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Genus

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Natronobiforma

Sorokin et al. 2019, VL187

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Na.tro.no.bi.for'ma. Gr. neut. n. *natron* arbitrarily derived from 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.

The genus *Natronobiforma*, classified within the family *Natrialbaceae*, order *Natrialbales*, in the class *Halobacteria*, currently consists of a single species, *Natronobiforma cellulositropha*. It is a moderately alkaliphilic, obligately aerobic, extreme halophile, forming pink colonies with large clearance zones on plates containing amorphous cellulose. The cells are pleomorphic flat motile rods or nonmotile coccoid cells. Multiple strains classified within this genus were isolated from alkaline hypersaline lakes in different locations. They grow optimally on insoluble native celluloses. Xylan, β-mannan, cellobiose, and maltose can also be used as carbon and energy sources. Other organic compounds used by most members of the *Halobacteria* do not support growth.

DNA G+C content (mol%): 65.4–65.5 (genome sequences).

Type species: ***Natronobiforma cellulositropha*** Sorokin et al. 2019, VL187.

Cells are pleomorphic, flat motile rods, or nonmotile coccoid. **Colonies are pink**. Motile cells have a single subpolar flagellum or several peritrichous flagella. **Fastest growth is obtained with insoluble native celluloses or cellobiose as carbon and energy source and ammonium ions as nitrogen source. Xylan, β-mannan, and maltose and maltose are also utilized.** Acid is produced during growth with both polymeric and soluble sugars. **Obligately aerobic**; no growth is observed anaerobically in the presence of arginine, nitrate, dimethyl sulfoxide, elemental sulfur, or fumarate. **All isolates are extremely halophilic and obligately alkaliphilic**. Polar membrane lipids are $C_{20}C_{20}$ and $C_{20}C_{25}$ glycerol diether derivatives of phosphatidylglycerol phosphate, phosphatidylglycerol, phosphatidylglycerophosphate, phosphatidylglycerophosphate methyl ether, phosphatidylglycoside, glycosyl phospholipid, and diglycosyl phospholipid.

DNA G+C content (mol%): 65.4–65.5 (genome sequences).

Type species: *Natronobiforma cellulositropha* Sorokin et al. 2019, VL187.

Number of species with validated names: 1.

Family classification: The genus *Natronobiforma* is classified within the family *Natrialbaceae* and the order *Natrialbales* in the class *Halobacteria*.

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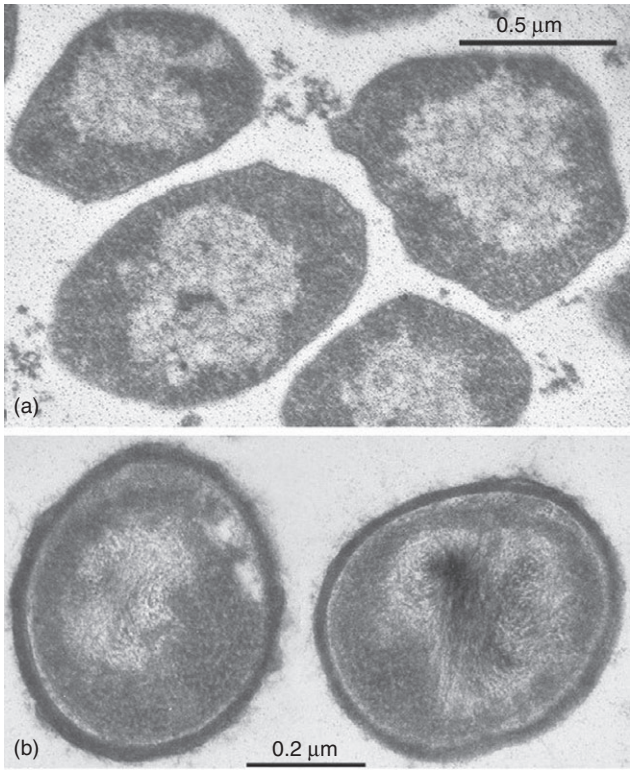
Further descriptive information

