phase re-extracted twice with DCM. All steps of the extraction were then repeated, but with a solvent mixture of methanol, DCM and trichloroacetic acid pH 2–3 (2:1:0.8, v:v:v). Finally, the combined extract was dried under a stream of N_2 gas. Before analysis, the extracts were redissolved in a mixture of MeOH:DCM (9:1, v:v) and aliquots were filtered through 0.45 μm regenerated cellulose syringe filters (4 mm diameter; Grace Alltech, Deerfield, IL, United States). Analysis of extracts was carried out using an Ultra High Pressure Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS) according to the reversed phase method [35] with modifications described in [2]. Identification was carried out by comparison of accurate masses and mass spectral fragmentation with published data for IPLs [3] and for quinones [7].

Genome sequencing and phylogenetic analysis

The genome of AArc-ST was sequenced, assembled as a single chromosome and analyzed as described previously [31]. The genome statistics is given in the Supplementary Table S1. For phylogenomic reconstructions, 122 archaeal single copy conserved marker proteins were used according to the Genome Taxonomy Database [23] and aligned using GTDB-Tk [4] or MAFFT [20] using G-INS-I algorithm. The trees were built with the IQ-TREE 2 program [18] with fast model selection via ModelFinder [14] and ultrafast bootstrap approximation [17] as well as approximate likelihood-ratio test for branches [1] or using PhyML 3.0 [9] with approximate likelihood-ratio test for branches [1], SPR type of tree improvement [13] and SMS algorithm for best substitution model selection [15]. 16S rRNA gene based phylogenetic tree was build using the IQ-TREE 2 program [20]. The whole genome comparison was conducted by using three different methods: Average Nucleotide Identity (ANIb and ANIm), using JSpeciesWS web server; Average Amino acid Identity (AAI) by the AAI calculator online of Kostas lab (https://enve-omics.ce.gatech.edu/aai/index) and DDH by the Genome-to-Genome Distance Calculator 2.1 online tool (https://ggdc.dsmz.de/ggdc.php) [22,24]. The 16S rRNA gene and the full genome sequences of strain AArc-ST are deposited in the GenBank under the numbers MZ297357 and CP064786, respectively.

Results and discussion

Pure culture isolation, cell morphology and chemotaxonomy

From the three different types of carbohydrate substrates (glucose, starch, glycerol) used for the primary anaerobic sulfur-reducing enrichments only a glucose-supplemented culture was dominated by archaea, while the other two were overrun by bacteria despite the use of antibiotics. However, serial dilutions in liquid culture were not sufficient to remove the bacterial component. Therefore, further purification was done via colony separation in soft agar. The colonies of sulfur-reducing archaea were recognizable by their pink color and a characteristic discoloration zone against yellow background due to the reduction of polysulfide sulfur to sulfide (Fig. 1). These colonies grew back in anaerobic liquid medium with glucose and sulfur. One of the cultures was designated strain AArc-S^T. The cells of AArc-S^T were polymorphic, from flat coccoids to board rods, motile with 1–3 archaella without any signs of polyhydroxyalkanoate-like refractive granule accumulation (also obvious from the lacking of the respective genes in the genome) characteristic of the neutraphilic *Halapricum desulfuricans* cells [33] (Fig. 1). The biomass in all growth conditions contained carotenoids, in contrast to the previously described facultatively aerobic natronarchaeal sulfur-reducing genera producing pigments only under aerobic growth conditions [28–30,32].

The core component of the membrane lipids of strain AArc-S^T consisted of two major dialkyl glycerol ether (DGE) components, common among haloarchaea: archaeol (C_{20}-$C_{20;}$ AR) and extended archaeol (C_{20}-$C_{25;}$ EXT-AR), including a high proportion of unsaturated EXT-AR. Also present was a lyso-AR (where one of the isoprenoidal alkyl chains is absent) (Supplementary Table S2). The lipid polar head groups in AArc-S^T were also typical of haloarchaea, dominated by phosphatidylglycerolphosphate methyl ester (PGP-Me), with lower levels of phosphatidylglycerol (PG). Neither glyco- or sulfo-lipids were detected. The dominant respiratory lipoquinone was identified as menaquinone MK-8:8, with only trace amounts of MK-8:7, MK-7:7 and MK-7:6 (Supplementary Table S2).

