Natronocalculus

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Genus

Halobacteriota/Halobacteria/Halobacteriales/Haloferacaceae/

Natronocalculus

Sorokin et al. 2022, VL211

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Na.tro.no.cal'cu.lus N.L. neut. n. *natron*, arbitrarily derived from the Arabic *natrun* or *natron*, soda; L. masc. n. *calculus*, pebble, gravel; N.L. masc. n. *Natronocalculus*, soda-loving pebble-shaped cells.

The genus *Natronocalculus* is a member of the family *Halorubraceae*, order *Haloferacales*, and class *Halobacteria*, according to the NCBI classification, while recent changes in the taxonomy of the *Halobacteria* based on the Genome Taxonomy Database phylogenomic classification place *Natronocalculus* into the family *Haloferacaceae*, order *Halobacteriales*. The genus members are aerobic, extremely halophilic and moderately alkaliphilic, and saccharolytic archaea, utilizing various alpha-glucans and sugars as carbon and energy sources. The genus currently includes a single species *Natronocalculus amylovorans* inhabiting hypersaline soda lakes. The genus three-letter abbreviation is Ncl.

DNA G + C content (mol%): 51.5 (whole-genome sequence of the type strain).

Type species: **Natronocalculus amylovorans** Sorokin et al. 2022, VL211.

Cells of *Natronocalculus* are polymorphic, mostly cocci, with a thin monolayer cell wall. Produce red carotenoids. The **core lipids** are **$C_{20}-C_{20}$ archaeol** and **$C_{20}-C_{25}$ extended archaeol** with the **polar head groups** as phosphatidylglycerol (PG) and **phosphatidylglycerol phosphate methylether** (PGP-Me). The **respiratory lipoquinone** is **MK-8:8**. It includes obligately aerobic **saccharolytic** heterotrophs narrowly specialized on **utilization of alpha-glucans as the growth substrate. They are extremely halophilic** (optimum at 4.0 M total Na^+), **moderately alkaliphilic** (pH range from 7.2 to 9.3), and **mesophilic** (maximum temperature 48°C). Thus far, the genus includes a single species represented by three closely related strains enriched either with amylopectin or beta-fructan inulin from oxic sediments and brines of hypersaline soda lakes in Kulunda Steppe (Altai, Russia) (Sorokin et al., 2022). The genus is a member of the ***Halobacteriales* order** in the **class *Halobacteria***. The genus three-letter abbreviation is Ncl.

DNA G + C content (mol%): 51.5 (whole-genome sequence of the type strain).

Type species: **Natronocalculus amylovorans** Sorokin et al. 2022, VL211.

Number of species with validly published name: 1.

Family classification: According to the most recent phylogenomic classification, the genus *Natronocalculus* is classified within the family *Haloferacaceae* (Cui et al., 2023).

Further descriptive information

The cells of the type species are mostly nonmotile cocci, 0.8 × 2 μm. The colonies are orange to pink, flat, and up to 3 mm (Figure 1).

