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Natronocalculus amylovorans gen. nov., sp. nov., and *Natranaeroarchaeum aerophilus* sp. nov., dominant culturable amylolytic natronoarchaea from hypersaline soda lakes in southwestern Siberia ☆

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ABSTRACT

Several pure cultures of alkaliphilic haloaloarchaea were enriched and isolated from hypersaline soda lakes in southwestern Siberia using amylopectin and fructans as substrates. Phylogenomic analysis placed the isolates into two distinct groups within the class *Halobacteria*. Four isolates forming group 1 were closely related to a recently described *Natranaeroarchaeum sulfidigenes* and the other three strains forming group 2 represent a novel genus-level phylogenetic lineage. All isolates are saccharolytic archaea growing with various starch-like alpha-glucans including soluble starch, amylopectin, dextrin, glycogen, pullulane and cyclodextrin. In addition, group 1 can use levan while group 2 – inulin (plant storage beta-fructans). Group 1 strains can also grow anaerobically with either glucose or maltose using elemental sulfur as the electron acceptor. Both groups are moderately alkaliphilic with a pH range for growth from 7.2 to 9.3 (optimum between 8.0–8.8) and low Mg-demanding extreme halophiles growing optimally at 4 M total Na^+. The major respiratory menaquinone is MK-8:8 and the core biphytanyl lipids are dominated by archaeol (C_{20}-C_{20}) and a less abundant extended archaeol (C_{20}-C_{25}) with PG and PGP-Me as polar groups. The four isolates of group 1 are suggested to be classified into a new species as *Natranaeroarchaeum aerophilus* sp. nov. (type strain AArc-St1-1^T = JCM 32519^T = UQM 41458^T). The three isolates of group 2 are proposed to form a new genus and species for which the name *Natronocalculus amylovorans* gen. nov., sp. nov. is suggested (type strain AArc-St2^T = JCM 32475^T = UQM 41459^T).

Introduction

Most of the known species of aerobic extremely halophilic and haloalkaliphilic euryarchaea (natronoarchaea) were enriched and isolated from hypersaline alkaline lakes on unspecific media containing rich soluble organic substrates, such as peptone, yeast extract or simple sugars [1–5]. Yet, these extremophilic, organoheterotrophic archaea are definitely more important in organic matter mineralization, in particular organic polymers, in hypersaline systems than is widely recognized. For example, a test for amylolytic activity with soluble starch is included into the minimal standards for taxonomy characterization of haloarchaea [6] but starch is rarely used for targeted isolation of amylolytic haloarchaea which would not only hydrolyze the polymer but also able to utilize it as growth substrate. And this situation is also true for other polysaccharides. So far, only a few examples of haloarchaea specialized on utilization of recalcitrant polysaccharides have been described in pure culture, which is particularly true for natronoarchaea living in alkaline hypersaline (soda) lakes. Our recent targeted enrichments from such lakes using insoluble forms of cellulose and chitin resulted in isolation of several groups of natronoarchaea highly specialized on utilization of either various forms of cellulose and xylan (genera *Natronobiforma* and *Natronolimnobius*) or chitin (genus *Natrarchaeobius*) [7–9]. Following further in this direction, we used insoluble starch (amylopectin) or beta-fructans as substrates to enrich for amylolytic or fructanolytic natronoarchaea from hypersaline soda lakes. So far, we are aware

☆ The draft genome sequences of strains AArc-St1-1^T and AArc-St2^T are deposited in the GenBank under the numbers JAKRVY000000000 and JAKRVX000000000, respectively.

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only of a single amylolytic natronarchaeon, *Natronococcus amylolyticus*, specifically isolated from the hypersaline soda lake Magadi in Kenya using starch as a growth substrate [10,11], while no natronoarchaea, growing on fructans are currently known.

Here we describe phenotypic, phylogenetic and genomic properties of two novel taxa of amylolytic natronoarchaea enriched from hypersaline soda lakes in southwestern Siberia, which specialized on utilizing various alpha-glucan and beta-fructan polysaccharides as growth substrates.

Materials and methods

Enrichment and cultivation conditions

The sources of inocula were mixed surface (0–3 cm deep) aerated sediments and the near bottom brines obtained from four hypersaline soda lakes in Kulunda Steppe (Tanatar and Bitter lake systems, Altai region). The lakes brines total salt concentration varied from 20 to 40%, the total soluble carbonate alkalinity – from 2.5 to 5 M and the pH – from 10.2 to 11.0. The top flocculant sediment layer together with the near bottom brines was sucked into 50 ml syringe through silicon tubing and placed into a sterile 50 ml Falcon tube, resulting in an approximate volumetric ratio between the solid and liquid fractions of 1:5. After transportation to the laboratory the samples were separated onto clean brine top layer and concentrated sediment, all of which then were kept at 4 °C. Before inoculation, the two fractions from each of four lakes were combined in equal proportions to make two master mixes (brine and sediments) used as inocula at 1% final (v/v).

For the enrichment and further cultivation of pure cultures two basic mineral media were used, both containing 4 M total Na^+. The neutral 4 M NaCl base medium included (g l^{-1}): NaCl, 240; K_2HPO_4 2.5 g l^{-1}; NH_4Cl 0.5 g l^{-1}, KCl 5 g l^{-1}, 20 mg l^{-1} yeast extract and was adjusted to pH 7 with 10% KH_2PO_4. The alkaline sodium carbonate base contained (g l^{-1}): Na_2CO_3 190, $NaHCO_3$ 45, NaCl 16, KCl 5, K_2HPO_4 1 and 20 mg l^{-1} yeast extract (pH 10). After sterilization, the basic media were supplemented with 1 ml l^{-1} of trace metal and vitamin solutions [12] and 1 mM $MgSO_4$. NH_4Cl (4 mM) was also added from 1 M sterile stock solution to the carbonate base after sterilization. To prepare final medium with a certain pH/alkalinity, the two ready to use bases were mixed in different proportions resulting in pH range from 8 to 10. For the enrichments from salt lakes, the neutral base medium was used as it is, while for the soda lake enrichments the neutral and alkaline base media were mixed 3:1 with the final pH 9.5. For the pH range from 7 to 8, the NaCl base was supplied with various amounts of 1 M filter-sterilized $NaHCO_3$, while for the pH below 7 it was titrated with sterile 1 M KH_2PO_4. Carbon and energy substrtates were added from sterile 10% stock solution.

For isolation of pure cultures, several rounds of enrichments were repeated at dilutions 1:100 and finally the sediment-free enrichments were plated onto solid medium obtained by mixing the liquid alkaline medium and 4% washed agar at 50 °C in ratio 3:2. To compensate for the decreased salinity, solid NaCl was added to the liquid medium before mixing with melted agar to bring the final salt concentration back to 4 M total Na^+. The isolation of pure cultures was achieved from separate colonies which grew back in liquid medium with the target polysaccharide and the purity was confirmed by 16S rRNA gene and genome sequencing.

