Natronobiforma cellulositropha gen. nov., sp. nov., a novel haloalkaliphilic member of the family Natrialbaceae (class Halobacteria) from hypersaline alkaline lakes

Sorokin, Dimitry Y.; Khijniak, Tatiana V.; Kostrikina, Nadezhda A.; Elcheninov, Alexander G.; Toshchakov, Stepan V.; Bale, Nicole J.; Damsté, Jaap S.Sinninghe; Kublanov, Ilya V.

DOI
10.1016/j.syapm.2018.04.002

Publication date
2018

Document Version
Final published version

Published in
Systematic and Applied Microbiology

Systematic and Applied Microbiology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Natronobiforma cellulositropha gen. nov., sp. nov., a novel haloalkaliphilic member of the family *Natrialbaceae* (class *Halobacteria*) from hypersaline alkaline lakes

Dimitry Y. Sorokin[a,b,*], Tatiana V. Khijniak[a], Nadezhda A. Kostrikina[a], Alexander G. Elcheninov[a], Stepan V. Toshchakov[c], Nicole J. Bale[d], Jaap S. Sinninghe Damsté[d,e], Ilya V. Kublanov[a,c]

[a] *Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia*
[b] *Department of Biotechnology, TU Delft, The Netherlands*
[c] *Immanuel Kant Baltic Federal University, Kaliningrad, Russia*
[d] *Department of Marine Microbiology and Biogeochemistry, NIOZ Netherlands Institute for Sea Research, and Utrecht University, The Netherlands*
[e] *Department of Earth Sciences – Geochemistry, Faculty of Geosciences, Utrecht University, Utrecht, The Netherlands*

ARTICLE INFO

Article history:
Received 11 March 2018
Received in revised form 13 April 2018
Accepted 18 April 2018

Keywords:
Hypersaline
Soda lakes
Haloalkaliphilic
Natronoarchaea
Cellulotrophic

ABSTRACT

Six strains of extremely halophilic and alkaliphilic euryarchaea were enriched and isolated in pure culture from surface brines and sediments of hypersaline alkaline lakes in various geographical locations with various forms of insoluble cellulose as growth substrate. The cells are mostly flat motile rods with a thin monolayer cell wall while growing on cellobiose. In contrast, the cells growing with cellulose are mostly nonmotile cocci covered with a thick external EPS layer. The isolates, designated AArcel, are obligate aerobic heterotrophs with a narrow substrate spectrum. All strains can use insoluble celluloses, cellobiose, a few soluble glucans and xylan as their carbon and energy source. They are extreme halophiles, growing within the range from 2.5 to 4.8 M total Na^+ (optimum at 4 M) and obligate alkaliphiles, with the pH range for growth from 7.5 to 9.9 (optimum at 8.5–9). The core archaeal lipids of strain AArcel5^T were dominated by C_{20}–C_{20} dialkyl glycerol ether (DGE) (i.e. archaeol) and C_{20}–C_{25} DGE in nearly equal proportion. The 16S rRNA gene analysis indicated that all six isolates belong to a single genomic species mostly related to the genera *Saliphagus-Natribaculum-Halovarius*. Taking together a substantial phenotypic difference of the new isolates from the closest relatives and the phylogenetic distance, it is concluded that the AArcel group represents a novel genus-level branch within the family *Natrialbaceae* for which the name *Natronobiforma cellulositropha* gen. nov., sp. nov. is proposed with AArcel5^T as the type strain (JCM 31939^T = UNIQEM U972^T).

Introduction

Hypersaline habitats, such as inland salt and soda lakes and salterns with salt concentrations close to saturation are usually inhabited by a dense population of haloarchaea, which represent the extremely halophilic branch of the phylum *Euryarchaeota*. According to the current knowledge, haloarchaea are mostly aerobic heterotrophs, with a few exceptions of facultative anaerobes capable of utilizing simple soluble organic monomers [7,8,1,16,17,6]. A few haloarchaeal species are capable of hydrolyzing polymeric substances, such as starch, proteins and olive oil [3,5,14,19,2]. However, the potential functioning of haloarchaea in the mineralization of insoluble organic polymers has not yet been considered and this function in hypersaline habitats is usually attributed to halophilic bacteria [1,16]. In particular, next to nothing is known about the ability of haloarchaea to utilize native insoluble cellulose as a growth substrate. Glycosyl-hydrolase (GH) genes encoding putative cellulases have been noted in several haloarchaeal genomes and the presence of functional endoglucanases were demonstrated in two genera of neutrophilic haloarchaea, i.e. *Halorhabdus* and *Haloarcula* [10,11,26]. However, it remains to be investigated whether these archaea are actually capable of using native forms of cellulose as growth substrates.

* Corresponding author at: Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia.
E-mail addresses: soroc@inmi.ru, d.sorokin@tudelft.nl (D.Y. Sorokin).

https://doi.org/10.1016/j.syapm.2018.04.002

Table 1
Cellulotrophic natronoarchae isolated from hypersaline alkaline lakes.

Strain	Isolated from:		Chemical parameters of brines			Cellulose form used in the enrichment
	Lake	Area	Salt ($g\,l^{-1}$)	pH	Alkalinity (M)	
AArcel 2	Bitter-1	Kulunda Steppe Altai, Russia	330	10.3	4.0	Amorphous
AArcel 4	Soda crystallizer		380	9.6	3.1	Avicel
AArcel 5^T	Tanatar-1		400	11.0	4.9	Sigma 20 μm
AArcel 9	Mixed from 3 lakes		330–400	9.6–11.0	3.1–4.9	Filter paper
AArcel 6	Shar-Burdiin, Hotontyn	n-e Mongolia	220–360	9.6–9.9	0.9–1.2	Amorphous
AArcel 8-1	Owens lake	California	180	9.7	1.0	Amorphous

So far, a single study focused on the functional aspect of cellulose degradation by haloarchaea has been published [22]. In that work, for the first time we were able to enrich and isolate in pure culture a number of haloarchaeal strains utilizing cellulose as the growth substrate. One of the most active groups included six natronarchaeal isolates from various alkaline hypersaline lakes which, to our knowledge, represent the first example of natronoarchaea with such a metabolic trait. This paper describes phenotypic and phylogenetic properties of the novel group and proposes to assign it into a novel genus and species *Natronobiforma cellulotropha*.

