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**Publication date**
2024

**Document Version**
Final published version

**Published in**
Systematic and Applied Microbiology

**Citation (APA)**
Sorokin, D. Y., Elcheninov, A. G., Kublanov, I. V., Bale, N. J., & Sinninghe Damsté, J. S. (2024). Natronosalvus hydrolyticus sp. nov., a beta-1,3-glucan utilizing natronoarchaeon from hypersaline soda lakes. *Systematic and Applied Microbiology*, *47*(2-3), 1-8. Article 126514.

Systematic and Applied Microbiology 47 (2024) 126514

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.com/locate/syapm

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

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ARTICLE INFO

*Keywords:*
Hypersaline soda lakes
Beta-1
3-glucans
Curdlan
Glycoside hydrolase GH81
*Natronosalvus*

ABSTRACT

Use of curldlan, an insoluble β-1,3-glucan, as an enrichment substrate under aerobic conditions resulted in the selection from hypersaline soda lakes of a single natronarchaeon, strain AArc-curdl1. 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-curdl1 represents a separate species in the recently described genus *Natronosalvus* within the family *Natrialbaceae.* 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-curdl1$^T$ 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 natronarchaeon, strain AArc-curdl1, 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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https://doi.org/10.1016/j.syapm.2024.126514
Received 5 March 2024; Received in revised form 23 April 2024; Accepted 26 April 2024
Available online 2 May 2024

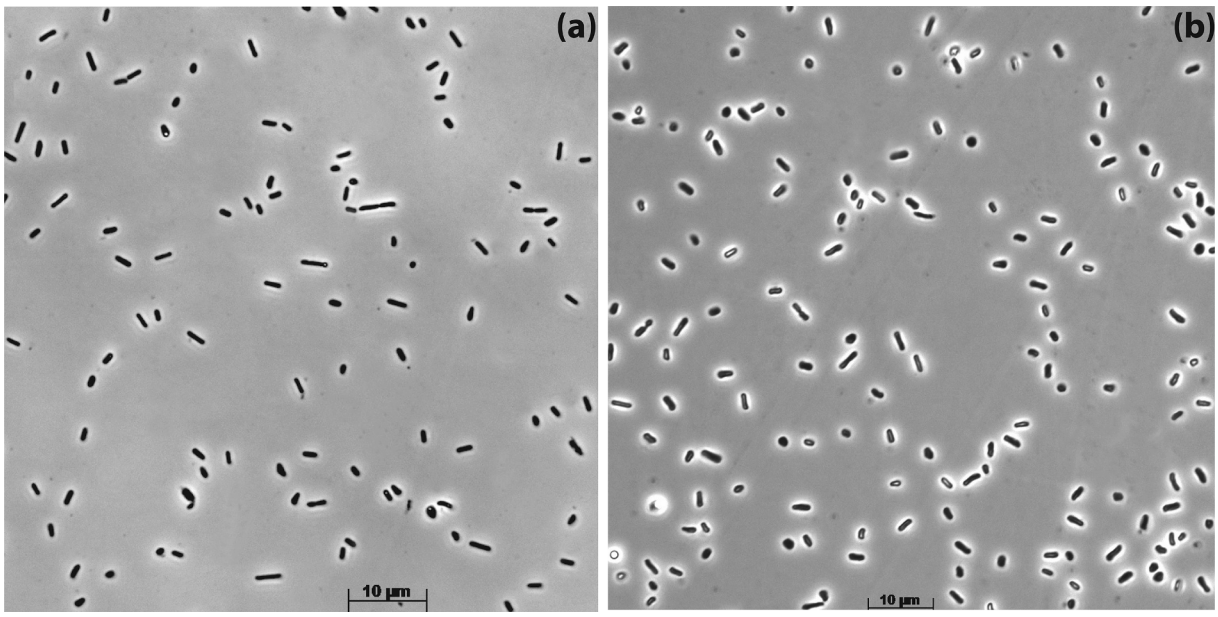


**Fig. 1.** Cell morphology of the beta-1,3-glucan-utilizing natronarchaeon AArc-curdl1 growing at 4 M total $Na^+$, pH 9.5 and 37 °C with either curdlan (a) or maltose (b).

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 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 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 $Na^+$ at pH 9.5 and 1 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 ($OD_{600}$) 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 $Na^+$ 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 $OD_{600}$. 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 $Na^+$ 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-curdl1 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 Ngen = 50000 and 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-curdl1 and in the representatives of *Natronosalvus* genus using dbCAN v.4 (Zheng et al., 2023). Presumable activities were predicted using BLAST search against Swiss-Prot/PDB databases. Gene syntheny was visualized using gggenes package (https://CRAN.R-project.org/package = gggenes).