Pure culture characterization

Cell morphology was examined by using phase contrast microscopy (Zeiss Axioplane Imaging 2, Germany). Substrate utilization profiles were performed in medium containing 1 part of the alkaline base and 7 parts of the NaCl base (final pH 9.0). For the pH profiling, the two media were mixed in various proportions as described above and soluble starch served as the substrate. The growth was measured by increase of OD_{600} with pH monitoring at each point. Anaerobic cultivation was performed as described previously [13]. Catalase and oxidase activity were tested with 3% (v/v) H_2O_2 and 0.1% *N,N,N,N* tetramethyl-p-phenylenediamine hydrochloride, respectively, using cell-free extract (obtained by sonication) from cells of type strains. The protease, esterase/lipase activities were tested on plates spotted with fully grown liquid cultures: using casein/gelatin (hydrolysis zones after flooding with 10% TCA) and emulsified tributyrin/olive oil (turbidity clearance), respectively. Antibiotic sensitivity of type strains AArc-St1-1 and AArc-St2 was tested in liquid medium at pH 9 with starch as substrate.

The intact polar lipids (IPLs) and respiratory quinones were analyzed as described previously [14]. Briefly, the lipid fraction was extracted from freeze-dried cells with sonication in methanol:dichloromethane:phosphate buffer (2:1:0.8, v:v), followed by phase separation by adjusting the solvent mix to a ratio 1:1:0.9. The lipids and quinones were analyzed by normal phase, high performance liquid chromatography-ion trap mass spectrometry (HPLC-ITMS) and identified by masses and mass spectral fragmentation according to literature [14,15].

Genome sequencing

Genomic DNA from the type strains AArc-St1-1 and AArc-St2 was extracted using DNeasy PowerLyzer Microbial Kit (Qiagen) according to manufacturer instructions. Quality and quantity of the DNA samples were measured with Trinean Xpose spectrophotometer (PLT Scientific Instruments) and Qubit 2.0 fluorometer (Thermo Fisher Scientific) DNA libraries were prepared using KAPA HyperPlus kit (KAPAbiosystems) according to manufacturer recommendations. Paired-end sequencing (2x100bp) was performed using Illumina NextSeq. Obtained reads were filtered (quality and length) with CLC Genomics Workbench v.10. Genomes of the strains were assembled using SPAdes v.3.15.2 [16] --isolate mode with --trusted-contigs option (contigs obtained from Unicycler v.0.4.9 [17] were used as trusted contigs). Contigs with length $\leq$ 500 bp or with low coverage were eliminated. Genome assemblies statistics were checked with Quast v.5.0.2 [18,19]. Completeness and contamination levels were detected using CheckM v.1.1.2 [20] with archaea-specific marker set.

Phylogenetic and genomic analyses

For 16S rRNA gene sequence-based phylogenetic analysis 16S rRNA gene sequences of the seven isolates were aligned with the sequences of type species of all genera within *Halobacteria* (as well as *Archaeoglobus fulgidus* VC-16, *Methanocella paludicola* SANAE, *Methanothermobacter thermautotrophicus* Delta H used as the outgroup). Multiple sequence alignment and phylogenetic tree construction were performed as described earlier [9]. For phylogenomic analysis based on the "ar122" set of conserved single copy archaeal proteins [21] the protein sequences were identified and aligned in *in silico* proteomes of type species of all genera within *Halobacteria* (nontype species were taken for *Halalkalicoccus*, *Halorbellus*, *Natronoarchaeum* and *Halohasta* genera because the genomes of type spices are not available) using the GTDB-tk v.1.7.0 with reference data v.202 [22]. The phylogenomic tree was constructed in the RAxML v.8.2.12 [23] with the PROTGAMMAILG model of amino acid substitution; local support values were 1000 rapid bootstrap replications. Phylogenetic trees were

visualized using iTOL v.6.5.2 [24]. The whole genome-based comparisons were done as described by Sorokin et al. [25].

For functional genome analysis, genes encoding carbohydrate-active enzymes (CAZymes) were searched in genomes of AArc-St2 and AArc-St1-1 strains using dbCAN v.3.0.2 [26]. Further manual checking of the specificity of discovered glycosidases and other CAZymes were performed using BLAST against Swiss-Prot/PDB database.

Results and discussion

Enrichment and isolation of pure cultures

The primary enrichments for amylolytic and fructan-utilizing natronoarchaea were performed with amylopectin (insoluble starch) or levan/inulin, respectively, in the presence of 200 mg l^{-1} streptomycin to suppress growth of bacteria. The enrichments from sediment fraction showed visible growth (after removal of the sediment particles by low speed centrifugation) after one week of incubation, while the brine enrichments became turbid and pinkish after 10–14 days of incubation. After several 1:100 transfers, the liquid cultures were plated and individual pink-colored colonies were transferred back to the corresponding liquid media. This procedure was repeated 2 more times to ensure the homogeneity of colony morphology. The purity and identity of obtained cultures were verified by the 16S rRNA gene sequencing. The list of isolates is shown in Table 1.

Cell morphology of the isolates grown at pH 9 and 4 M total Na^+ with soluble starch is shown on Fig. 1. Cultures of all strains were dominated by nonmotile (with only a few occasionally showing slow motility) flattish cocci with irregular contour, typical for haloarchaea, with a small fraction of flat roads (“boards”). The colonies of all isolates were colored from orange to red and concentrated cell pellets were bright red, what is also typical for aerobic haloarchaea.

Chemotaxonomy

Membrane polar lipids and respiratory lipoquinones were analyzed in two strains, one of which represents a group 1 (AArc-St1-1^T), while another a group 2 (AArc-St2^T). In both, the core lipids were dominated by archaeol (AR; C_{20}-C_{20}) with a smaller proportion of extended archaeol (Ext-AR; C_{20}-C_{25}). The polar head groups of the intact polar lipids were phosphatidylglycerophosphate methylether (PGP-Me) and phosphatidylglycerol (PG). Both the core lipids and the polar head groups are very similar to the closest phylogenetic relatives of the amylolytic natronoarchaea (see below in comparative tables). The major difference is the absence of glycolipids and sulfolipids (such as phosphatidylglycerosulfate and sulfated glycosyl diethers) in natronoarchaea, which are more common in neutrophilic haloarchaea. The only respiratory lipoquinone species detected in both strains was the fully saturated MK-8:8, one of the most common in haloarchaea [15].

Phylogenetic and genomic analyses

The genome of AArc-St2 was assembled to 20 scaffolds including one circular plasmid, while the genome of AArct-St1-1 was assembled into 32 scaffolds with no plasmids in it (Suppl. Table S1). Genome size was 3.26 Mbp (GC content was 51.5%) for strain AArc-St2 and 3.29 Mbp (GC content was 61%) for strain AArc-St1-1. Completeness and contamination levels for AArc-St2 genome were 100%/0% and for AArc-St1-1 – 99.07%/1.87%. Genomic sequences are available in NCBI GenBank database with accession numbers JAKRVX000000000 (AArc-St2) and JAKRVY000000000 (AArc-St1-1).