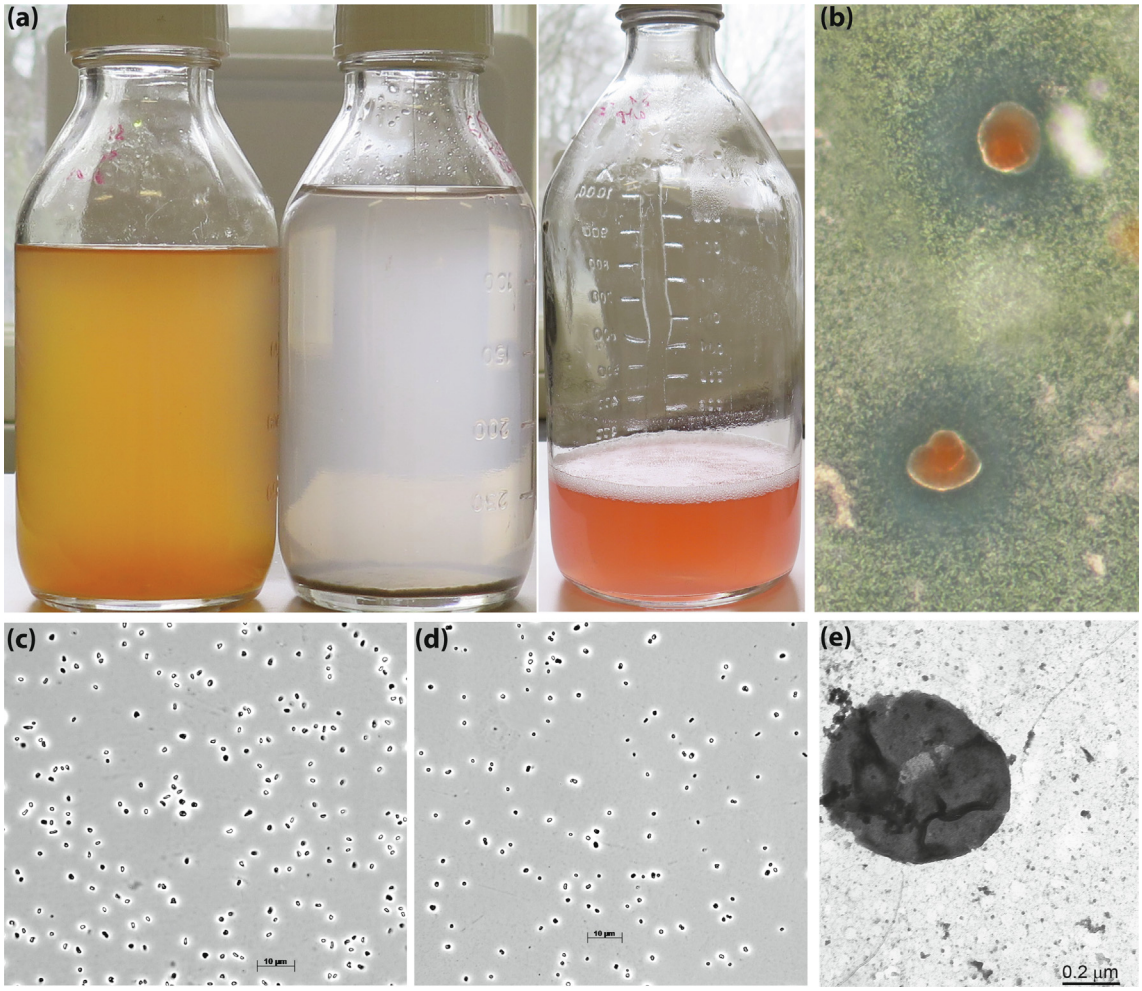

Fig. 1. Macro- and micro- morphology of carbohydrate-utilizing sulfur-respiring natronoarchaeon AArc-S growing at 4 M total Na^+ and pH 9.7. (**a**), appearance of liquid cultures at different incubation conditions: left, sulfur-reducing culture showing intensive polysulfide formation; middle, thiosulfate-reducing culture forming sulfide; right, microaerobic culture showing intensive carotenoid production. (**b**), anaerobic colonies inside sulfur/polysulfide agar with polysulfide-consumption zone. (**c-d**) phase contract microphotographs of cells from sulfur- and thiosulfate-reducing cultures, respectively. (**e**) transmission electron microphotographs of a positively stained cell showing flagellation.

