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Natrarchaeobius chitinivorans gen. nov., sp. nov., and *Natrarchaeobius halalkaliphilus* sp. nov., alkaliphilic, chitin-utilizing haloarchaea from hypersaline alkaline lakes☆

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ABSTRACT

Two groups of alkaliphilic haloarchaea from hypersaline alkaline lakes in Central Asia, Egypt and North America were enriched and isolated in pure culture using chitin as growth substrate. These cultures, termed AArcht, were divided into two groups: group 1 which includes eleven isolates from highly alkaline soda lakes and group 2 which contains a single isolate obtained from the alkaline hypersaline Searles Lake. The colonies of chitin-utilizing natronoarchaea were red-pigmented and surrounded by large zones of chitin hydrolysis. The free cells of both groups were mostly flat nonmotile rods, while the cells that attached to chitin or formed colonies on chitin plates were mostly coccoid. The isolates are obligate aerobic saccharolytic archaea utilizing chitin and chitosane (less actively) as the only sugar polymers as well as a few hexoses as their carbon and energy source. Both groups are extremely halophilic, growing optimally at 3.5–4 M total Na^+, but they differ in their pH profiles: the main group 1 isolates are obligately alkaliphilic, while the single group 2 strain (AArcht-SI^T) is alkalitolerant. The core archaeal lipids in both groups are dominated by C_{20}–C_{20} and C_{20}–C_{25} dialkyl glycerol ethers (DGE) in approximately equal proportion. Phylogenetic analysis indicated that the isolates form an independent genus-level lineage within the family *Natrialbaceae* with 3 species-level subgroups. The available genomes of the closest cultured relatives of the AArcht strains, belonging to the genera *Natrialba* and *Halopiger*, do not encode any chitinase-related genes. On the basis of their unique phenotypic properties and distinct phylogeny, we suggest that the obligate alkaliphilic AArcht isolates (group 1) with an identical phenotype are classified into a new genus and species *Natrarchaeobius chitinivorans* gen. nov., sp. nov., with strain AArcht4^T as the type strain (JCM 32476^T = UNIQEM U966^T), while the facultatively alkaliphilic strain AArcht-SI^T (group 2) — as a new species *Natrarchaeobius halalkaliphilus* sp. nov. (JCM 32477^T = UNIQEM U969^T).

Introduction

Extremely halophilic euryarchaea form a dominant group within the prokaryotic microbial communities in various types of salt-saturated terrestrial brines, such as athalassic lakes and sea solar salterns. In contrast to other classes of Euryarchaeota, they are mostly aerobic heterotrophs, which utilize soluble organic substrates, such as sugars, organic acids and complex rich amino acid-containing substrates such as peptons, and yeast extract [2,4,12,26–27,29]. Until recently the polymer-degrading potential of cultivated haloarchaeal species was limited to a few examples, such as starch, proteins and olive oil [1,3,10,25,34]. As for the recalcitrant insoluble polysaccharides, such as cellulose or chitin, almost nothing has been described to date in the literature. However, due to the increased availability of multiple genome sequencing data over the past few years, it has become apparent that some of the haloarchaea belonging to the genera *Haloarcula*, *Halobacterium*, *Halalkalicoccus*, *Haloferax*, *Halorhabdus*, *Halovivax*,

Abbreviations: DGE, dialkyl glycerol ether; MGE, monoalkyl glycerol ether; PG, phosphatidyl glycerol; PGP-Me, phosphatidylglycerophosphate methylester; PE, phosphatidylethanolamine; PGP, phosphatidylglycerophosphate.

☆ The whole genome shotgun projects of strains AArcht4^T, AArcht7 and AArcht-SI^T have been deposited at DDBJ/ENA/GenBank under the accessions SAMN10160502, SAMN10160503 and SAMN10160504, respectively.

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Halostagnicola, *Haloterrigena-Natrinema* and *Natronococcus* may possess the potential to hydrolyze sugar polymers, including cellulose and hemicelluloses (GH family 3, 5 and 9). This inference was recently validated by phenotypic studies of some of the afore mentioned genera [20–22,36]. Indeed, the chitinase genes (GH family 18) are present in the genomes of the genera *Halobacterium*, *Halomicrobium*, *Natrinema*, *Haloferax* and *Salinarchaeum.* Furthermore, recent physiological studies of pure cultures belonging to these genera have confirmed their ability to use chitin as growth substrate [37,14–15,8,9,24], substantially changing previous concepts of the ecological role of these extremophilic archaea.

In our recent work we were able to enrich and isolate in pure culture for the first time a number of alkaliphilic haloarchaea (i.e. natronoarchaea) from hypersaline alkaline lakes which utilize chitin as their growth substrate [37]. These included two phylogenetic groups: a dominant group with multiple isolates from soda lakes and a single strain from a less alkaline Searles Lake. In this paper we describe the phenotypic and phylogenetic properties of these two groups of chitin-utilizing natronoarchaea and propose to classify them into two species within a new genus *Natrarchaeobius*.

Material and methods

Samples

To enrich for chitin-utilizing natronoarchaea, surface sediments and near-bottom brines from the following alkaline hypersaline inland lakes were used: soda lakes in Kulunda Steppe (Altai region, Russia, 2011–2012); soda lakes in north-eastern Mongolia (1999) and Inner Mongolia (2013); soda Owens Lake in California (2008); alkaline lakes in Wadi al Natrun (Egypt, 2000) and alkaline Searles Lake in California (2005). The chemical parameters of the brines and their location coordinates are given in a previous publication [37]. Before use, the sediment slurries were homogenized by vortexing and the coarse sediment fraction was removed by a low speed centrifugation, while the remaining colloidal fraction was used as inoculum (2% v/v). The brines were used directly (10% v/v).