Seven natronoarchaeal isolates formed two clusters on 16S rRNA gene sequence-based phylogenetic tree. Four strains of the group 1 clustered with the recently described facultatively anaerobic sulfur-reducing natronarchaeon *Natranaeroarchaeum sulfidigenes* which can also use starch as a substrate for growth either aerobically or anaerobically [27,28]. The three closely related isolates from the group 2 formed a novel genus-level lineage with the genera “*Halalkalirubrum*” and *Halohasta* as the nearest neighbors (around 93 and 92% sequence identity to its type species, respectively). This potentially new genus lineage also includes multiple uncharacterized isolates from various hypersaline habitats whose 16S rRNA gene sequences were deposited recently in the GenBank. However, the 16S rRNA gene sequence-based phylogenetic tree (Fig. 2a) had relatively low bootstrap support of its inner nods within the class *Halobacteria*. Phylogenomic tree based on 122 archaeal conserved single-copy protein markers of the strains AArc-St1-1 and AArc-St2 and other haloarchaea supported the branching, obtained in the 16S rRNA gene sequence-based tree but showed a better resolution of the inner nods (Fig. 2b). Strain AArc-St2 formed a novel-genus branch in a distinct cluster containing genera “*Halalkalirubrum*”, *Halohasta* and *Halonotius*. The latter three genera are currently classified in the order *Haloferacales*, family *Halorubraceae* [29]. Strain AArc-St1-1 was closely related to *Natranaeroarchaeum sulfidigenes* AArc-S and formed a potential new species in the genus which is currently classified within the family *Natranoarchaeacea* [28]. Strains AArct-St2 and AArc-St1-1 were proposed to be type species within their lineages.

For additional support of phylogenetic and phylogenomic analyses, ANI and AAI values were calculated for the genomes of AArc-St1-1 and AArc-St2 and the nearest relatives (Suppl. Table S2 and S3). The AAI values between strain AArc-St2 and the species of three related haloarchaeal genera with available genome sequences ranged from 62.7% to 70.1%. These values are below the level of AAI for the representatives of the majority of genera, for which AAI results were compared [30], but it is similar to the intergenera level within the whole cluster. The ANI values varied from 70.7% to 73%. Same calculations for strain AArc-St1-1 and *Natranaeroarchaeum sulfidigenes* AArc-S showed AAI and ANI values of 90.1% and 88.8%, respectively, confirming the separate species status of AArc-St1-1 within the genus *Natranaeroarcheum*. Although, according to the 16S rRNA gene sequence phylogeny,

Table 1
List of pure cultures of natronoarchaea enriched and isolated from hypersaline soda lakes in southwestern Siberia with amylopecting and fructans.

Strain	Source	Enrichment substrate	Phylogenetic group	Closest relative
AArc-St1-1^T	Sediments	Amylopectin	Group 1	*Natranaeroarchaeum sulfidigenes*
AArc-St1-2	Brines			
AArc-St1-3	Brines			
AArc-lev1	Sediments	Levan		
AArc-St2^T	Sediments	Amylopectin	Group 2	“*Halalkalirubrum halophilum*”
AArc-St3	Brines			
AArc-in2	Sediments	Inulin		

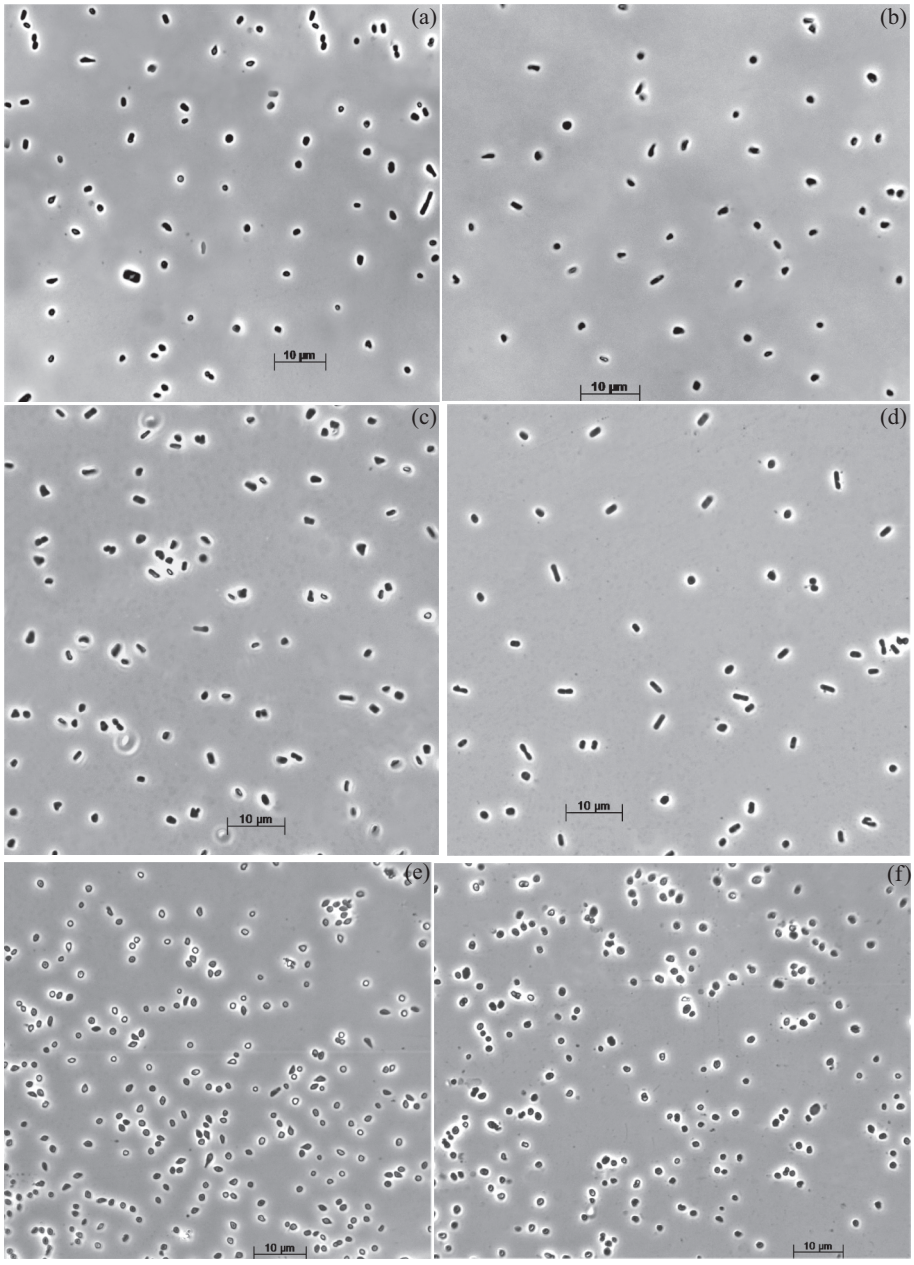

Fig. 1. Cell morphology (phase contrast microphotograps) of starch-utilizing natronoarchaea growing aerobically at 4 M total Na^+, pH 9 and 37 °C. (a-d), group 1, including strains AArc-St1-1^T, AArc-St1-2, AArc-St1-3 and AArc-lev1, respectively. (e-f), group 2, including strains AArc-St2^T and AArc-in2.

strain AArc-St1-2 might be sufficiently distant from the other three members of the group 1 and the type strain AArc-S^T of *Natranaeroarchaeum sulfidigenes* (98.6–98.8 and 98.5%, respectively), the proposed type species of the genus *Natranaeroarchaeum*, its phenotypic properties were quite similar to the other group 1 strains implying it would be more practical to classify all four isolates of this group in a single species.