The genus *Natronobiforma* differs from nearly all other described genera of the class *Halobacteria* by its preference for cellulose as carbon and energy source and its inability to grow on many simple sugars and other soluble organic compounds (organic acids, peptone, and yeast extract) that are the preferred growth substrates for most other members of the class (Andrei et al., 2012). Although the presence of genes encoding putative cellulases has been noted in several haloarchaeal genomes, the presence of functional endoglucanases was earlier demonstrated only in two genera of neutrophilic haloarchaea: *Haloarcula* and *Halorhabdus* (Li and Yu, 2013a, 2013b; Zhang et al., 2011), but not in any alkaliphilic haloarchaea. The six *Natronobiforma* strains isolated from different alkaline hypersaline lakes are the first alkaliphilic extremely halophilic Archaea described to specialize in the utilization of cellulose (Sorokin et al., 2015, 2018). All isolates also grew well using birch and beech wood xylanes and cellobiose. No growth was observed on carboxymethylcellulose. During growth on cellulose, no accumulation of reducing sugars could be demonstrated in the medium, showing that the cellulose degradation products formed during growth in the medium are efficiently taken up by the cells. The *Natronobiforma* cellulases are exclusively cell-bound. In spite of the use of media strongly buffered with carbonates, a drop in pH was observed during growth, showing that the metabolism of the degradation products of cellulose is accompanied by acid production.

On agarose plates containing amorphous cellulose as growth substrate, all strains formed pin-point pink colonies with large cellulose clearance zones around them after 4–6 weeks of incubation, indicative of cellulose hydrolysis. Cells aggregated with the cellulose particles in liquid medium and cells grown on plates with amorphous cellulose were nonmotile cocci covered with a thick electron dense external layer, while the free suspended cells in the second growth phase of massive cellulose degradation were dominated by motile thin flat rods with a thin cell wall (Figure 1).

In addition to cellulose, all isolates were found to grow on xylan (from birch and beech) and on barley β-glucan. Some strains, including the type strain of *Natronobiforma cellulositropha*, also slowly grew on lichenan, glucomannan, and β-1,4-mannan. The artificial soluble analogue carboxymethylcellulose is not used as growth substrate. α-Glucans such as starch and starch-like polymers, are not utilized. The only simple sugars shown to support growth are dimers cellobiose and, to a lesser extent, maltose. Other simple compounds

FIGURE 1. Thin-section electron microphotographs showing cell ultrastructure of *Natronobiforma cellulositropha* AArcel5^T grown with amorphous cellulose: (a) free cells with a thin cell wall from the stage of massive cellulose degradation and (b) cells attached to cellulose with a thick extracellular matrix layer.



such as glycerol and mannitol; organic acids such as acetate, lactate, and pyruvate; amino acids such as glutamate and aspartate; peptones; and yeast extract are not used as growth substrates. Ammonium is used as nitrogen source; nitrate and urea are not.

Fermentative growth with arginine, cellobiose, or maltose does not occur. Anaerobic respiration with nitrate, sulfur, fumarate, or dimethylsulfoxide as electron acceptors and cellobiose as the energy source was not observed.

The genomes of AArcel2 and AArcel5^T have been sequenced, but the sequences are not yet released. The genomes have the size of 3,732,973 and 3,782,872, containing 3,776 and 3,860 genes encoding 3,583 and 3,632 proteins, respectively. The genomes contain multiple genes encoding proteins of the glycosyl-hydrolases family, including GH5 (17–18 copies) and GH9 (4 copies; not found previously in any archaeal genomes) encoding putative cellulases, and GH10 (5–6 copies) encoding putative endo-xylanases.