Phylogenetic analyses

The genome of AArc-S^T has two identical *rrn* operons with the 16S rRNA gene most related to the genus *Natronoarchaeum* which currently includes four species [19]. In all cases, the level of 16S rRNA gene identity to the closest related species were below 95%, indicating a novel genus level (Fig. 2**a**). A search of the metagenomic database did not identify any close relatives of AArc-S^T. However, we recently did obtain several strains of aerobic amylolytic natronoarchaea enriched with amylopectin (insoluble starch) and levan (beta-fructan) from surface brines and sediments of the same hypersaline soda lakes, with their 16S rRNA gene sequence identity to AArc-S^T ranging from 98.2 to 99% (Fig. 2**a**). This level of relation indicates a separate species status in the same genus. Results of extended phylogenomic analysis based on 122 conserved archaeal protein markers were consistent with the placement of AArc-S^T by the 16S rRNA gene-based phylogeny into a novel genus (Fig. 2**b**). This conclusion was also obvious from calculations of the whole genome comparison indexes, ANI, AAI and DDH (Supplementary Table S3). Furthermore, based on our phylogenomic reconstruction of phylogeny of the class *Halobacteria* (261 genomes), AArc-S^T represents a genus level branch in a monophyletic cluster including the genera *Natronoarchaeum* [19], *Salinarchaeum* [5–6,16] and *Halostella* [11–12,27] (Fig. 2**b**). These genera are currently classified as members of the families *Halobacteriaceae* and *Natrialbaceae* [10]. This monophyletic cluster is closest to the family *Natrialbaceae* and has the same branching depth, thus forming a novel family group within the order *Halobacteriales* for which we propose the name *Natronoarchaeaceae* (according to the genus *Natronoarchaeum* as the earliest described in the cluster). This result has been confirmed using various algorithms for alignment and tree construction and is consistent with the GTDB taxonomy except for the placement of the genus *Salinarchaeum* in a separate family [4]. Our analysis does not support such a placement, but it must be stressed that for the final classification it would be necessary to include the genome of the recently described *S. chitinilyticum* not yet available in the public databases.

Metabolic properties

The key physiological property of strain AArc-S^T is the ability to utilize sugars, starch, and glycerol as the energy and carbon source for anaerobic sulfidogenic growth in presence of either elemental sulfur or thiosulfate as the electron acceptors [31], the potential that has not been demonstrated for the previously described sulfur-reducing natronoarchaea. Interestingly, the anaerobic car-