Metabolic properties

All isolates were capable of utilizing alpha-1,4/1,6 glucans as growth substrates, including soluble starch, amylopectin (insoluble starch), glycogen, dextrin, cyclodextrin and pullulan. Furthermore, the group 1 isolates can also grow with levan (polyfructose with beta-2,6 backbond) while the group 2 strains utilized another fructan – inulin (polyfructose with beta-2,1 backbond). Two other alpha-bonded polysaccharides tested, including dextran from *Leuconostoc* and arabinan were not utilized by any of the seven isolates, as well as various beta-glucans (amorphous cellulose, xylan, xyloglucan, chitin, mannan, glucomannan, galactomannan, lichenan, laminarin and galactan). All strains can also grow on three sugar dimers including maltose, α,α-trehalose and cellobiose. In addition, the group 2 strains were able to grow with glycerol. None of the other tested substrates, except for a weak growth with mannose, gave positive results (glucose, sucrose, galactose, arabinose, rhamnose, raffinose, aminosugars, uronic acids, xylose and arabinose, sugar alcohols, C2-C6 organic acids, pepton). Such limited substrate profile characterize the isolates as narrow-specialized saccharolytics. Anaerobic fermentative growth with either starch, maltose or arginine was not observed. None of the isolates were capable of anaerobic respiratory growth with soluble starch or maltose as substrates, using thiosulfate, DMSO, fumarate

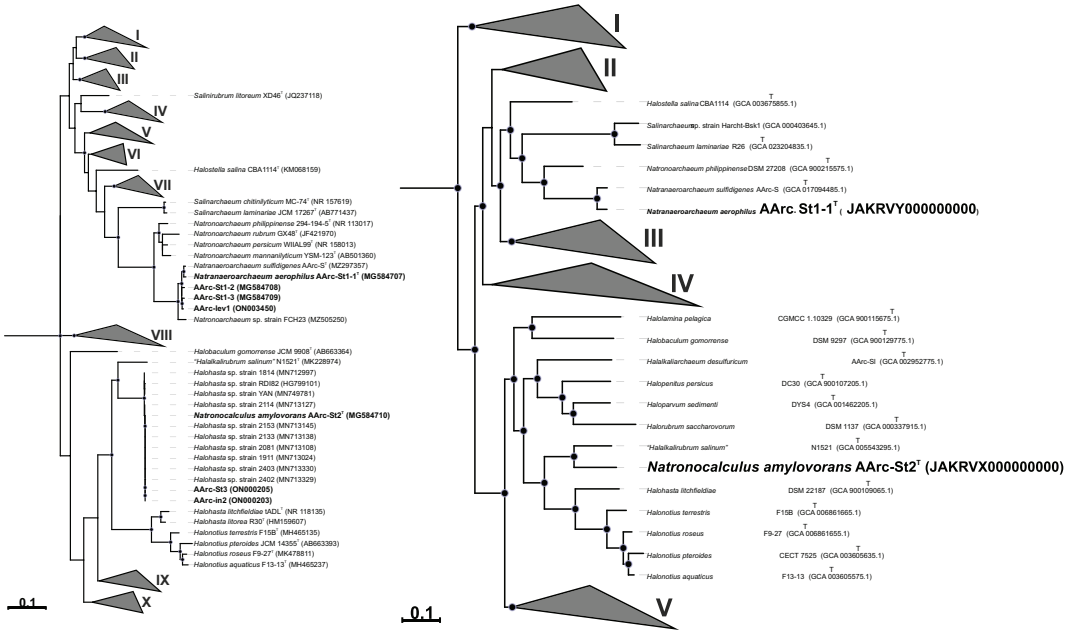

Fig. 2. Phylogeny of amylolytic natronoarchaea. (a) Figure X. (a) 16S rRNA gene sequence-based maximum-likelihood phylogenetic tree, showing the position of AArc-St2^T and AArc-St1-1^T (in bold) within the *Halobacteria* class. The black circles at nodes indicate that the percentage of corresponding support values was above 50. *Archaeoglobus fulgidus* VC-16^T, *Methanocella paludicola* SANAE^T, *Methanothermobacter thermautotrophicus* Delta H^T were used as an outgroup (not shown). Species in clusters: I (*Halodesulfurarchaeum*, *Halanaeroarchaeum*, *Halarchaeum*, *Halobacterium*, *Salarchaeum*, *Halocalculus*); II (*Halomicroarcula*, *Haloarcula*, *Halorientalis*, *Halorhabdus*, *Halococcoides*, *Halapricum*, *Salinirussus*, *Halovenus*); III (*Haloglomus*, *Natronomonas*, *Halosegnis*, *Salinirubellus*, *Halomarina*, *Halocatena*); IV (*Halopelagius*, *Haloferax*, *Halogeometricum*, *Haloquadratum*, *Halobellus*); V (*Halococcus*, *Halalkalicoccus*, *Haloarchaeobius*, *Halorubellus*); VI (*Halorussus*, *Halomicrococcus*, *Haladaptatus*); VII (*Saliphagus*, *Natribaculum*, *Halovarius*, *Natrononococcus*, *Halovivax*, *Natronobiforma*, *Halostagnicola*, *Natronobacterium*, *Halopiger*, *Halobiforma*, *Natrarchaeobaculum*, *Natronolimnohabitans*, *Natronolimnobius*, *Natronorubrum*, *Natrinema*, *Haloterrigena*, *Natrialba*, *Natrarchaeobius*, *Salinadaptatus*); VIII (*Halosimplex*, *Salinibaculum*, *Halosiccatus*, *Halomicrobium*); IX (*Salinigranum*, *Haloplanus*, *Halobium*, *Halegenticoccus*, *Halogranum*, *Haloprofundus*, *Halolamina*); X (*Halalkaliarchaeum*, *Halopenitus*, *Halorubrum*, *Haloparvum*). (b) Maximum likelihood phylogenetic tree based on concatenated alignment of 122 conserved archaeal proteins and showing position of strains AArc-St2^T and AArc-St1-1^T (in bold) within the class *Halobacteria*. The branch lengths correspond to the number of substitutions per site with corrections associated with the models. The black circles at nodes indicate that the percentage of corresponding support values was above 50. *Archaeoglobus fulgidus* VC-16^T, *Methanocella paludicola* SANAE^T and *Methanothermobacter thermautotrophicus* Delta H^T were used as an outgroup (not shown). Species in clusters: I (*Halanaeroarchaeum*, *Halodesulfurarchaeum*, *Halarchaeum*, *Halobacterium*, *Salarchaeum*, *Halocalculus*); II (*Halalkalicoccus*, *Halorussus*, *Halomicrococcus*, *Haladaptatus*); III (*Haloarchaeobius*, *Halorubellus*, *Halovivax*, *Saliphagus*, *Natronobiforma*, *Halostagnicola*, *Natrarchaeobius*, *Natrarchaeobaculum*, *Salinadaptatus*, *Halopiger*, *Natronolimnobius*, *Natronobacterium*, *Halobiforma*, *Natrialba*, *Natrononococcus*, *Natrinema*, *Haloterrigena*, *Natronorubrum*, *Natronolimnohabitans*); IV (*Halococcus*, *Halocatena*, *Halomarina*, *Natronomonas*, *Haloglomus*, *Halosegnis*, *Halorientalis*, *Halapricum*, *Salinirussus*, *Salinibaculum*, *Halovenus*, *Halosimplex*, *Halococcoides*, *Halorhabdus*, *Halomicrobium*, *Halosiccatus*, *Halomicroarcula*, *Haloarcula*); V (*Haloplanus*, *Salinirubrum*, *Haloprofundus*, *Halegenticoccus*, *Halogranum*, *Salinigranum*, *Haloferax*, *Halopelagius*, *Halogeometricum*, *Halobellus*, *Haloquadratum*).