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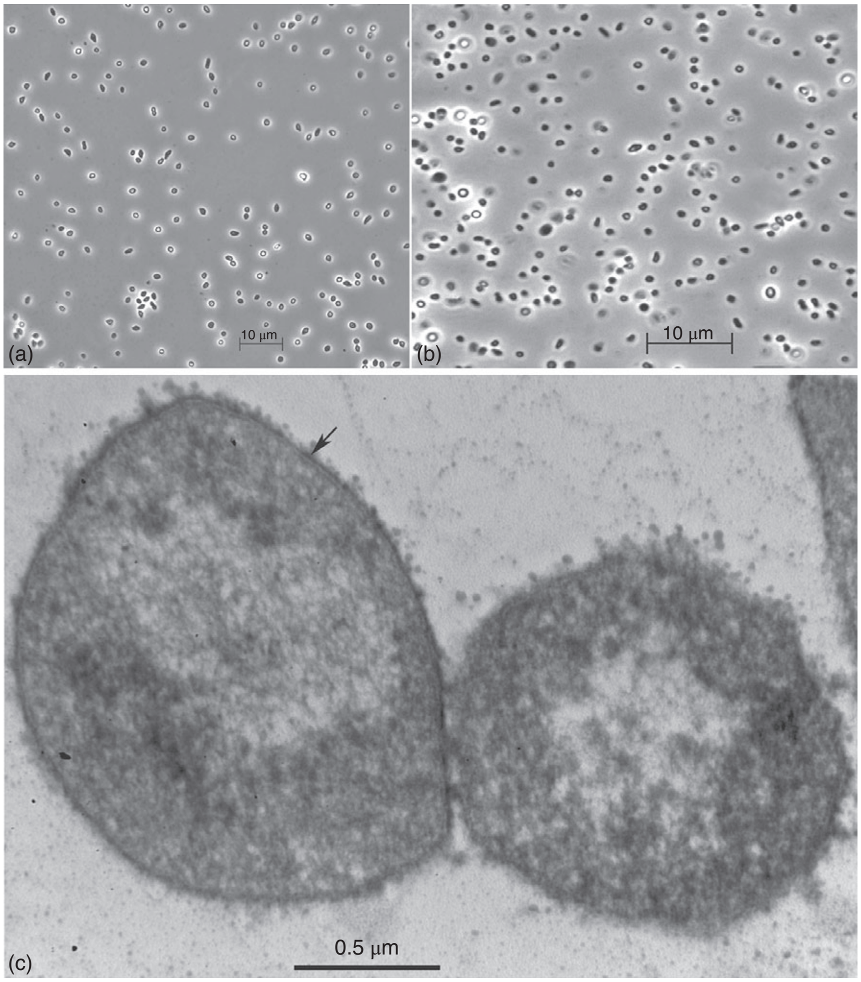
FIGURE 1. Cell morphology of *Ncl. amylovorans* grown at 4 M total Na^+, pH 9, and 37°C. (a and b) Phase-contrast microphotographs of cells of strain AArc-St2^T grown on soluble starch and strain AArc-in2 grown on inulin, respectively. (c) Thin-section electron microscopy of AArc-St2^T cells showing thin *S*-layer cell wall (arrow).



The most characteristic feature of the genus is its capacity to utilize various alpha-glucans and a beta-fructan inulin as growth substrates (Sorokin et al., 2022). While multiple haloarchaeal species have tested positive for amylase activity included in the Minimal Standards (Oren et al., 1997), currently, only a single other species, *Natranaeroarchaeum aerophilus*, has shown the ability to grow with amylopectin or a beta-fructan levan (Sorokin et al., 2022).

Habitat, enrichment, and isolation

Thus far, the genus *Natronocalculus* has been represented by three strains that originated from surface sediments and brines of hypersaline soda lakes in Kulunda Steppe (Altai, Russia). Two of them were enriched with amylopectin, and the third one with inulin. The basic enrichment medium contained 4 M total Na^+ at pH 9.5 (produced from one part of sodium carbonate base containing 4 M total Na^+ at pH 10 and three parts of 4 M NaCl-containing base medium at pH 7) and included a mixture of three antibiotics, streptomycin, kanamycin, and vancomycin, 100 mg/l each, to suppress bacterial growth. The final isolation of pure cultures was achieved by plating maximal serial dilutions on solid medium (4 M total Na^+, pH 9) with the same composition containing either soluble starch or inulin. Since high concentrations of sodium carbonate suppress agar solidification, the total fraction of sodium carbonate in plates was reduced to 0.5 M total Na^+ with 3.5 M NaCl to balance. This liquid base medium was heated to 50°C with addition of solid NaCl to compensate for dilution with agar and mixed in 3:2 proportion with 5% melted washed agar before pouring.