Cultivation and phenotypic tests

The composition of the basic mineral medium and the details of enrichment and cultivation of chitin-utilizing natronoarchaea are described in a previous publication [37]. Briefly, two basic media, one containing 4 M NaCl at pH 7 and another one containing 4 M Na as carbonates at pH 10 were mixed 1:1, resulting in a Cl/carbonate basic mineral medium with a pH 9.5. Mixing these two bases in different proportions also allowed for the investigation of the growth pH range in the range 8–9.5. For the lower pH (6–8) the NaCl base medium buffering capacity was increased by adding 4 g l^{-1} of HEPES (higher concentrations inhibited growth) and, instead of the alkaline base, the pH was adjusted by adding 1 M filter-sterilized sodium bicarbonate with pH 8. For the pH above 9.5, 1 part of the NaCl base was mixed with three parts of the soda base and was further titrated using 4 M NaOH to reach the pH values up to 11. It must be stressed that during growth on carbohydrates, despite the high buffering capacity of the carbonate base medium, the pH shifted significantly at the extremes (below 8.5 and above 10). We note therefore that it is essential, under such conditions, to monitor the actual pH while testing the influence of pH on growth. For the salt profiling, two media, containing an equal Na molar ratio of NaCl and sodium carbonate at pH 9 were mixed in different proportions to create a range of salinities from 1 to 5 M with steps of 0.5 M Na. The temperature profile was tested within the range 20–60 °C (at 5 °C increments). This profile was carried out at pH 8.5 (to avoid cell lysis at an extreme alkaline range in combination with high temperature), with 4 M total Na^+ while *N*-acetylglucosamine was used as the substrate. The details of the preparation of the solid medium with amorphous chitin are described in our previous publication [37]. For anaerobic growth, 10 ml portions of liquid medium at pH 9.1–9.5 (4 M total Na^+ and 10 mM *N*-acetylglucosamine as carbon and N-source) were dispensed into 23 ml sterile serum bottles which were closed with butyl rubber stoppers and made anoxic through three cycles of evacuation/flushing with sterile argon. The utilization of inorganic N-sources was tested using sucrose as a substrate. Carbon substrate profiling was done for strains AArcht4^T, AArcht7 (at pH 9.5) and AArcht-Sl^T (at pH 9) at 4 M total Na^+ using ammonium as the N-source. Proteolytic and lipolytic activities were tested on solid medium with casein (clearance after flooding with 10% TCA) and emulgated olive oil (direct clearance), respectively. Catalase and oxidase activity were detected by colony assay using 3% H_2O_2 and 0.1% *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) hydrochloride, respectively.

Analyses

The phase contrast and epifluorescence microscopy and photography were performed using the Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany). Cells absorbed on chitin were visualized using live-dead staining with SYTO9 (Invitrogen kit L7012). For the electron microscopy of thin sections, the cells of strains AArcht4^T and AArcht-Sl^T grown with amorphous chitin were fixed in 1% (w/v) OsO_4 containing 3 M NaCl for 1 week at 4 °C, washed and resuspended in 3 M NaCl, stained overnight with 1% (w/v) uranyl acetate, dehydrated in ethanol series and embedded in Epon resin. After thin sectioning, the preparations were post-stained with 1% (w/v) lead citrate and examined using the JEOL-100 TEM (Japan).

The core membrane lipids were obtained by acid hydrolysis (5% HCl in methanol by reflux for 3 h) of the freeze-dried cells and subsequent analysis by HPLC-MS for membrane-spanning lipids and archaeol derivatives according to Ref. [40]. Intact polar lipids were obtained by Bligh Dyer extraction of freeze-dried cells and subsequent HPLC-MS analysis as described in Ref. [35].

Respiratory quinones were recovered from wet biomass by three consecutive extractions with cold acetone for 1 h on a magnetic stirrer. The cumulative extract was concentrated by evaporation and the quinone fraction was separated from carotenoids by TLC (Sorbofil, Russia) in hexane-diethyl ether (85:15). The obtained quinone band (Rf = 0.52) was recovered by extraction with CCl_4–CH_3OH (1:1) and subjected to mass-spectroscopy with chemical ionization at atmospheric pressure using quadrupol massspectrometer Finnigan LCQ Advantage MAX (Germany) [7].

Phylogenetic analysis

For strains AArcht4^T, AArcht7 and AArcht-Sl^T the 16S rRNA and *rpoB*′ gene nucleotide sequences were obtained from the draft genome assemblies, while for the rest of the AArcht strains partial 16S rRNA gene sequences were previously available [37]. The phylogenetic analysis was performed in Mega 7 package [18]. The 16S rRNA gene sequences of all type strains of the *Natrialbaceae* family and *Halomarina oriensis* JCM 16495 (as an outgroup) obtained from the Genbank were aligned together with the sequences of AArcht strains using G-INS-i method in MAFFT server v. 7 [17]. The phylogenetic analysis was performed using Maximum Likelihood method and the General Time Reversible (GTR) model (*G* + *I*, 4 categories) [31]. For the *rpoB*′-based phylogenetic analysis, the full-length nucleotide sequences of all type strains from the *Natrialbaceae* and *Halomarina oriensis* JCM 16495 (as outgroup) were obtained from the GenBank and IMG and aligned using the

Table 1
Natronarchaeal strains isolated from brines and surface sediments of hypersaline alkaline lakes with chitin as substrate.

Strain	Isolated from:		Phylogenetic group	Culture collections numbers
	Lake	Area		
AArcht1	Soda crystallizer (2012)	Kulunda Steppe	Group 1	UNIQEM U967
AArcht5	Tanatar-1	Altai, Russia		UNIQEM U968
AArcht6	Soda crystallizer (2003)			
AArcht7				
AArcht8	Bitter-3			
AArcht-St	Stamp Lake			
AArcht3	Mixed brine-sediments from 6 lakes	Wadi Natrun		UNEQEM U966
AArcht4^T		Egypt		JCM 32476
AArcht-Mg	Shar-Burdiin	*N–E* Mongolia		
AArcht-Ow	Owens Lake	California, USA		
AArcht-Bj	Badain Jaran	Inner Mongolia		
AArcht-Sl^T	Searles Lake	California, USA	Group 2	UNIQEM U969 JCM 32477

Bold text means the type strains of type species.

G-INS-i method in MAFFT server v. 7. A phylogenetic tree was constructed using Maximum Likelihood method with GTR model (G + *I*, 4 categories). For the conserved proteins phylogeny, amino acid sequences of 33 single-copy proteins derived from the respected genes present in 49 genomes of *Natrialbaceae* species (Supplementary Table S1), including AArcht4^T, AArcht7 and AArcht-Sl^T and *Natronomonas pharaonis* DSM 2160 as an outgroup, were obtained from IMG [6]. The 33 sets of protein sequences were aligned in MAFFT v. 7 using L-INS-i algorithm, the alignments were concatenated using FaBox joiner alignment [39] and the phylogenetic tree was constructed using Maximum Likelihood method and the LG model (G + *I*, 4 categories) [20].

Pairwise ANI comparison was performed using pyani module v0.2.7 [32] with MUMmer [19] and BLASTn+ [5] as alignment methods. The DDH values between the three genome-sequenced AArcht strains and the type strains of *Natrialbaceae* were calculated using the Genome-to-Genome Distance Calculator 2.1 (GGDC) [23] with the BLAST+ as a local alignment tool.