or nitrate as the electron acceptors. However, all four group 1 isolates grew anaerobically with maltose as the electron donor and carbon source and elemental sulfur as the electron acceptor, similar to the closely related type species of the genus *Natranaeroarchaeum*. In 15 d incubation (4 M Na^+, pH 9, 30 °C) the following amount of sulfide was produced: 15.6 mM by AArc-St1-1, 5.8 mM by AArc-St1-2; 19 mM by AArc-St1-3 and 10.5 mM by AArc-St-lev1. In comparison, *Natranaeroarchaeum sulfidigenes* formed 30 mM sulfide in 6 days of cultivation at pH 9.5.

Two type strains tested positive for catalase reaction and TMPD-oxidase. The protease, esterase and lipase activities were negative in all strains in the spot-plate tests. Ammonium and yeast extract (but not nitrate) can serve as the N-source in cultures grown with soluble starch for both strains. Urea was only utilized by strain AArc-St1-1 consistent with the presence of the *ureABC-DEFG* urease operon in the genome. Indole formation from tryptophan (Kovac's reagent test) showed a weak positive result only for strain AArc-St2. The type strains grown in liquid culture at pH 9 with soluble starch were insensitive to streptomycin, penicillin G, ampicillin, kanamycin, vancomycin and gentamicin up to 200 mg l^{-1}. Rifampicin and chloramphenicol inhibited growth at 50 mg l^{-1}, and tetracycline – at 100 mg l^{-1}.

All isolates grew well at as low Mg concentration as 1 mM, while in their sodium requirement they are typical extreme halophiles, growing optimally at 4 M total Na^+ and within the range from 3 to 5 M (tested at pH 8.8). The cells of isolates in both groups lyzed at salinity downshift below 2 M total Na^+. The pH profiling of four cultures at 4 M total Na^+ showed that they are moderate alkaliphiles with optimal growth within a pH range from 8.0 to 8.8

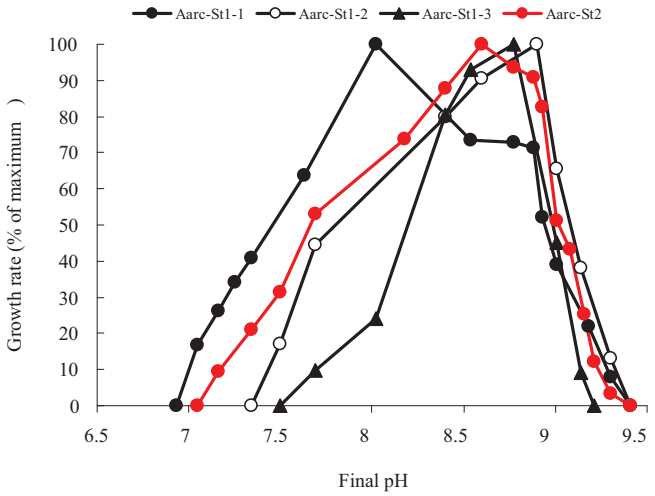

Fig. 3. pH profiles for growth with soluble starch in amylolytic natronoarchaea at 4 M total Na^+ and 37 °C. Actual final pH are shown. The results are average from two parallel incubations.

(Fig. 3). The maximum growth temperature of type strains grown with soluble starch at pH 8.5 and 4 M total Na^+ was 50 °C for the group 1 strains and 48 °C for the group 2 strains.

Comparative properties of the group 1 and group 2 isolates with their nearest phylogenetic relatives are shown in Tables 2 and 3.

The main difference of the two groups from each other was in utilization of two different fructans and the phylogeny. The key difference of the group 1 isolates from the type species of the genus *Natrananaeroarchaeum* was their inability to grow at extremely high pH values above 9.3. The main difference of the group 2 isolates from the nearest related genera is that they are the only ones isolated from soda lakes. The two out of three related genera (*Halohasta* and *Halonotius*) are definitely neutrophilic, while, despite the reported ability of "Halalkalirubrum salinum" to grow up to pH 10.5, there is a doubt about it. First, the organism is isolated from a salt lake with pH 8.5, thus being only a slightly alkaline salt (but not soda) lake. Even natronoarchaea isolated from hypersaline soda lakes with permanent pH above 10, seldom grow above pH 10.2. Secondly, the final pH values were apparently not measured during the pH profiling, which makes the reported values for the maximum pH unverified. Hence, the newly isolated amylolytic strains from soda lakes can still be considered as first obligate alkaliphilic (albeit only moderate) representatives of this group of related genera. They also differs from the other three genera in their alpha-glucan/fructan substrate specialization and the absence of glyco- and sulfo-lipids in their membranes.

Genomic analysis

The genome search the two type strains (dbCAN) identified a set of genes typically encoding alpha-amylases and alpha-glucosidases (GH13 and 15 families) in both representatives of two groups, although in AArc-St1-1 the total number and the fraction of putative extracellular amylases are much more abundant (Suppl. Table S4, consensus results from HHMER/DIAMOND tools). This is also in agreement with the growth and amylase activity results (Suppl. Fig. 1). These enzymes would allow utilization of a spectrum of alpha-linked glucans such as amylopectin, soluble starch, dextrin, glycogen, pullulan, maltose and trehalose as sole carbon and energy sources. In this respect strain AArc-St1-1 is highly similar to the type species of the genus *Natranaeroarchaeum* [16,17]. Furthermore genomes of both type strains contain genes coding for beta-fructosidases of the GH families 32 and 68, which is in agreement with their ability to use fructans as growth substrate. As for the other glucanases encoded in two genomes, such as the beta-endo-1,3/1,4-glucanases of GH16 and 81 families (in AArc-St1-1), and pectin lyase (PL family) in AArc-St2, none of the tested potential polysaccharide substrates for these hydrolases supported growth (laminarin, lichenan, xylan, beta-glucan, glucomannan, mannan, curdlan, pachyman or pectin).