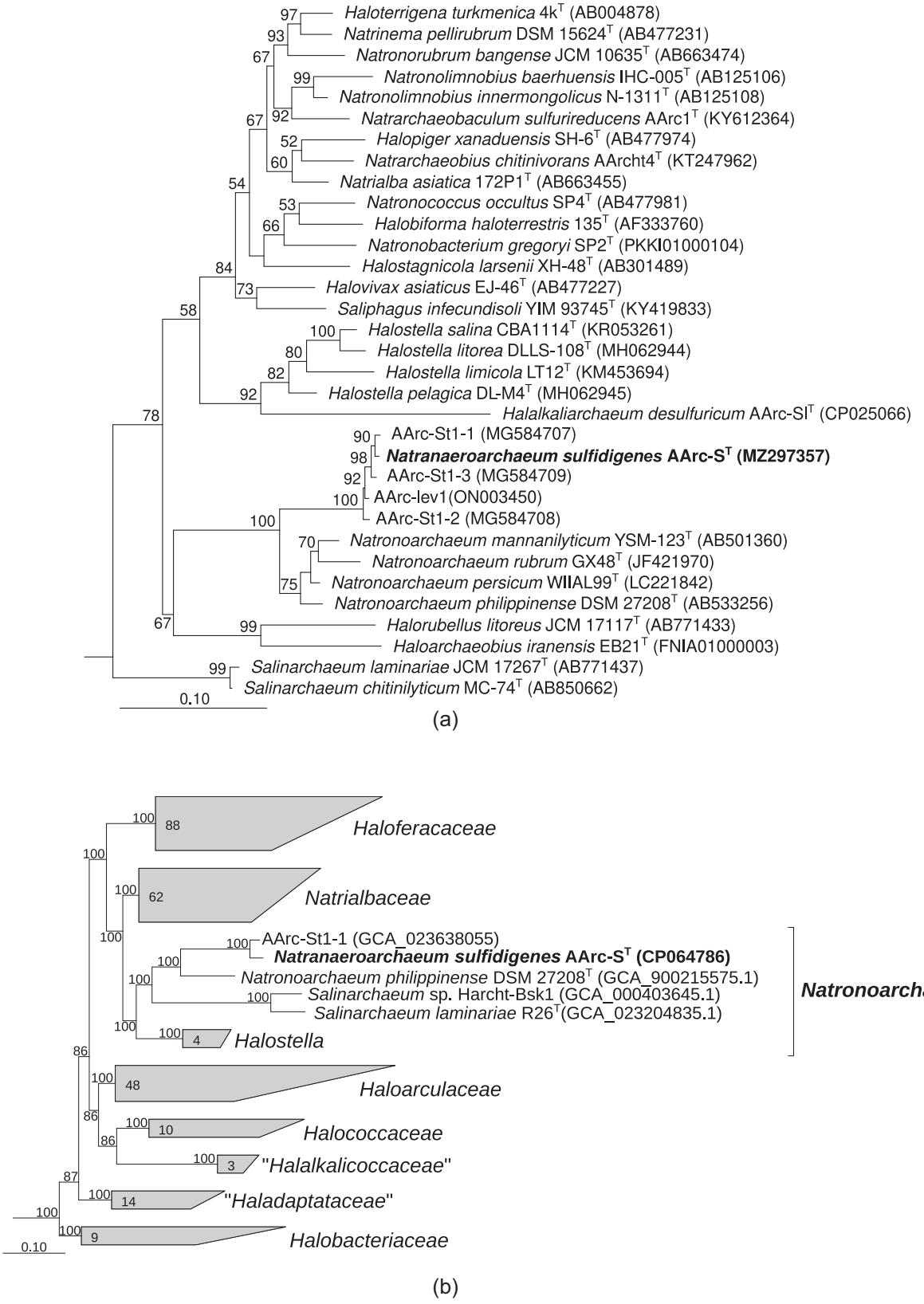

Fig. 2. Phylogenetic placement of *Natranaeroarchaeum sulfidigenes* AArc-S^T within the class *Halobacteria* based on (**a**) 16S rRNA gene sequences and (**b**) on concatenated amino acid sequences of 122 archaeal single copy conserved marker proteins with taxonomic designations according to the Genome Taxonomy Data Base. *Archaeoglobus fulgidus* DSM 4304 was used as an outgroup for phylogenetic reconstruction based on the 16S rRNA gene and representatives of many other deep phylogenetic groups including other phyla were used as an outgroup for phylogenetic reconstruction based on 122 archaeal single copy conserved marker proteins. Bootstrap consensus tree is shown with values above 70 (**a**) and 90 (**b**) % placed at the nodes. Bar, 0.1 change per position.

bohydrate utilization in AArc-S^T was strictly respiratory (i.e. no growth occurred without *e*-acceptors) in contrast to a group of neutrophilic carbohydrate-utilizing sulfur-reducing haloarchaea which grew fermentatively with H_2 formation in the absence of sulfur [31,33]. We also tested a potential ability for anaerobic growth with sulfur and thiosulfate as acceptors and glucose as donor of the type species of the genus *Natronoarchaeum, N. mannanilyticum*, and the result was negative. This is corroborated by the absence of genes encoding the Psr/Phs family polysulfide/thiosulfate reductases in the genome of *N. philippinense* [26] (since the genome of the type species is not available yet).

Concerning the utilized spectrum of carbohydrates as the *e*-donor/C source, apart from a limited number of mono- and disugars and glycerol, AArc-S^T was also able to grow with soluble starch. This potential is confirmed by the presence of multiple gene copies encoding alpha-amylases of the GH13 (12) and GH15 (2) families in the genome of AArc-S^T [31]. It is also worth to note, that the type species of the closest relative of strain AArc-S^T, *N. mannanilyticum*, is a hydrolytic organism able to grow aerobically with galactomannan and starch [25].

The genome of AArc-S^T encodes catalase/peroxidase and several types of quinol oxidases supporting positive catalase (assay with 3% H_2O_2) and oxidase (with 0.1% *N,N,N,N* tetramethyl-p-phenylendiamine hydrochloride) tests with cells grown microaerobically (10% air/90% argon gas phase, liquid:gas = 1:10). The protease, esterase and lipase activities in microaerobic culture of AArc-S^T incubated on plates with casein/gelatin (after flooding with 10% trichloroacetic acid) and emulsified tributyrin/olive oil (turbidity clearance), respectively, were negative. Ammonium and urea (but not nitrate) can serve as the N-source in cultures grown anaerobically with glucose and sulfur (genome contains a full [Ni]-urease operon UreABCEFGH). Indole formation from tryptophan (Kovac's reagent test) showed negative results with cells grown microaerobically. Antibiotic sensitivity was tested aerobically in liquid medium with glucose and yeast extract at pH 9.7 and showed growth inhibition by rifampicin and chloramphenicol above 50 mg l^{-1}, and resistance to streptomycin, ampicillin, kanamycin, vancomycin and gentamicin at up to 100 mg l^{-1}.

The Mg-demand was low, with an optimum growth at 1–2 mM. AArc-S^T is an extreme halophile, with an optimal growth (at pH 9.5) occurring at 3.5–4 M total Na^+ and with the range from 2.5 to 4.5 M (tested aerobically). The pH profiling at microaerobic conditions (pH 6–8, HEPES/$NaHCO_3$/NaCl; pH 8–8.5, HEPES/Na_2CO_3/NaCl; pH 9–10.5, $NaHCO_3$/Na_2CO_3/NaCl) showed that AArc-S^T is a typical obligate natronophile, growing optimally at pH around 9.5 and up to 10.2 in a sodium carbonate-based buffer system. Genome analysis shows complete absence of genes encoding export of external organic osmolytes but a large representation of the potassium-trafficking proteins, including K^+/H^+ symporter of the Trk family (8 copies of the *trkA* and 2 copies of *trkH*), two copies of *kefB* [K^+/H^+ symporter (K^+ efflux)], potassium ion channel Pch and Na/K:proton antiporter NhaP. The intracellular pH homeostasis at extreme outside alkalinity is apparently maintained by the activity of a multisubunit Na^+/H^+ antiporter MrpB1B2CD1D2D3EFG.