Enrichment and isolation procedures

Isolates of *Natronobiforma cellulositropha* were enriched from surface sediments and near-bottom brines from various hypersaline alkaline inland lakes with salt concentration of 200–400 g/l, pH 9.3–11, and soluble carbonate alkalinity of 0.1–4 M. The type strain AArcel5^T was obtained from lake Tanatar-1, Kulunda Steppe, Altai region, SW Siberia, by enrichment on cellulose (20 µm, Sigma). Strain AArcel2 was recovered from Bitter-1 Lake, Kulunda Steppe, Altai, Russia, by enrichment on amorphous cellulose. Strain AArcel4 was obtained from a soda crystallizer (Tanatar system) using Avicel 101 as the substrate (Sigma); strain AArcel9 was enriched with filter paper slurry from a mixed inoculum from three Kulunda Steppe lakes; strains AArcel6 and AArcel8-1 came from Shar-Burdiin, Hotontyn (NE Mongolia) and from Owens Lake in California, respectively, both following enrichment on amorphous cellulose.

Enrichment media contained a total of 4 M Na^+ as an equal mix of sodium carbonates and NaCl on the basis of Na^+ molarity, pH 9.5, and was supplemented with 1–2 g/l of various forms of insoluble cellulose with different degrees of crystallinity. The sodium carbonate base included (g/l): Na_2CO_3, 185; $NaHCO_3$, 45; NaCl, 16; and K_2HPO_4, 1.0, pH 10–10.1 after sterilization. The NaCl base contained (g/l): NaCl, 240; K_2HPO_4, 2.5; NH_4Cl, 0.4; and $(NH_4)_2SO_4$, 0.1 (pH adjusted to 7.0). Both bases also included KCl (5 g/l), and after sterilization they were supplemented with $MgCl_2$ (1 mM), yeast extract (20 mg/l), trace metal solution, and vitamin mix, added from separate sterile solutions (Sorokin et al., 2015). The ready-to-use medium was prepared by 1:1 mixing of the two bases (pH 9.5). Addition of streptomycin (200 mg/l) served to inhibit growth of bacteria. Before inoculation, the sediments were resuspended 1:10 in the basic medium, and after 5–10 min settling 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 at 120 rpm. The development of cells was monitored by the visual extent of cellulose degradation, appearance of a pink color, and by microscopy.

Solid medium was prepared by mixing three parts of liquid medium and two parts of 5% extensively washed agar at 55°C. Before mixing, the liquid medium was supplemented with solid NaCl to compensate for dilution with agar to bring the salinity up to 4 M of total Na^+. Amorphous cellulose was added from 5% stock at final concentration of 1 g/l. Growth on plates is slow, and visible cellulose degradation and colony formation becomes evident after 2–6 weeks incubation in closed plastic bags. Clearance zones are observed around cellulolytic colonies. Pure cultures are isolated by combining iterative dilutions to extinction with colony purifications, because dilutions alone did not exclude growth of contaminating nonhydrolytic archaea fed by scavenging soluble hydrolysis products.

Maintenance procedures

Strains of *Natronobiforma cellulosilytica* can be preserved by deep-freezing at 80°C in growth medium supplemented with 15% glycerol (v/v) as cryoprotectant. Active cultures remained viable at 4–10°C up to 2 months.