In respect to the osmoprotection and pH homeostasis, both genomes encode a range of typical potassium import complexes (but variable in copy numbers) and a multysubunit Na^+/H^+ antiporter

Table 2
Comparative properties of amylolytic natronoarchaea of group 1 with the type species of the nearest phylogenetically related genera [28,33].

Property	*"Natranaeroarchaeum aerophilus"* (4 strains)	*Natranaeroarchaeum sulfidigenes* JCM 34033^T	*Natronoarchaeum mannanilyticum* JCM 16328^T
Cell morphology	flat pleomorphic, motility not observed	flat pleomorphic, motile	pleomorphic, nonmotile
Pigmentation	red	red (aerobic); pink (anaerobic)	red
PHA accumulation	-	-	-
Aerobic growth	+	+	+
Anaerobic growth by:			
sugar fermentation	-	-	-
sulfur/thiosulfate respiration	+ (with sulfur)	+ (with sulfur and $S_2O_3^{2-}$)	-
sulfoxide respiration	-	-	-
Number of Psr/Phs operons in genomes	3	2	0*
e-donors for anaerobic growth	glucose, maltose	sugars, starch, glycerol	-
Substrates for aerobic growth	starch-like alpha-glucans, levan, maltose, cellobiose, trehalose	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 tryptophan	-	-	+
Salinity range (opt.) (M Na^+)	3.0–5.0 (4.0)	2.5–4.5 (3.5)	1.6–4.2 (2.5–3.2)
pH range (opt.)	7.2–9.3 (8.0–8.8)	8.5–10.2 (9.5–9.7)	6.0–9.5 (8.5–9.0)
Temperature max (°C)	50 (at pH 8.5)	45 (at pH 9)	55
Core lipids	C_{20}-C_{20}, C_{20}-C_{25} DGE	C_{20}-C_{20}, C_{20}-C_{25} DGE	NR
Intact membrane polar lipids:			
phospholipids	PG, PGP-Me	PG, PGP-Me	PG, PGP-Me, PGP,
glycolipids/sulfolipids	-	-	S_2-DGDE
Respiratory lipoquinones	MK-8:8	MK-8:8	NR
DNA G + C	61.0 (genome)	60.8% (genome)	63.0 (mol%)
Type of hypersaline habitat	Hypersaline soda lakes		Marine solar saltern

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

Table 3
Comparative properties of group 2 isolates with nearest phylogenetic relatives [34–36].

Property	*"Natranocalculus amylovorans"* (3 isolates)	*"Halalkalirubrum halophilum"*	*Halohasta* (2 species)	*Halonotius* (4 species)
Cell morphology	flat pleomorphic nonmotile	pleomorphic, nonmotile	rods, motile	polymorphic rods,motility (V)
Pigmentation	red	red	red	red
PHA accumulation	-	NR	NR	
Anaerobic growth by:				
sugar fermentation	-	NR	NR	NR
sulfur respiration	-	NR	NR	NR
DMSO respiration	-	-	-	+(1 species)
Growth substrates				
carbohydrates:	starch-like alpha-glucans, inulin, maltose, cellobiose, trehalose, glycerol	glucose, maltose, fructose, sorbose, lactose, xylose, mannitol, sorbitol	glucose, sucrose; mannose, galactose, lactose, maltose (all V)	glucose, arabinose, fructose, galactose, sucrose, maltose, raffinose, xylose, mannitol, sorbitol, glycerol, (all V)
organic acids:	none	acetate, pyruvate, lactate, fumarate, succinate, citrate	pyruvate, lactate, succinate, malate, fumarate, citrate (V);	pyruvate, citrate, tartrate (all V)
Amylase	+ (soluble starch)	- (soluble starch)	- (soluble starch)	- (soluble starch)
Esterase/lipase	- (tributyrin/olive oil)	- (Tweens)	- (Tween 80)	- (Tween 80)
Protease	- (gelatin, casein)	- (gelatin, casein)	- (gelatin, casein)	- (gelatin, casein)
Catalase/oxidase	+/+	+/ +	+/V	V/V
Indole from tryptophan	+(w)	+	-	NR
Salinity range (opt.) M Na^+	3–5 (4.0)	1.9–4.2 (2.5)	2.0–4.7 (2.5–3.0)	2.5–6.0** (3.0–4.0)
Mg^{2+} demand	low	low	high	high
pH range (opt.)	7.2–9.3 (8.5–8.8)	7.0–10.5* (8.5–9.5)	5.5–9.0* (7.0–7.5)	5.0–9.0 (7.0–7.5)
Temperature max (°C)	48 (at pH 8.5)	42	45–50	45–50
Core lipids	C_{20}-C_{20}, C_{20}-C_{25} DGE	NR	NR	NR
Intact membrane polar lipids:				
phospholipids:	PG, PGP-Me	PG, PGP-Me	PG, PGP-Me, PA	PG, PGP-Me
glycolipids:	-	1, unidentified	S-DGD-1	S-DGD-1
sulfolipids:	-	PGS		PGS (V)
Respiratory lipoquinones	MK-8:8	NR	NR	NR
DNA G + C (% genomic)	51.5 (type strain)	58.4 (type strain)	58.8 (type species)	59.7–62.7 (4 species)
Type of hypersaline habitat	soda lakes	salt lake	solar saltern	solar saltern, saline soils

NR, not reported; (V), variable property in different species of the same genus.
PA, phosphatidic acid; PGS, phosphatidylglycerol sulfate; S-DGD-1, sulfated mannosyl glucosyl diether; other abbreviations (see Table 2).
* Actual final pH values were not measured.
** reported for the type species, but not verified in any further research.

of the Mrp family. Both genomes lacks genes for organic osmolyte import and synthesis, indicating that the organisms rely solely on the potassium accumulation strategy. Both strains produce catalase/peroxidase and have a haem-copper family cytochrome *c* terminal oxidase of the aa_3 type. In addition, strain AArc-St2 has another terminal oxidase of the ba_3 type (Supplementary Table S5). These also agree with the positive tests for catalase and oxidase in both strains.

A major difference between the two type strains was found in the presence of two types of other respiratory complexes. Strain AArc-St2 genome contains a locus apparently coding for the aerobic type of CO-dehydrogenase (Cox, most probably of the type II) lacking in AArc-St1-1. Although the capacity to oxidize CO at low concentration has been demonstrated for several haloarchaeal species [31,32], the physiological role of such potential is still unclear. One of the possibilities is CO detoxification.

