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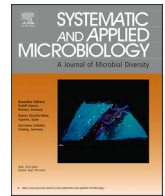
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## *Natronosalvus hydrolyticus* sp. nov., a beta-1,3-glucan utilizing natronoarchaeon from hypersaline soda lakes

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### ABSTRACT

Use of curdlan, an insoluble β-1,3-glucan, as an enrichment substrate under aerobic conditions resulted in the selection from hypersaline soda lakes of a single natronoarchaeon, strain AArc-curd11. This organism is an obligately aerobic saccharolytic, possessing a poorly explored (in Archaea) potential to utilize beta-1–3 glucans, being only a second example of a haloarchaeon with this ability known in pure culture. The main phenotypic property of the isolate is the ability to grow with insoluble β-1,3-backboned glucans, i.e. curdlan and pachyman. Furthermore, the strain utilized starch family α-glucans, beta-fructan inulin and a limited spectrum of sugars. The major ether-bound membrane polar phospholipids included PGP-Me and PG. The glyco- and sulfolipids were absent. The major respiratory menaquinone is MK-8:8. According to phylogenomic analysis, AArc-curd11 represents a separate species in the recently described genus *Natronosalvus* within the family *Natrialbaeae*. The closest related species is *Natronosalvus amylolyticus* (ANI, AAI and DDH values of 90.2, 91.6 and 44 %, respectively). On the basis of its unique physiological properties and phylogenomic distance, strain AArc-curd11<sup>T</sup> is classified as a novel species *Natronosalvus hydrolyticus* sp. nov. (=JCM 34865 = UQM 41566).

### Introduction

In the last decade the knowledge on the metabolic potential of extremely halophilic aerobic archaea from the archaeal class *Halobacteria* has been significantly broadened. It now includes polysaccharide utilizers capable of growth on various recalcitrant, insoluble polymers, such as chitin and cellulose and a wide spectrum of other neutral glucans (Sorokin et al., 2015; 2018; 2019; 2022a). Among the latter, the most difficult to degrade were two variety of insoluble glucans with the beta-1,3-linked backbone - curdlan and pachyman, produced by bacteria and mushrooms, respectively. In contrast to straight-chain curdlan, pachyman may have some branching points with the same type of the glycoside bond (Aquinas et al., 2022; Hoffmann et al., 1971; Zhang and Edgar, 2014). These polysaccharides can be hydrolyzed by fungal and bacterial glycoside hydrolases (GH) belonging to families 81 and 128 (Kumar et al., 2018; Martín-Cuadrado et al., 2008; Pluvinage et al., 2017). According to the CAZy database (<http://www.cazy.org>), some of their homologues are also encoded in several haloarchaeal genomes, in

particular GH81. However, until now, there was no clear evidence of the utilization of curdlan as a substrate in the Archaeal Kingdom. Currently, there are only two cultured haloarchaeal isolates with a proven ability to utilize such polysaccharides as growth substrates. One originated from neutral hypersaline lakes and has recently been described as *Halapricum hydrolyticum* (Sorokin et al., 2023). The other is a natronoarchaeon, strain AArc-curd11, which was enriched from hypersaline soda lakes with curdlan as substrate (Sorokin et al., 2022a). This paper provides its phenotypic and functional genomic characteristics and the strain is proposed to be classified as a novel species within the recently described genus *Natronosalvus* (Tan et al., 2023).

### Materials and methods

#### Media and cultivation conditions

Top oxic sediment and its overlying brines from four hypersaline soda lakes in Kulunda Steppe (Altai, Russia) served as the source for

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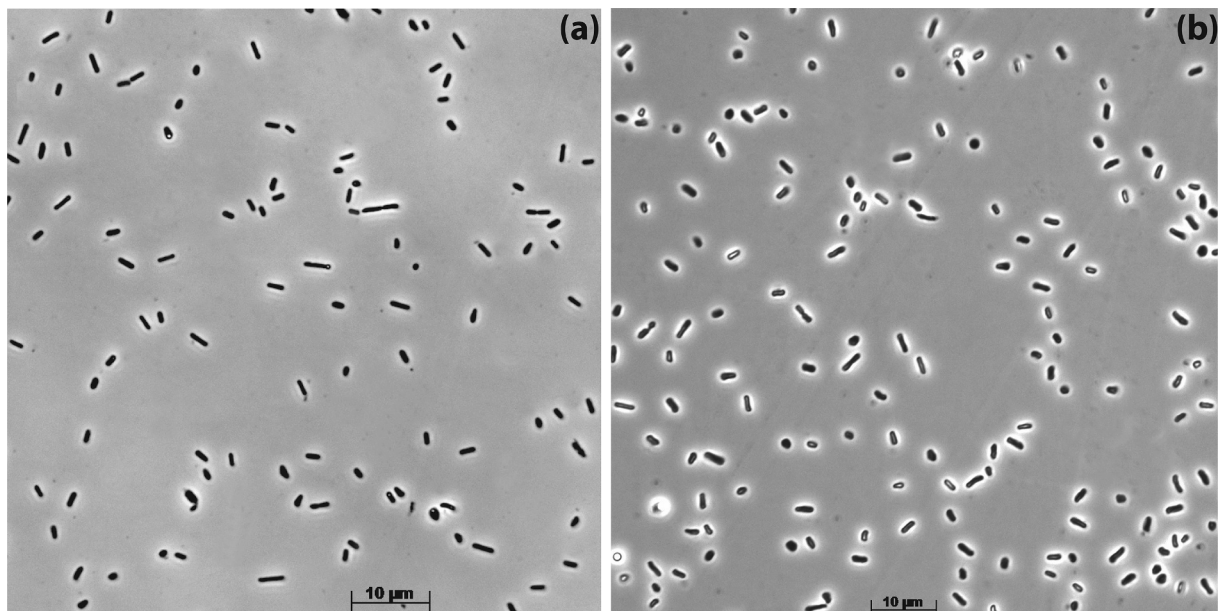
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**Fig. 1.** Cell morphology of the beta-1,3-glucan-utilizing natronarchaeon AArc-curdI1 growing at 4 M total  $\text{Na}^+$ , pH 9.5 and 37 °C with either curdlan (a) or maltose (b).

enrichment. The brine pH ranged from 9.8 to 11, the sodium carbonate alkalinity from 2 to 5 M and the total salinity from 180 to 350  $\text{g l}^{-1}$ . Acquisition of the primary liquid enrichment culture using curdlan as substrate and subsequent purification of the dominant curdlan-utilizing natronoarchaea have been described previously (Sorokin et al., 2022a). Briefly, the mineral base medium (without substrate) was inoculated with a fine sediment suspension (5 % v/v) and preincubated for 3 days at 28 °C on a rotary shaker with addition of 200  $\text{mg l}^{-1}$  of both ampicillin and streptomycin to suppress bacteria. After centrifugation the pellet was resuspended into a sodium carbonate-NaCl base medium containing 4 M total  $\text{Na}^+$  at pH 9.5 and 1  $\text{g l}^{-1}$  of curdlan (Megazyme, Ireland; prepared as a 10 % hydrated suspension in sterile distilled water) and incubated on a rotary shaker at 150 rpm and 37 °C until visible substrate degradation and appearance of pink turbidity in the liquid phase. Pure culture isolation was achieved from a single colony after plating of a final positive serial dilution, which successfully grew in liquid medium with curdlan as the sole carbon and energy source.