Results and discussion

Isolation, morphology and chemotaxonomy

Overall, eleven strains of natronoarchaea capable of using chitin (both amorphous and crystalline, originated either from crab or shrimp shells) as the growth substrate were purified from enrichment cultures inoculated with brines and surface sediments from hypersaline soda lakes (AArcht strains group 1). In addition, a single strain AArcht-Sl^T (group 2) was isolated from a less alkaline hypersaline Searles Lake (Table 1). All AArcht isolates formed red-orange pigmented colonies with a large clearance zones of amorphous chitin around them, allowing for their recognition amongst multiple non-chitinolytic natronarchaeal satellites (Fig. 1a, b). Growth in liquid culture with chitin had two phases: the initial phase was characterized by an absorption of the cells on chitin, resulting in the aggregation of amorphous chitin particles in larger conglomerates or the formation of biofilms on crystalline chitin particles. During this phase (as well as in the colonies on plates with amorphous chitin), the cells were in the coccoid form (Fig. 1e, f). In the second stage of massive chitin hydrolysis, free cells started to accumulate in the culture broth and were mostly in the form of nonmotile flat rods (Fig. 1c, d). The same type of "dimorphism" has recently been observed in another group of hydrolytic natronoarchaea from soda lakes, *Natronobiforma cellulosivorans*, which utilize insoluble celluloses as a substrate [36]. Thin section electron microscopy of strains AArcht4^T and AArcht-Sl^T revealed the presence of a thin monolayer cell wall, typical for most of the haloarchaeal species, and a large nucleoid. Many of the cells examined contained electron-transparent inclusion bodies, who, although their nature was not investigated further, are speculated to be of either polyhydroxyalkanoate or glycogen origin (Fig. 1g, h). The rod-phase cells lyzed after resuspension in solutions containing less than 1.5 M NaCl, while the cells in coccoid stage were more resistant to hypoosmosis and only started to lyze in distilled water.

The core membrane lipids in strains AArcht4^T and AArcht-Sl^T were represented by two dominant components commonly found in haloarchaea: archaeol [C_{20}–C_{20} dialkyl glycerol ether (DGE)] and extended archaeol (C_{20}–C_{25} DGE), approximately at equal proportions. In addition, unsaturated forms of both C_{20}–C_{20} DGE and C_{20}–C_{25} DGE were detected as minor components. The C_{25}–C_{20} DGE species was not detected. The intact polar lipids in both strains were dominated by phosphatidylglycerophosphate methylester (PGP-Me) and phosphatidylglycerol (PG), which are both common in haloarchaeal species, including members of the family *Natrialbaceae*. Minor amounts of phosphatidylethanolamine (PE) were detected in AArcht4^T, while minor amounts of phosphatidylglycerophosphate (PGP) were detected in both AArcht4^T and AArcht-Sl^T. The glyco- and sulfo-lipids were not detected (Suppl. Fig. S1).

The respiratory quinone analysis in the two type strains (AArcht4^T and AArcht-Sl^T) representing the two groups of natronarchaeal chitinolytics revealed in both the presence of a single menaquinon species, identified as MK-8:0 (Suppl. Fig. S2), which is commonly detected in haloarchaea [11].

Phylogenetic analysis

BLAST analysis of the 16S rRNA gene sequence of the twelve AArcht strains showed that all of them fell into the family *Natrialbaceae* and that they formed two subgroups divided by a 3-species cluster of the genus *Natrialba* (Fig. 2a). Group 1 included ten isolates from soda lakes with a high sequence identity (above 98%), while group 2 contained only strains AArcht7 and AArcht-Sl^T. However, the 16S rRNA gene-based phylogeny seemed to be unreliable because the bootstrap values in the key nodes were very low and thus most of the branches were not resolved. Therefore, to further clarify the AArcht phylogeny, two additional analyses were performed for the genome-sequenced strains AArcht4^T, AArcht7 and AArcht-Sl^T together with the *Natrialbaceae* representatives based on the RNA polymerase B′-subunit (*rpoB′*) gene nucleotide sequences (Fig. 2b) and on the concatenated alignment of 33 single-copy conserved proteins (Fig. 2c). In sharp contrast to the results of the 16S rRNA gene-based phylogeny, the latter approaches were coherent and reliably showed that the AArcht strains form an