The maximum temperature for aerobic growth determined at optimal pH-salt conditions was 45°C. Comparative properties of the new isolate with the type species of the closest related genus *Natronoarchaeum* are given in Table 1. The soda lake isolate AArc-S^T can be easily differentiated from the related species of the genus *Natronoarchaeum* by its ability for sulfidogenic growth and the obligate alkaliphily. Functional annotation of the anaerobic

Table 1
Comparative properties of strain AArc-S^T and *Natronoarchaeum mannanilyticum* [25].

Property	***Natranaeroarchaeum sulfidigenes* AArc-S^T**	*Natronoarchaeum mannanilyticum* JCM 16328^T
Cell morphology	flat pleomorphic, motile	pleomorphic, nonmotile
Pigmentation	red (aerobic); pink (anaerobic)	red
PHA accumulation	-	-*
Aerobic growth	+	+
Anaerobic growth by:		
sugar fermentation	-	-*
sulfur/thiosulfate respiration	+	-*
sulfoxide respiration	-	-*
Number of Psr operons in genomes	1	0**
e-donors for anaerobic growth	sugars, starch, glycerol	-*
Substrates for aerobic growth	sugars, starch, yeast extract	lactose, raffinose, sucrose, maltose, cellobiose, starch, galactomannan, pyruvate, lactate, glutamate,yeast extract, peptone
Amylase	+-	+-
Esterase/lipase	(tributyrin/olive oil)-	(Tween 80)-
Protease	(gelatin, casein)	(gelatin)
Catalase/oxidase	+/+(w)	-/ +w *
Indole from tryptophane	-	+
Salinity range (opt.) M Na^+	2.5–4.5 (3.5)	1.6–4.2 (2.5–3.2)
pH range (opt.)	8.5–10.2 (9.5–9.7)	6.0–9.5 (8.5–9.0)
Temperature max (°C)	45	55
Core lipids	C_{20}-C_{20}, C_{20}-C_{25} DGE	NR
Intact membrane polar lipids:		
phospholipids:	PG, PGP-Me	PG, PGP-Me, PGP,
glycolipids:	absent	S_2-DGDE
Respiratory lipoquinones	MK-8:8 (major); MK-7:7/MK8:7 (minor)	NR
DNA G + C	60.8% (genome)	63.0 (mol%)
Type of hypersaline habitat	Soda lakes	Marine solar saltern

NR, not reported; (v) - variable property in different species of the same genus; w (weak); Psr - polysulfide reductase;
* determined in this study.
** genome of *N. phillipinensis* [26]. Lipids: (PG) phosphatidylglycerol, phosphatidylglycero-phosphate (PGP), (PGP-Me) phosphatidylglycerophosphate methyl ester, disulfated diglycosyl diether (S_2-DGDE), (DGE) - dialkyl glycerol ether.

and aerobic respiratory systems in AArc-S^T have already been provided in details by Sorokin et al., 2021 [31].

Comparative properties of the novel genus with the other three related genera suggested to be classified together with *Natranaeroarchaeum* in the new family *Natronoarchaeaceae* (*Natronoarchaeum*, *Salinarchaeum* and *Halostella*) are presented in Table 2. The common properties of these four genera include extreme halophily, phosphatidylglecerol (PG) and phosphatidylglycerophosphate methyl ester (PGP-Me) as the dominant membrane phospholipids, menaquinone MK8:8 as the dominant respiratory lipoquinone (although the data are incomplete) and the ability to grow aerobically with starch, sugars and glycerol. *Natranaeroarchaeum* differs from the other three genera by obligate alkaliphily, consistent with its different habitat (soda lakes in contrast to NaCl-dominated salt lakes/salterns with neutral pH) and by its ability to grow by anaerobic sulfur/thiosulfate-dependent respiration. The only genus among the rest of *Natronoarchaeaceae* members which can tolerate highly alkaline conditions (but still being neutrophile in its optimum growth pH) is the closest relative of *Natranaeroarchaeum*, the genus *Natronoarchaeum*. Although tolerance to pH up to 10 has also been reported for two species of the genus *Salinarchaeum* [16], the results were apparently not verified by checking the final pH values. Taking into account our own results with *Salinarchaeum* sp. HArcht-Bsk and the nature of habitats from which the two described species of *Salinarchaeum* were obtained, the three representatives of this genus known so far in pure culture are typical neutraphilic haloarchaea. A unique feature of the genus *Natronoarchaeum* is its ability to use heteropolysaccharide galactomannan (arabic gum) as the growth substrate. So far, this potential has only been reported for one another haloarchaeon - *Haloarcula mannanilytica* [8]. Another discriminating feature of all four known species of *Natronoarchaeum* is the presence of disulfated glycolipid S2-DGDE as one of the dominant membrane lipids. A unique feature of all three known strains of the genus *Salinarchaeum* is their potential to grow with chitin as substrate. The only available genome of *Salinarchaeum* sp. HArcht-Bsk contains five gene copies of the GH18-family endochitinase [6]. Two out of four described species of the genus *Halostella* (*H. litorea* and *H. limicola*) are capable of anaerobic respiration using dimethylsulfoxide as the electron acceptor and, furthermore, *H. limicola* can grow by arginine fermentation and has a full denitrification pathway [11].