#### Phenotypic characterization

Phase contrast microscopy was performed using Zeiss Axioplane Imaging 2 (Germany). Growth in liquid cultures was monitored spectrophotometrically ( $\text{OD}_{600}$ ) after 30 min gravity sedimentation of insoluble polysaccharide particles, or directly in the case of soluble substrates. The potential for anaerobic growth was tested in serum bottles sealed with butyl rubber stoppers and subjected to vacuum cold boiling to remove both dissolved and gaseous oxygen followed by 3 cycles of flushing with sterile argon gas-evacuation. Aerobic substrate utilization, pH-salt profiling and other standard phenotypic testing were performed as described previously (Sorokin et al., (2022b); Sorokin et al., (2022c) in 30 ml screw-cap (with a rubber septum) bottles with 10 ml medium placed on a rotary shaker at 150 rpm and 37 °C. The temperature range was investigated at 4 M total  $\text{Na}^+$  and pH 9 (optimal conditions) within the range from 20 to 60 °C with 5 °C steps. Maltose served as a substrate for the pH, salt and temperature profiling. Basic mineral medium without substrate was used as a control and the growth was monitored by measuring  $\text{OD}_{600}$ . In the case of insoluble polysaccharides, in particular curdlan and pachyman (Megazyme), the particles were allowed to settle by gravity for 30 min. Microscopy showed that most of cells were not attached to the polysaccharide particles.

Membrane polar lipids and respiratory menaquinones were extracted from freeze-dried biomass grown with starch at 37 °C, 4 M total  $\text{Na}^+$  and pH 9.5 until late exponential growth phase and analyzed by Ultra High Pressure Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS), using conditions described previously (Bale et al., 2021; Sorokin et al., 2022b).

#### Genome sequencing, phylogenomic and functional genomic analyses

The genome of AArc-curdI1 was sequenced using the MiSeq Illumina platform and assembled as described previously (Sorokin et al., 2022a). The draft assembly is deposited in the GenBank under the number GCA\_025517525.

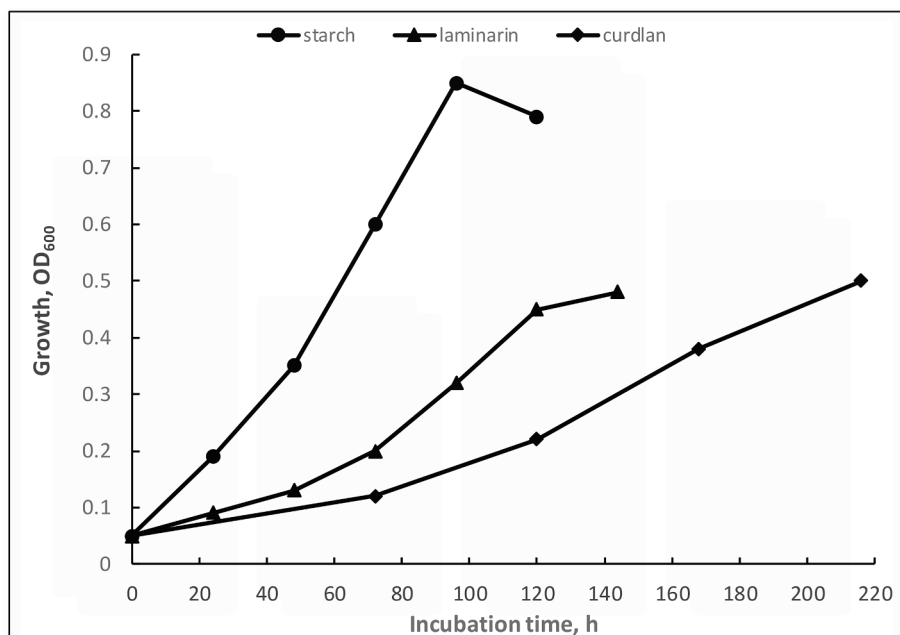
Phylogenomic analysis based on “ar122” set of conserved proteins with usage maximum likelihood approach and phylogenetic tree decoration were performed as described earlier (Sorokin et al., 2024). Additional Bayesian phylogenetic analysis of the obtained “ar122”-based alignment was done using MrBayes v.3.2.6 with  $\text{Ngen} = 50000$  and  $\text{Samplefreq} = 100$  (Ronquist et al., 2012). Average nucleotide identity (ANI), average amino acid identity (AAI) and digital DNA-DNA hybridization (dDDH) were calculated using pyani module v.0.2.8 (Pritchard et al., 2016), aai-martix.sh script (<https://github.com/lmrodriguezr/enveomics>) and GGDC v.3.0 (Meier-Kolthoff et al., 2022), respectively.

Genes of glycosidases were identified in the genome of strain AArc-curdI1 and in the representatives of *Natronosolus* genus using dbCAN v.4 (Zheng et al., 2023). Presumable activities were predicted using BLAST search against Swiss-Prot/PDB databases. Gene synteny was visualized using gggenes package (<https://CRAN.R-project.org/package=ggenes>).

## Results and discussion

#### Enrichment and isolation of pure culture

A primary enrichment with curdlan became positive after three weeks of incubation and was dominated by flattish rods typical for haloarchaea, although rod-shaped motile bacterial component was also abundant. It was not possible to eliminate the bacteria in further serial dilutions (positive up to  $10^{-9}$ ). Hence, final purification of the archaeal



**Fig. 2.** Comparative growth dynamics of strain AArc-curd11<sup>T</sup> at 4 M total Na<sup>+</sup>, pH 9.5 and 37°C with three different polysaccharides. Data shown represent average values of a duplicate experiment.

component was achieved after plating of the maximum serial dilution, which resulted in two dominant colony types: pink-colored (archaeal) and whitish (bacterial, identified as *Halomonas* sp.). Only pink-colored colonies grew back in liquid medium with curdian and one of the lineages resulted in a pure culture designated as strain AArc-curd11 as demonstrated by 16S-rRNA gene and whole genome sequence analyses.