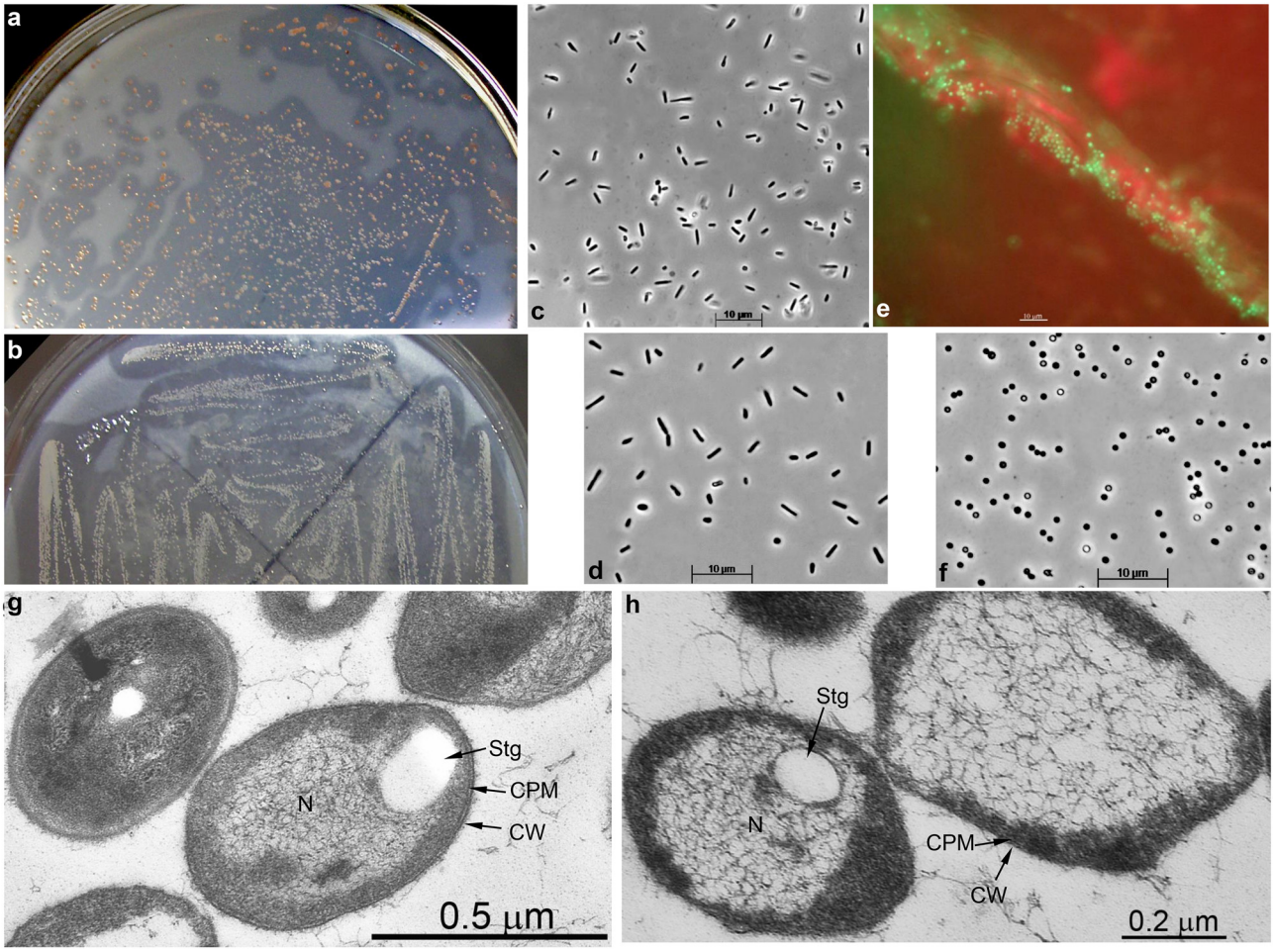

Fig. 1. Morphology of strains AArcht4^T (a, c, e, g) and AArcht-Sl^T (b, d, f, h) growing at 4 M total Na^+, pH 9.2 and 37 °C with chitin. (a–b) colonies on amorphous chitin plates forming hydrolysis zones; (c–d) phase contrast microphotograph of cells grown with amorphous chitin in liquid culture; (e) epifluorescence image of coccoid cells of AArcht4^T forming biofilm on crystalline chitin fiber; (f) phase contrast microphotograph of coccoid cells of AArcht-Sl^T from a colony on amorphous chitin plate; (d) electron microscopy of thin sections of cells grown with amorphous chitin. CW, cell wall; CPM, cytoplasmic membrane; N, nucleoid, Stg, storage granule.

independent monophyletic lineage within the *Natrialbacea*, with AArcht4^T and 7 clustering together and AArcht-Sl^T — as a separate branch. It also demonstrated that the genus *Natrialba* most probably consists of two different genera and needs a taxonomic revision.

Further genomic comparison of the 3 genome-sequenced AArcht strains 4, 7 and Sl between each other and with the members of *Natrialbacea* were performed using two standard indexes, ANI and DDH. Two variations of the ANI calculation showed that the similarity level between the AArcht strains and the members of the family is higher (but only marginally) than the average intragenus level (0.86 versus 0.85) (Supplementary Tables S2 and S3). Likewise, the DDH analysis showed low values of similarity of the 3 AArcht strains (below 24%) with members of the *Natrialbacea* (Supplementary Table S4) and between each other (below 26%).

Growth physiology

The AArcht isolates are obligately aerobic saccharolytic natronoarchaea (growth by fermentation, nitrate, DMSO and sulfur reduction with *N*-acetylglucosamine as substrate was not observed). All isolates grew with chitin and (less actively) with chitosane in their amorphous or crystalline forms. Genome analysis of 3 representative strains showed a presence of 3–7 endochitinase genes of the GH18 family consistent with the physiology of the AArcht isolates (Table 2). In contrast, none of those genes were found in the available genomes from the related genera within the *Natrialbaceae* family except the neutrophilic *Salinarchaeum*, for which the potential to utilize chitin for growth has also recently been demonstrated [24,37]. We tested type strains of *Natrialba asiatica* and *Halopiger xanaduensis* for their ability to grow with chitin and chitosane and the results were negative. Both AArcht groups also utilized chitin and chitosane monomers (*N*-acetylglucosamine and glucosamine, respectively) along with a few other hexoses and glycerol (Table 2). No growth was detected with the following polysaccharides: amorphous cellulose, CMC, various beta- and alpha-glycans, beta-mannan, beta-galactan, beta glyco- and galactomannans, pectin, alginate. The soluble sugar compounds which tested negative included glucose, galactose, mannose, arabinose, rhamnose, glucuronic and galacturonic acids, xylose, ribose, maltose, lactose, trehalose, melibioze, sorbitol and mannitol. No growth was detected with organic acids (C_2–C_8 fatty acids, lactate, pyruvate, malate, succinate, fumarate) nor complex organic amino acid substrates, such as various peptons and yeast extract. Lipase and protease were negative. Anaerobic growth with *N*-acetylglucosamine was not observed either by fermentation, or in the presence of electron acceptors, including nitrate/nitrite, sulfur, thiosulfate, DMSO, fumarate, (10 mM each), arsenate, selenate (5 mM each). Utilization of N-sources was tested for three strains: AArcht4^T, AArcht7 and AArcht-Sl^T using sucrose as the carbon and energy source. All strains were able to grow only with ammonium,

