

**Delft University of Technology** 

Natronospira bacteriovora sp. nov., and Natronospira elongata sp. nov., extremely salttolerant predatory proteolytic bacteria from soda lakes and proposal to classify the genus Natronospira into Natronospiraceae fam. nov., and Natronospirales ord. nov., within the class Gammaproteobacteria

Sorokin, Dimitry Y.; Merkel, Alexander Y.; Kolganova, Tatyana V.; Bale, Nocile J.; Sinninghe Damsté, Jaap S.

# **DOI** 10.1016/j.syapm.2024.126519

Publication date 2024 Document Version

Final published version

## Published in

Systematic and Applied Microbiology

## Citation (APA)

Sorokin, D. Y., Merkel, A. Y., Kolganova, T. V., Bale, N. J., & Sinninghe Damsté, J. S. (2024). Natronospira bacteriovora sp. nov., and Natronospira elongata sp. nov., extremely salt-tolerant predatory proteolytic bacteria from soda lakes and proposal to classify the genus Natronospira into Natronospiraceae fam. nov., and Natronospirales ord. nov., within the class Gammaproteobacteria. *Systematic and Applied Microbiology*, *47*(4), Article 126519. https://doi.org/10.1016/j.syapm.2024.126519

### Important note

To cite this publication, please use the final published version (if applicable). Please check the document version above.

Copyright

Other than for strictly personal use, it is not permitted to download, forward or distribute the text or part of it, without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license such as Creative Commons.

Takedown policy

Please contact us and provide details if you believe this document breaches copyrights. We will remove access to the work immediately and investigate your claim.



Contents lists available at ScienceDirect

# Systematic and Applied Microbiology



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

# Natronospira bacteriovora sp. nov., and Natronospira elongata sp. nov., extremely salt-tolerant predatory proteolytic bacteria from soda lakes and proposal to classify the genus Natronospira into Natronospiraceae fam. nov., and Natronospirales ord. nov., within the class Gammaproteobacteria

Dimitry Y. Sorokin<sup>a, c, \*</sup>, Alexander Y. Merkel<sup>a</sup>, Tatyana V. Kolganova<sup>b</sup>, Nicole J. Bale<sup>d</sup>, Jaap S. Sinninghe Damsté<sup>d</sup>

<sup>a</sup> Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia

<sup>b</sup> Skryabin Insitutute of Bioengineering, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia

<sup>c</sup> Department of Biotechnology, TU Delft, The Netherlands

<sup>d</sup> Department of Marine Microbiology and Biogeochemistry, NIOZ Royal Netherlands Institute for Sea Research, and Utrecht University, Den Burg, Texel, The Netherlands

ARTICLE INFO

Keywords: Natronospira Predatory Proteolytic Haloalkaliphilic Soda lakes

### ABSTRACT

The genus Natronospira is represented by a single species of extremely salt-tolerant aerobic alkaliphilic proteolytic bacterium, isolated from hypersaline soda lakes. When cells of Gram-positive cocci were used as a substrate instead of proteins at extremely haloalkaline conditions, two new members of this genus were enriched and isolated in pure culture from the same sites. Strains AB-CW1 and AB-CW4 are obligate aerobic heterotrophic proteolytic bacteria able to feed on both live and dead cells of staphylococci and a range of proteins and peptides. Similar to the type species, N. proteinivora, the isolates are extremely salt-tolerant obligate alkaliphiles. However, N. proteinivora was unable to use bacterial cells as a substrate. Electron microscopy showed direct contact between the prey and predator cells. Functional analysis of the AB-CW1 and AB-CW4 genomes identified two sets of genes coding for extracellular enzymes potentially involved in the predation and proteolysis, respectively. The first set includes several copies of lysozyme-like GH23 peptidoglycan-lyase and murein-specific M23 [Zn]-dipeptidase enabling the cell wall degradation. The second set features multiple copies of secreted serine and metallopeptidases apparently allowing for the strong proteolytic phenotype. Phylogenomic analysis placed the isolates into the genus Natronospira as two novel species members, and furthermore indicated that this genus forms a deep-branching lineage of a new family (Natronospiraceae) and order (Natronospirales) within the class Gammaproteobacteria. On the basis of distinct phenotypic and genomic properties, strain AB-CW1<sup>T</sup> (JCM 335396 = UQM 41579) is proposed to be classified as *Natronospira elongata* sp. nov., and AB-CW4<sup>T</sup> (JCM 335397 = UQM 41580) as Natronospira bacteriovora sp. nov.