#### Phenotypic properties

The cells of AArc-curd11 were mostly nonmotile flat rods with less frequently occurring flat coccoids (Fig. 1). In contrast to its previously described neutrophilic counterpart, *Halapricum hydrolyticum* HArc-curd15-1 (Sorokin et al., 2023), the cells of natronarchaeal isolate did not aggregate with the curdian particles and were mostly located in the liquid phase. From the tested range of various polysaccharides, AArc-curd11 was able to grow with other two beta-1-3-backbone glucans, the insoluble curdian analogue pachyman and the soluble mixed beta-1,3/1,6-linkage laminarin. Weak growth was also observed with galactomannan, but since mannan was not utilized, most probably only the side chains of galactomannan were used. Furthermore, the isolate grew slowly with beta-fructan inulin (from Chicory; Megazyme), while the fastest growth was detected on alpha-glucans, such as soluble starch and pullulan. The comparative growth dynamics on three different types of polysaccharides is shown on Fig. 2. It is noteworthy that both neutrophilic and soda lake curdian-utilizing haloarchaeal isolates (*Halapricum hydrolyticum* and AArc-curd11) are strong amyolytics, while lacking affinity for beta-1,4-linked glucans. Other utilized organic substrates included trehalose, sucrose, raffinose, maltose, cellobiose, pyruvate and acetate. While growing with acetate, many cells became highly refractive. Although thin sectioning electron microscopy was not performed, the presence of an operon coding for archaeal PHA-synthase (*phaCEP*) suggests that the observed cell refractivity was due to the accumulation of polyhydroxybutyrate. A similar operon is present in the genome of *Natronosaurus amyolyticus*, closely related to AArc-curd11 (see below), although it does not grow with acetate. Anaerobic growth, either fermentative (maltose, arginine), or respiratory (with pyruvate as the electron donor and sulfur, thiosulfate, DMSO and nitrate as the electron acceptors) was not observed.

AArc-curd11 is a typical extreme halophile in its salt profile with an

**Table 1**

Composition of intact polar lipids identified in strain AArc-curd11. The cells were grown with starch at 4 M total Na<sup>+</sup>, pH 9.5 and 37 °C until late exponential growth phase.

Polar head group	Core	[M + H] <sup>+</sup>	Assigned elemental composition	Percent
PGP-Me	EXT-AR	971.7449	C <sub>52</sub> H <sub>109</sub> O <sub>11</sub> P <sub>2</sub>	14.1
	Uns(1)-EXT-AR	969.7286	C <sub>52</sub> H <sub>107</sub> O <sub>11</sub> P <sub>2</sub>	1.0
	Uns(2)-EXT-AR	967.7138	C <sub>52</sub> H <sub>105</sub> O <sub>11</sub> P <sub>2</sub>	0.1
	Uns(3)-EXT-AR	965.6976	C <sub>52</sub> H <sub>103</sub> O <sub>11</sub> P <sub>2</sub>	0.9
	Lyso-EXT-AR	691.3527	C <sub>32</sub> H <sub>69</sub> O <sub>11</sub> P <sub>2</sub>	0.6
	Lyso-AR	621.3527	C <sub>27</sub> H <sub>59</sub> O <sub>11</sub> P <sub>2</sub>	2.2
	AR	901.6658	C <sub>47</sub> H <sub>99</sub> O <sub>11</sub> P <sub>2</sub>	32.8
	<b>Total</b>			<b>52</b>
PG	EXT-AR	877.7623	C <sub>51</sub> H <sub>106</sub> O <sub>8</sub> P	13.4
	Uns(1)-EXT-AR	875.7461	C <sub>51</sub> H <sub>104</sub> O <sub>8</sub> P	1.6
	AR	807.6837	C <sub>46</sub> H <sub>96</sub> O <sub>8</sub> P	31.9
	Lyso-AR	527.3704	C <sub>26</sub> H <sub>56</sub> O <sub>8</sub> P	1.3
	<b>Total</b>			<b>48</b>
PGP	AR			0.1
	<b>Sum AR</b>			<b>65</b>
	<b>Sum EXT-AR</b>			<b>27</b>
	<b>Sum uns-EXT-AR</b>			<b>4</b>
	<b>Sum lyso-AR</b>			<b>4</b>
	<b>Sum lyso-EXT-AR</b>			<b>1</b>

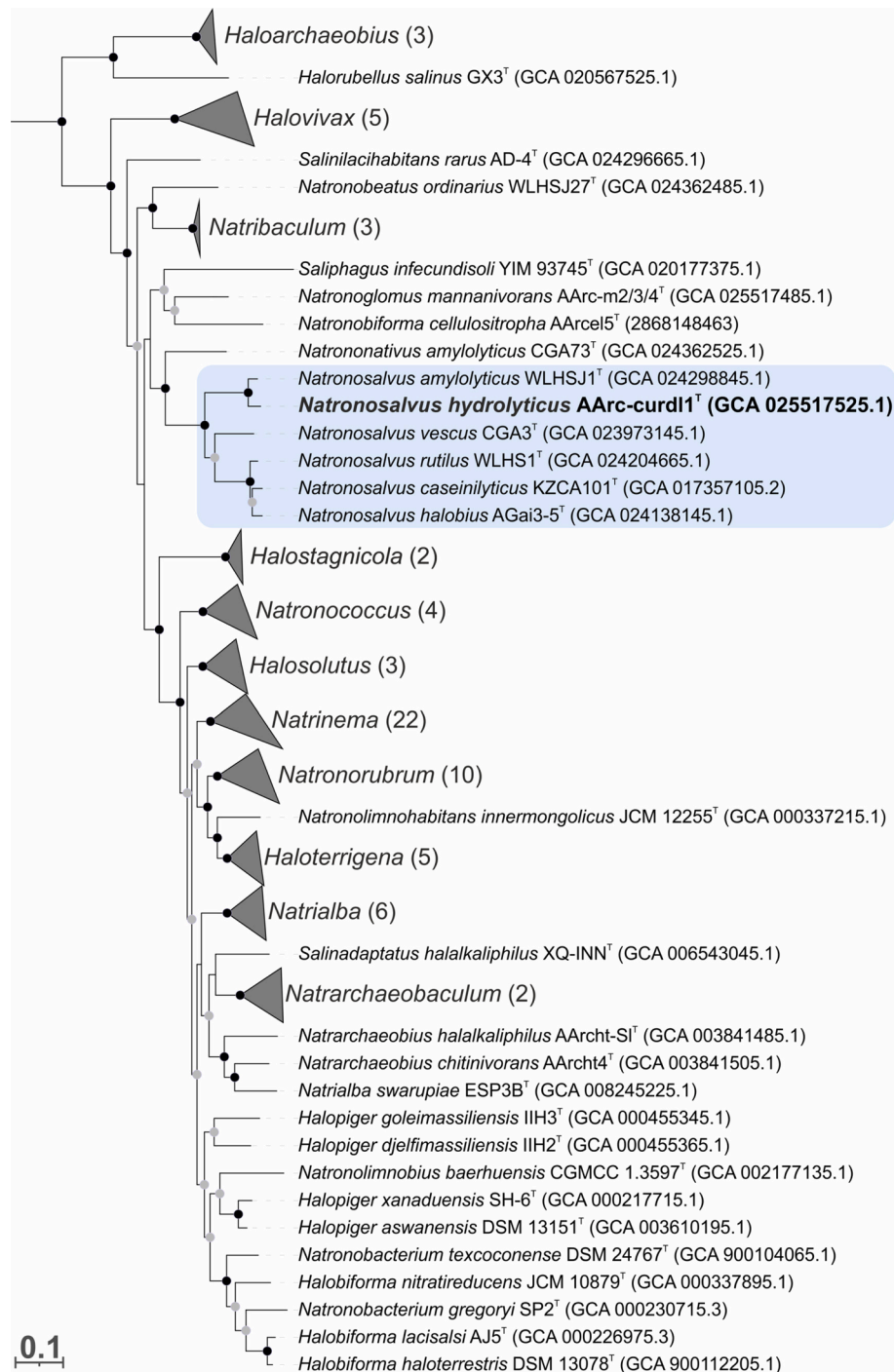
PGP-Me = phosphatidylglycerolphosphate methyl ester; PG = phosphatidylglycerol; PGP=.