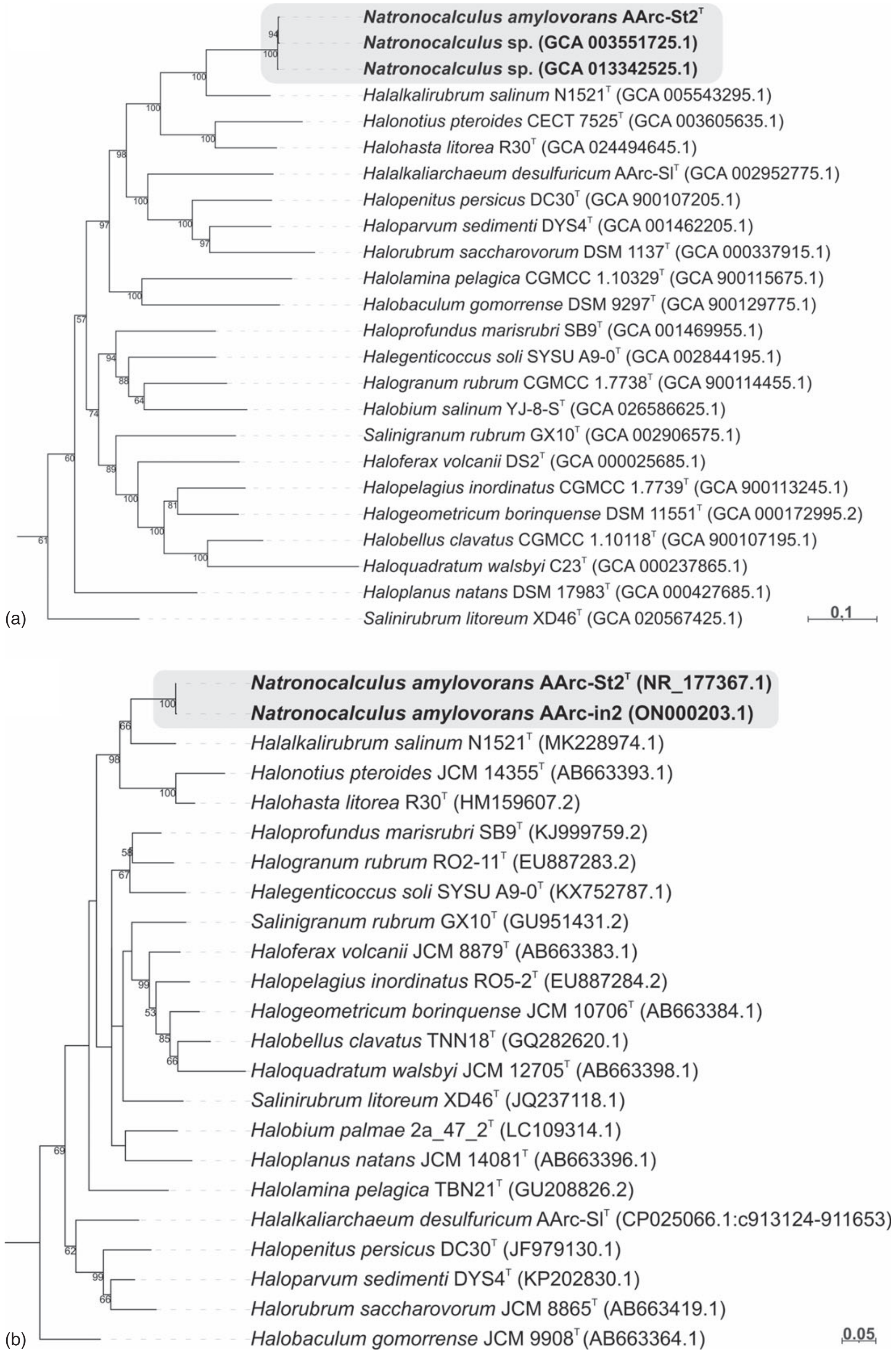

Natronocalculus amylovorans AArc-St2^T
Natronocalculus sp. (GCA 003551725.1)
Natronocalculus sp. (GCA 013342525.1)
Halalkalirubrum salinum N1521^T (GCA 005543295.1)
Halonotius pteroides CECT 7525^T (GCA 003605635.1)
Halohasta litorea R30^T (GCA 024494645.1)
Halalkaliarchaeum desulfuricum AArc-Sl^T (GCA 002952775.1)
Halopenitus persicus DC30^T (GCA 900107205.1)
Haloparvum sedimenti DYS4^T (GCA 001462205.1)
Halorubrum saccharovorum DSM 1137^T (GCA 000337915.1)
Halolamina pelagica CGMCC 1.10329^T (GCA 900115675.1)
Halobaculum gomorrense DSM 9297^T (GCA 900129775.1)
Haloprofundus marisrubri SB9^T (GCA 001469955.1)
Halegenticoccus soli SYSU A9-0^T (GCA 002844195.1)
Halogranum rubrum CGMCC 1.7738^T (GCA 900114455.1)
Halobium salinum YJ-8-S^T (GCA 026586625.1)
Salinigranum rubrum GX10^T (GCA 002906575.1)
Haloferax volcanii DS2^T (GCA 000025685.1)
Halopelagius inordinatus CGMCC 1.7739^T (GCA 900113245.1)
Halogeometricum borinquense DSM 11551^T (GCA 000172995.2)
Halobellus clavatus CGMCC 1.10118^T (GCA 900107195.1)
Haloquadratum walsbyi C23^T (GCA 000237865.1)
Haloplanus natans DSM 17983^T (GCA 000427685.1)
Salinirubrum litoreum XD46^T (GCA 020567425.1)
0.1
(a)
Natronocalculus amylovorans AArc-St2^T (NR_177367.1)
Natronocalculus amylovorans AArc-in2 (ON000203.1)
Halalkalirubrum salinum N1521^T (MK228974.1)
Halonotius pteroides JCM 14355^T (AB663393.1)
Halohasta litorea R30^T (HM159607.2)
Haloprofundus marisrubri SB9^T (KJ999759.2)
Halogranum rubrum RO2-11^T (EU887283.2)
Halegenticoccus soli SYSU A9-0^T (KX752787.1)
Salinigranum rubrum GX10^T (GU951431.2)
Haloferax volcanii JCM 8879^T (AB663383.1)
Halopelagius inordinatus RO5-2^T (EU887284.2)
Halogeometricum borinquense JCM 10706^T (AB663384.1)
Halobellus clavatus TNN18^T (GQ282620.1)
Haloquadratum walsbyi JCM 12705^T (AB663398.1)
Salinirubrum litoreum XD46^T (JQ237118.1)
Halobium palmae 2a_47_2^T (LC109314.1)
Haloplanus natans JCM 14081^T (AB663396.1)
Halolamina pelagica TBN21^T (GU208826.2)
Halalkaliarchaeum desulfuricum AArc-Sl^T (CP025066.1:c913124-911653)
Halopenitus persicus DC30^T (JF979130.1)
Haloparvum sedimenti DYS4^T (KP202830.1)
Halorubrum saccharovorum JCM 8865^T (AB663419.1)
Halobaculum gomorrense JCM 9908^T (AB663364.1)
0.05
(b)