#### Introduction

Hypersaline soda lakes with salt concentration reaching saturation represent an unique type of inland salt lakes with molar concentrations of sodium carbonate/bicarbonate as a soluble alkalinity buffer, maintaining stable pH values at ca. 9.5–11. In contrast to chloride/sulfate hypersaline lakes and solar salterns, soda lakes are often characterized by a dense population of primary producers, including haloalkaliphilic cyanobacteria and unicellular algae, and by a highly productive and functionally diverse prokaryotic community (Krienitz and Schagerl, 2016; Oduor and Schagerl, 2007; Samylina et al., 2014). The functional microbial diversity of the soda lake communities in these unique habitats has been studied by both intensive culturing and phenotypic characterization of pure cultures and molecular biology studies over the past 20 years, fueled by both fundamental interest in life at double extreme conditions (reviewed by Sorokin, 2017; Sorokin et al., 2014; 2015; Grant and Jones, 2016) and the biotechnological potential of alkali-stable hydrolytic enzymes (Fujinami and Fujisawa, 2010; Sarethy et al.,

\* Corresponding author at: Winogradsky Institute of Microbiology, Research Centre of Biotechnology, Russian Academy of Sciences, Moscow, Russia; Department of Biotechnology, TU Delft, The Netherlands.

https://doi.org/10.1016/j.syapm.2024.126519

Received 20 March 2024; Received in revised form 14 May 2024; Accepted 14 May 2024 Available online 15 May 2024

0723-2020/© 2024 The Author(s). Published by Elsevier GmbH. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

E-mail addresses: soroc@inmi.ru, d.sorokin@tudelft.nl (D.Y. Sorokin).



**Fig. 1.** Colony (**a**-**b**) and cell morphology (**c**-**f**) of predatory *Natronospira* strains AB-CW1 (right column) and AB-CW4 (left column) grown pH 9.5, 2 M total Na<sup>+</sup> and 37 °C. (**a** and **b**), surface colonies forming clearance lytic plaques on a solid medium with *Staphylococcus* cells, scale bar 5 mm; (**c** and **d**) electron microscopy microphotographs of cells grown on peptone; (**e** and **f**) electron microscopy microphotographs from cultures grown with *Staphylococcus* cells.

#### 2011; Uma et al., 2020).

However, proteolytic prokaryotes from these soda lake environments are poorly represented in these studies. Only a single dedicated proteolytic anaerobe has ever been obtained in pure culture from soda lakes represented by a haloalkaliphilic *Bacillota* member, *Proteinivorax tanatarense* (Kevbrin et al., 2013). Aerobic proteolytic bacteria isolated from soda lakes include extremely salt-tolerant alkaliphilic *Bacteroidota* members, *Natronotalea proteinilytica* and *Longimonas alkaliphilus*, and a member of the *Gammaproteobacteria* – *Natronospira proteinivora* (Sorokin et al., 2017; Sorokin and Merkel, 2022). These isolates were not capable of microbial cell predation, although *Proteinivorax* originated from an enrichment with decaying cyanobacterial biomass. Recently, we used cells of Gram-positive cocci (possessing thick murein cell wall) to enrich potentially biomass-degrading aerobic proteolytic bacterial predators from hypersaline soda lakes at pH 10. The enrichments at low salt (0.6 M total Na<sup>+</sup>) resulted in isolation of a moderately salt-tolerant proteolytic alkaliphile *Wenzhouxiangella* sp. AB-CW3, a gammaproteobacterium capable of killing its prey by producing peptide-based lantibiotic, which is unusual for Gram-negative bacteria, and lyzing the prey cells in direct contact using its abundant extracellular proteolytic complex (Sorokin et al., 2020). Similar type of enrichments but at higher salinity (2 and 4 M total Na<sup>+</sup>) selected for two extremely salt-tolerant alkaliphilic proteolytic strains belonging to the genus *Natronospira* able to use bacterial cells as food, apparently using not only their proteases but also peptidoglycan hydrolases.

Here we describe the phenotypic and genomic properties of these

bacteria and propose to classify them as two new species of the genus *Natronospira*, which, in turn, is suggested to form a new family and order in the class *Gammaproteobacteria*.