Phosphatidylglycerolphosphate. AR = archaeol (C<sub>20</sub>-C<sub>20</sub>); EXT-AR = extended archaeol.

(C<sub>20</sub>-C<sub>25</sub>); lyso = one alkyl chain is absent; uns = unsaturated.

optimal growth (with maltose) at 3.5–4 M total Na<sup>+</sup> and a growth range of 2.5–4.5 M. It is obligately alkaliphilic, with the pH range for growth from 8.0 – 10.0 (optimum at 9.0–9.3) and mesophilic, with the temperature range for growth at pH 9 between 25 and 45°C (optimum at





**Fig. 3.** Maximum likelihood phylogenetic tree based on concatenated alignment of 122 conserved archaeal proteins, showing the position of strain AArc-curd11 (in bold) within the family Natrionalaceae. 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. Species of some genera are collapsed and numbers of species are given in brackets. *Archaeoglobus fulgidus* VC-16<sup>T</sup>, *Methanocella paludicola* SANAE<sup>T</sup> and *Methanothermobacter thermautotrophicus* Delta H<sup>T</sup> were used as an outgroup (not shown).

35). The Mg requirement is low, with a maximal growth rate already at 1 mM. The minimal Cl<sup>-</sup> concentration for growth (at 4 M total Na<sup>+</sup> balanced by carbonates at pH 9.5) was 0.5 M. Ammonium or yeast extract, but not urea or nitrate, served as the nitrogen source. Colony tests showed positive results for catalase and oxidase activities and Kovach test was also positive for the indole formation from tryptophane. Colony assays for lipase and protease (with emulsified olive oil and casein, respectively) were negative, while amylase activity was strongly positive. The AArc-curd11 culture grown with maltose at pH 9.5 was

tolerant to streptomycin, ampicillin, kanamycin and vancomycin up to 200 mg l<sup>-1</sup> and to tetracycline up to 100 mg l<sup>-1</sup>. The growth was completely suppressed by rifampicin and chloramphenicol at concentrations above 50 mg l<sup>-1</sup>.

The core of the membrane phospholipids in AArc-curd11 consisted of four archaeol modifications: C<sub>20</sub>-C<sub>20</sub> archaeol and C<sub>20</sub>-C<sub>25</sub> extended archaeol as dominant constituents and small proportions of unsaturated extended archaeol and lyso-archaeol. The intact polar ether lipids were dominated by phosphatidylglycerolphosphate methyl ester (PGP-Me)

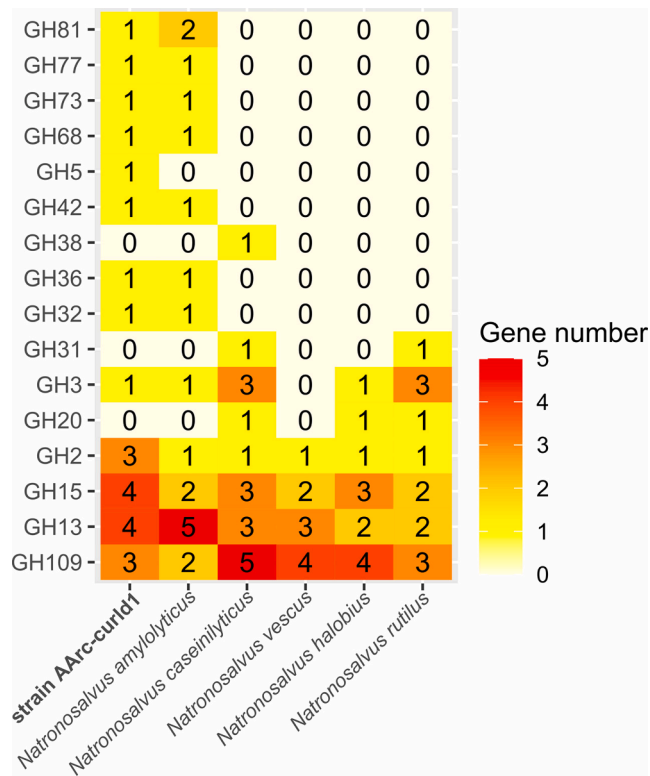


Fig. 4. Glycosidases repertoires of strain AArc-curd1 and other representatives of *Natronosalmus* genus.

and phosphatidylglycerol (PG) in equal proportion (Table 1). Glyco- and sulfo-lipids, commonly found in neutrophilic haloarchaea, were not detected. The dominant respiratory lipoquinone was MK-8:8 (94.3 %), with a minor proportion of MK-8:7 (5.6 %).

#### Phylogenetic analysis

The genome of AArc-curd1 has a single *rrn* operon with the 16S rRNA gene closely related (99.5 % sequence identity) to a recently described species *Natronosalmus amylolyticus* isolated from a hypersaline alkaline lake in Inner Mongolia (Tan et al., 2023). The maximum likelihood phylogenomic tree based on comparison of 122 archaeal conserved single-copy protein markers (Fig. 3) confirmed the assignment of strain AArc-curd1 as a member of the genus *Natronosalmus*. Moreover, the Bayesian phylogenomic tree possessed almost same topology and also showed belonging of novel isolate to *Natronosalmus*

(Supplementary Fig. S1). Comparison of the genomes showed that the novel isolate differs from *Nsv. amylolyticus* and other *Natronosalmus* species on the level of a separate species (Supplementary Table S1). The ANI, AAI and DDH values between AArc-curd1 and *Natronosalmus* species were 77.4–90.2 %, 71.1–91.6 % and 22.6–42 %, with the highest values to *Nsv. amylolyticus*. All values are lower than the proposed interspecies thresholds, i.e. 95 % for ANI (Kim et al., 2014) and AAI (Konstantinidis and Tiedje, 2005) and 70 % for DNA-DNA hybridization (Wayne et al., 1987).