Differentiation from closely related genera

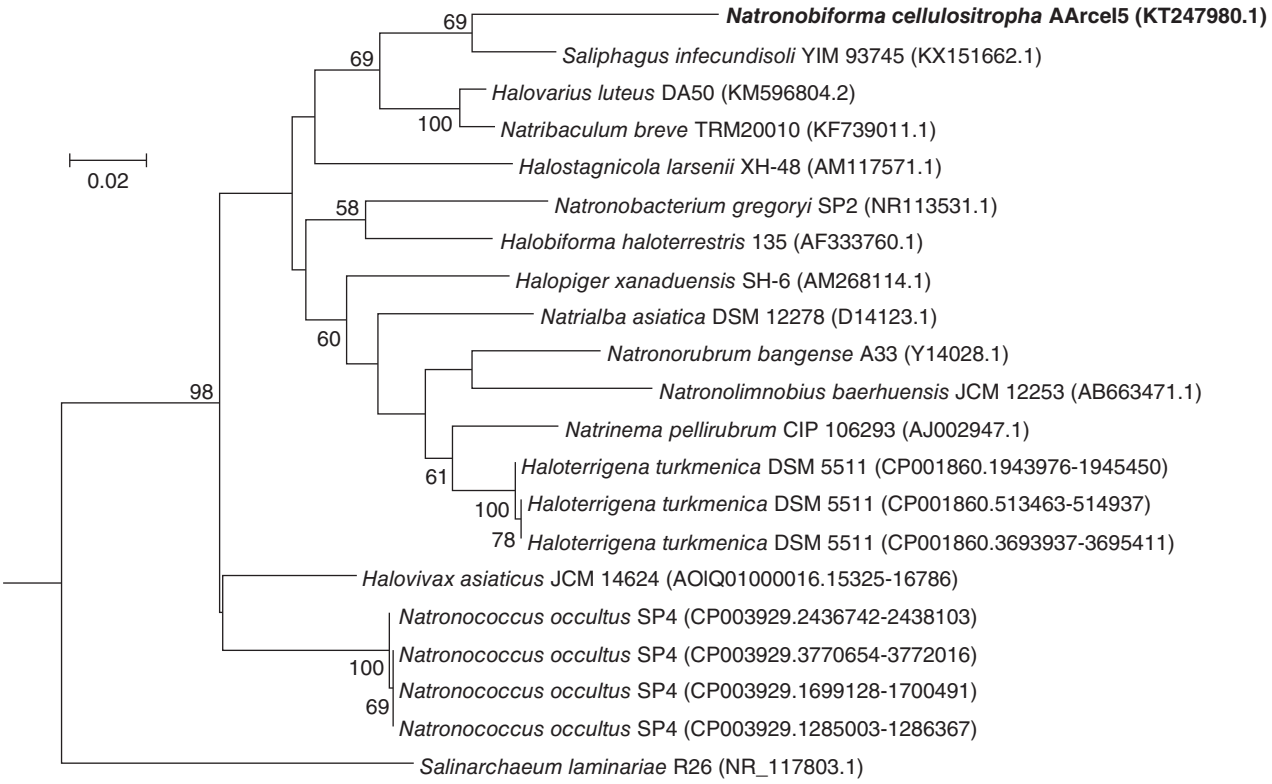
On the basis of the phylogenetic analysis of different conservative markers (16S rRNA gene, RpoB', and ribosomal proteins, Sorokin et al., 2018), the genus *Natronobiforma* belongs to the family *Natrialbaceae*. The nearest relatives are genera *Saliphagus* (one species), *Natribaculum* (two species), and *Halovarius* (one species) (Figure 2). Table 1 shows the main differences between the single species of the genus *Natronobiforma* and the type species of the related genera from the *Natrialbaceae* family.

Whether the closest relatives are also able to grow on cellulose on which *Natronobiforma cellulositropha* grows best was never tested. However, the available genomes from those genera lack the genetic determinants for cellulose degradation. *Natronobiforma* is the only alkaliphile in this group. Whether sustained growth of *Natribaculum breve* (optimum growth at pH 7.0–7.5) is also possible at pH 10, reported as the highest pH for growth, remains to be ascertained, since the medium used was not properly buffered and the final pH was not monitored.

Taxonomic comments

The genus *Natronobiforma* (Sorokin et al., 2018, 2019) was described as a member of the family *Natrialbaceae*, order *Natrialbales* (Gupta et al., 2015). On the basis of the 16S rRNA gene sequence phylogenetic analysis, its closest relatives are the genera *Saliphagus*, *Halovarius*, *Natribaculum*, and *Halovarius*. Trees based on sequences of the *rpoB'* gene and the RpoB' protein confirm a close relationship with the abovementioned genera *Natribaculum* and *Halovarius*, the genus *Saliphagus* being more distantly related (Sorokin et al., 2018).

FIGURE 2. Maximum-Likelihood 16S rRNA gene-based tree showing phylogentic position of *Natronobiforma cellulositropha* AArcel5^T (in bold) within the family *Natrialbaceae* (only type strains are included). Branch lengths (see scale) correspond to the number of substitutions per site with corrections, associated with the model (GTR, G + *I*, 4 categories). Numbers at nodes indicate bootstrap values of 1,000 repetitions, values below 50% are not shown. The 16S rRNA gene of *Halomarina oriensis* KeC-11^T (AB519798) was used as an outgroup.