Material and methods

Samples

Surface sediments and near-bottom brines from various hypersaline alkaline inland lakes from Central Asia, Egypt and USA with salt concentration of 200–400 $g\,l^{-1}$, pH from 9.3 to 11 and soluble carbonate alkalinity from 0.1 to 4 M were used to enrich for cellulotrophic natronoarchaea [22].

Enrichment, isolation and cultivation conditions

The alkaline (pH 9.5) base medium, containing 4 M total Na^+ (2 M Na^+ as sodium carbonates + 2 M NaCl) also included 1 $g\,l^{-1}$ K_2HPO_4 and 5 g/l KCl and was supplemented after sterilization with 1 $ml\,l^{-1}$ of trace metal solution and vitamin mix [18], 1 mM $MgCl_2$, 2 mM $(NH_4)_2SO_4$ and 20 $mg\,l^{-1}$ of yeast extract. Addition of 200 $mg\,l^{-1}$ of streptomycin served to inhibit growth of bacteria. Various forms of insoluble cellulose were used as the only carbon and energy source at a final concentration of 1 $g\,l^{-1}$ (Table 1). Before inoculation, the sediments were resuspended 1:10 in the basic medium and after 5–10 min precipitation of the course fractions, a 1 ml portion from the top fraction containing mostly colloidal sediments and microbial cells was used to inoculate 20 ml cultures in 100 ml closed serum bottles placed on a rotary shaker at 37 °C and at 120 rpm. The development of cells was monitored by the visual extent of cellulose degradation, appearance of pink color and by microscopy. After visible cellulose degradation and biomass growth became evident (20–40 days), the cultures were serially diluted in the same medium but with amorphous cellulose as substrate and the maximal positive dilutions were plated onto a solid medium prepared by mixing 3 parts of the liquid medium (with additional solid NaCl addition to compensate for dilution with agar) and 2 parts of 5% extensively washed agar at 55 °C. After 2–6 weeks of incubation in closed plastic bags at 37 °C the colonies with clearance zones were transferred to the liquid media with amorphous cellulose and the positive cultures were further purified by several rounds of plating-liquid culture cultivation with amorphous cellulose. This, eventually, resulted in isolation of 6 pure cultures of cellulotrophic natronoarchaea with a common designation as AArcel (Table 1). The purity was checked microscopically (Zeiss Axioplan Imaging 2 microscope, Göttingen, Germany) and by the 16S rRNA gene sequencing.

Phenotypic characterization

For the total cell electron microscopy, the cells were centrifuged and resuspended in 3 M NaCl, fixed with paraformaldehyde (final concentration 3%, v/v) for 2 h at room temperature, then washed again with the same NaCl solutions. The fixed cells were positively contrasted with 1% (w/v) uranyl acetate. For thin sectioning, the cell pellets were fixed in 1% (w/v) OsO_4 containing 3.0 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. Thin sections were post-stained with 1% (w/v) lead citrate.

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 GDGTs and archaeol derivatives according to [24]. Intact polar lipids were obtained by Bligh Dyer extraction of freeze-dried cells and subsequent HPLC-MS analysis as described in Ref. [20].

Genome sequencing

Fragment genomic libraries of strains AArcel5^T and AArcel2 were prepared by NEBNext® Ultra™ kit (New England Biolabs, USA) according to the manufacturer's instructions and sequenced on Illumina Miseq™ System using 2*150 bp paired-end read cartridge. Preliminary assembly and gene prediction was performed by CLC Genomics Workbench 10.5 (Qiagen, Germany) with recommended parameters. Draft genome assemblies were used only for obtaining 16S rRNA and *rpoB′* gene sequences and will be published upon the improvement of assemblies by sequencing of long insert (jumping) genomic libraries.

Phylogenetic analysis

16S rRNA and *rpoB′* gene sequences were extracted from the draft genome assemblies and deposited in the Genbank under the accession numbers: (MG938052-MG938053 for 16S rRNA and MG940906 and MG940907 for *rpoB′* genes of strains AArcel5 and AArcel2, respectively).

To perform 16S rRNA gene sequence-based phylogenetic analysis, the sequences of all type species of the *Natrialbaceae* genera were obtained from the Genbank and aligned together with complete sequences of strains AArcel5^T and AArcel2 and nearly complete sequences of strains AArcel9 and AArcel8-1 in Muscle, implemented in Mega 6 package [23]. The phylogenetic analysis was performed in Mega 6 using Maximum Likelihood algorithm and the General Time Reversible (GTR) model (*G + I*, 4 categories) [15]. Partial sequences of AArcel4 and AArcel6 were identical to

Please cite this article in press as: D.Y. Sorokin, et al., *Natronobiforma cellulositropha* gen. nov., sp. nov., a novel haloalkaliphilic member of the family *Natrialbaceae* (class *Halobacteria*) from hypersaline alkaline lakes, Syst. Appl. Microbiol. (2018), https://doi.org/10.1016/j.syapm.2018.04.002