#### Environmental occurrence

To determine the ecological distribution of the *Natronosalmus*, both metagenome-assembled genomes (MAGs) and 16S rRNA gene sequences were analyzed. Besides five described species of *Natronosalmus* isolated from sediments of various hypersaline lakes in China (Tan et al., 2023), there are currently four MAGs belonging to this genus in the databases. All of them are from metagenomes obtained in hypersaline soda lake sediments of Kulunda Steppe (Altai, Russia) (Vavourakis et al., 2018). These MAGs possessed ANI values of around 77 % when compared with the genome of AArc-curd1. Only five clones/undescribed strains relatively close to AArc-curd1 (16S rRNA sequences identities > 97.3 %) were found in Genbank nr/nt database. These sequences were obtained from salt lakes and coastal salt soils in China. Thus, the current geographical distribution of *Natronosalmus* seems to be limited to hypersaline locations in Central Asia.

#### Functional genome analysis

All representatives of *Natronosalmus* genus have a relatively narrow range of encoded GH family glycosidases (10 to 23). The most abundant families were GH13 and GH15, whose members are responsible for hydrolysis of alpha-linked glucans (Fig. 4). The genome of AArc-curd1 encodes a secreted beta-1,3-endoglucanase of GH81 family, but, in contrast to the homologous protein in the neutrophilic curdian-utilizing *Halapricum hydrolyticum*, it lacked the GH81-specific CBM6 (carbohydrate-binding domain) or any other CBMs that are crucial for hydrolysis of insoluble beta-1,3-glucans (Hettle et al., 2017). However, right upstream of the GH81 gene in the AArc-curd1 genome a bicistronic operon is present encoding two putative translocated integrin proteins related to CBM/Fibronectin III/PKD involved in cell adhesion (Fig. 5A). They might substitute to the absence of the CBM domain in the AArc-curd1 GH81, although it contradicts the abovementioned lack of visible cell attachment to the glucan particles. Gene encoding beta-glucosidase from GH3 family and ABC transporter with substrate-binding subunit homologous to sugar- and oligopeptide-binding proteins (Ghimire-Rijal et al., 2014) were also the neighborhood of GH81 gene. Phylogenetic analysis performed earlier showed that the haloarchaeal GH81

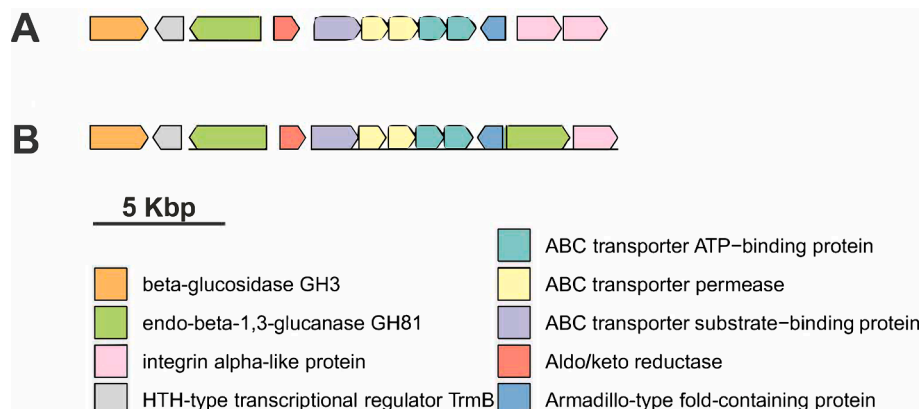


Fig. 5. Clusters containing gene of *endo*-beta-1,3-glucanase from GH81 family in genome of strain AArc-curd1 (A) and *Natronosalmus amylolyticus* (B).

**Table 2**

Glycoside hydrolase family proteins encoded in the genome of strain AArc-curd11 (functional domains are in parentheses).

Locus tag	HMMER	Signal peptide	Putative function
MCU475			
+			
<b>endo-beta-1,3/1,4-glucanase</b>			
1269	<b>GH81</b> (61–708)*	Tat/SPI	<b>endo-1,3-beta-glucanase</b>
1304	GH5_13(34–294)	–	unknown
<b>alpha-glucanases</b>			
1886	GH13(353–689) + CBM13 (856–996)	Tat/SPI	alpha-amylase
2681	GH13(238–521)	–	alpha-amylase
4306	GH13(305–655)	Tat/SPI	alpha-amylase
0398	CBM34(8–130) + GH13_20(177–473)	–	neopullulanase
0402	GH15(317–728)	–	glucoamylase
0404	GH15(281–639)	–	alpha-1,4-glucosidase
2682	GH15(277–653) + GH15 (995–1386)	–	glucoamylase
2868	GH15(235–593)	–	trehalase
2683	GH77(15–494)	–	amylomaltase
<b>beta-fructosidases</b>			
2688	GH32(296–592)	–	endo/exo-levanase
0559	GH68(30–407)	–	levan/inulo-sucrase
<b>other glycosidases</b>			
0702	GH2(3–594)	–	beta-mannosidase
1300	GH2(15–502)	–	beta-galactosidase
1305	GH2(4–751)	–	beta-galactosidase
4213	GH36(56–504)	–	alpha-galactosidase
4214	GH42(6–390)	–	beta-galactosidase
1271	GH3(90–311)	–	beta-xylosidase/ glucosidase

\* The **GH81** gene is preceded by a bicistron operon (1260–1261) encoding secreted alpha-beta integrin subunits, usually involved in cell adhesion.

homologues are forming a separate cluster on this family tree and include two types of proteins: large bimodal with CBM, and smaller proteins with only a catalytic GH81 domain (Sorokin et al., 2023). The enzyme from AArc-curd11 is clustering with a homologue from its closest relative, i.e. *Nsv. amylolyticus*, that, in addition, also have another, dissimilar GH81 gene copy (Cluster III in the tree from Sorokin et al., 2023) located nearby in the genome. Moreover, a beta-glucosidase, ABC transporter and closely related homologue of one of the two integrins present in vicinity of the GH81 from AArc-curd11 is also following the second GH81 gene copy in *Nsv. amylolyticus*, while the second integrin of AArc-curd11 is absent (Fig. 5B).

A second important functional group of glycosidases encoded in the AArc-curd11 genome, as well as in its closest relative *Nsv. amylolyticus*, includes a large repertoire of glycosidases involved in the breakdown of alpha-glucans (GH13 and GH15 families). This corroborates the potential of both species to utilize starch and its homologues as growth substrates. In particular, an extracellular GH13 alpha-amylase with a CBM13 module and a glucoamylase with a double GH15 domain are encoded by both genomes. Furthermore, they also encode two beta-fructan hydrolases from the GH families 32 and 68, which corroborate with their ability to grow with a fructan inulin. A list of the glycoside hydrolases encoded in the genomes of AArc-curd11 is presented in Table 2.