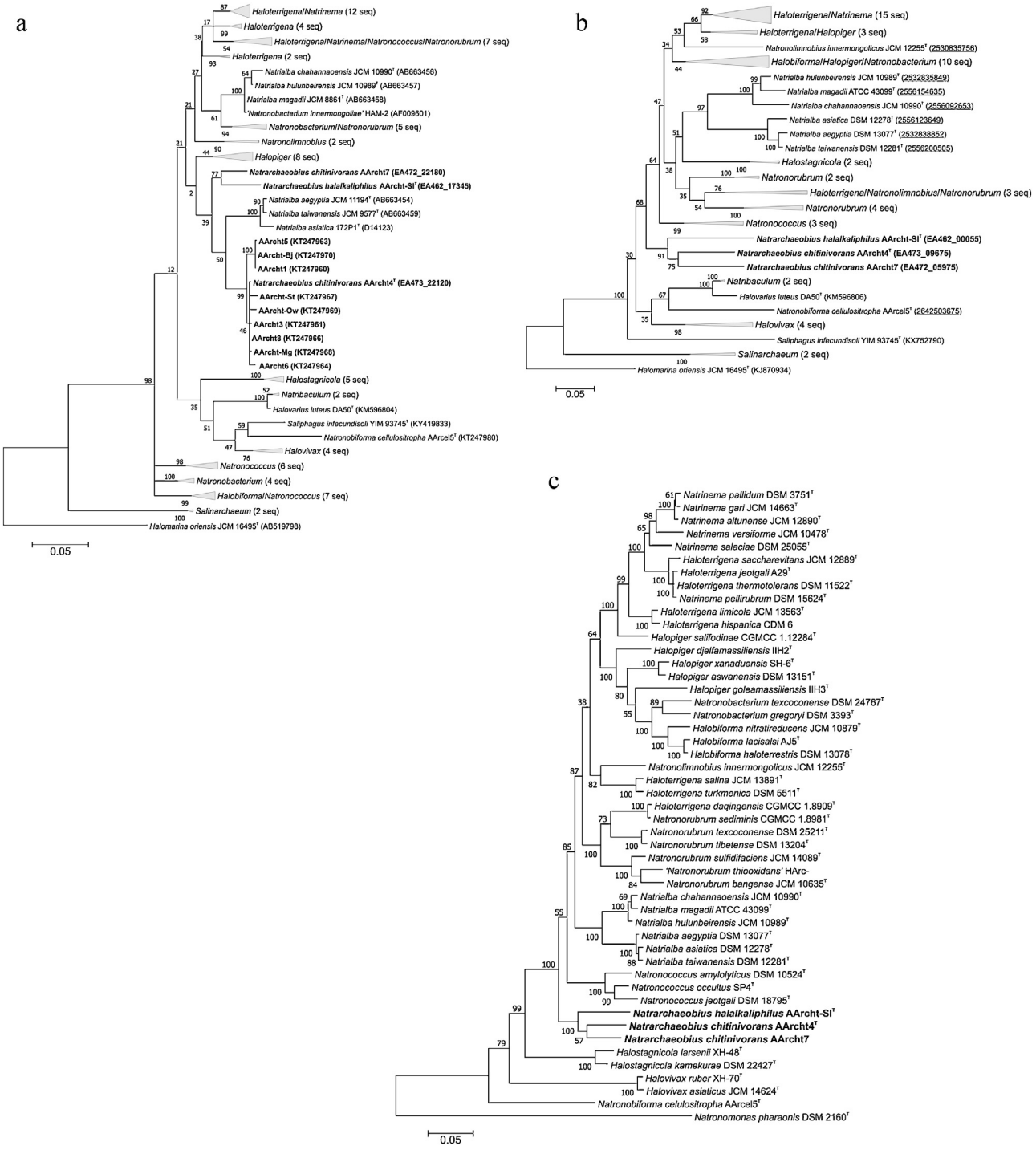

Fig. 2. Phylogeny of the AArcht strains.

(a) Maximum Likelihood 16S rRNA gene-based phylogenetic tree of AArcht strains (in bold) within the family *Natrialbaceae* with *Halomarina oriensis* as an outgroup. Branch lengths correspond to the number of substitutions per site with corrections, associated with the model (GTR, G + *I*, 4 categories). All positions with less than 95% site coverage were eliminated. Totally 1359 positions were used in the alignment of 93 sequences (Supplementary Table S5a). Numbers at nodes indicate bootstrap values of 1000 repetitions.

(b) Maximum Likelihood phylogenetic tree based on *rpoB'* gene sequences of *Natrialbaceae* representatives together with AArcht4^T, AArcht7 and AArcht-Sl^T strains (in bold) with *Halomarina oriensis* as an outgroup. Branch lengths correspond to the number of substitutions per site with corrections, associated with the model (GTR, G + *I*, 4 categories). All positions with less than 95% site coverage were eliminated. Totally 1830 positions were used in the alignment of 64 sequences (Supplementary Table S5b). Numbers at nodes indicate bootstrap values of 1000 repetitions. Gene accession numbers obtained from the IMG database are underlined.

(c) Maximum Likelihood tree based on concatenated amino acid sequences of 33 single-copy conserved proteins showing position of the AArcht lineage (in bold) within the *Natrialbaceae* family. *Natronomonas pharaonis* was used as an outgroup. Branch lengths correspond to the number of substitutions per site with corrections, associated with the model (LG, G + *I*, 4 categories). All positions with less than 95% site coverage were eliminated. Totally 6342 positions were used in the alignment of 49 amino acid sequences. Numbers at nodes indicate bootstrap values of 1000 repetitions. Gene accession numbers obtained from the IMG database are underlined.

Table 2
Comparative property of chitin-utilizing natronoarchaea with the related genera from the family *Natrialbaceae* containing alkaliphilic species. Cumulative comparative data are taken from Ref. [30]. Number of species are indicated in parenthesis.

Property	**"Natrarchaeobius" (2)**	*Natrialba* (6)	*Natronolimnobius* (2)	*Natronobacterium* (2)	*Natronorubrum* (6)	*Natronococcus* (4)	"Natronobiforma" (1)
Cell morphology	Dimorphic	Dimorphic	Pleomorphic, flat	Rods or cocci	Pleomorphic, flat	Cocci in clusters	Dimorphic
Motility	–	V	–	–	V	–	+
Pigmentation	Red-orange	No pigment or red-orange	Red-orange	Red	Pink-red	Pink, red, orange or brown	Red
Cell lyzis in distilled water	+	+	+	+	+	–	+
Growth with chitin	+	–(G)	–[a]	–(G)	-(G)	–(G)	–
Growth with insoluble cellulose	–	–(G)	V[a]	–(G)	-(G)	–(G)	+
Proteolysis	–	V	V	V	V	V	–
Starch hydrolysis	–	V	V[a]	–	–	V	–
Anaerobic growth with nitrate	–	nd	–	–	–	–	–
Minimal salinity M Na^+	3.0	1.6	Above 2.5	1.7	1.7	1.5	2.5
pH type	Facultative or obligate alkaliphilic	Facultative alkaliphilic or alkalitolerant	Obligate alkaiphilic	Obligate alkaiphilic	Facultative or obligate alkaliphilic alkalitolerant	Obligate alkaiphilic	Obligate alkaiphilic
Temperature max.	55 °C (at pH 8)	50-60	54	40	50–55 °C	50–55	53 (at pH 8.5)
Major core lipids	C_{20}–C_{20}, C_{20}–C_{25}	C_{20}–C_{20}, C_{20}–C_{25}	C_{20}–C_{20}, C_{20}–C_{25}	C_{20}–C_{20}, C_{20}–C_{25}	C_{20}–C_{20}, C_{20}–C_{25}	C_{20}–C_{20}, C_{20}–C_{25}	C_{20}–C_{20}, C_{20}–C_{25}
Intact membrane phospholipids	PGP-Me, PG (minor): PE, PGP	PGP-Me, PG	PGP-Me, PG	PGP-Me, PG	PGP-Me, PG	PGP-Me, PG	PGP-Me, PG, PGP
Glycolipids	–	S_2-DGD (in alkalitolerant species)	–	V (unidentified)	TGA-1 (in a neutrophilic species)	–	GL-PG, 2GL
G+C, mol%	61.9–62.3	61.5–64.3	59–64	62.5–65.9	59.9–63.3	62.1–64.0	65.4–65.5
Habitat	Hypersaline alkaline lakes	Soda and salt lakes	Soda lake	Soda lakes	Soda and salt lakes	Soda lakes	Soda lakes