## 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

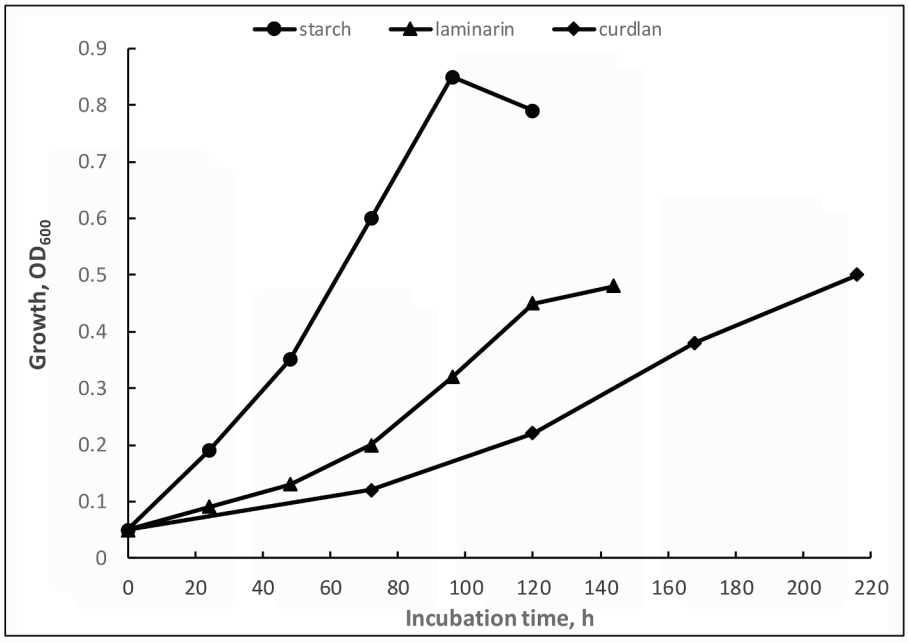


**Fig. 2.** Comparative growth dynamics of strain AArc-curdl1 $^{T}$ at 4 M total $Na^{+}$, pH 9.5 and 37°C with three different polysaccharides. Data shown represent average values of a duplicate experiment.

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 curdlan and one of the lineages resulted in a pure culture designated as strain AArc-curdl1 as demonstrated by 16S-rRNA gene and whole genome sequence analyses.

*Phenotypic properties*

The cells of AArc-curdl1 were mostly nonmotile flat rods with less frequently occurring flat coccoids (Fig. 1). In contrast to its previously described neutrophilic counterpart, *Halapricum hydrolyticum* HArc-curdl5-1 (Sorokin et al., 2023), the cells of natronarchaeal isolate did not aggregate with the curdlan particles and were mostly located in the liquid phase. From the tested range of various polysaccharides, AArc-curdl1 was able to grow with other two beta-1–3-backbone glucans, the insoluble curdlan 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 polysacchirdes is shown on Fig. 2. It is noteworthy that both neutrophilic and soda lake curdlan-utilizing haloarchaeal isolates (*Halapricum hydrolyticum* and AArc-curdl1) are strong amylolytics, 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 *Natronosalvus amylolyticus*, closely related to AArc-curdl1 (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-curdl1 is a typical extreme halophile in its salt profile with an optimal growth (with maltose) at 3.5–4 M total $Na^{+}$ 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

**Table 1**
Composition of intact polar lipids identified in strain AArc-curdl1.The cells were grown with starch at 4 M total $Na^{+}$, pH 9.5 and 37 °C until late exponential growth phase.

| Polar head group | Core | [M + H]$^{+}$ | Assigned elemental composition | Percent |
|---|---|---|---|---|
| **PGP-Me** | EXT-AR | 971.7449 | $C_{52}H_{109}O_{11}P_2$ | **14.1** |
| | Uns(1)-EXT-AR | 969.7286 | $C_{52}H_{107}O_{11}P_2$ | 1.0 |
| | Uns(2)-EXT-AR | 967.7138 | $C_{52}H_{105}O_{11}P_2$ | 0.1 |
| | Uns(3)-EXT-AR | 965.6976 | $C_{52}H_{103}O_{11}P_2$ | 0.9 |
| | Lyso-EXT-AR | 691.3527 | $C_{32}H_{69}O_{11}P_2$ | 0.6 |
| | Lyso-AR | 621.3527 | $C_{27}H_{59}O_{11}P_2$ | 2.2 |
| | AR | 901.6658 | $C_{47}H_{99}O_{11}P_2$ | **32.8** |
| | **Total** | | | **52** |
| **PG** | EXT-AR | 877.7623 | $C_{51}H_{106}O_8P$ | **13.4** |
| | Uns(1)-EXT-AR | 875.7461 | $C_{51}H_{104}O_8P$ | 1.6 |
| | AR | 807.6837 | $C_{46}H_{96}O_8P$ | **31.9** |
| | Lyso-AR | 527.3704 | $C_{26}H_{56}O_8P$ | 1.3 |
| | **Total** | | | **48** |
| **PGP** | AR | | | **0.1** |
| | **Sum AR** | | | **65** |
| | **Sum EXT-AR** | | | **27** |
| | **Sum *uns*-EXT-AR** | | | **4** |
| | **Sum lyso-AR** | | | **4** |
| | **Sum lyso-EXT-AR** | | | **1** |

PGP-Me = phosphatidylglycerolphosphate methyl ester; PG = phosphatidylglycerol; PGP=.
Phosphatidylglycerolphosphate. AR = archaeol ($C_{20}$-$C_{20}$); EXT-AR = extended archaeol.
($C_{20}$-$C_{25}$); lyso = one alkyl chain is absent; uns = unsaturated.