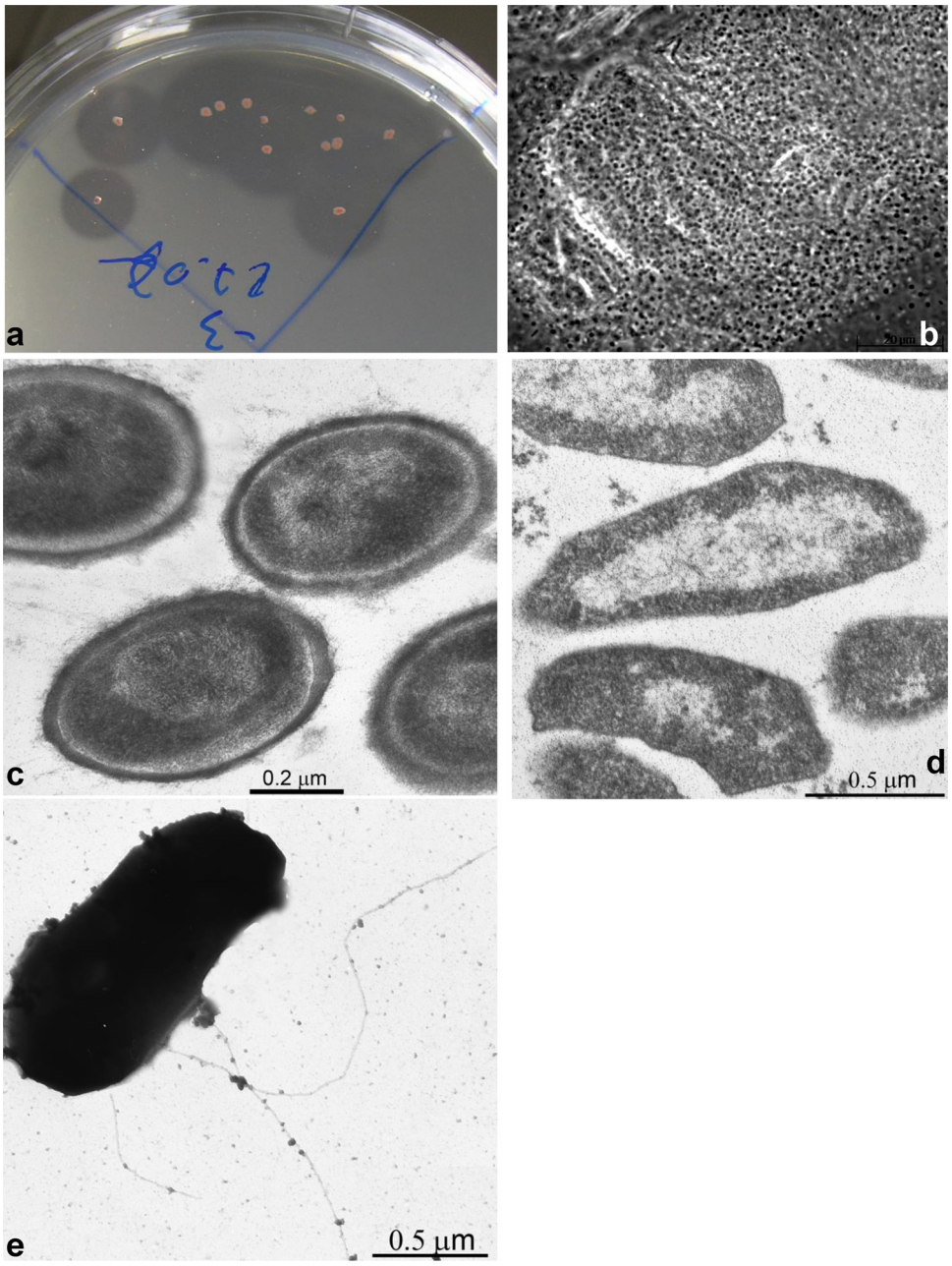

Fig. 1. Cell morphology of strain AArcel5 growing on amorphous cellulose at pH 9.5, 4 M total Na^+ and 37 °C. (a) colonies; (b) phase contrast and (c) electron microscopy of thin sections of cells during absorption phase on cellulose; (d) electron microscopy of thin sections and (e) and whole free suspended cells from the second growth phase on cellulose.

AArcel5 sequence and were not analyzed but placed on the tree together with the type strain.

To perform the *rpoB'*-based phylogenetic analysis, full-length nucleotide sequences of the *rpoB'* gene of type species from the *Natrialbaceae* were obtained from GenBank or IMG and aligned using the G-INS-i method in MAFFT server v7 [9]. Phylogenetic tree was constructed in Mega 6 using Maximum Likelihood algorithm with GTR model (G + *I*, 4 categories. For phylogeny reconstruction based on RpoB′ proteins, the nucleotide sequences were translated, aligned in MAFFT server v7 using the G-INS-i algorithm, and the tree was constructed using Maximum Likelihood algorithm with the LG model. To estimate the *rpoB′* gene distances of all validly published *Natrialbaceae* representatives, the pairwise distances matrix based on percentage of sequence identities was constructed using Mega 6.

Results and discussion

Phenotypic properties

On plates with amorphous cellulose, all strains formed pin-point pink colonies after 4–6 weeks incubation with a large clearance around them, indicative of cellulose hydrolysis (Fig. 1a). Growth in liquid culture with all forms of celluloses started with a massive attachment of cells to the solid phase cellulose surface, followed by gradual dissolution of cellulose and appearance of cells in the liquid phase. A dramatic change in cell morphology was observed in those two phases. The cells aggregated with the cellulose particles were non-motile cocci (Fig. 1b) covered with a thick electron dense external layer (Fig. 1c), while the free suspended cells in the second growth phase were dominated by motile thin flat rods with a thin

Please cite this article in press as: D.Y. Sorokin, et al., *Natronobiforma cellulositropha* gen. nov., sp. nov., a novel haloalkaliphilic member of the family *Natrialbaceae* (class *Halobacteria*) from hypersaline alkaline lakes, Syst. Appl. Microbiol. (2018), https://doi.org/10.1016/j.syapm.2018.04.002