V, variable property in different specoes; nd, not detrmined; (G) — genomic data.
PGP-Me, phosphatidylglycerophosphate methylester; PG, phosphatidylglycerols; PE, phosphatidylethanolamine; PGP, phosphatidylglycerophosphate; GL-PG, phosphatidylglycose; 2GL — diglycosyl; S2-DGD — disulfated mannosyl glucosyl diether; TGA-1 — triglycosylarchaeol.
Bold text means the organisms described in this article in contrast to the reference organisms used for comparison.
[a] Our data (*Natronolimnobius innermongolicus and Nl. baerhaense* can not grow on chitin, but the latter can weakly grow with insoluble cellulose and grow well with xylane and starch).

while urea, nitrate and nitrite did not support growth in several progressive passages.

Antibiotic resistance was tested for AArcht4^T (at pH 9.5) and AArcht-SI^T (at pH 9) in liquid culture with the chitin monomer as substrate. Both were resistant to 100 μg ml^{-1} of penicillin G, ampicilline, kanamycin, streptomycin, gentamicin, erythromycin and vancomicin. Rifampicin and chloramphenicol inhibited growth of AArcht4^T at 100 μg/ml, while AArcht-SI^T did not grow already at 50 μg/ml of those two antibiotics.

The salt profile for growth in the representative strains AArcht4^T and AArcht-SI^T was investigated using *N*-acetylglucosamine as the substrate at pH 9. Both strains grew within a narrow salt range from 3 to 5 M total Na^+ with an optimum at 3.5–4 M, which classifies them as extreme halophiles. In respect to the pH range for growth (tested at 4 M Na^+), the two groups were clearly different. Five tested soda lake AArcht isolates (1, 3, 4^T, 5, 7) started to grow only at pH above neutral with an optimum at 9.1–9.3 and maximum (final) pH up to 9.9–10, thus belonging to the obligate alkaliphilic type. In contrast, strain AArcht-SI^T showed growth at pH as low as 6.5, although it still grew optimally at pH 8–8.5 and up to 9.5, characterizing it as a facultative alkaliphile. The difference in high pH response between the two AArcht groups correlated with their different Cl^- dependence: the AArcht strains from group 1 demanded at least 1 M Cl^-, while AArcht-SI^T did not grow at Cl^- concentrations below 2.5 M. Strains from both groups grew equally well at Mg^{2+} concentrations from 1 to 5 mM (at higher concentration magnesium started to precipitate at high pH). Both groups showed mesophilic temperature profiles typical for many haloarchaeal species with a somewhat elevated maximum up to 50 (AArcht4^T) and 55 °C (AArcht-SI^T) which decreased with increasing pH. The probable reason for the latter is protein instability at a high temperature-high pH combination.

The *Natrialbaceae* family (the only family in the order *Natrialbales*) currently includes twelve recognized genera [30] and a recently described genus "Natronobiforma" [36]. Most of the genera in this family include alkaliphilic or alkalitolerant species [30]. However, as described above, doubt exists as to the reliability of reported maximum pH values as, in most cases, the actual pH changes during growth were not tested. Most probably, only those species that originated from soda lakes are true alkaliphiles, including species from the genera *Natrialba*, *Natronobacterium*, *Natronococcus*, *Natronolimnobius*, *Natronorubrum* and "Natronobiforma". Hence, Table 2 provides phenotypic comparison of the AArcht isolates with those genera of the family that contain mainly species originating from alkaline habitats. The main phenotypic property of the novel group which discriminates it from the other natronoarchaea in the *Natrialbaceae* is the ability to utilize chitin as the growth substrate. The only other genus in the *Natrialbaceae* with such capability is the genus *Salinarchaeum* [24,37] which is a neutrophilic halophile. Another prominent difference of the AArcht strains is the high values of their minimal salt concentration for growth (3 M Na^+). In respect to cell morphology, the novel group shares a tendency for dimorphism (rods and cocci) with genera *Natrialba* and "Natronobiforma". In respect to the lipid composi-

Table 3
Comparative property of chitin-utilizing natronoarchaea with the related species from the family *Natrialbaceae*: **1**, *Natrialba asiatica* [16]; **2**, *Natrialba chahannaoensis* [41]; **3**, *Halopiger xanaduensis* [13].

Property	Group 1 (11 strains)	AArcht-Sl^T	**1**	**2**	**3**
Cell morphology	Non-motile flat rods in free state; coccoids in chitin-attached state	Pleomorphic, from rods to coccoids, non-motile	Rods, motile	Rods, non-motile	Dimorphic
Pigmentation	Red–orange	Orange	No	Red	Red
Growth substrates:					
Sugar polymers					
Chitin, chitosane	+	+	–[d]		–[d]
Other glycans	–	–	–	starch	
Sugars	Glucosamine, *N*-acetylglucosamine, sucrose, maltose, trehalose, melizitose, cellobiose, glycerol	Glucosamine, *N*-acetylglucosamine, sucrose, maltose, trehalose, melizitose, fructose, glycerol	–[d] –[d] Glucose, galactose, xylose	– – Glucose, fructose, maltose	–[d] –[d] Glucose, arabinose, xylose
Proteins, peptides	–	–	+	+	+
Number of chitinase GH18 genes in the genome	AArcht4 (5) AArcht7 (7)	3	0	0	0
Anaerobic growth	–	–	–	Nitrate to nitrite reduction	+ (denitrification)
Catalase/oxidase	+/+	+/+	+/+	+/+	+/+
Salinity range (opt.) M Na^+	3.0–5.0 (4.0)	3.0–5.0 (3.5)	2.0–5.0 (4.0)	1.6–5.2 (2.5)	2.5–5.0 (4.3)
pH range (opt.)	7.0–10.0 (9.1–9.3)	6.5–9.5 (8.0–8.5)	6.0–8.0 (6.6–7.0)	8.5–10.5[a] (9.0)	6–11[b] (7.5–8.0)
Temperature (°C)[c]	20–50 (opt. 43)	25–55 (opt. 45)	max. 50 (30–40)	20–55 (50)	28–45 (37)
Core lipids	C_{20}–C_{20}, C_{20}–C_{25} (dominant) 1- and 2-C_{20} MGE and 2-C_{25} MGE (minor)	C_{20}–C_{20}, C_{20}–C_{25}	C_{20}–C_{20}, C_{20}–C_{25}	C_{20}–C_{20}, C_{20}–C_{25}	C_{20}–C_{20}, C_{20}–C_{25}
Intact membrane polar lipids	(major): PGP-Me, PG (minor): PE, PGP	(major): PGP-Me, PG (minor): PGP	PGP-Me, PG, S_2-DGD	PGP-Me, PG	PGP-Me, S_2-DGD
G + C, mol%	AArcht4^T:61.9 (genome) AArcht7:62.3 (genome)	61.1 (genome)	62.4 (genome)	64.3 (T_m)	65.2 (genome)
Habitat	Hypersaline alkaline lakes in Central Asia and Africa	Hypersaline alkaline Searles Lake (California)	Sea salt evaporites	Soda lakes, Inner Mongolia	