#### Materials and methods

#### Inoculum and enrichment conditions

The upper 1 cm of oxic sediments and near-bottom brines from four hypersaline soda lakes were obtained from the south of Kulunda Steppe (Altai region, Russia) in July 2022. The salt concentration of the brines ranged from  $250 - 430 \text{ g l}^{-1}$ , the pH from 10.2 - 10.8 and the carbonate alkalinity from 3.5 - 4.0 M. The 1:1 sediment:brine slurries from individual lakes were mixed in equal proportions and were used as an inoculum (5 % v/v). The enrichment media containing either 2 or 4 M total Na<sup>+</sup> was based on sodium carbonate/bicarbonate buffer, each also containing 0.2 M of Na<sup>+</sup> as NaCl, with a final pH of 10. Cells of *Staph*ylococcus aureus DSM 20231 were grown in the LB medium, separated by centrifugation and washed 2 times with sterile 0.1 M NaCl. The concentrated cell preparation was divided in two parts: one was kept alive at 4 °C and the second was autoclaved at 120 °C for 20 min and once again subjected to centrifugation and two washing steps to remove released soluble proteins. Both preparation were added to the final cultivation medium to an  $OD_{600}$  of 2.0. The enrichments were incubated at 37 °C on a rotary shaker at 150 rpm until visible decrease of turbidity and microscopic evidence of prey cell degradation and appearance of new morphotypes. These primary cultures were then serially diluted up to  $(10^{10})$  in the same media and maximum positive dilutions  $(10^8-10^{10})$ were surface-plated onto a solid medium prepared by 3:2 mixing of the liquid medium and melted 4.5 % washed agar at 50 °C (Daishin, Brunschwig Chemie BV, Amsterdam) resulting in plates in which Staphylococcus cells formed an uniform opaque background. The plates were incubated at 37 °C for 3-4 weeks until appearance of colonies forming clearance plaques (Fig. 1 a, b). Those were transferred into the liquid medium with 2 M total Na<sup>+</sup> (pH 9.5) with Staphylococcus cells, and eventually resulted in isolation of two pure bacterial cultures, strains AB-CW1 and AB-CW4, capable of predating on Staphylococcus cells.

Apart from *Staphylococcus*, *Micrococcus luteus* DSM 20030 with the same type of cell wall but with larger cells (also pregrown in the LB medium) was also used as a prey for the isolates. In addition, three pure cultures of haloalkaliphilic prokaryotes from soda lakes were tested: *Isoptericola* sp. (*Actinobaceteria*), a Gram-negative gammaproteobacterium *Halomonas alkaliphilus*, and a natronoarchaeon *Natronococcus amylolyticus* (all from a personal collection). The bacteria were grown on soluble starch at 1 M Na<sup>+</sup> and *Natronococcus* – at 4 M Na<sup>+</sup> (pH 9.5) and the cells were prepared in a similar way as for *Staphylococcus*.

#### Microscopy and chemotaxonomy

The progress of growth at predatory conditions was examined by phase contrast microscopy (Zeiss Axioplan Imaging 2 microscope, Göttingen, Germany) and electron microscopy was used to examine flagellation and cell–cell interaction. For the latter, the cells were centrifuged, resuspended in 2 M NaCl and fixed with *para*-formaldehyde (final concentration 3 %, v/v) at room temperature for 2 h, then washed again with the same NaCl solution, positively contrasted with 1 % (w/v) uranyl acetate and examined under a JEOL 100 electron microscope (Japan).

Membrane polar lipids and respiratory quinones were extracted from freeze-dried cells grown at 37 °C at optimal salt/pH conditions with peptone from casein until the late exponential growth phase. Intact polar lipids were extracted with a modified Bligh-Dyer procedure and analysed by Ultra High Pressure Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS<sup>n</sup>), as described previously (Bale et al., 2021). For the polar lipid fatty acids profiling, the material was

hydrolyzed in HCl/MeOH (1.5 N) followed by three successive extractions with dichloromethane. Fatty acids were derivatized with diazomethane (CH<sub>2</sub>N<sub>2</sub>) and alcohol groups were silylated with BSTFA. Identification and quantitation of (hydroxy) fatty acids was performed using an Agilent Technologies 7890B GC equipped with a silica column (CP Sil-5, 25 × 0.32 mm) coupled to an Agilent Technologies 5975C VL MSD mass spectrometer operated at 70 eV, with a mass range *m*/*z* 50–800 and a scan rate of 3 scans s<sup>-1</sup> (Bale et al., 2019).