However, the main functional property of the new isolate is its potential to utilize insoluble beta-1,3-glucans as growth substrate. The genome of AArc-curd11 encodes a translocated protein containing the GH81 hydrolytic domain specific for the beta-1,3-endoglucanases, but, in contrast to the homologous protein in neutrophilic *Halapricum hydrolyticum*, it lacked the GH81-specific CBM6 (carbohydrate-binding domain). However, right upstream of the GH81 gene in the AArc-curd11 genome a bicistronic operon is present coding for two putative translocated *integrin* proteins related to CBM/Fibronectin III/PKD involved in cell adhesion. They might substitute to the absence of the CBM domain

**Table 3**

Comparative properties of the beta-1,3-glucan-utilizing strain AArc-curd11 with the related species from the genus *Natronosolus* (Tan et al., 2023).

Property	AArc-curd11 <sup>T</sup>	<i>Natronosolus amylolyticus</i> WLHSJ1 <sup>T</sup>	<i>Natronosolus halobius</i> AGai3-5 <sup>T</sup>
Cell morphology	nonmotile flat rods	motile, pleomorphic	motile, pleomorphic
Pigmentation	pink + (on acetate)	pink to red - (on pyruvate)	pink to red
PHA accumulation	+	*	nd
Anaerobic growth	–	–	+
Denitrification genes	–	–	(denitrification)
Nitrate reductase	+	–	..**
<i>narGHJ</i>	–	–	..**
Cu-nitrite reductase <i>nirK</i>	+	–	..**
NO-reductase <i>qNor</i>	–	–	–
N <sub>2</sub> O reductase cluster	starch, glycogen, pullulan, laminarin, curdlan, pachyman, inulin	starch*, pullulan*, glycogen*, laminarin*, curdlan*, pachyman*, inulin (w)	–
Substrates for aerobic growth: polysaccharides	–	*	–
sugars/sugar alcohols	trehalose, raffinose, sucrose, maltose, cellobiose	galactose, sucrose; mannose(w*), trehalose*, maltose*, cellobiose*, melezitose*, raffinose (w)*	mannose, galactose, ribose, lactose, mannitol, glycerol pyruvate, lactate, malate, fumarate
organic acids	acetate, pyruvate	pyruvate	–
beta-1,3-glucanase GH81 genes	1	2	– (Tweens 80) - casein
Amylase	+/-	+	–
Esterase/lipase	(tributyryl/olive oil)-	(Tween 80)+	–
Protease	(casein; gelatin)	(casein)	–
Catalase/oxidase	+/+	+/w+	+/–
Indole from tryptophane	+	–	+
Salinity range (opt.)	2.5–4.4 (3.5–4.0)	0.9–4.8 (3.5)	0.9–4.8 (3.9)
M Na <sup>+</sup>	–	–	–
Mg requirement (optimum)pH range (opt.)	low (1 mM) 8.0–10.0 (9.0–9.3)	low (1 mM)* 6.0–9.5 (8.5)	nd 7.0–9.0 (8.5)
Temperature max (°C)	45 (at pH 9.0)	55	50
Core lipids (archaeols)	C <sub>20</sub> -C <sub>20</sub> , C <sub>20</sub> -C <sub>25</sub> DGE	C <sub>20</sub> -C <sub>20</sub> , C <sub>20</sub> -C <sub>25</sub> DGE	C <sub>20</sub> -C <sub>20</sub> , C <sub>20</sub> -C <sub>25</sub> DGE
Intact membrane polar lipids: phospholipids glycolipids	PG, PGP-Me -	PG, PGP-Me, PA DGD-1, S-DGD-1, S-TGD-1	PG, PGP-Me, PA DGD-1, S-DGD-1
Respiratory lipoquinones	MK-8:8 (major) MK-8:7 (minor)MK-7:7 (traces)	nd	nd
DNA G + C (% chromosome)	59.2	59.5	63.1
Genome size (Mbp)	4.54	4.22	3.85
Plasmids	0	4	2
Plasmids	hypersaline soda lakes	hypersaline alkaline lake	hypersaline salt lake
Isolation source	–	–	–



\*, determined in this study; \*\* full denitrification is reported, but three genes required for the denitrification process are absent in the genome of this species; nd, not determined; w (weak). Lipids: (PA) phosphatidic acid, (PG) phosphatidylglycerol, (PGP) phosphatidylglycerol-phosphate, (PGP-Me) phosphatidylglycerophosphate methyl ester, (DGD-1) mannosyl glucosyl diether, (S-DGD-1) sulfated mannosyl glucosyl diether, (S-TGD-1) sulfated galactosyl mannosyl glucosyl diether, (DGE) dialkyl glycerol ether. (MK) menaquinone.

in the AArc-curd11 GH81, although it contradicts the abovementioned lack of visible cell attachment to the glucan particles. Phylogenetic analysis of the GH81 protein from AArc-curd11 showed a close relation with a homologue from its closest relative *Nsv. amylolyticus*, which, in addition, also have another, dissimilar GH81 gene copy located close by in the genome. Moreover, a closely related homologue of one of the two *integrins* present in vicinity of the GH81 from AArc-curd11 is also following the second GH81 gene copy in *Nsv. amylolyticus*, while the second integrin of AArc-curd11 is absent.

Taking into account the direct prove of existence of halo(natrono) archaea capable of growth with insoluble beta-1,3-glucans and the presence of genes encoding homologous GH81 family enzymes in many other characterized haloarchaeal species, it might be reasonable to speculate that there should be a source for beta-1,3-glucans in hypersaline habitats, both NaCl and sodium carbonate-based, and that haloarchaea may actively participate in their mineralization.

Other functionally important proteins from the genome of AArc-curd11 are listed in Supplementary Table S2, including the salt-pH homeostasis, energy-related complexes and oxidative stress response systems. Of particular interest is the presence of an incomplete denitrification pathway, including Cu-nitrite reductase NirK and N<sub>2</sub>O reductase but lacking nitrate reductase Nar and NO-reductase qNor. This explains the negative anaerobic growth test for AArc-curd11 with nitrate. However, the strain also did not grow in presence of 5 mM nitrite or 10 % N<sub>2</sub>O in the gas phase, despite the presence of necessary genetic potential. Interestingly, the opposite situation occurs with the type species of the genus *Natronosaurus* – *Nsv. halobius*: full denitrification of nitrate with gas production has been reported for this organism (Tan et al., 2023), while the only recognizable denitrification protein (by Blast search using *Haloferax mediterranei* proteins as the references) encoded in its genome is a Cu-nitrite reductase NirK.