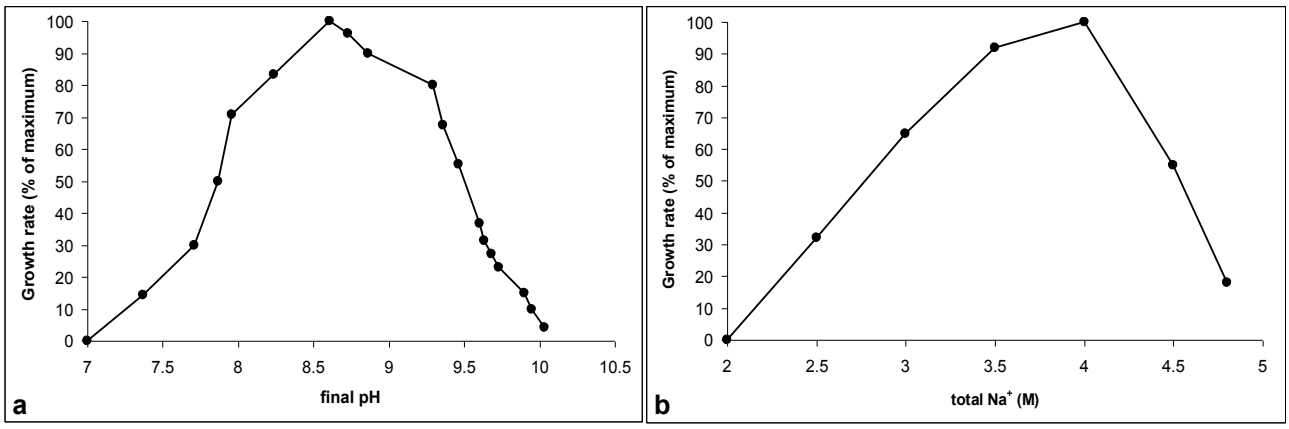

Fig. 2. Influence of pH at 4 M total Na^+ (a) and Na^+ at pH 9 (b) on growth of strain AArcel5 with cellobiose at 37 °C. The results are mean values from two biological replicate experiments.

cell wall (Fig. 1d,e). The cells in the colonies resembled the coccoid cells from the first phase in liquid cultures, while the cells grown on cellobiose in liquid culture were similar to those from the second growth phase on cellulose.

The polar membrane lipids were analyzed in the type strain AArcel5^T grown with cellobiose at 37 °C. 4 M total Na^+ and pH 9.3 harvested in the mid-exponential growth phase. The core membrane lipids were represented by two dominant components: archaeol (C_{20}–C_{20} dialkyl glycerol ether (DGE), 58% of the total) and extended archaeol (C_{20}–C_{25} DGE, 40% of the total). Traces of the monoglycerol ether (MGE) lipids (1-C_{20} MGE, 2-C_{20} MGE, and 2-C_{25} MGE) were also detected. The intact polar lipids were dominated (in order of abundance) by phosphatidylglycerophosphate methylester (PGP-Me), phosphatidylglycerol (PG), a phosphatidylglycose (GL-PG), a diglycosyl (2GL), and phosphatidylglycerophosphate (PGP) (Supplementary Fig. S1).

The AArcel strains are obligately aerobic saccharolytic archaea with a limited range of substrates supporting growth. They are the first natronoarchaea reported as being specialized in the utilization of native insoluble celluloses (but they cannot use an artificial soluble analogue carboxymethyl cellulose CMC) [22]. All strains can grow with xylan (from birch and beech) and barley beta-glucan. AArcel strains 2, 4, and 5 also utilized lichenan, glucomannan and β-1,4-mannan, but growth was much slower. Amorphous chitin also seems to be a substrate for these 3 strains, but the growth was unstable: the transition from cellulose to chitin was only randomly successful, failing on many occasions. Apparently growth on this polymer is not optimal for these archaea. Alpha-glucans, such as starch and starch-like polymers, were not utilized. Among the sugars, only two dimers were used — cellobiose and maltose (less actively). Sugars not utilized included: glucose, fructose, galactose, mannose, rhamnose, arabinose, raffinose, sucrose, trehalose, maltose, glucosamin, *N*-acetylglucosamine, glucouronic acid, halacturonic acid, lactose, ribose, xylose, melezitose and melibiose. Sugar alcohols which tested negative included glycerol, sorbitol and mannitol. Negative organic acids were C_2–C_8 saturated fatty acids, lactate, pyruvate, succinate, malate and fumarate. The organic nitrogen compounds not utilized were glutamate, aspartate meat and casein peptons and yeast extract. Ammonium was utilized as the N-source with cellobiose as carbon and energy substrate, while nitrate and urea were negative. Anaerobic fermentative growth with arginine, cellobiose or maltose was not observed, nor was anaerobic respiration (with cellobiose as the electron donor) with nitrate, sulfur, fumarate or DMSO as electron acceptors.

These archaea belong to the group of extreme halophiles, growing optimally at 4 M total Na^+ (Fig. 2a) and are moderate alkaliphiles with a pH optimum around 9 (Fig. 2b). It is important to stress that, when working with alkaliphiles, the pH profiling should be performed with an obligatory check of the pH change during growth [21]. For this particular group of natronoarchaea mineralizing sugars and producing metabolic acids, we observed a drop of the pH from 11 to 9.5 even when using a highly buffered sodium carbonate system. In our experience, if a natronarchaeon is growing at pH 6 it will not grow at pH 10, which leads to the impression that in the case, for example, of *Natribaculum breve*, the final pH check at the highest pH range was not performed [12]. The type strain AArcel5^T grew within a relatively wide temperature range from 20 to 53 °C with an optimum at 40 °C. However, it should be mentioned that the growth at high temperature (above 43 °C) was only possible at the lowest growth pH (8.5). The most probable explanation is that a combination of high temperature and high alkalinity results in instability of the haloarchaeal *S*-layer.

Phylogenetic analysis

The results of 16S rRNA gene sequence analysis demonstrated that the AArcel isolates formed a separate, single species-level group within the family *Natrialbaceae* with a recently described neutrophilic *Saliphagus infecundisoli* as the closest relative (Fig. 3a). Strain AArcel2 was most remote from the type strain AArcel5^T (99.1% of sequence identity), but still within the currently recognized species border. Calculated of the Average Nucleotide Identity (ANI) between these two genome-sequenced strains gave a value close to the statistically average species border (95%). Taking into account practically identical phenotypes, it can be concluded that all six AArcel cellulotrophic isolates belong to a single species.