### Growth physiology

A sodium carbonate/bicarbonate buffer containing 0.2–4.5 M total Na<sup>+</sup> with a pH of 9.5–10 was used for routine cultivation experiments and for testing the salinity range. To examine the pH, range the media contained 2 M total Na<sup>+</sup> as NaCl and 50 mM HEPES/50 mM K-P buffer (pH from 6 to 8), bicarbonate/NaCl (for pH 8–8.5) and bicarbonate/ carbonate (for pH 8.5–11). The measured pH at the end of experiments was considered representative for the whole experiment. The temperature range for growth was measured at optimal Na<sup>+</sup>-pH with peptone from casein as substrate within the range from 20 to 55 °C. All media were supplemented with 1 mM Mg sulfate and 1 ml/L of acidic trace metal solution (Pfennig and Lippert, 1966).

# Genome sequencing, phylogenomic analysis and functional genome analysis

Genomic DNA was obtained from the freshly grown cells of AB-CW1 and AB-CW4 using the FastDNA<sup>™</sup> SPIN Kit for Soil (MP Biomedicals, United States). A shotgun WGS library preparation and sequencing were performed using KAPA HyperPlus Library Preparation Kit (KAPA Biosystems, UK) and NovaSeq 6000 system (Illumina, San Diego, CA, USA). The genomes were assembled with Unicycler v.0.5.0 (Wick et al., 2017) and submitted for automatic annotation to the PGAP (Tatusova et al., 2016) in GenBank. The genome statistics are given in Supplementary Table S1. For phylogenomic reconstructions, 120 single copy conserved bacterial marker proteins were used according to the Genome Taxonomy Database (Rinke et al., 2021), aligned using GTDB-Tk v2.3.0 (Chaumeil et al., 2022) and trimmed by trimAl 2.rev0 build 2019-08-05 using "-automated1" (optimized for Maximum Likelihood phylogenetic tree reconstruction) and "-gt 0.98" modes (Capella-Gutiérrez et al., 2009) resulting in 17,657 aa length alignment. The trees were built with the IQ-TREE2 program v2.2.0.3 (Minh et al., 2020) with fast model selection via ModelFinder (Kalyaanamoorthy et al., 2017) and ultrafast bootstrap approximation (Minh et al., 2013) as well as approximate likelihood-ratio test for branches (Anisimova and Gascuel, 2006). Relative evolutionary divergence (RED) were calculated according to Parks et al. (2018) https://github.com/donovan-h-parks/PhyloRank) and bac120\_r214 tree from GTDB repository.

The whole genome comparison included Average Nucleotide Identity (ANI), using Pyani 0.2.12 (Pritchard et al., 2016); Average Amino acid Identity (AAI) by the EzAAI v1.1 (Kim et al., 2021) and digital DNA-DNA hybridization (DDH) by the Genome-to-Genome Distance Calculator 3.0 online tool (https://ggdc.dsmz.de/ggdc.php). The genome assemblies of strains AB-CW1 and AB-CW4 are deposited in the GenBank under accession numbers GCA\_034931365 and GCA\_030848495, respectively.

Both genomes were blast-searched for lysozyme-like glycoside hydrolases in dbCAN3 (Zheng et al., 2023) and for extracellular peptidasesproteases in MEROPS 12.5 (Rawlings et al., 2018) databases and the selected protein sequences functionality were further manually checked in UniProt release 2023\_05. Proteins potentially involved in haloalkaline adaptations were also identified.



Fig. 2. Phylogenetic placement of strains AB-CW1 and AB-CW4 within the genus *Natronospira* and of the genus *Natronospira* as a separate family-order lineage within the class *Gammaproteobacteria* based on concatenated amino acid sequences of 120 bacterial single copy conserved marker proteins with taxonomic designations according to the Genome Taxonomy DataBase. The length of the alignment is 17,657 aa. Bootstrap consensus tree is shown with values placed at the nodes. Bar, 0.1 change per position.