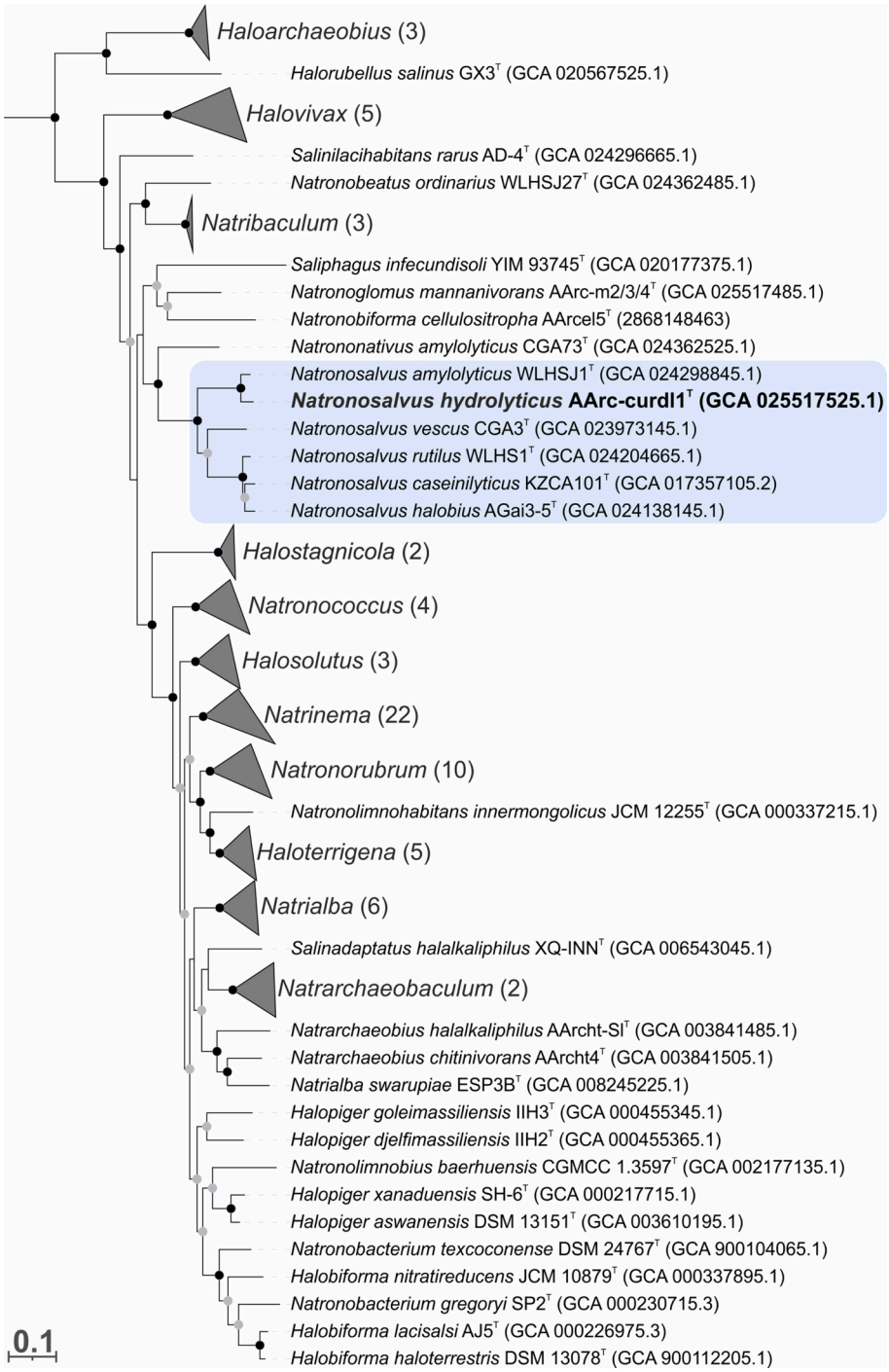


**Fig. 3.** Maximum likelihood phylogenetic tree based on concatenated alignment of 122 conserved archaeal proteins, showing the position of strain AArc-curdl1 (in bold) within the family *Natrialbaceae*. The branch lengths correspond to the number of substitutions per site with corrections associated 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 $^{T}$, *Methanocella paludicola* SANAE$^{T}$ and *Methanothermobacter thermautotrophicus* Delta H$^{T}$ were used as an outgroup (not shown).

35). The Mg requirement is low, with a maximal growth rate already at 1 mM. The minimal $Cl^-$ concentration for growth (at 4 M total $Na^+$ 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-curdl1 culture grown with maltose at pH 9.5 was tolerant to streptomycin, ampicillin, kanamycin and vancomycin up to 200 mg $l^{-1}$ and to tetracycline up to 100 mg $l^{-1}$. The growth was completely suppressed by rifampicin and chloramphenicol at concentrations above 50 mg $l^{-1}$.

The core of the membrane phospholipids in AArc-curdl1 consisted of four archaeol modifications: $C_{20}$-$C_{20}$ archaeol and $C_{20}$-$C_{25}$ 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)