Another marker, widely used for phylogenetic reconstructions of *Halobacteria*, is the RNA-polymerase subunit B′ gene [13]. The phylogenetic trees based on comparative analysis of *rpoB′* gene and RpoB′ protein sequences of the type species from *Natrialbaceae* revealed that AArcel5^T and AArcel2 formed a branch positioned separately from the closely related genera (Fig. 3b,c). The *rpoB′* gene sequences of AArcel5 and AArcel2 strains were 92.2% similar to each other and had 82.5–88.8% identity with the members of *Natrialbaceae* (Supplementary Table S1 and Fig. S2). Phylogenetic analysis of the *rpoB'*-gene of all validly published *Natrialbaceae* was done to reveal all inter- and intra-genus clustering (Supplementary Fig. S2). It showed that only *Halovivax*, *Halostagnicola*, *Natrialba*, *Natronococcus*, *Saliphagus* and *Salinarchaeum* genera formed monophyletic clusters. These genera are similar to AArcel5^T-AArcel2 in the intrageneric/intergeneric distances, calculated basing on the percentage of sequence identities (Supplementary Table S1): *Halovivax* 89.9–97.8%/82.4–89.6%; *Halostagnicola* 94.4%/81.2–89%; *Natrialba*

Please cite this article in press as: D.Y. Sorokin, et al., *Natronobiforma cellulositropha* gen. nov., sp. nov., a novel haloalkaliphilic member of the family *Natrialbaceae* (class *Halobacteria*) from hypersaline alkaline lakes, Syst. Appl. Microbiol. (2018), https://doi.org/10.1016/j.syapm.2018.04.002

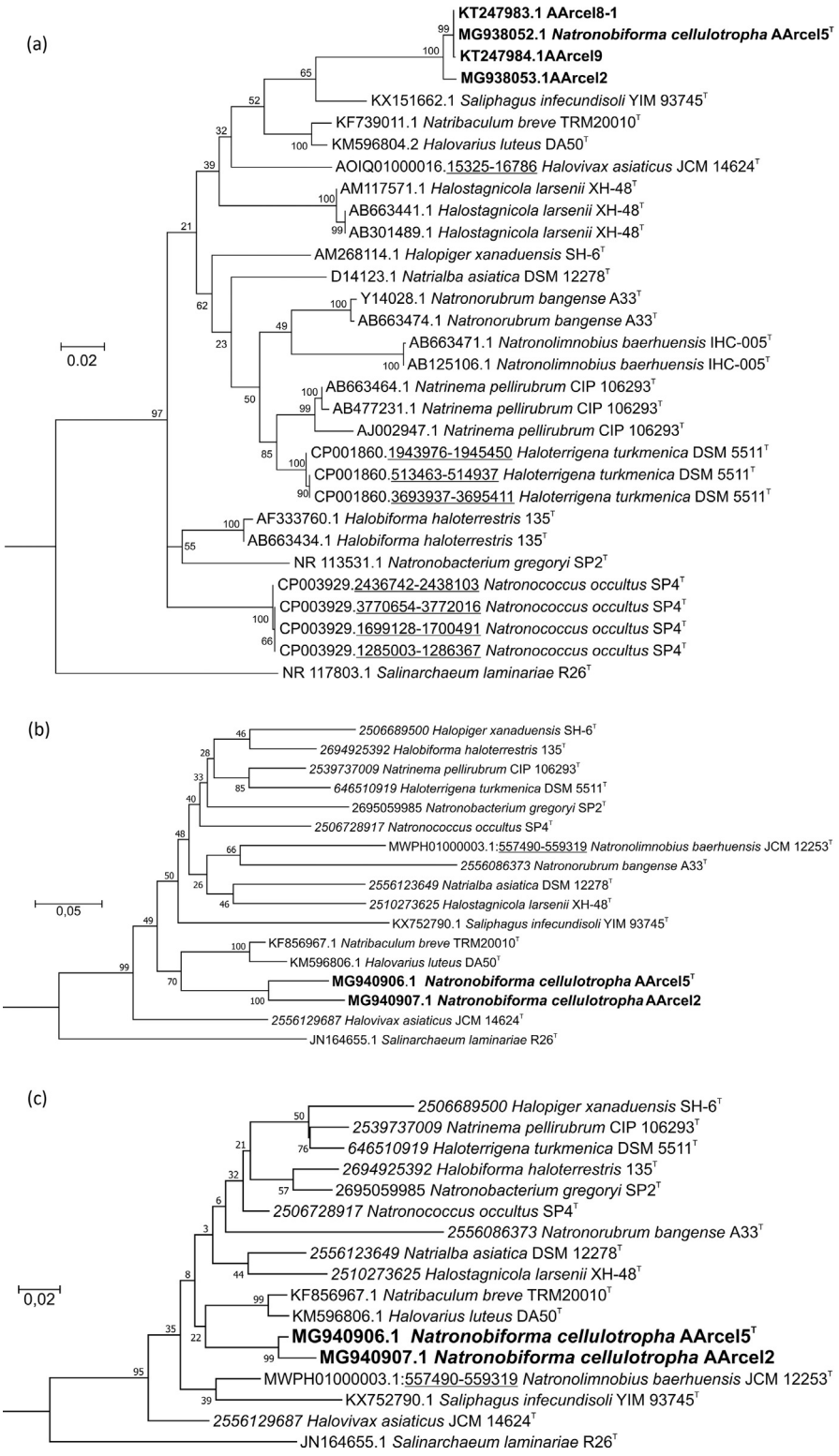

Fig. 3. Phylogeny of the AArcel strains. (a) Maximum Likelihood 16S rRNA gene sequence-based phylogenetic tree showing position of the AArcel strains (in bold) within the family *Natrialbaceae*. Branch lengths (see scale) correspond to the number of substitutions per site with corrections, associated 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 32 sequences (except for the partial AArcel4 and AArcel6 sequences, 100% identical to AArcel5^T). Numbers at nodes indicate bootstrap values of 1000 repetitions. *Halomarina oriensis* strain JCM 16495 (AB663390.1) was used as an outgroup. (b) Maximum Likelihood *rpoB'* gene sequence-based tree showing position of the AArcel2 and AArcel5 strains (in bold) within family *Natrialbaceae*. Totally 1827 positions were used in the alignment of 18 sequences. *Halomarina oriensis* JCM 16495 (KJ870934.1) was used as an outgroup. (c): Maximum Likelihood RpoB' protein sequence-based tree showing position AArcel2 and AArcel5 strains (in bold) within the family *Natrialbaceae*. Totally 608 positions were used in the alignment of 18 sequences. *Halomarina oriensis* JCM 16495 (KJ870934.1) was used as an outgroup. Sequences with accession numbers in italic were obtained from IMG, in roman – from the Genbank.