FIGURE 2. Phylogenetic analyses based on comparison of concatenated alignment of 122 single-copy conserved archaeal proteins (a) and 16S rRNA genes (b) reveal position of *Natronocalculus* (in bold) within the family *Haloferacaceae*. The two metagenomically assembled genomes (MAGs) immediately clustering with the *Natronocalculus* isolates (Figure 2a) were retrieved from the same soda lakes in Kulunda Steppe (Vavourakis et al., 2018). The "ar122"- and 16S rRNA gene-based tree were constructed using the RAxML program v.8.2.12 with PROTGAMMAILG model (Stamatakis, 2014) and MEGA7 with GTR +G +*I* model (Kumar et al., 2016), respectively. Support values were 1,000 (rapid) bootstrap replications (percentage values higher than 50% are given at nodes). *Methanocella paludicola* SANAE and *Halobacterium salinarum* DSM 3754 were used as the outgroup (not shown).

Genome analysis

The draft genome of *Ncl. amylovorans* AArc-St2^T is 3.35 Mb and contains 3,346 genes encoding 3,265 proteins. The detailed genome analysis was published previously (Sorokin et al., 2022). The observed major physiological features are corroborated by the presence in the genome of several genes encoding for various extra- and intracellular alpha-glucanases and glucosidases belonging to the GH families 15 and 13 and of two families of beta-fructan hydrolases: GH32 and GH68. The pH-ion homeostasis membrane complexes are represented by a multisubunit and two types of single-subunit Na$^+$:H$^+$ antiporters, MrpEFGB1B2CD1D2D3, NhaA and NhaP, respectively. The potassium homeostasis is enabled by the TrkAH K$^+$:H$^+$ symporter (three copies), CPA2 and KhtT K$^+$:H$^+$ antiporters, KefBC potassium efflux (two copies), and a potassium channel Kch. The terminal cytochrome oxidase includes the aa_3-type CoxABC, while the ba_3-type common among haloarchaea seems to be absent.

Maintenance and preservation

Active liquid cultures of *Natronocalculus* are viable at 4°C for up to 6 months. Long-term preservation by deep freezing is possible with 15% glycerol as a cryoprotectant.

Taxonomy

According to the 16S rRNA gene comparison and genome-based phylogenetic analyses, the genus *Natronocalculus* forms a subcluster including genera *Halohasta*, *Halonotius*, and *Halalkalirubrum* within the family "*Halorubraceae*" (Figure 2) (Sorokin et al., 2022). Thus, it was classified in the NCBI database as a member of this family within the order *Haloferacales* according to the classification proposed by Gupta et al. (2015, 2016). However, according to the Genome Taxonomy Database and the recent proposal for reorganization of the hierarchical structure of the class *Halobacteria*, the former order *Haloferacales* has been regarded as a heterotypic synonym of the order *Halobacteriales*, and the family "*Halorubraceae*" as the heterotypic synonym of the family *Haloferacaceae*. Therefore, the correct placement of the genus *Natronocalculus* should change to the family *Haloferacaceae* within the order *Halobacteriales* (Cui et al., 2023).