Comparative properties of the novel isolates with the two *Natronosaurus* species are shown in Table 3. AArc-curd11 has more features in common with *Nsv. amylolyticus*, which was also isolated from an alkaline lake, but is more distant from the neutrophilic type species of the genus *Nsv. halobius*. The especially important similarity of the new isolate and *Nsv. amylolyticus* lays in the genomic potential encoding beta-1,3-glucanases (absent in the neutrophilic species of *Natronosaurus*) and alpha-glucanases. Since the original testing of hydrolytic potential of *Nsv. amylolyticus* was limited to starch, we examined the ability to utilize insoluble curdlan and pachyman and soluble laminarin in the type strain JCM 33563 using soluble starch as a positive control. The results were strongly positive (after adaptative 5 days lag phase) for all three beta-1,3-glucans and also showed lack of cell attachment to polysaccharide particles similar to AArc-curd11 (Supplementary Fig. S2). Furthermore, *Nsv. amylolyticus* also tested positive for growth with three more (soluble) polysaccharides, including alpha-glucans pullulan and glycogen and beta-fructan inulin. So, in this particular case, the genome functional prediction was quite accurate. Among the obvious difference of AArc-curd11 from its closest related *Nsv. amylolyticus* is the obligate alkaliphily and absence of proteolytic activity and glycolipids.

#### Taxonomic conclusion

Considering the distinct phylogeny and phenotypic properties, the beta-1,3-glucan-utilizing strain AArc-curd11 isolated from hypersaline soda lakes is suggested to be classified as a new species *Natronosaurus hydrolyticus* sp. nov. The species protologue is presented in Table 4.

**Table 4**  
*Natronosaurus hydrolyticus*: protologue.

Parameter	Species: <i>Natronosaurus hydrolyticus</i> sp. nov.
Species name	<i>hydrolyticus</i>
Genus name	<i>Natronosaurus</i>
Species status	sp. nov.
Etymology	<a href="https://www.hydro.ro.ly">hydro.ro.ly</a> 'ti.cus Gr. neut. n. <i>hydor</i> , water; Gr. masc. adj. <i>lytikos</i> , dissolving, splitting; N.L. masc. adj. <i>hydrolyticus</i> , hydrolyzing
Description of the new taxon	The cells are nonmotile flat rods, 0.7–1 x 1–3 μm, producing red pigments. The cells lyse in distilled water. The core membrane diether lipids are dominated by C <sub>20</sub> -C <sub>20</sub> DGE (archaeol) and C <sub>20</sub> -C <sub>25</sub> DGE (extended archaeol) with 0–3 double bonds. The polar lipid head groups include phosphatidylglycerolphosphate methyl ester (PGP-Me) as a major component and less abundant phosphatidylglycerol (PG). Glyco- and sulfolipids were not detected. The dominant respiratory quinone is MK-8:8 with a small proportion of monounsaturated MK-8:7. It is obligate aerobic saccharolytic heterotroph. Represents first example of natronoarchaea capable of utilizing insoluble beta-1,3-glucans (curdlan and pachyman) for growth. Can also grow with soluble beta-1,3/1,6-glucan laminarin, beta-fructan inulin and alpha-glucans, including starch, glycogen and pullulan. The spectrum of utilized sugars includes raffinose, trehalose, sucrose, maltose and cellobiose. From tested C <sub>2</sub> -C <sub>6</sub> organic acids only acetate and pyruvate supported growth. Ammonium, but not nitrate or urea, served as the N-source. Oxidase and catalase activity are positive. Mesophilic, with a temperature range from 25 to 45 °C (optimum at 35–40 °C). It is a low Mg-demanding, extreme halophile, with a range of total Na <sup>+</sup> for growth from 2.5 to 4.5 M (optimum at 3.5–4 M) and an obligate alkaliphile growing within the pH range from 8 to 10 (optimum at 9–9.3). The G + C content of the DNA is 59.2 % (genome). Habitat - hypersaline soda lakes. The type strain (AArc-curd11 <sup>T</sup> = JCM 34865 = UQM 41566) was isolated from oxic sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia).
Authors	Dimitry Y. Sorokin, Alexander G. Elcheninov, Nicole J. Bale, Jaap S. Sinnighe Damsté and Ilya V. Kublanov
Title	<i>Natronosaurus hydrolyticus</i> sp. nov., a beta-1,3-glucan utilizing natronoarchaeon from hypersaline soda lakes
Journal	Systematic and Applied Microbiology
Corresponding author	Dimitry Y. Sorokin
E-mail of corresponding author	soroc@inmi.ru; d.sorokin@tudelft
Designation of the type strain	AArc-curd11
Strain collection numbers	JCM 34865; UQM 41566
16S rRNA gene accession number	PP697474
Genome assembly accession number	GCA_025517525
Genome status	Draft
Country/region of origin	Russian Federation/Altai region
Date of isolation	2017
Source of isolation	Surface sediments and brines from 3 hypersaline soda lakes
Sampling dates	2015–08-05
Geographic location	south-western Siberia, Kulunda Steppe
Latitude	51°39' N
Longitude	79°48' E
Depth	0.05 m
Temperature of the sample	22 °C
pH of the sample	9.8–11.0
Salinity of the sample	22–35 ‰
Number of strains in study	1
Growth medium, incubation conditions	4 M total Na <sup>+</sup> , pH 9.5; incubation – 37 °C; shaker 150 rpm
Conditions of preservation	Deep freezing in 15 % glycerol (v/v) directly in growth medium (4 M total Na <sup>+</sup> , pH 9.5)
Colony morphology	pink, flat, smooth, up to 2 mm

(continued on next page)

Table 4 (continued)

Parameter	Species: <i>Natronosalvus hydrolyticus</i> sp. nov.
Optimal temperature for growth	35–40
Salinity category	Extremely halophilic
Relation to oxygen	Obligate aerobe
O <sub>2</sub> conditions for strain testing	Fully aerobic
Specific compounds	beta-1,3-glucans curdlan and pachyman

#### CRedit authorship contribution statement

**Dimitry Y. Sorokin:** Writing – original draft, Methodology, Investigation, Conceptualization. **Alexander G. Elcheninov:** Writing – original draft, Methodology, Investigation. **Nicole J. Bale:** Writing – original draft, Methodology, Investigation. **Jaap Sininghe Damsté:** Writing – review & editing. **Ilya V. Kublanov:** Writing – review & editing, Conceptualization.

#### Data availability

No data was used for the research described in the article.

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#### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.syapm.2024.126514>.

#### References

- Aquinas, N., Bhat, M.R., Selvaraj, S., 2022. A review presenting production, characterization, and applications of biopolymer curdlan in food and pharmaceutical sectors. *Polym. Bull.* 79, 6905–6927.
- Bale, N.J., Ding, S., Hopmans, E.C., Arts, M.G.I., Villanueva, L., Boschman, R.C., Haas, A. F., Schouten, S., Sininghe Damsté, J.S., 2021. Lipidomics of environmental microbial communities. I: Visualization of specific niches using untargeted analysis of high-resolution mass spectrometry data. *Front. Microbiol.* 12, 659302.
- Ghimire-Rijal, S., Lu, X., Myles, D.A., Cuneo, M.J., 2014. Duplication of genes in an ATP-binding cassette transport system increases dynamic range while maintaining ligand specificity. *J. Biol. Chem.* 289, 30090–30100.
- Hettle, A., Fillo, A., Abe, K., Massel, P., Pluvinaige, B., Langelaan, D.N., Smith, S.P., Boraston, A.B., 2017. Properties of a family 56 carbohydrate-binding module and its role in the recognition and hydrolysis of alpha-1,3-glucan. *J. Biol. Chem.* 292, 16955–16968.
- Hoffmann, G.C., Simson, B., Timell, T.E., 1971. Structure and molecular size of pachyman. *Carbohydr. Res.* 20, 185–188.
- Kim, M., Oh, H.-S., Park, S.-C., Chun, J., 2014. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int. J. Syst. Evol. Microbiol.* 64, 346–351.