The genome of AArc-St1-1 contains loci apparently encoding enzymes for sulfur-dependent anaerobic respiration, which are lacking in AArc-St2: two of them are highly homologous to the PsrABCD/SseA and PhsABCD complexes encoded in the genome of type species of genus *Natranaeroarchaeum* responsible for sulfur- and thiosulfate-dependent anaerobic respiration, respectively [27]. In addition, there is a second encoded PsrABCD lacking sulfur transferase more homologous to the one present in sulfur-respiring *Halalkaliarchaeum desulfuricum* (Supplementary Table S5). In our experience, such genomic potential must enable the anaerobic sulfur respiration in strain AArc-St1-1, similar to the type species *Natranaeroarchaeum sulfidigenes*. And this, indeed, is directly confirmed by the growth experiments, although the activity of sulfur reduction, in general, was lower in the novel isolates than in the type species of the genus *Natranaeroarchaeum*. Furthermore, none of the novel group 1 isolates were capable of

Table 4
Natranaeroarchaeum aerophilus: protologue.

Parameter	Species: *Natranaeroarchaeum aerophilus* sp. nov.
Author	Dimitry Y. Sorokin
Species name	*aerophilus*
Genus name	*Natranaeroarchaeum*
Specific epithet	*aerophilus*
Species status (SPST)	sp. nov.
Etymology	a.e.ro'phi.lus Gr. masc. n. *aer*, air; N.L. masc. adj. *philus* (from Gr. masc. adj. *philos*), friend, loving; N.L. masc. adj. *aerophilus*, air-loving.
Description of the new taxon	The cells are angular, flat, polymorphyc coccoids or rods, mostly nonmotile, variable in size from 1 to 3 μm. The cells lyze in hypotonic solutions below 1 M NaCl. produces red carotenoids. The core membrane diether lipids are composed of C_{20}-C_{20} DGE (archaeol) and C_{20}-C_{25} DGE (extended archaeol). The polar lipid head groups include phosphatidylglycerolphosphate methyl ester (PGP-Me) and phosphatidylglycerol (PG). The dominant respiratory quinone is MK-8:8. Saccharolytic with limited substrate profile including several starch-like alpha-glucans, levan, maltose, trehalose and cellobiose. Capable of anaerobic sulfogenic growth with glucose and maltose as electron donors and sulfur as electron acceptor. Ammonium, urea and yeast extract serve as the N-source. Oxidase and catalase positive. Maximum growth temperature is 50 °C. Extremely halophilic with a range of total Na^+ for growth from 3 to 5 M (optimum at 4 M) and moderately alkaliphilic, with a pH range for growth from 7.2 to 9.3 (optimum at 8.0–8.8). The G + C content of the DNA is 61.0% (genome of the type strain). Habitat - aerobic sediments and brines of hypersaline soda lakes. The type strain (AArc-St1-1^T = JCM 32519^T = UQM 41458^T) was isolated aerobic sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia). The species also includes other three closely related strains isolated from the same area. The draft genome of type strain is deposited in the GenBank under accession number JAKRVY000000000.
Authors	Dimitry Y. Sorokin, Alexander G. Elcheninov, Tatjana V. Khizhniak, Michel Koenen, Nicole J. Bale, Jaap S. Sinninghe Damsté, Ilya V. Kublanov
Title	*Natronocalculus amylovorans gen.* nov., sp. nov., and *Natranaeroarchaeum aerophilus* sp. nov., dominant culturable amylolytic natronoarchaea from hypersaline soda lakes in southwestern Siberia.
Journal	Systematic and Applied Microbiology
Corresponding author	Dimitry Y. Sorokin
of corresponding author	soroc@inmi.ru; d.sorokin@tudelft
Designation of the type strain	AArc-St1-1
Strain collection numbers	JCM 32519^T = UQM 41458^T
16S rRNA gene accession numbers	MG584707-MG584709; ON003450
Genome accession numbers	JAKRVY000000000 (type strain)
Genome status	Draft
G + C, %	61.0 (genome of type strain)
Country of origin	Russian Federation
Region of origin	Altai region
Date of isolation	2016
Source of isolation	Surface aerobic sediments from hypersaline soda lakes
Sampling dates	2015–07-07
Geographic location	*S-W* Siberia, Kulunda Steppe; southern Russia
Latitude	51°39′ N; 49°10′ N; 48°14′ N
Longitude	79°48′ E; 46°39′ E; 46°35′ E
Depth	0–2 cm
Temperature of the sample	20 °C
pH of the sample	10–11
Salinity of the sample	18–36%
Number of strains in study	4
Source of isolation of non-type strains	Surface aerobic sediments and brines from hypersaline soda lakes, *S-W* Siberia, Kulunda Steppe; southern Russia
Growth medium, incubation conditions	4 M total Na^+, pH 9; incubation – 37 °C; starch as substrates; aerobic
Conditions of preservation	Deep freezing in 15% glycerol (v/v)
Gram stain	Negative
Cell shape	Pleomorphic flat coccoids
Cell size	0.8–2 μm in diameter
Motility (MOTY)	Mostly nonmotile
Sporulation	None
Colony morphology	Flat, compact, max. 2 mm, red
Temperature range for growth	nd
Lowest temperature for growth	nd
Highest temperature for growth	50
Optimal temperature for growth	37–40
Lowest pH for growth	7.2
Highest pH for growth	9.3
Optimum pH for growth	8–8.8
pH category	Moderately alkaliphilic
Lowest Na^+ concentration for growth	3.0 M
Highest Na^+ concentration for growth	5.0 M
Optimum salt concentration for growth	4.0 M total Na^+
Other salts important for growth	KCl; Na-carbonates

Salinity category	Extremely halophilic
Relation to oxygen	Facultatively anaerobic
O_2 conditions for strain testing	Fully aerobic
Carbon source used (class)	Carbohydrates
Specific compounds	Starch-like alpha-glucans, levan
Nitrogen source	Ammonium, urea, yeast extract
Terminal electron acceptor	O_2 and S_8
Energy metabolism	Chemoorganotrophic
Phospholipids	Core membrane lipids are C_{20}-C_{20} DGE (archaeol) and C_{20}-C_{25} DGE (extended archaeol).Polar head groups are phosphatidylglycerophosphate methylester (PGP-Me) and phosphatidylglycerol (PG)
Respiratory lipoquinones	MK-8:8
Glycolipids (GLYC)	-
Habitat (HABT)	Hypersaline soda lakes
Extraordinary features (EXTR)	Narrowly specialized amylolytics

Table 5
Natronocalculus amylovorans: protologue.