#### **Results and discussion**

### Phylogenomic analysis and classification

According to the 16S rRNA gene sequences analysis, strains AB-CW1 and AB-CW4 belonged to the genus Natronospira on the level of two new species with the sequence identity to *N. proteinivora* BSker1<sup>T</sup> of 97.09 % and 96.76 %, respectively, and 98.58 % between each other (Supplementary Fig. S1). ANI, AAI, DDH and comparative phylogenomic analysis using 120 bacterial conserved protein markers confirmed this placement. ANI values of AB-CW1 and AB-CW4 compared with *N. proteinivora* BSker1<sup>T</sup> were 84.1 % and 84.2 %, respectively, and 85.9 % when compared with each other. AAI values of AB-CW1 and AB-CW4 compared with N. proteinivora BSker1<sup>T</sup> were 74.6 % and 74.1 %, respectively, and 76.1 % when compared with each other. The digital DDH values of AB-CW1 and AB-CW4 compared with N. proteinivora BSker1<sup>T</sup> are 19.9 % and 20.3 %, respectively, and 22.3 % compared with each other. These values are in agreement with a new-species status of the predatory isolates within the genus Natronospira. Strains AB-CW1 and AB-CW4 have closely related MAGs assembled from the same soda lakes (Fig. 2) (Vavourakis et al., 2018; Vavourakis et al., 2019). All three MAGs were assembled from sediments where they accounted for 0.16 % to 0.36 % of the prokaryotic community.

Phylogenetic reconstruction based on 120 bacterial conserved protein markers (Parks et al., 2018) placed all three strains as a single genus-level cluster with 100 % statistically branch support (Fig. 2). This is in agreement with our earlier suggestion (Sorokin and Merkel, 2022) that this genus, which is formerly classified as a member of the family *Ectothiorhodospiraceae* (REF), is a part of a deep independent branch within the class *Gammaproteobacteria* at the level of a separate order. Genus *Natronospira*, together with a MAG GCA\_007133205.1 from a surface sediment of a hypersaline soda lake of Kulunda Steppe (Vavourakis et al., 2019), form a family-level lineage that is called f\_SLND01 in GTDB 08-RS214 and has 100 % branch support in our and GTDB reconstructions. Consequently, we propose the Natronospiraceae family for this cluster (Fig. 2). The Natronospiraceae and a phylogenetic cluster called f REEB76 in GTDB 08-RS214, which includes three MAGs from acid mine drainage and one MAG from an alkaline soil, together form an order-level lineage that has 73% branch support in the GTDB reconstruction, but 100% in our reconstruction (Fig. 2). Consequently, we propose Natronospirales for this new order-level cluster. It has 0.655 relative evolutionary divergence (RED) value, which is much closer to the median value of 0.610 for bacterial orders in GTDB 08-RS214, than in case of separate orders o SLND01 (RED value 0.827) and o REEB76 (RED value 0.862) proposed in GTDB 08-RS214. Thus, f\_REEB76 is a group of yet uncultured bacteria belonging to the order Natronospirales. It consists of a group of MAGs assembled from acid mine drainage (Gao et al., 2022) and one deep-branching MAG from alkaline desert soil (Mandakovic et al., 2020), while the entire family Natronospiraceae consists only of isolates and MAGs from the hypersaline soda lakes of Kulunda Steppe. This would suggests a limited environmental occurrence of members of the order Natronospirales. However, 32 16S rRNA gene sequences that were >95 % similar to those of the Natronospira species were identified. All of them were from alkaline coastal soils from the Gulf of Cambay, Gujarat, India (Keshri et al., 2015) or from alkaline saline soils of the former lake Texcoco (Valenzuela-Encinas et al., 2009). This indicates a wider environmental occurrence of the members of the Natronospirales although they seem to be restricted to alkaline environments.

#### Morphology and predatory behaviour

Cells of both isolates grown on peptones were vibrio to short spirilla with a single polar flagellum (Fig. 1 c, d). Cells grown on peptones, both colonial and in liquid culture, contained a yellow membrane-bound pigment with an absorption maximum at 480 nm in methanol:

acetone (7:3, v:v) extract, similar to the type species of Natronospira.