Phospholipids: (PG) phosphatidylglycerol, (PGP-Me) phosphatidylglycerophosphate methylester, (PE) phosphatidylethanolamine, PGP (phosphatidylglycerophosphate); (S_2-DGD) disulfated mannosyl glucosyl diether.
Bold text means the organisms described in this article in contrast to the reference organisms used for comparison.
[a] Final pH is not measured therefore the max. growth pH is not validated.
[b] Final pH is not measured, therefore the max. growth pH is not validated, especially taking into account low pH optimum.
[c] Tested at pH 8.5.
[d] Tested in this work.

tion all compared genera are similar in their major core and intact phospholipids. The novel group has two additional minor components, both in the core and intact lipids, but it is possible that these were not detected in other genera because of less sensitive analysis (TLC versus HPLC).

Further, more detailed phenotypic comparison of the chitin-utilizing natronoarchaea with related species of the genera *Natrialba* and *Halopiger* is given in (Table 3).

The AArcht isolates represent the first example of alkaliphilic haloarchaea enriched and isolated from hypersaline alkaline lakes with chitin as their growth substrate. They are highly specialized in the utilization of chitin (which is reflected in the presence of multiple chitinase genes in their genomes) and can only utilize a few soluble sugars for growth. Their presence in hypersaline alkaline (soda) lakes on three different continents indicate that chitin must be abundant in such habitats — a fact not well recognized to date. A mass development of chitin-producing brine shrimp *Artemia monica* in soda lake Mono (California) is the only example of chitin-producing invertebrates reported in the literature [8]. However, the authors have noticed their presence *en masse* in highly alkaline soda lakes of the Kulunda Steppe (unpublished, Suppl. Fig. S3a). Another massive source of chitin in soda lakes could be from the soda fly larvas *Ephydra hians* [38] which was also often observed by the authors on the littoral of the Kulunda Steppe soda lakes (Suppl. Fig. S3b,c).

One of the peculiarities of the AArcht strains described here, that apparently draws attention, is the ineffectiveness of the 16S rRNA gene, which normally provides reliable phylogenetic reconstructions. This marker has been, so far, an indisputable basis for phylogenetic reconstructions, although, for haloarchaea in particular, several problematic examples of multiple dissimilatory copies of this gene have been documented [28]. This inability to use *rrn* genes for phylogenetic reconstructions could be overcome by involving phylogenomic analysis of conserved single-copy protein markers [32]. In the case of the AArcht strains, this approach gave much more consistent results indicating a monophyletic genus-level group with three species-level subgroups: ten isolates from hypersaline soda lakes, AArcht7 from a soda crystallizing pool in Kulunda Steppe and AArcht-Sl^T from the moderately alkaline Searles Lake. However, on the phenotypic level, strain AArcht7 can not be distinguished from the main soda lake group, in contrast to strain AArcht-Sl^T, which clearly differentiated by its lower pH optimum and maximum and much higher chloride dependence.

In conclusion, taking into account unique phenotypic properties and results of phylogenomic analysis, we propose to classify the major soda lake group 1 (11 isolates) in a new genus and species

Table 4
Natrarchaeobius chitinivorans and *Natrarchaeobius haloalkaliphilus*: protologue.