The proposed novel family *Natronoarchaeaceae*, according to our phylogenetic reconstruction based on 122 conserved single-copy protein markers, is surrounded by families that are part of the *Halobacteriales* order according to GTDB whose archaeal taxonomy structure is based on the same 122 conserved markers. Although families *Haloferacaceae* and *Natrialbaceae* are currently not recognized as the *Halobacteriales* members, we are unambiguously convinced that they are a part of the order *Halobacteriales* as well. This conclusion is confirmed both by the high reliability of our genomic-based phylogenetic reconstructions and by the GTDB. The families *Halobacteriaceae*, *Haloarculaceae* and *Halococcaceae* are recognized as the genuine members of *Halobacteriales* both in the GTDB and the NCBI taxonomies and these three families lie at the base of the monophyletic cluster present on our phylogenomic tree (Fig. 2**b**). In contrast, the newly proposed *Natronoarchaeaceae* and the families *Natrialbaceae* and *Haloferacaceae* seem to be a more recently evolved cluster of the *Halobacteriales*. However, taxonomic reevaluation of the whole *Halobacteriales* order is outside of the scope of present work and would need a more focused deep phylogenomic analysis.

Table 2
Comparative properties of the members of family *Natronoarchaeaceae*. The data are from [25–31;33–34] and this work.

Property	***Natranaeroarchaeum***	*Natronoarchaeum*	*Salinarchaeum*	*Halostella*
Number of species	1	4	2	4
Number of available genomes	1	1	2	5
Genome size (Mbp)	3.04	3.16	3.26	3.74–4.12
G + C genomic/mol%	60.8	65.2/63.0–66.7	66.6/66.4–68.2	63.2–68.1
Number of the 16S-rRNA genes	2 (identical)	1–2 (dissimilar)	1	2–3 (dissimilar)
Cell morphology	Irregular coccoids, motile	Rods, motility (v)	Rods, motile	Cocci or rods, motile
Core membrane lipids	C_{20}-C_{20}, C_{20}-C_{25} DGE	nrPG, PGP-Me, PGP	C_{20}-C_{20}	nrPG, PGP-Me; PA
Identified membrane phospholipids	PG, PGP-Me	(v)	PG, PGP-Me, PGS	(v)+
Glycolipids	-	S2-DGDE	-	(probably sulfated glycosyl-mannosyl glycolipids)MK8:8 and MK8:7
Menaquinones	MK8:8 (major)	nr	nr	(3 species)
Anaerobic respiration	+ (growth experiments and genomic content)	- (growth experiment* and genomic content**)	- (growth experiments* and genomic content**)	(v) (growth experiments)
Electron acceptors for anaerobic respiration	sulfur and thiosulfate	-	-	DMSO (v);nitrate (full denitrification) (v)
Fermentative growth	-	- (growth experiment*)	nr	+ arginine (growth experinent) (v)
Aerobic growth with:	Sugars, glycerol, starch	Starch, sugars;galactomannan, glycerol, acetate and lactate (v)	Chitin, sugars; laminarin*, starch, glycerol, pyruvate, lactate (v)	Starch, sugars, acetate, pyruvate, succinate, malate, fumarate, glycerol, mannitol (v)
pH range (opt.)	8.5–10.2 (9.5–9.7)	5.5–9.5 (7.0–9.0)	6–10*** (7.0)	7–9 (7–8)
Salinity range (opt.), M Na^+	2.5–4.5 (3.5)	1.4–5.3 (2.6–4.5)	1.4–5.1 (2.6–4.3)	1.4–5.1 (2.6–3.4)
Mg^{2+} requirement	low	high	high	v
Temperature range (opt.), ºC	25–45 (35–40)	20–55 (37–45)	20–50 (37–40)	20–50 (40)
Amylase/protease/lipase	Amylase	Amylase	Protease (v)	Protease (v)
Catalase/oxidase	+/+(w)	v/v	v/v	+/-
Indole from tryptophane	-	v	-	v
Hypersaline habitat type	Soda lakes	Salt lakes and salterns, salted marine algae		

* Tested in this work in *N. mannanilyticum and Salinarchaerum* sp. HArc-Bsk.
** Absence of the psrA/phsA genes in the genome of *N. philippinense, Salinarchaeum* sp. HArc-Bsk, *H. salina.*
*** The highest values are not proven properly because final pH values at alkaline range are not reported in [16]. PA - phosphatidic acid; other lipid abbreviations - as in Table 1. v - variable in different species; w - weak reaction; nr - not reported.