Please cite this article in press as: D.Y. Sorokin, et al., *Natronobiforma cellulositropha* gen. nov., sp. nov., a novel haloalkaliphilic member of the family *Natrialbaceae* (class *Halobacteria*) from hypersaline alkaline lakes, Syst. Appl. Microbiol. (2018), https://doi.org/10.1016/j.syapm.2018.04.002

Table 2
Comparative property of cellulotrophic natronoarchaea with the nearest phylogenetic relatives in *Natrialbaceae*: *Saliphagus infecundisoli* [25], *Natribaculum breve* [13] and *Halovivax asiaticus* [4]. PGP-Me – phosphatidylglycerophosphate methylester; PG – phosphatidylglycerols; GL-PG – phosphatidylglycose; 2GL – diglycosyl; PGS – phosphatidylglycerol sulfate; PGP – phosphatidylglycerophosphate; GL – glycolipid; PL – phospholipid; glycolipids: TGD-1 (galactosyl mannosyl glucosyl diether), S_2-DGD (disulfated mannosyl glucosyl diether). Antibiotics: s, streptomycin; k, kanamycin; a, ampicillin; t, tetracyclin; v, vancomycin; g, gentamycin; r, rifampicin;. e, erythromycin, c, chrolarmphenicol.

Property	"Natronobiforma cellulositropha" (6 strains)	*Saliphagus infecundisoli*	*Natribaculum breve*	*Halovivax asiaticus*
Cell morphology	Thin flat motile rods on cellobiose; cocci with thick cell wall on cellulose	Cocci	Motile pleomorphic rods	Pleomorphic nonmotile, from rods to discs
Pigmentation	Pink	Pink	Red	Pale-pink
Anaerobic growth	- (With cellobiose as substrate)	–	Contradictory[a]	–
Growth substrates:				
Polymers	Insoluble cellulose, xylane, chitin (3 strains), β-1,4 glucans and mannan	Starch, dextrin	Starch[b], gelatin hydrolysis	Proteolytic[c]
Sugars	Cellobiose, maltose	Glucose, mannose, raffinose, sucrose, trehalose	Glucose	Lactose, raffinose, xylose, trehalose
Amino acids	–	Glutamate, aspartate, ornithine, lysine		
Organic acids	–	Pyruvate, succinate	Pyruvate	Acetate
Esterase activity	–	Tween-20	–	Tween-80
Catalase/oxidase	+/+	+/+		+/Weak
Antibiotic resistance	s, k, a, t, v, g, e, p (50–100 mg l^{-1}) r, c (<50 mg l^{-1})	a, v, g, e, c, p (discs)	s, k, a, t, v, g, e, c, p, r (discs)	s, k, a, t, v, g, e, c, p (discs)
Salinity range (opt.) M Na^+	2.5–4.8 (4.0)	2–6 (2.5–3.0)	0.9–5.1 (2.6)	1.6–4.8 (2.5)
Mg requirement	Low	Low	Low	Low
pH range (opt.)	7.5–9.9 (8.5–9.0)	6.0–8.5 (7.0–7.5)	6.0–10[e] (7.0–7.5)	6.5–8.5
Temperature (°C)	18–53[d] (opt. 43)	25–50 (opt. 37)	30–62 (opt. 37)	25–45 (opt. 37)
Core lipids	C_{20}–C_{20}, C_{20}–C_{25}	nd	nd	C_{20}–C_{20}, C_{20}–C_{25}
Polar lipids	PG, PGP-Me, GL-PG, 2GL, PGP	PG, PGP-Me, PGS, three GL	PG, PGP-Me, TGD-1, S_2-DGD	PG, PGP-Me, two PL, four GL
G+C, mol%	65.4–65.5	64.4	63.9	60.3
Habitat	Hypersaline alkaline lakes (s–w Siberia, n–e Mongolia, California)	Saline soil (China)	Saline soil (China)	Hypersaline lake (Inner Mongolia)

nd – no data.
[a] It is stated that it grows anaerobically with nitrate, and next – that it can not reduce nitrate to nitrite or nitrite to N_2.
[b] Since this organism did not utilize maltose, its capability to grow with starch is questionable.
[c] Growth on polymers was not investigated.
[d] At pH 8.5.
[e] The final pH is not measured, the high pH limit is not justified.

Table 3
Natronobiforma cellulositropha: protologue.

Parameter		
Date created	2018-03-04	2018-03-04
Taxon number (TXNR)	TA00433	TA00433
Author (AUTE)	Dimitry Y. Sorokin	
Species name (SPNA)		*Natronobiforma cellulotropha*
Genus name (GENA)	*Natronobiforma*	
Specific epithet (SPEP)		*"Cellulotropha"* from *Natronobiforma cellulotropha*
Species status (SPST)		sp. nov.
Etymology (GETY/SPTY)	*Natronobiforma* (Na.tro.no.bi.for' ma Gr. neutral n. *natron*, arbitrarily derived from the Arabic n. *natrun* or *natron*, soda; L. adv. num. *bis*, *twice*; L. fem. n. *forma*, form, shape; N.L. fem. n. *Natronobiforma*, the dimorphic natronoarchaeon	*Cellulositropha* (cel.lu.lo.si.tro' pha N.L. n. *cellulosum*, cellulose; N.L. fem. n. from Gr. n. fem. *trophê*, nourishment, food; N.L. fem. adj. *cellulositropha*, utilizer of cellulose)
Authors (AUT)	Dimitry Y. Sorokin, Tatiana V. Khijniak, Nadezhda A. Kostrikina, Alexander G. Elcheninov, Stepan V. Toshchakov, Nicole J. Bale, Jaap S. Sinninghe Damsté, Ilya V. Kublanov	
Title (TITL)	*Natronobiforma cellulotropha* gen. nov., sp. nov., a novel haloalkaliphilic member of the family *Natrialbaceae* (class *Halobacteria*) 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)		AArcel5
Strain collection numbers (COLN)		JCM31939; UNIQEM U972
16S rRNA gene accession number (16 SR)		KT247980
Alternative house-keeping genes: gene [accession numbers] (HKGN)		*rpoB′* [MG940906]
Genome status (GSTA)		Draft
GC mol% (GGCM)		65.4–65.5 (genomes of AArcel5^T and AArcel2)
Country of origin (COUN)		Russian Federation