- Konstantinidis, K.T., Tiedje, J.M., 2005. Towards a genome-based taxonomy for Prokaryotes. *J. Bacteriol.* 187, 6258–6264.
- Kumar, K., Correia, M.A.S., Pires, V.M.R., Dhillon, A., Sharma, K., Rajulapati, V., Fontes, C.M.G.A., Carvalho, A.L., Goyal, A., 2018. Novel insights into the degradation of beta-1,3-glucans by the cellulosome of *Clostridium thermocellum* revealed by structure and function studies of a family 81 glycoside hydrolase. *Int. J. Biol. Macromol.* 117, 890–901.
- Martín-Cuadrado, A.B., Encinar del Dedo, J., de Medina-Redondo, M., Fontaine, T., del Rey, F., Latgé, J.P., Vázquez de Aldana, C.R., 2008. The *Schizosaccharomyces pombe* endo-1,3-beta-glucanase Eng1 contains a novel carbohydrate binding module required for septum localization. *Mol. Microbiol.* 69, 188–200.
- Meier-Kolthoff, J.P., Sardá Carbasse, J., Peinado-Olarte, R.L., Göker, M., 2022. TYGS and LPSN: a database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes. *Nucleic Acid Res* 50, D801–D807.
- Pluvinaige, B., Fillo, A., Massel, P., Boraston, A.B., 2017. Structural analysis of a family 81 glycosidehydrolase implicates its recognition of beta-1,3-glucan quaternary structure. *Structure* 25, 1348–1359.
- Pritchard, L., Glover, R.H., Humphris, S., Elphinstone, J.G., Toth, I.K., 2016. Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. *Anal. Methods* 8, 12–24.
- Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61 (3), 539–542.
- Sorokin, D.Y., Toshchakov, S.V., Kolganova, T.V., Kublanov, I.V., 2015. Halo(natrono) archaea isolated from hypersaline lakes utilize cellulose and chitin as growth substrates. *Front. Microbiol.* 6, 942.
- Sorokin, D.Y., Khijniak, T.V., Kostrikina, N.A., Elcheninov, A.G., Toshchakov, S.V., Bale, N.J., Sininghe Damsté, J.S., 2018. *Natronobiforma cellulositropha* gen. nov., sp. nov., a novel haloalkaliphilic member of the family Natrialbaeaceae (class Halobacteria) from hypersaline alkaline lakes. *Syst. Appl. Microbiol.* 41, 355–362.
- Sorokin, D.Y., Khijniak, T.V., Kostrikina, N.A., Elcheninov, A.G., Toshchakov, S.V., Bale, N.J., Sininghe Damsté, J.S., 2019. *Halococcoides cellulovorans* gen. nov., sp. nov., an extremely halophilic cellulose-utilizing haloarchaeon from hypersaline lakes. *Int. J. Syst. Evol. Microbiol.* 69, 1327–1335.
- Sorokin, D.Y., Elcheninov, A.G., Khijniak, T.V., Kolganova, T.V., Kublanov, I.V., 2022a. Selective enrichment on a wide polysaccharide spectrum allowed isolation of novel metabolic and taxonomic groups of haloarchaea from hypersaline lakes. *Front. Microbiol.* 13, 1059347.
- Sorokin, D.Y., Yakimov, M.M., Messina, E., Merkel, A.Y., Koenen, M., Bale, N.J., Sininghe Damsté, J.S., 2022b. *Natronaerarchaeum sulfidigenes* gen. nov., sp. nov., carbohydrate-utilizing sulfur-respiring haloarchaeon from hypersaline soda lakes, a member of a new family *Natronaerarchaeaceae* fam. nov. in the order *Halobacteriales*. *Syst. Appl. Microbiol.* 45, 126356.
- Sorokin, D.Y., Elcheninov, A.G., Khijniak, T.V., Koenen, M., Bale, N.J., Sininghe Damsté, J.S., Kublanov, I.V., 2022c. *Natronocalculus amylovorans* gen. nov., sp. nov., and *Natronaerarchaeum aerophilus* sp. nov., dominant culturable amylolytic natronaerarchaea from hypersaline soda lakes in southwestern Siberia. *Syst. Appl. Microbiol.* 45, 126336.
- Sorokin, D.Y., Elcheninov, A.G., Merkel, A.Y., Bale, N.J., Sininghe Damsté, J.S., Kublanov, I.V., 2023. *Halapricum hydrolyticum*, a beta-1,3-glucan utilizing haloarchaeon from hypersaline lakes. *Syst. Appl. Microbiol.* 46, 126471.
- Sorokin, D.Y., Elcheninov, A.G., Bale, N.J., Sininghe Damsté, J.S., Kublanov, I.V., 2024. *Natronoglomus mannanivorans* gen. nov., sp. nov., beta-1,4-mannan utilizing natronaerarchaea from hypersaline soda lakes. *Front. Microbiol.* 15, 1364606.
- S. Tan M. Cheng X.X. Li Y. Hu X. Ma J. Hou H.L. Cui *Natronosalvus halobius* gen. nov., sp. nov., *Natronosalvus caseinilyticus* sp. nov., *Natronosalvus vesicus* sp. nov., *Natronosalvus rutilus* sp. nov. and *Natronosalvus amylolyticus* sp. nov., halophilic archaea isolated from salt lakes and soda lakes *Int. J. Syst. Evol. Microbiol.* 73 2023 006036.
- Vavourakis, C.D., Andrei, A.-S., Mehrshad, M., Ghai, R., Sorokin, D.Y., Muyzer, G., 2018. A metagenomics roadmap to the uncultured genome diversity in hypersaline soda lake sediments. *Microbiome* 6, 168.
- Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Krichevsky, M.I., Trüper, H. G., 1987. Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Evol. Microbiol.* 37, 463–464.
- Zhang, R., Edgar, K.J., 2014. Properties, chemistry, and applications of the bioactive polysaccharide curdlan. *Biomacromolecules* 15, 1079–1096.
- Zheng, J., Ge, Q., Yan, Y., Zhang, X., Huang, L., Yin, Y., 2023. dbCAN3: automated carbohydrate-active enzyme and substrate annotation. *Nucleic Acids Res.* 51, W115–W121.