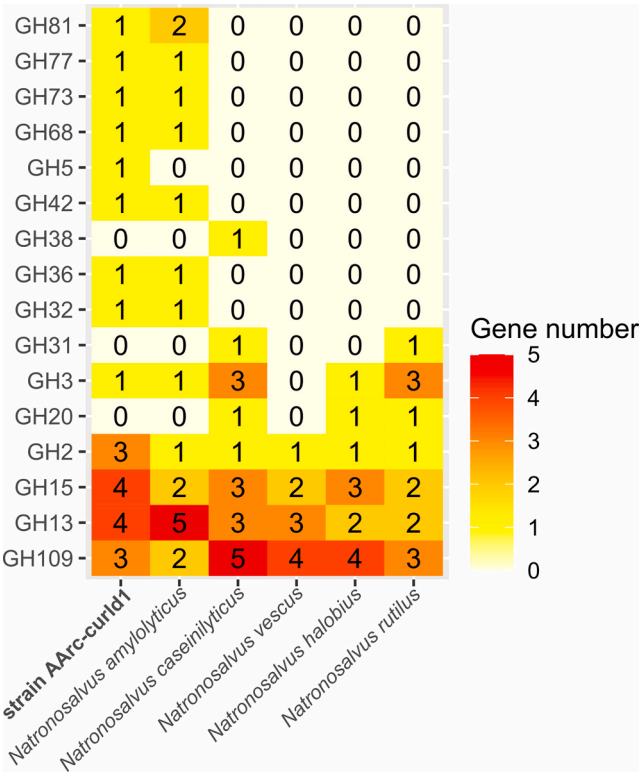


**Fig. 4.** Glycosidases repertoires of strain AArc-curld1 and other representatives of *Natronosalvus* genus.

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-curdl1 has a single *rrn* operon with the 16S rRNA gene closely related (99.5 % sequence identity) to a recently described species *Natronosalvus 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-curdl1 as a member of the genus *Natronosalvus*. Moreover, the Bayesian phylogenomic tree possessed almost same topology and also showed belonging of novel isolate to *Natronosalvus* (Supplementary **Fig. S1**). Comparison of the genomes showed that the novel isolate differs from *Nsv. amylolyticus* and other *Natronosalvus* species on the level of a separate species (Supplementary **Table S1**). The ANI, AAI and DDH values between AArc-curdl1 and *Natronosalvus* 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 *Natronosalvus*, both metagenome-assembled genomes (MAGs) and 16S rRNA gene sequences were analyzed. Besides five described species of *Natronosalvus* 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-curdl1. Only five clones/undescribed strains relatively close to AArc-curdl1 (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 *Natronosalvus* seems to be limited to hypersaline locations in Central Asia.

### *Functional genome analysis*

All representatives of *Natronosalvus* 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-curdl1 encodes a secreted beta-1,3-endoglucanase of GH81 gamily, but, in contrast to the homologous protein in the neutrophilic curdlan-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-curdl1 genome a bicistronic operon is present encoding two putative translocated *integrin* proteins related to CBM/Fibronectin III/PKD involved in cell adhesion (Fig. 5**A**). They might substitute to the absence of the CBM domain in the AArc-curdl 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

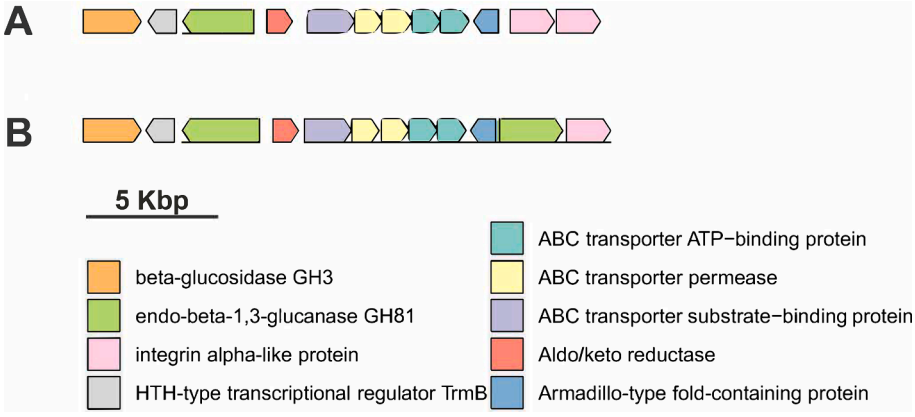


**Fig. 5.** Clusters containing gene of *endo*-beta-1,3-glucanase from GH81 family in genome of strain AArc-curdl1 (A) and *Natronosalvus amylolyticus* (**B**).

**Table 2**
Glycoside hydrolase family proteins encoded in the genome of strain AArc-curdl1 (functional domains are in parentheses).

| Locus tag MCU475 + | HMMER | Signal peptide | Putative function |
|---|---|---|---|
| ***endo*-beta-1,3/1,4-glucanase** | | | |
| 1269 | **GH81**(61–708)* | Tat/SPI | ***endo*-1,3-beta-glucanase** |
| 1304 | GH5_13(34–294) | – | unknown |
| **alpha-glucanases** | | | |
| 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 |
| **beta-fructosidases** | | | |
| 2688 | GH32(296–592) | – | endo/*exo*-levanase |
| 0559 | GH68(30–407) | – | levan/inulo-sucrase |
| **other glycosidases** | | | |
| 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-curld1 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-curdl1 is also following the second GH81 gene copy in *Nsv. amylolyticus*, while the second integrin of AArc-curdl1 is absent (Fig. 5**B**).

A second important functional group of glycosidases encoded in the AArc-curdl1 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-curdl1 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-curdl1 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-curdl1 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 β-1,3-glucan-utilizing strain AArc-curdl1 with the related species from the genus Natronosalvus (Tan et al., 2023).