Please cite this article in press as: D.Y. Sorokin, et al., *Natronobiforma cellulositropha* gen. nov., sp. nov., a novel haloalkaliphilic member of the family *Natrialbaceae* (class *Halobacteria*) from hypersaline alkaline lakes, Syst. Appl. Microbiol. (2018), https://doi.org/10.1016/j.syapm.2018.04.002

Table 3 (*Continued*)

Region of origin (REGI)		Altai region
Date of isolation (DATI)		2013-08-15
Source of isolation (SOUR)	Surface sediments and brines of hypersaline alkaline lakes	Surface sediments from hypersaline soda lake Tanatar-1
Sampling dates (DATS)		2013-07-07
Geographic location (GEOL)	South Siberia, *N–E* Mongolia, California	*S–W* Siberia, Kulunda Steppe
Latitude (LATI)		51°39′N
Longtitude (LONG)		79°48′E
Depth (DEPT)		0.1 m
Temperature of the sample (TEMS)		25 °C
pH of the sample (PHSA)		11.0
Salinity of the sample (SALS)		40%
Number of strains in study (NSTR)	6	
Source of isolation of non-type strains (SAMP)	Hypersaline alkaline lakes in Russia, Mongolia and California	
Growth medium, incubation conditions (CULT)	Alkaline medim containing 4 M Na^+ with pH 9–9.5 and cellulose 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 cellulose or cellobiose as C and energy source
Conditions of preservation (PRES)	Deep freezing in 15% glycerol (v/v)	
Gram stain (GRAM)		Negative
Cell shape (CSHA)	Pleomorphic, from flat motile rods to nonmotile coccoid cells	
Cell size (CSZI)		0.5–0.8 μm in diameter, length is variable
Motility (MOTY)		Motile
Motility type (MOTK)		Flagellar
Type of flagellation (TFLA)		Variable, from single subpolar to several peretrichous flagella
Sporulation (SPOR)	None	
Colony morphology (COLM)		Pink, up to 2 mm
Temperature range for growth (TEMR)		20–53 °C
Lowest temperature for growth (TEML)		20
Highest temperature for growth(TEMH)		53
Optimal temperature for growth (TEMO)		43
Lowest pH for growth (PHLO)		7.5
Highest pH for growth (PHHI)		9.9
Optimum pH for growth (PHOP)		9.0
pH category (PHCA)	Alkaliphile (optimum > 8.5)	
Lowest NaCl concentration for growth (SALL)		2.5
Highest NaCl concentration for growth (SALH)		4.8
Optimum salt concentration for growth (SALO)		4.0
Other salts important for growth	Sodium carbonates	
Salinity category (SALC)	Extreme halophilic (optimum & gt; 15% NaCl)	
Relation to oxygene (OREL)	Aerobe	
O_2 conditions for strain testing (OCON)	Aerobic	
Carbon source used (class) (CSUC)	Carbohydrates	
Specific compounds (CSUC)	Cellulose, xylan, mannan, cellobiose, maltose	
Nitrogen source (NSOU)	Ammonium	
Terminal electron acceptor (ELAC)	O_2	
Energy metabolism (EMET)	Chemoorganotrophic	
Phospholipids (PHOS)	Core membrane lipids are archaeol (C20–C20 DGE) and C20–C25 DGE in equal proportion	Phosphatidylglycerophosphate methylester (PGP-Me), phosphatidylglycerol (PG), phosphatidylglycerol sulfate (PGS) and phosphatidylglycerophosphate (PGP)
Glycolipids (GLYC)		Phosphatidylglycose (GL-PG), diglycosyl (2GL)
Habitat (HABT)	Hypersaline alkaline lakes	
Extraordinary feautres (EXTR)	Growth with native insoluble cellulose	Fast growth with insoluble native celluloses; more than 30 GH glucosyl-hydrolases genes in the genome

88.6–98.6%/82–89%; *Natronococcus* 91.4–93%/82.4–90.8%; *Saliphagus* nd/81.5–87.1% and *Salinarchaeum* 92.4%/80.2–85.1%.

Furthermore, phenotypic comparison shows a clear physiological differentiation of the AArcel isolates from the three closest relatives in *Natrialbaceae* (Table 2).

In conclusion, the six AArcel strains isolated from hypersaline alkaline lakes represent a first example of natronoarchaea specialized in utilization of native insoluble celluloses as growth substrate. Taking into account their unique phenotypic properties and the phylogenetic distances (based on two conservative phylogenetic markers) from the nearest genera in *Natrialbaceae*, we propose to classify the group as a novel genus and species *Natronobiforma cellulotropha* with strain AArcel5 as the type strain. The novel genus and species protologue (diagnosis) is provided in Table 3.

Funding

This work was supported by the Russian Science Foundation (grant 16-14-00121) and by the Russian Academy of Sciences and Federal Agency of Scientific Organizations (project 0104-2018-0033). This project also received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No. 694569 – MICROLIPIDS).

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.2018.04.002.

References

[1] Andrei, A.S., Banciu, H.L., Oren, A. (2012) Living with salt: metabolic and phylogenetic diversity of archaea inhabiting saline ecosystems. FEMS Microbiol. Lett. 330, 1–9.
[2] Amoozegar, M.A., Siroosi, M., Atashgahi, S., Smidt, H., Ventosa, A. (2017) Systematics of haloarchaea and biotechnological potential of their hydrolytic enzymes. Microbiology 163, 623–645.