Parameter	Genus: *Natrarchaeobius* gen. nov.	Species: *Natrarchaeobius chitinivorans* sp. nov.	Species: *Natrarchaeobius halalkaliphilus* sp. nov.
Date created	2018-09-24	2018-09-24	2018-09-24
Taxon number (TXNR)	GA00091		
Author (AUTE)	Dimitry Y. Sorokin		
Species name (SPNA)		***Natrarchaeobius chitinivorans***	***Natrarchaeobius halalkaliphilus***
Genus name (GENA)	***Natrarchaeobius***		
Specific epithet (SPEP)	–	*chitinivorans*	*haloalkaliphilus*
Species status (SPST)	–	sp. nov.	sp. nov.
Etymology (GETY/SPTY)	*Natr.ar.chae.o'bi.us* [N.L. n. *natron* (arbitrarily derived from Arabic n. *natrun* or *natron*) soda, sodium carbonate; N.L. pref. *natr-* pertaining to soda; Gr. adj. *archaios* ancient; Gr. masc. n. *bios* life; N.L. masc. n. *Natrarchaeobius*, soda-philic archaeon]	*chitinivorans* [chi.ti.ni.vo'rans N.L. neut. n. *chitinum* chitin; L. pres. part. *vorans* devouring; N.L. part. adj. *chitinivorans* chitin devouring]	*halalkaliphilus* [hal.al.ka.li.phi'lus Gr. n. *hals halos* salt; N.L. n. *alkali* soda ash (from Arabic al-qalyi the ashes of saltwort); N.L. adj. *philus* (from Gr. adj. philos *-ê −on*) friend, loving; N.L. masc. adj. *halalkaliphilus* salt and alkali-loving].
Authors (AUT)	Dimitry Y. Sorokin, Alexander G. Elcheninov, Stepan V. Toshchakov, Nicole J. Bale, Jaap S. Sinninghe Damsté, Tatiana V. Khijniak, Ilya V. Kublanov		
Title (TITL)	*Natrarchaeobius chitinivorans* gen. nov., sp. nov., and *Natrarchaeobius halalkaliphilus* sp. nov., alkaliphilic, chitin-utilizing haloarchaea from hypersaline alkaline lakes		
Journal (JOUR)	Systematic and Applied Microbiology		
Corresponding author (COAU).	Dimitry Y. Sorokin		
E-mail of corresponding author (EMAU)	d.sorokin@tudelft; soroc@inmi.ru		
Designation of the type strain (TYPE)	–	**AArcht4**	**AArcht-SI**
Strain collection numbers (COLN)	–	JCM 32476; UNIQEM U966	JCM 32477; UNIQEM U969
16S rRNA gene accession number (16 SR)	–	KT247962	KT247971
Alternative house-keeping genes: gene [accession numbers] (HKGN)	–	*rpoB′*	
		33 single-copy conservative protein genes	
Genome status (GSTA)	–	Draft: AArcht4^T (accession SAMN10160502) AArcht7 (accession SAMN10160503)	Draft: (accession SAMN10160504)
GC mol % (GGCM)	–	61.9–62.3 (genomes of AArcht4^T and AArcht7)	61.1 (genome)
Country of origin (COUN)	Russian Federation, Mongolia, China, Egypt, USA	Russian Federation, Mongolia, China, Egypt, USA	USA
Region of origin (REGI)	–	Altai region; *N-E* Mongolia, Inner Mongolia, Wadi al Natrun, California	California
Date of isolation (DATI)	–	2011–2013	2012
Source of isolation (SOUR)	Surface sediments and brines of hypersaline alkaline lakes	Surface sediments and brines of hypersaline soda lakes	Surface sediments of hypersaline alkaline Searles Lake
Sampling dates (DATS)	1999–2013	1999–2013	2005
Geographic location (GEOL)	*S–W* Siberia, *N–E* Mongolia, Inner Mongolia, Northern Africa, North America	*S–W* Siberia, *N–E* Mongolia, Inner Mongolia, Northern Africa, North America	North America
Latitude (LATI)	–	–	N35°44′
Longtitude (LONG)	–	–	W117°20′
Depth (DEPT)	0–0.1 m	0–0.1 m	0–0.1m
Temperature of the sample (TEMS)	15–25 °C	15–25 °C	20 °C
pH of the sample (PHSA)	9–11.0	9.5–11.0	9.0
Salinity of the sample (SALS)	18–40%	18–40%	35%
Number of strains in study (NSTR)	12	11	1
Source of isolation of non-type strains (SAMP)	–	Hypersaline alkaline lakes in Russia, Mongolia, China and USA	–
Growth medium, incubation conditions (CULT)	Alkaline medium containing 4 M Na^+ with pH 9–9.5 and chitin as substrate	4 M total Na^+, equal mix of sodium carbonate and NaCl on the basis of Na molarity, pH 9.5; incubation −37 °C; amorphous chitin as C, energy and N-source	4 M total Na^+, 1:3 mix of sodium carbonate and NaCl on the basis of Na molarity, pH 9; incubation −37 °C; amorphous chitin as C, energy and N-source
Conditions of preservation (PRES)	Deep freezing in 15% glycerol (v/v)		
Gram stain (GRAM)	Negative		
Cell shape (CSHA)	Pleomorphic, from flat rods to cocci		
Cell size (CSZI)	–	0.6–1 μm in diameter, length is variable from 1 to 4 μm	0.6–1.2 μm in diameter, length is variable from 1 to 5 μm
Motility (MOTY)	–	nonmotile	
Motility type (MOTK)	–		
Type of flagellation (TFLA)	–		
Sporulation (SPOR)	none		
Colony morphology (COLM)	Pink–orange	Pink–orange, up to 2 mm	Pale orange, up to 1.5 mm
Temperature range for growth (TEMR)	20–55 °C	20–53 °C	25–55 °C
Lowest temperature for growth (TEML)	20 °C	20 °C	25 °C

Table 4 (*Continued*)

Parameter	Genus: *Natrarchaeobius* gen. nov.	Species: *Natrarchaeobius chitinivorans* sp. nov.	Species: *Natrarchaeobius halalkaliphilus* sp. nov.
Highest temperature for growth(TEMH)	55	50 (at pH 9)	55 (at pH 8.5)
Optimal temperature for growth (TEMO)	43–45 °C	43 °C	45 °C
Lowest pH for growth (PHLO)	6.5	7.0	6.5
Highest pH for growth (PHHI)	10	10	9.5
Optimum pH for growth (PHOP)	8.5–9.3	9.1–9.3	8.5
pH category (PHCA)	Alkaliphile (optimum > 8.5)		
Lowest NaCl concentration for growth (SALL)	3.0 M total Na^+		
Highest NaCl concentration for growth (SALH)	5 M total Na^+		
Optimum salt concentration for growth (SALO)	3.5–4.0 M total Na^+	4.0 M total Na^+	3.5 M total Na^+
Other salts important for growth	Sodium carbonates		
Salinity category (SALC)	extreme halophilic (optimum 3.5–4 M Na^+)		
Relation to oxygene (OREL)	Aerobe		
O_2 conditions for strain testing (OCON)	Aerobic		
Carbon source used (class) (CSUC)	Carbohydrates		
Specific compounds (CSUC)	Chitin, chitosane, hexoses	Glucosamine, *N*-acetylglucosamine, sucrose, maltose, trehalose, melizitose, cellobiose, glycerol	Glucosamine, *N*-acetylglucosamine, sucrose, maltose, trehalose, melizitose, fructose, glycerol
Nitrogen source (NSOU)	Ammonium		
Terminal electron acceptor (ELAC)	O_2		
Energy metabolism (EMET)	Chemoorganotrophic		
Phospholipids (PHOS)	Core membrane lipids are archaeol (C_{20}–C_{20} DGE) and C_{20}–C_{25} DGE Polar lipids are phosphatidylglycerophosphate methyl ester (PGP-Me), phosphatidylglycerol (PG)		
Glycolipids (GLYC)	–	Phosphatidylglycose (GL-PG), diglycosyl (2GL)	
Respiratory quinons	MK8:0	MK8:0	MK8:0
Habitat (HABT)	Hypersaline alkaline lakes		
Extraordinary feautres (EXTR)	Fast growth with chitin and chitosane in hypersaline alkaline brines Multiple chitinase genes (GH18 family) in the genomes		

(–), not fixed for the taxon.
Bold text means the organisms described in this article in contrast to the reference organisms used for comparison.

Natrarchaeobius chitinivorans, and the Searles Lake isolate AArcht-SI^T in a new species *Natrarchaeobius halalkaliphilus*. The protologue summarizing properties of these three novel taxa is presented in Table 4.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2019.01.001.

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