| Property | AArc-curdl1$^T$ | ***Natronosalvus amylolyticus*** WLHSJ1$^T$ | ***Natronosalvus halobius*** AGai3-5 $^T$ |
|---|---|---|---|
| Cell morphology | nonmotile flat rods | motile, pleomorphic | motile, pleomorphic |
| Pigmentation | pink | pink to red | pink to red |
| PHA accumulation | + (on acetate) | + - (on pyruvate) * | nd |
| Anaerobic growth | – | – | + (denitrification) |
| Denitrification genes | - | - | -** |
| Nitrate reductase *narGHJ* | + - | - - | + -** |
| Cu-nitrite reductase *nirK* | + | - | -** |
| NO-reductase *qNor* | - | - | - |
| $N_2O$ reductase cluster | - | - | - |
| Substrates for aerobic growth: polysaccharides | starch, glycogen, pullulan, laminarin, curdlan, pachyman, inulin | starch*, pullulan*, glycogen*, laminarin*, curdlan*, pachyman*,inulin (w) * | - |
| sugars/sugar alcohols | trehalose, raffinose, sucrose, maltose, cellobiose | galactose, sucrose; mannose(w*), trehalose*, maltose*, cellobiose*, melezitose*, raffinose (w)* | mannose, galactose, ribose, lactose, mannitol, glycerol |
| organic acids | acetate, pyruvate | pyruvate | pyruvate, lactate, malate, fumarate - |
| beta-1.3-glucanase GH81 genes | 1 | 2 | – |
| Amylase | +-/- | +- | (Tweens 80) - casein |
| Esterase/lipase | (tributyrin/ | (Tween 80)+ | |
| Protease | olive oil)- | (casein) | |
| | (casein; gelatin) | | |
| Catalase/oxidase | +/+ | +/w+ | +/- |
| Indole from tryptophane | + | – | + |
| Salinity range (opt.) M $Na^+$ | 2.5–4.4 (3.5–4.0) | 0.9–4.8 (3.5) | 0.9–4.8 (3.9) |
| Mg requirement | low (1 mM) | low (1 mM)* | nd |
| (optimum)pH range (opt.) | 8.0–10.0 (9.0–9.3) | 6.0–9.5 (8.5) | 7.0–9.0 (8.5) |
| Temperature max (°C) | 45 (at pH 9.0) | 55 | 50 |
| Core lipids (archaeols) | $C_{20}$-$C_{20}$, $C_{20}$-$C_{25}$ DGE | $C_{20}$-$C_{20}$, $C_{20}$-$C_{25}$ DGE | $C_{20}$-$C_{20}$, $C_{20}$-$C_{25}$ DGE |
| Intact membrane polar lipids: phospholipids | PG, PGP-Me | PG, PGP-Me, PA DGD-1, S-DGD-1, S-TGD-1 | PG, PGP-Me, PA DGD-1, S-DGD-1 |
| glycolipids | - | | |
| Respiratory lipoquinones | MK-8:8 (major) MK-8:7 (minor)MK-7:7 (traces) | nd | nd |
| DNA G + C (%, | 59.2 | 59.5 | 63.1 |
| chromosome) | 4.54 | 4.22 | 3.85 |
| Genome size (Mbp) | 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 glycosyl diether, (DGE) dialkyl glycerol ether. (MK) menaquinone.

in the AArc-curdl1 GH81, although it contradicts the abovementioned lack of visible cell attachment to the glucan particles. Phylogenetic analysis of the GH81 protein from AArc-curdl1 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-curdl1 is also following the second GH81 gene copy in *Nsv. amylolyticus*, while the second integrin of AArc-curdl1 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-curdl1 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 $N_2O$ reductase but lacking nitrate reductase Nar and NO-reductase qNor. This explains the negative anaerobic growth test for AArc-curdl1 with nitrate. However, the strain also did not grow in presence of 5 mM nitrite or 10 % $N_2O$ in the gas phase, despite the presence of necessary genetic potential. Interestingly, the opposite situation occurs with the type species of the genus *Natronosalvus* – *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 *Natronosalvus* species are shown in Table 3**.** AArc-curdl1 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 *Natronosalvus*) 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-curdl1 (**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-curdl1 from its closest related *Nsv. amylolyticus* is the obligate alkalophily and absence of proteolytic activity and glycolipids.

### *Taxonomic conclusion*

Considering the distinct phylogeny and phenotypic properties, the beta-1,3-glucan-utilizing strain AArc-curdl1 isolated from hypersaline soda lakes is suggested to be classified as a new species *Natronosalvus hydrolyticum* sp. nov. The species protologue is presented in Table 4.

**Table 4**
*Natronosalvus hydrolyticus:* protologue.

| Parameter | Species: *Natronosalvus hydrolyticus* sp. nov. |
|---|---|
| Species name | *hydrolyticus* |
| Genus name | *Natronosalvus* |
| Species status | sp. nov. |
| Etymology | hyd.ro.ly'ti.cus Gr. neut. n. *hydor*, water; Gr. masc. adj. *lytikos*, dissolving, splitting; N.L. masc. adj. *hydrolyticus*, 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 $C_{20}$-$C_{20}$ DGE (archaeol) and $C_{20}$-$C_{25}$ 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 $C_2$-$C_6$ 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 $Na^+$ 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-curdl1$^T$ = 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 | *Natronosalvus hydrolyticus* 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-curdl1 |
| 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 |
| Longtitude | 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 $Na^+$, 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 $Na^+$, pH 9.5) |
| Colony morphology | pink, flat, smooth, up to 2 mm |

(*continued on next page*)

**Table 4** (*continued*)

| Parameter | Species: *Natronosalvus hydrolyticus* sp. nov. |
|---|---|
| Optimal temperature for growth | 35–40 |
| Salinity category | Extremely halophilic |
| Relation to oxygen | Obligate aerobe |
| $O_2$ 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.

## Acknowledgements

We thank Michel Koenen, Monique Verweij and Denise Dorhout (NIOZ) for analytical support.