Table 3
Natranaeroarchaeum sulfidigenes protologue.

Parameter	**Family:** ***Natranaeroarchaeaceae* fam. nov.**	**Genus:** ***Natranaeroarchaeum* gen. nov.**	**Species:** ***Natranaeroarchaeum sulfidigenes* sp. nov.**
Author	Dimitry Y. Sorokin		
Family name	*Natronoarchaeaceae*		
Genus name		*Natranaeroarchaeum*	
Species name			*sulfidigenes*
Status	fam.nov	gen. nov.	sp. nov.
Etymology	Na.tro.no.ar.chae.a.ce'ae N.L. neut. n. *Natronoarchaeum,* the type genus of the family; *-aceae* ending to denote a family; N.L. fem. pl. n. *Natronoarchaeaceae,* the family of the genus *Natronoarchaeum*	*Natranaeroarchaeum* Natr.an.aer.o.ar.chae'um N.L. n. *natron* (derived from Arabic n. *natrun* or *natron*), soda, sodium carbonate; Gr. pref. *an-*, not; Gr. masc. n. *aêr* (gen. aeros), air; Gr. masc. adj. *archaios,* ancient; N.L. neut. n. *Natranaeroarchaeum,* anaerobic natronophilic archaeon	sul.fi.di'ge.nes. N.L. neut. n. *sulfidum*, sulfide; Gr. suff. *-genes*, producing; from Gr. ind. v. *gennaô*, to produce; N.L. part. adj. *sulfidigenes*, sulfide-producing
Type species of the genus		***Natranaeroarchaeum sulfidigenes***	yes
Description of new taxon	The family *Natronoarchaea-ceae* includes obligately aerobic and facultatively anaerobic, extremely halophilic or haloalkaliphilic organoheterotrophic archaea living in hypersaline habitats of marine or terrestrial origin. Most of the members are saccharolytic, utilizing simple sugars and starch for aerobic or anaerobic growth and some are able to utilize polysaccharides as growth substrates, including galactomannan, chitin and laminarin. The family includes four genera, *Natranaero-archaeum*, *Natronoarchaeum*, *Salinarchaeum* and *Halostella*, forming a monophyletic phylogenomic clusuter within the order *Halobacteriales.* The type genus is *Natronoarchaeum*.	Extremely halophilic and obligately alkaliphilic archaea capable of anaerobic growth by carbohydrate-dependent sulfur/thiosulfate respi-ration. Found in sulfidic sediments of hypersaline soda lakes. Recommended three-letter abbreviation: Naa. The type species is *Natranaeroarchaeum sulfidigenes.* Family classification: the genus *Natranaeroarchaeum* is classified in a novel family *Natronoarchaeaceae,* order *Halobacteriales* within the class *Halobacteria*.	The cells are motile, polymorphous, from flat rods to coccoids, 0.5–1 × 0.8–2.5 μm, depending on the growth conditions. The cells lyze in hypotonic solutions below 1.5 M NaCl. Red carotenoids are produced both during aerobic and anaerobic growth. The core membrane diether lipids are dominated by the C_{20}-C_{20} DGE (archaeol) and the C_{20}-C_{25} DGE (extended archaeol) with 0–4 double bonds. The polar lipid head groups are phosphatidylglycerol-phosphate methyl ester (PGP-Me) and phosphatidylglycerol (PG). The dominant respiratory lipoquinone is MK-8:8. Facultatively anaerobic. Anaerobic growth is strictly respiratory, with sulfur compounds as *e*-acceptors, including elemental sulfur (reduced to sulfide) and thiosulfate (two-electron reduction to sulfide and sulfite). Aerobic growth occurs at microoxic conditions. The utilized substrates include hexoses, (glucose, fructose, mannose, galactose, raffinose, trehalose and maltose), glycerol and starch. The substrates tested but not utilized include arabinose, rhamnose, sucrose, lactose, raffinose, melibiose, melezitose, ribose, xylose, cellobiose, glucuronic and galacturonic acids, mannitol, arabitol, inosi-tol, inulin, levan, acetate, lactate, pyruvate, glycine, glutamate and aspartate. Ammo-nium and urea are utilized as the N-source. Oxidase is weakly positive, catalase is positive. Maximum growth temperature is 45 °C. Extremely halophilic with a range of total Na^+ for growth from 3 to 5 M (optimum at 3.5–4 M) and obligately alkaliphilic, with a pH range from 8.5 to 10.2 (optimum at 9.5–9.7). The temperature range for aerobic growth is 25–45 °C (optimum at 35–40). The G + C content of the genomic DNA is 60.8%. The type strain (AArc-ST = JCM 34033^T = UNIQEM U1000^T) was isolated from anaerobic sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia). The 16S rRNA gene and the full genome sequences are deposited in the GenBank under accession numbers MZ297357 and CP064786, respectively.
Authors	Dimitry Y. Sorokin, Michail M. Yakimov, Enzo Messina, Alexander Y. Merkel, Michel Koenen, Nicole J. Bale, Jaap S. Sinninghe Damsté		
Title	*Natranaeroarchaeum sulfidigenes* gen. nov., sp. nov., carbohydrate-utilizing sulfur-respiring haloarchaeon from hypersaline soda lakes and proposal of a new family *Natronoarchaeaceae* fam. nov. in the order *Halobacteriales*.		