Please cite this article in press as: D.Y. Sorokin, et al., *Natronobiforma cellulositropha* gen. nov., sp. nov., a novel haloalkaliphilic member of the family *Natrialbaceae* (class *Halobacteria*) from hypersaline alkaline lakes, Syst. Appl. Microbiol. (2018), https://doi.org/10.1016/j.syapm.2018.04.002

[3] Bhatnagar, T., Boutaiba, S., Hacene, H., Cayol, J.-L., Fardeau, M.-L., Ollivier, B., Baratti, J.C. (2005) Lipolytic activity from *Halobacteria*: screening and hydrolase production. FEMS Microbiol. Lett. 248, 133–140.
[4] Castillo, A.M., Gutiérrez, M.C., Kamekura, M., Ma, Y., Cowan, D.A., Jones, B.E., Grant, W.D., Ventosa, A. (2006) *Halovivax asiaticus* gen. nov., sp. nov., a novel extremely halophilic archaeon isolated from Inner Mongolia, China. Int. J. Syst. Evol. Microbiol. 56, 765–770.
[5] Enache, M., Kamekura, M. (2010) Hydrolytic enzymes of halophilic microorganisms and their economic values. Rom. J. Biochem. 47, 47–59.
[6] Grant, B.D., Jones, B.E. (2016) Bacteria, archaea and viruses of soda lakes. In: Schagerl, M. (Ed.), Soda lakes of East Africa, Springer, Switzerland, pp. 97–147.
[7] Halophiles and hypersaline environments. Current research and future trends. Ventosa, A., Oren, A., Ma, Y. (Eds.) (2011) Springer, Heidelberg, Dordrecht, London, New York, 387 pp.
[8] Halophiles, Part III. In: Extremophiles Handbook, Horikoshi, K. (Ed.) (2011) vol. 1, Springer, Tokyo, pp. 255–402.
[9] Katoh, K., Rozewicki, J., Yamada, K.D. (2017) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinform, bbx108.
[10] Li, T.X., Yu, H.-Y. (2013) Halostable cellulase with organic solvent tolerance from *Haloarcula* sp. LLSG7 and its application in bioethanol fermentation using agricultural wastes. J. Ind. Microbiol. Biotechnol. 13, 1357–1365.
[11] Li, X., Yu, H.-Y. (2013) Characterization of a halostable endoglucanase with organic solvent-tolerant property from *Haloarcula* sp. G10. Int. J. Biol. Macromol. 62, 101–106.
[12] Liu, Q., Ren, M., Zhang, L.-L. (2015) *Natribaculum breve* gen. nov., sp. nov. and *Natribaculum longum* sp. nov., halophilic archaea isolated from saline soil. Int. J. Syst. Evol. Microbiol. 65, 604–608.
[13] Minegishi, H., Kamekura, M., Itoh, T., Echigo, A., Usami, R., Hashimoto, T. (2011) Further refinement of the phylogeny of the *Halobacteriaceae* based on the full-length RNA polymerase subunit B′ (*rpo*B′) gene. Int. J. Syst. Evol. Microbiol. 60, 2398–2408.
[14] Moshfegh, A.R.M., Shahverdi, G., Zarrini, M.A. (2013) Faramarzi Biochemical characterization of an extracellular polyextremophilic α-amylase from the halophilic archaeon *Halorubrum xinjiangense*. Extremophiles 17, 677–687.
[15] Nei, M., Kumar, S. 2000 Molecular Evolution and Phylogenetics, Oxford University Press, New York.
[16] Oren, A. (2015) Halophilic microbial communities and their environments. Curr. Opin. Biotechnol. 33, 119–124.
[17] Oren, A. (2013) Life at high salt concentrations. In: Rosenberg, E., et al. (Eds.), The Prokaryotes. Ecophysiology and Biochemistry, 4th ed., Springer, NewYork, pp. 429–440.
[18] Pfennig, N., Lippert, K.D. (1966) Über das Vitamin B12-Bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. 55, 245–256.
[19] Selim, S., Hagagy, N., Azis, M.A., Meleigy, T.S., Pessione, E. (2014) Thermostable alkaline halophilic-protease production by *Natronolimnobius innermongolicus* WN18. Nat. Prod. Res. 28, 1476–1479.
[20] Sinninghe Damsté, J.S., Rijpstra, W.I.C., Hopmans, E.C., Jung, M.Y., Kim, J.G. (2012) Intact polar and core glycerol dibiphytanyl glycerol tetraether lipids of group I. 1a and I. 1b *Thaumarchaeota* in soil. Appl. Environ. Microbiol. 78, 6866–6874.
[21] Sorokin, D.Y. (2005) Is there a limit for high-pH growth? Int. J. Syst. Evol. Microbiol. 55, 1405–1406.
[22] Sorokin, D.Y., Toschakov, S.V., Kolganova, T.V., Kublanov, I.V. (2015) Halo(natrono)archae isolated from hypersaline lakes utilize cellulose and chitin as growth substrates. Front. Microbiol. 6, article 942.
[23] Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
[24] Weijers, J.W.H., Panoto, E., van Bleijswijk, J., Schouten, S., Balk, M., Stams, A.J.M., Rijpstra, W.I.C., Sinninghe Damsté, J.S. (2009) Constraints on the biological source(s) of the orphan branched tetraether membrane lipids. Geomicrobiol. J. 26, 402–414.
[25] Yin, X.-Q., Liu, B.-B., Chu, X., Salam, N., Li, X., Yang, Z.-W., Zhang, Y., Xiao, M., Li, W.-J. (2018) *Saliphagus infecundisoli* gen. nov., sp. nov., an extremely halophilic archaeon isolated from a saline soil. Int. J. Syst. Evol. Microbiol. 67, 4154–4160.
[26] Zhang, S., Datta, J., Eichler, N., Ivanova, S.D., Axen, C.A., Kerfeld, E. (2011) Rubin Identification of a haloalkaliphilic and thermostable cellulose with improved ionic liquid tolerance. Green Chem. 13, 2083–2090.

Please cite this article in press as: D.Y. Sorokin, et al., *Natronobiforma cellulositropha* gen. nov., sp. nov., a novel haloalkaliphilic member of the family *Natrialbaceae* (class *Halobacteria*) from hypersaline alkaline lakes, Syst. Appl. Microbiol. (2018), https://doi.org/10.1016/j.syapm.2018.04.002