*Funding information*

The Russian authors were supported by the Russian Ministry of Higher Education and Science. DS and JSSD were also supported by the Gravitation-SIAM Program of the Dutch Ministry of Education and Sciences (grant 24002002).

## Appendix A. Supplementary material

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

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**Table S1.** Whole genome comparisons of strain AArc-curdl1 and representatives of *Natronosalvus*

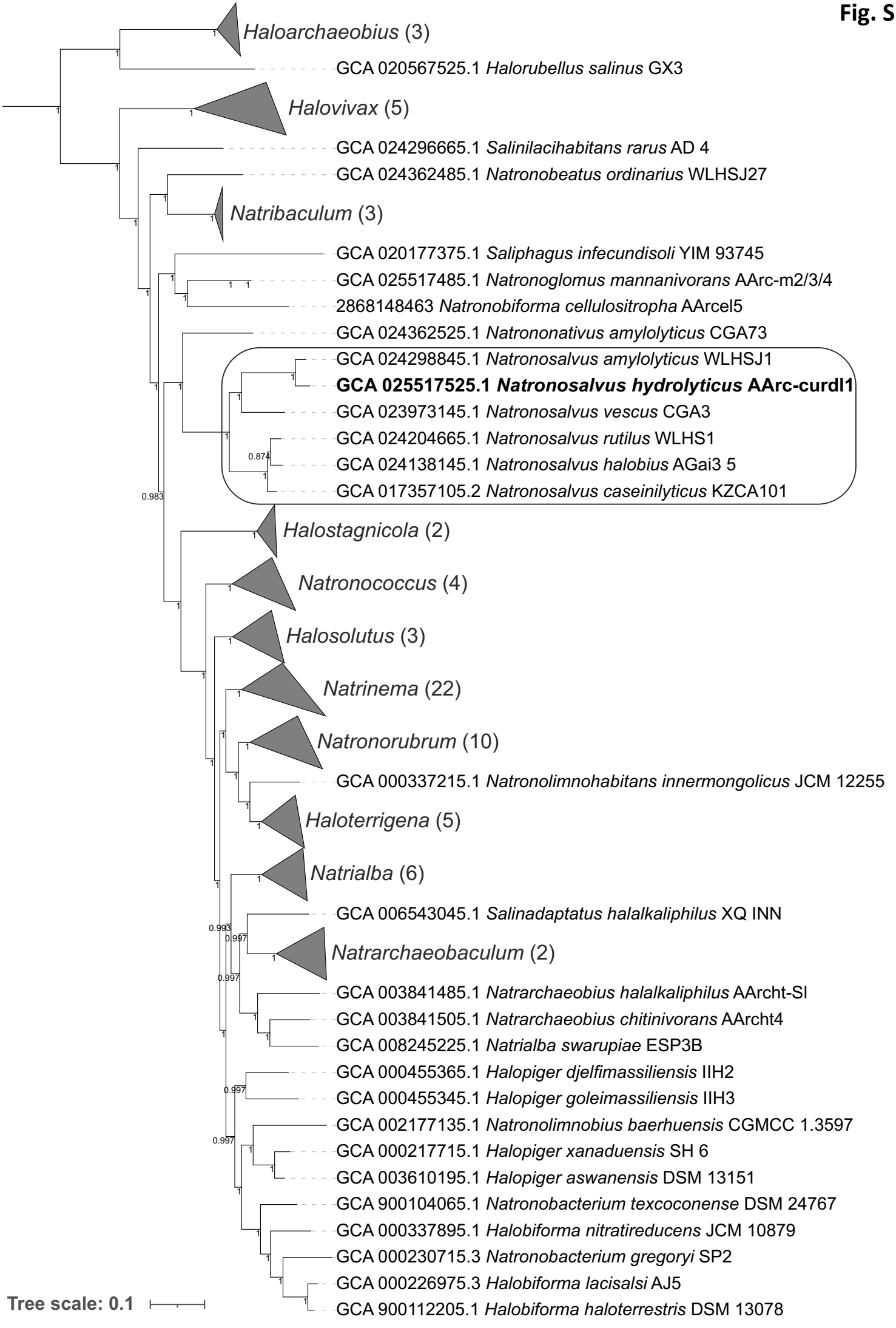
| **strain AArc-curld1 vs** | **ANI** (%) | **AAI** (%) | **dDDH** (%) |
|---|---|---|---|
| GCA_024298845.1_*Natronosalvus_amylolyticus*_WLHSJ1 | 90.24 | 91.57 | 42.0 |
| GCA_023973145.1_*Natronosalvus_vescus*_CGA3 | 78.34 | 74.26 | 24.2 |
| GCA_017357105.2_*Natronosalvus_caseinilyticus*_KZCA101 | 77.47 | 71.29 | 22.8 |
| GCA_024204665.1_*Natronosalvus_rutilus*_WLHS1 | 77.44 | 71.12 | 22.6 |
| GCA_024138145.1_*Natronosalvus_halobius*_AGai3-5 | 77.33 | 71.39 | 22.5 |