The six isolates of the single species share >99.1% 16S rRNA gene sequence identity. The genome of strain AArcel2, the isolate with the lowest 16S rRNA sequence identity with the type strain AArcel5^T, shows 95% Average Nucleotide Identity with the type strain, which is close to the statistically accepted average species border.

The protolog in the species description (Sorokin et al., 2018) erroneously gave the name *Natronobiforma cellulotropha* for the type species. The name was later corrected to *Natronobiforma cellulositropha* (Sorokin et al., 2019).

List of species of the genus *Natronobiforma*

Natronobiforma cellulositropha Sorokin et al. 2019, VL187

cel.lu.lo.si.tro'pha. N.L. neut. n. *cellulosum* cellulose; Gr. n. *trophein* to feed; N.L. fem. adj. *cellulositropha* feeding on cellulose.

Cells are pleomorphic, flat motile rods or nonmotile coccoid, measuring 0.5–0.8 µm in diameter, and with variable length, and stain Gram-negative. Colonies are pink, up to 2 mm in diameter. Motile cells have a single subpolar flagellum or several peritrichous flagella. Fastest growth is obtained with insoluble native celluloses and cellobiose as carbon and energy source and ammonium ions as nitrogen source. Xylan, β-mannan, and maltose are also used. Acid is produced from sugars. Anaerobic growth was never observed. Isolates are extremely halophilic, growing at 2.4–4.8 M NaCl with an optimum at 4.0 M and alkaliphilic (pH range for growth is 7.5–9.9 with an optimum at 8.5–9.0); sodium carbonates are required for growth. The temperature range for growth is 18–53°C, with an optimum at 43°C. The core membrane lipids are $C_{20}C_{20}$ archaeol and $C_{20}C_{25}$ glycerol diether (extended archaeol) in equal proportion. Traces of 1-C_{20}, 2-C_{20}, and 2-C_{25} monoglycerol ethers were also detected. The intact

TABLE 1. Characteristics differentiating the genus *Natronobiforma* from the closest relatives[a]

Characteristics	*Natronobiforma*	*Halovarius*	*Natribaculum*	*Saliphagus*
Cell morphology	Thin flat rods or cocci	Pleomorphic	Pleomorphic rods	Cocci
Pigmentation	Pink	Orange	Red to pale red	Yellowish-pink
Motility	+	+	+	−
NaCl range and optimum (M)	2.4–4.8 (4.0)	2.5–5.0 (4.0)	0.9–5.1 (2.6–3.4)	0.9–5.2 (2.6–3.4)
pH range and optimum	7.5–9.9 (8.5–9.0)	6.5–8.0 (7.0)	6.0–10.0 (7.0–7.5)	6.0–8.5 (7.0–7.5)
Temperature range and optimum	18–53 (43)	25–50 (45)	30–62 (37)	25–50 (37)
Oxidase	+	+	+	+
Compounds used as carbon and energy sources by all species the genus	Cellulose, xylane, β-1,4-glucans, β-1,4-mannan, cellobiose, and maltose	D-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, maltose, sucrose, and glycerol D-mannitol	D-glucose and pyruvate	D-glucose, D-mannose, sucrose, D-sorbitol, L-aspartate, L-glutamate, and L-lysine
Compounds not used as carbon and energy source	Arabinose, fructose, galactose, glucose, lactose, mannose, rhamnose, ribose, xylose, maltose, sucrose, melibiose, trehalose, melezitose, raffinose, glycerol, mannitol, sorbitol, glucosamine, glucuronic acid, fumarate, lactate, malate, pyruvate, and succinate	D-ribose, D-xylose, lactose trehalose	D-fructose, L-sorbose, D-xylose, lactose, maltose, sucrose, glycerol, D-mannitol, D-sorbitol, acetate, citrate. fumarate, DL-lactate, L-alanine, L-arginine, L-glutamate, L-lysine, and L-ornithine	D-xylose, D-mannitol, glycerol
Hydrolysis of starch	−	−	+	+
Hydrolysis of cellulose	+	NR	NR	NR
Major glycolipids[b]	Phosphatidylglycoside And phosphatidyldiglycoside	Four unidentified glycolipids	TGD-1 and S2-DGD	S-DGD and two unidentified glycolipids
DNA G + C content (mol%)	65.4–65.5 (genomes)	62.3 (HPLC)	63.8–63.9 (T_m)	64.4–64.6 (HPLC)