The comparative properties of *Natronocalculus* and its closest related genera are presented in Table 1. *Natronocalculus* is the only one originating from soda lakes. The genera *Halohasta* and *Halonotius* are definitely neutrophiles, while *Halalkalirubrum salinum* reported growth maximum pH of 10.5 is very doubtful. In our experience, haloarchaea isolated from chloride-sulfate hypersaline habitats with neutral pH might be alkalitolerant or mildly alkaliphilic at best, but they would never grow at pH above 9.5 in well-buffered and pH-controlled conditions. Furthermore, the final pH in the profiling was apparently not measured. Since strains can lower the pH during growth, the starting pH may not correspond to the actual growth pH. Another important difference is the narrow metabolic specialization of the *Natronocalculus* isolates on alpha-glucans and the absence of glyco- and sulfolipids in their membranes.

List of species of the genus *Natronocalculus*

Natronocalculus amylovorans
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a.my.lo.vo'rans. Gr. neut. n. *amylon*, starch; L. inf. v. *vorare*, to devour; N.L. masc. part. adj. *amylovorans*, eating starch.

The cells are mostly nonmotile polymorphic cocci from 0.8 to 2 µm. The cells lyse in hypotonic solutions below 1 M NaCl. Produces red-orange carotenoids. The core membrane diether lipids are $C_{20}-C_{20}$ DGE (archaeol) and $C_{20}-C_{25}$ DGE (extended archaeol). The polar lipid head groups include PGP-Me and PG. Glyco- and sulfolipids are absent. The dominant respiratory quinone is MK-8:8. The available isolates are obligately aerobic and saccharolytic, with a

TABLE 1. Comparative properties of the genus *Natronocalculus* with the nearest phylogenetic relatives

Property	*Natronocalculus amylovorans* (three isolates)	*Halalkalirubrum salinum* (one isolate)[a]	*Halohasta* (two species)[b]	*Halonotius* (four species)[c]
Cell morphology	Polymorphic cocci, nonmotile	Polymorphic, nonmotile	Rods, motile	Polymorphic rods, motility (v)
Pigmentation	Red	Red	Red	Red
Anaerobic growth with DMSO	–	–	–	+ (one 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	Glucose, arabinose, fructose, galactose, sucrose, maltose, raffinose, xylose, mannitol, sorbitol, glycerol
Organic acids	None	Acetate, pyruvate, lactate, fumarate, succinate, citrate	Pyruvate, lactate, succinate, malate, fumarate, citrate (v)	Pyruvate, citrate, tartrate
Amylase	+	–	–	–
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)	+	–	Nd
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	Nd	Nd	Nd
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	Nd	Nd	Nd
DNA G + C (*mol%*, genomic)	51.5 (type strain)	58.4 (type strain)	58.8 (type species)	59.7–62.7 (four species)
Type of hypersaline habitat	Soda lakes	Salt lake	Solar saltern	Solar saltern; saline soils

Nd, not reported; (v), variable property in different species of the same genus; [*], actual final pH values were not measured; [**], for the type species, but not verified in any further research. Lipids: PA, phosphatidic acid; PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; S-DGD-1, sulfated mannosyl glucosyl diether; DGE, dialkyl glycerol ether.

[a] Zuo et al. (2021).

[b] Mou et al. (2012).

[c] Burns et al. (2010), Durán-Viseras et al. (2019a, 2019b).

limited substrate spectrum including several starch-like alpha-glucans, inulin (beta-fructan), maltose, trehalose, cellobiose, and glycerol. Oxidase- and catalase-positive. Indole production from tryptophan is weakly positive. Mesophilic, with a maximum growth temperature of 50°C (at pH 8.5). 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 8.5–9.0). The G+C content of the DNA is 51.5 *mol%* (genome sequence of the type strain). Apart from the type strain, the species also includes two closely related strains AArc-St3 and AArc-in2. All three strains were isolated from surface sediments and brines of hypersaline soda lakes in Kulunda Steppe (Altai, Russia).

Type strain: AArc-St2 (JCM 32475 = UQM 41562).

EMBL/GenBank accession number (16S rRNA gene): MG 584710 (type strain).

EMBL/GenBank accession number (genome assembly): GCF_023638035 (type strain).

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