**Table S2**. Functional redox and pH-salt homeostasis proteins encoded in the genome of strain AArc-curdl1

| Locus tag MCU475 + | Gene | Protein | Function |
|---|---|---|---|
| ***Anaerobic respiration**** | | | |
| 3226-3227 | *hcy/hcp-nirK* | Halocyanin/Cu-Nir | Dissimilatory nitrite reductase |
| 3640-3644 | *nosL1Y1FDL2* | Copper incorporation chaperons | Nitrous oxide reductase maturation |
| 3645 | *nosZ* | Nitrous oxide reductase | Dissimilatory $N_2O$ reductase |
| 2516-2519 | *dmsABCD* | Mo-oxidoreductase (translocated) | Putative DMSO |
| ***Aerobic respiration*** | | | |
| 0455-0465 | *nuoABCDHIJ1J2KLMN* | NADH-quinone oxidoreductase | Respiratory complex I |
| 0780-0782 | *petABC* | Cytochrome $bc_1$ | Respiratory complex II |
| 1288-1291 | *cbaABCD* | Oxidase | Quinol oxidase $ba_3$ (1) |
| 2438-2444 | *cbaABCDE* | Oxidase | Quinol oxidase $ba_3$ (2) |
| 2852-2853/2860 | cyoA/BC/D | Oxidase | Quinol oxidase $bo_3$ |
| 3212-3215/3222 | *ctaBCD/cbaD* | Oxidase | Heme-copper quinol oxidase $aa_3$ |
| ***Oxidative stress response*** | | | |
| 1312 | *catG* | Catalase-peroxidase HPI | Bifunctional catalase/peroxidase |
| 4004 | *catE* | Haem-containing catalase | Monofunctional catalase |
| 2091 | *ahpD* | Alkyl-peroxidase | Peroxidase |
| 4432 | *dyp* | DyP-type peroxidase | Broad specificity extracellular peroxidase |
| 1051<br>2139<br>2716 | *bcp*<br>*bcp*<br>*ahpC* | Thiol-dependent peroxiredoxins | Antioxidant activities |
| 2083 | *sodA* | Mn-superoxide reductase | Oxidative stress response |
| ***pH-salt homeastasis*** | | | |
| 4164-4172 | *mrpB1B2CD1D2D3EFG* | Multisubunite $Na^+/H^+$ antiporter | Alkaline pH homeostasis |
| 1883-1885 | *mrpD1D2D3* | Fragment of Mrp | |
| 3280 | *nhaC* | $Na^+/H^+$ antiporter | |
| 2783-2784 | *trkA/CPA1* | $K^+/Na^+/H^+$ antiporters | Potassium trafficking |
| 2807-2808 | 2 x *CPA1* | | |
| 4183<br>4204 | *CPA2* | | |
| 1559<br>4007<br>4008<br>4523 | *trkA* | Potassium transporter monosubunit | |
| 4199-4201 | *trkAH1H2* | $K^+/H^+$ symporter | |
| 1700 | *kch* | Potassium voltage-gate channel | |
| 0728; 0736 | CaCA | $Na^+/Ca^{2+}$ antiporters | Ca uptake |