(*continued on next page*)

Table 3 (*continued*)

Parameter	Family: *Natranaeroarchaeaceae* fam. nov.	Genus: *Natranaeroarchaeum* gen. nov.	Species: *Natranaeroarchaeum sulfidigenes* sp. nov.
Journal	Systematic and Applied Microbiology		
Corresponding author	Dimitry Y. Sorokin		
E-mail of corresponding author	d.sorokin@tudelft; soroc@inmi.ru		
Strain collection numbers			JCM 34033; UNIQEM U1000
16S rRNA gene accession number			MZ297357
Genome accession numbers			CP064786
Genome status			closed
G + C %		60.8–61.0	60.8 (genome type strain)
Country of origin		Russian Federation	Russian Federation
Region of origin		Altai region	Altai region
Date of isolation		2016	2016
Source of isolation		Hypersaline soda lakes	Anaerobic sediments of hypersaline soda lakes in southwestern Siberia
Sampling dates		2015	2015
Geographic location		*S-W* Siberia	*S-W* Siberia
Latitude			51°39′ N; 49°10′ N; 48°14′ N
Longtitude			79°48′ E; 46°39′ E; 46°35′ E
Depth			0–10 cm
Temperature of the samples			20 °C
pH of the samples			10–11
Salinity of the samples			18–36%
Number of strains in study		1	1
Growth medium, incubation conditions			4 M total Na^+, pH 9.7; incubation – 30 °C; glucose + S_8; anaerobic
Conditions of preservation		Deep freezing in 15% glycerol (v/v)	
Gram stain		negative	
Cell shape		Pleomorphic, from flat rods to coccoids, motile	
Cell size			0.8–2 μm in diameter
Motility			Non-motile
Colony morphology		Pink-orange	Pink-orange, up to 2 mm, discs with polysulfide clearance zones (anaerobic)
Highest temperature for growth		50 °C	45 °C (at pH 9.0)
Optimal temperature for growth		35–40 °C	35–40 °C
Lowest pH for growth		7.2	8.5
Highest pH for growth		10.2	10.2
Optimum pH for growth		8.8–9.7	9.5–9.7
pH category		Obligate alkaliphiles	
Lowest salt concentration for growth		2.5 M total Na^+	2.5 M total Na^+
Highest salt concentration for growth		5 M total Na^+	4.5 M total Na^+
Optimum salt concentration for growth		4.0 M total Na^+	3.5 M total Na^+
Other salts important for growth		Sodium carbonates	
Salinity category		extremely halophilic	
Relation to oxygene)		facultative anaerobes	
Carbon source used (class)		carbohydrates	
Specific compounds		Starch-like alpha glucans, levan	Starch
Nitrogen source		Ammonium, urea	
Terminal electron acceptor		O_2, S_8	O_2, S_8, $S_2O_3^{2-}$
Energy metabolism		chemoorganotrophic	
Phospholipids		Core membrane lipids are archaeol (C_{20}-C_{20} DGE) and extended archaeol (C_{20}-C_{25} DGE)Polar lipids are phosphatidylglycerophosphate methyl ester (PGP-Me) and phosphatidylglycerol (PG)	
Glycolipids		-	
Respiratory lipoquinones		MK8:8 (major); MK-7:7/Mk-8:7 (minor)	
Habitat		Hypersaline soda lakes	

Overall, on the basis of distinct phenotypic and genomic features, strain AArc-S^T is proposed to be classified as *Natranaeroarchaeum sulfidigenes* gen. nov., sp. nov. in a new family *Natronoarchaeacea* fam. nov. The protologue of the proposed new taxa is presented in Table 3.

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Conflict of interests

The authors declare that there is no conflict of interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2022.126356.

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