[a]Data taken from Mehrshad et al. (2015), Liu et al. (2015), and Yin et al. (2017).
[b]S-DGD = a sulfated diphytanyl diether lipid; S2-DGD = glycolipid chromatographically identical to bis-sulfated mannosyl glucosyl diether lipid; TGD-1 = glycolipid chromatographically identical to galactosyl mannosyl glucosyl diether lipid.

polar lipids include phosphatidylglycerophosphate, phosphatidylglycerol, phosphatidylglycerophosphate methyl ester, and two glycolipids—phosphatidylglycoside and phosphatidyldiglycoside derivatives of glycerol diethers (Table 2).

Source: The type strain was isolated from surface sediments of the hypersaline soda lake Tanatar-1, Kulunda Steppe, Altai region, SW Siberia. Additional strains were obtained from other hypersaline alkaline lakes in Russia, Mongolia, and California.

DNA G+ C content (mol%): 65.4–65.5 (genomes of strains AArcel5^T and AArcel2).

Type strain: AArcel5 (JCM 31939, UNIQEM U972).

GenBank/EMBL/DDBJ accession number (16S rRNA gene): KT247980.

TABLE 2. Characteristics of the single species of the genus *Natronobiforma*[a]

Characteristics	*Natronobiforma cellulositropha*
Cell shape	Pleomorphic, from flat motile rods to nonmotile coccoid cells
Cell size	0.5–0.8 µm in diameter, length variable
Motility	Variable; motile cells may have a single subpolar flagellum or several peritrichous flagella
Colonies on agar	Pink, up to 2 mm in diameter
Gram stain	Negative
Relation to oxygen	Aerobic
Catalase reaction	+
Oxidase reaction	+
Anaerobic growth with NO_3^-	–
Reduction of NO_3^- to NO_2^- or to gaseous products	–
Anaerobic growth with DMSO	–
Anaerobic growth with arginine	–
Salt concentration range for growth	2.4–4.8 M NaCl (optimum: 4.0 M); sodium carbonates are also required
$MgCl_2$ requirement for growth	Low
Temperature range for growth	18–53°C (optimum: 43°C)
pH range for growth	7.5–9.9 (optimum: 8.5–9.0)
Growth substrates	Insoluble native celluloses, xylan, mannan, cellobiose, and maltose; some strains degrade chitin
Substrates not supporting growth	Arabinose, fructose, galactose, glucose, lactose, mannose, rhamnose, ribose, xylose, maltose, sucrose, melibiose, trehalose, melezitose, raffinose, glycerol, mannitol, sorbitol, glucosamine, glucuronic acid, fumarate, lactate, malate, pyruvate, and succinate
Acid formation from carbohydrates	+
Starch hydrolysis	–
Resistant to:	Ampicillin, erythromycin gentamicin, kanamycin, penicillin, streptomycin, tetracycline, vancomycin (50–100 mg/l); chloramphenicol, and rifampicin (<50 mg/l)
Polar lipids	$C_{20}C_{20}$ and $C_{20}C_{25}$ glycerol diether derivatives of phosphatidylglycerol phosphate, phosphatidylglycerol, phosphatidylglycerophosphate, phosphatidylglycerophosphate methyl ether, phosphatidylglycoside, and phosphatidyldiglycoside
DNA G + C content (mol%)	65.4–65.5 (genomes of strains AArcel5^T and AArcel2)
Habitat	The type strain was isolated from surface sediments from the hypersaline soda lake Tanatar-1, Kulunda Steppe, S–W Siberia. Additional isolates were obtained from other hypersaline alkaline lakes in Russia, Mongolia, and California

[a]Sorokin et al. (2018). Reproduced with permission of Elsevier.

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