Parameter	Genus: ***Natronocalculus* gen. nov.**	Species: ***Natronocalculus amylovorans* sp. nov.**
Author (AUTE)	Dimitry Y. Sorokin	
Species name (SPNA)		*amylovorans*
Genus name (GENA)	*Natronocalculus*	
Specific epithet (SPEP)	-	*amylovorans*
Species status (SPST)	-	sp. nov.
Etymology (GETY/ SPTY)	Na.tro.no.cal'cu.lus N.L. neut. n. *natron*, arbitrarily derived from the Arabic n. natrun or natron soda; L. masc. n. *calculus*, pebble, gravel; N.L. masc. n. *Natronocalculus*, soda loving pebble-shaped cells	a.my.lo.vo'rans. Gr. neut. n. *amylon*, starch; L. inf. v. *vorare*, to devour; N.L. part. adj. *amylovorans*, eating starch
Type species of the genus (TYPE)	***Natronocalculus amylovorans***	yes
Description of new taxon	Obligately aerobic and organotrophic member of *Halobacteriales* narrowly specialized on utilization of starch-like polymers. Core lipids are dominated by archaeol and extended archaeol with PGP-Me and PG polar groups. Glycolipids are not present. MK-8:8 is the dominant lipoquinone. Extremely halophilic and moderately alkaliphilic inhabitants of hypersaline soda lakes. The three-letter abbreviation is Ncl.	The cells are angular, flat, polymorphyc coccoids or rods, nonmotile, from 0.8 to 2.0 μm. The cells lyze in hypotonic solutions below 1 M NaCl. Colonies are orange-red. The core membrane diether lipids include of C_{20}-C_{20} DGE (archaeol) and C_{20}-C_{25} DGE (extended archaeol). The polar lipid head groups consists of hosphatidylglycerolphosphate methyl ester (PGP-Me) and phosphatidylglycerol (PG). The dominant respiratory menaquinone is MK-8:8. Obligately aerobic saccharolytic with limited substrate profile including several starch-like alpha-glucans, inulin, maltose, trehalose, cellobiose and glycerol. Ammonium and yeast extract serve as the N-source. Oxidase and catalase positive. Maximum growth temperature is 48 °C. Extremely halophilic with a range of total Na^+ for growth from 3 to 5 M (optimum at 4 M) and moderately alkaliphilic, with a pH range for growth from 7.2 to 9.3 (optimum at 8.5–8.8). The G + C content of the DNA is 51.5% (genome of the type strain). Habitat - aerobic sediments and brines of hypersaline soda lakes. The type strain (AArc-St2^T = JCM 32475^T = UQM 41459^T) was isolated from aerobic sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia). The species also includes 2 other closely related strains isolated from the same area. The draft genome of type strain is deposited in the GenBank under accession number JAKRVX000000000.
Authors (AUT)	Dimitry Y. Sorokin, Alexander G. Elcheninov, Tatjana V. Khizhniak, Michel Koenen, Nicole J. Bale, Jaap S. Sinninghe Damsté, Ilya V. Kublanov	
Title (TITL)	*Natronocalculus amylovorans gen.* nov., sp. nov., and *Natranaeroarchaeum aerophilus* sp. nov., dominant culturable amylolytic natronoarchaea from hypersaline soda lakes in southwestern Siberia.	
Journal (JOUR)	Systematic and Applied Microbiology	
Corresponding author (COAU)	Dimitry Y. Sorokin	
of corresponding author (EMAU)	d.sorokin@tudelft; soroc@inmi.ru	
Strain collection numbers (COLN)	-	JCM 32475; UQM 41459
16S rRNA gene accession number (16 SR)	-	MG584710; ON000203; ON000205
Genome accession numbers		JAKRVX000000000
Genome status (GSTA)		Draft
GC mol % (GGCM)	-	51.5 (genome type strain)

(*continued on next page*)

Country of origin (COUN)	Russian Federation	Russian Federation
Region of origin (REGI)	-	Altai region
Date of isolation (DATI)	-	2016
Source of isolation (SOUR)	Hypersaline soda lakes	Surface sediments and brines of hypersaline soda lakes in southwestern Siberia
Sampling dates (DATS)	2015	2015
Geographic location (GEOL)	*S-W* Siberia	*S-W* Siberia
Latitude (LATI)	-	51°39′ N; 49°10′ N; 48°14′ N
Longitude (LONG)	-	79°48′ E; 46°39′ E; 46°35′ E
Depth (DEPT)		0–2 cm
Temperature of the sample (TEMS)		20 °C
pH of the sample (PHSA)		10–11
Salinity of the sample (SALS)		18–36%
Number of strains in study (NSTR)	3	3
Source of isolation of non-type strains (SAMP)	-	Surface sediments and brines of hypersaline soda lakes in southwestern Siberia
Growth medium, incubation conditions (CULT)		4 M total Na^+, pH 9; incubation – 37 °C; starch as substrates; aerobic
Conditions of preservation (PRES)	Deep freezing in 15% glycerol (v/v)	
Gram stain (GRAM)	negative	
Cell shape (CSHA)	Pleomorphic, from flat irregular coccoids	
Cell size (CSZI)	-	0.8–2 μm in diameter
Motility (MOTY)	-	nonmotile
Sporulation (SPOR)	none	
Colony morphology (COLM)		Pink-orange, up to 2 mm, flat
Temperature range for growth (TEMR)		
Lowest temperature for growth (TEML)		
Highest temperature for growth(TEMH)		48 (at pH 8.5)
Optimal temperature for growth (TEMO)		37–40 °C
Lowest pH for growth (PHLO)		7.2
Highest pH for growth (PHHI)		9.3
Optimum pH for growth (PHOP)		8.5–8.8
pH category (PHCA)	alkaliphile (optimum > 8.5)	
Lowest NaCl concentration for growth (SALL)	3.0 M total Na^+	
Highest NaCl concentration for growth (SALH)	5 M total Na^+	
Optimum salt concentration for growth (SALO)	4.0 M total Na^+	
Other salts important for growth	Sodium carbonates	
Salinity category (SALC)	extremely halophilic	

Relation to oxygen (OREL)	aerobe
O_2 conditions for strain testing (OCON)	aerobic
Carbon source used (class) (CSUC)	carbohydrates
Specific compounds (CSUC)	Starch-like alpha glucans and inulin
Nitrogen source (NSOU)	Ammonium, yeast extract
Terminal electron acceptor (ELAC)	O_2
Energy metabolism (EMET)	chemoorganotrophic
Phospholipids (PHOS)	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 (GLYC)	-
Respiratory lipoquinones	MK8:8
Habitat (HABT)	Hypersaline soda lakes

thiosulfate-dependent anaerobic respiration, which was a prominent trait in the type species. On the other hand, the amylolytic isolates grew much better at fully aerobic conditions, while *N. sulfidigenes* needed transition via microaerophilly before it started to grow at fully aerobic conditions. This reflects the difference in enrichment conditions used to isolate these closely related but still differentially specialized species of the same genus.

Overall, on the basis of distinct phenotypic, phylogenetic and genomic features, the group 1 isolates from hypersaline soda lakes are proposed to be classified in a novel species within the genus *Natranaeroarchaeum* as *Natranaeroarchaeum aerophilus* sp. nov. (type strain AArc-St1-1), while the group 2 isolates are forming a new species in a new genus for which the name *Natronocalculus amylovorans* gen. nov., sp. nov. (type strain AArc-St2) is proposed. The protologues for the new taxa are presented in Tables 4 and 5.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2022.126336.

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