*Nitrate reductases (Nar/Nap) and archaeal NO-reductase (qNor) are not encoded

**Fig. S1**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Natribaculum (3)
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2868148463 Natronobiforma cellulositropha AArcel5
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GCA 023973145.1 Natronosalvus vescus CGA3
GCA 024204665.1 Natronosalvus rutilus WLHS1
GCA 024138145.1 Natronosalvus halobius AGai3 5
GCA 017357105.2 Natronosalvus caseinilyticus KZCA101
Halostagnicola (2)
Natronococcus (4)
Halosolutus (3)
Natrinema (22)
Natronorubrum (10)
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Haloterrigena (5)
Natrialba (6)
GCA 006543045.1 Salinadaptatus halalkaliphilus XQ INN
Natrarchaeobaculum (2)
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GCA 003841505.1 Natrarchaeobius chitinivorans AArcht4
GCA 008245225.1 Natrialba swarupiae ESP3B
GCA 000455365.1 Halopiger djelfimassiliensis IIH2
GCA 000455345.1 Halopiger goleimassiliensis IIH3
GCA 002177135.1 Natronolimnobius baerhuensis CGMCC 1.3597
GCA 000217715.1 Halopiger xanaduensis SH 6
GCA 003610195.1 Halopiger aswanensis DSM 13151
GCA 900104065.1 Natronobacterium texcoconense DSM 24767
GCA 000337895.1 Halobiforma nitratireducens JCM 10879
GCA 000230715.3 Natronobacterium gregoryi SP2
GCA 000226975.3 Halobiforma lacisalsi AJ5
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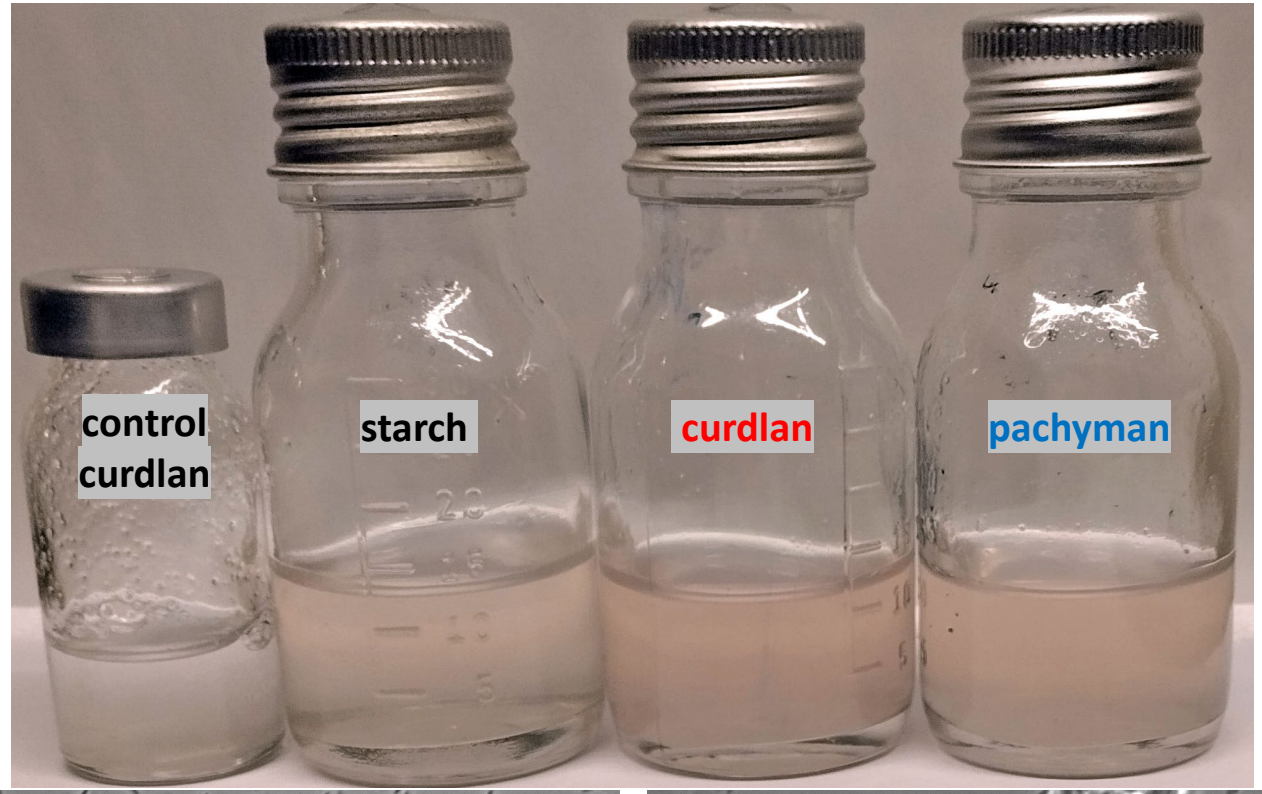
Fig. S2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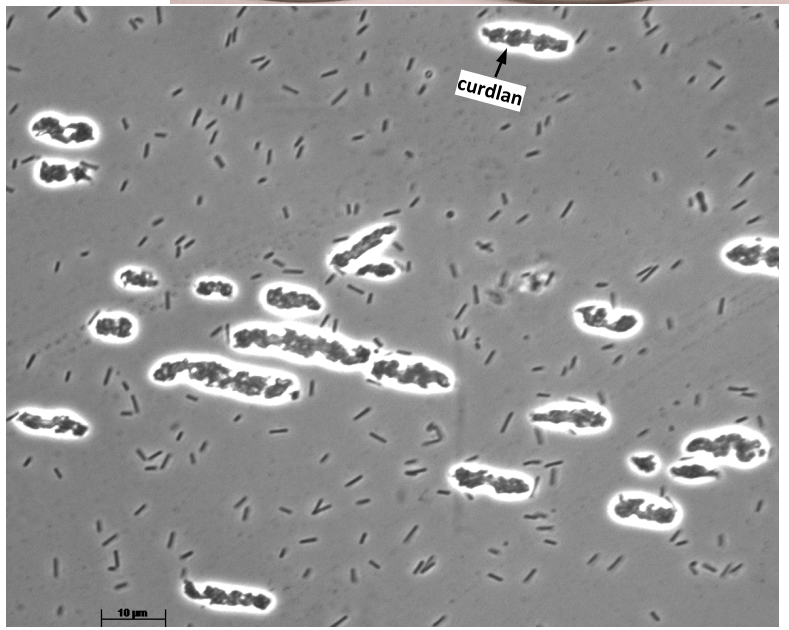
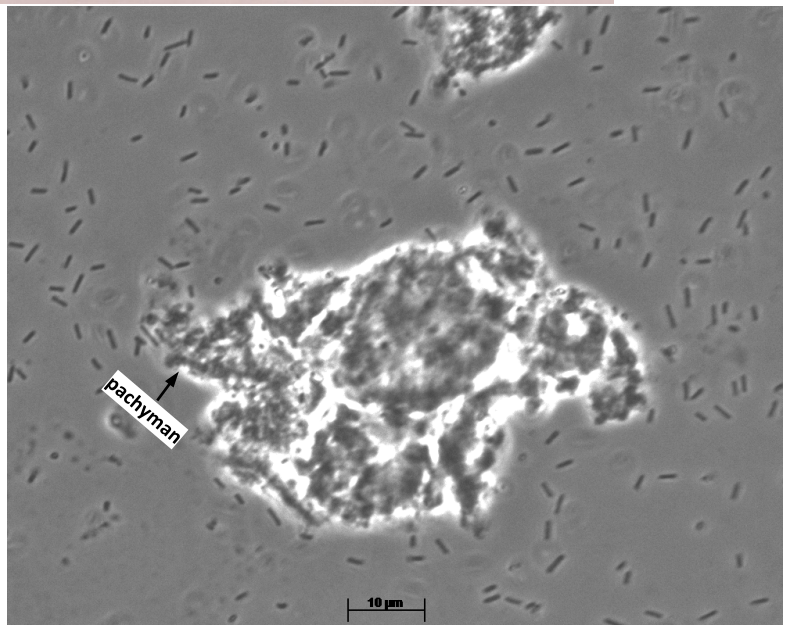
curdlan
starch
curdlan
pachyman


curdlan
10 µm


pachyman
10 µm