When grown with Staphylococcus cells as substrate, cells of AB-CW4 remained short, while AB-CW1 formed elongated, loosely coiled spirilla. Electron microscopy revealed that the predatory activity of both strains was accomplished by direct contact with the prey cells (Fig. 1 e, f). This has been similarly observed in a moderately salt-tolerant predatory Wenzhouxiangella sp. AB-CW3 (Sorokin et al., 2020). While cells of the latter produced multiple fimbria-like filaments, most probably responsible for the adhesion to prey and also encoded a large fimbrial protein of 3,068 aa (WP\_190974951), neither the fimbria and the gene were present in the cells and genomes of the AB-CW1 and AB-CW4 isolates. Apparently, AB-CW strains are using another mechanism for the prey cell attack. A possible option are the pili (part of the secretion systems type II and IV), which are also microtubular surface structures but shorter than fimbria and serving multiple purposes, such as secretion of toxins and extracellular hydrolases (type II) and cell adhesion and uptake of extracellular DNA (type IV) (de Masi et al., 2013). Both genomes contain several loci encoding such systems (Supplementary Table S2).

The respiratory quinone and membrane lipid composition of both strains is similar. The only quinone species present is ubiquinone UQ-8. The dominant identified membrane phospholipids in both isolates include phosphatidylcholines (PC) and less abundant phosphatidyleth-anolamines (PE) and phosphatidylglycerols (PG). The dominant polar lipid fatty acids included *i*-C17:0 and *i*-C17:1 $\omega$ 9c, similar to the type *Natronospira* species. In addition, novel isolates also have a significant fraction of the *i*-C19:1 $\omega$ 9c and a much higher proportion of the C16:0 (Supplementary Table S3).

#### Growth physiology

Similar to *N. proteinivorans*, AB-CW1 and AB-CW4 are obligately aerobic organoheterotrophs utilizing various proteins and peptides as the growth substrate, including alpha-keratin (fine powdered fraction), gelatin, casein, filter-sterilized bovine serum albumin and lactalbumin, soy protein, bovine collagen, and various peptones and yeast extract. Furthermore, a weak growth was observed with soluble starch for AB-CW1 and, for both strains, with maltose. None of the other tested single carbon compounds including C2-C6 organic acids, alcohols (glycerol, methanol, ethanol) and C5-C6 sugars supported growth. Tests for anaerobic growth, either fermentative with maltose or respiratory with maltose as substrate and nitrate or sulfur as electron acceptors were negative. Ammonium and amino acid nitrogen can be used as the Nsource by both isolates, while urea and nitrate did not support growth with maltose as the carbon and energy substrate.

A major phenotypic property of the new isolates is their ability to predate on bacterial cells. The most active growth was observed on heatsterilized cells of Staphylococcus, followed by live cells of the latter with nearly full prey digestion within 1 and 2 weeks, respectively. They were also able to grow on cells of two haloalkaliphilic bacteria isolated from the same soda lakes belonging to Actinobacteria and Gammaproteobacteria, although much less actively and with incomplete degradation of prey cells. No proliferation and cell degradation occurred when the prey cells were represented by a natronarchaeon Natronococcus amylolyticus. A possible reason for this may be that the sarcina-like tetracocci formed by Natronococcus are covered with a thick polysaccharide matrix and do not lyse even in distilled water, in contrast to most known haloarchaeal species (Albers and Meyer, 2011), making them inaccessible for the proteolytic complex produced by the predatory AB-CW isolates. It would be interesting to test other natronoarchaea with different type of cell wall as a prey, but it probably would need a separate enrichment to select for a bacterial predator capable of degrading the haloarchaeal Slayer glycoproteins. In contrast to the AB-CW strains, none of the predatory activity was observed in the type species N. proteinivorans.

Salt (as sodium carbonates) and pH (at 2 M total Na<sup>+</sup>) profiles of AB-CW1 and AB-CW4 grown on peptone were, in general, similar to what has been reported for the type strain of *Natronospira*. This characterizes

#### Table 1

Comparative properties of predatory strains AB-CW1 and AB-CW4 and the type strain of the genus *Natronospira*.

Property	AB-CW1	AB-CW4	Natronospira proteinivora BSker1 <sup>T</sup>
Cell morphology	Motile curved rod	Motile short spirillum	Motile spirillum
Yellow pigment	+	+	+
Relation to	Obligate aerobe	Obligate aerobe	Obligate aerobe
owngon	obligate aerobe	obligate delobe	obligate derobe
Oxygen			
Growth			
substrates	G + -cocci	G + -cocci	_*
bacterial cells	Isoptericola sp.	Isoptericola sp.	_*
	(partial)	(partial)	-*
	Halomonas	Halomonas	
	alkaliphilus	alkaliphilus (partial)	gelatine, casein.
proteins		, , , , , , , , , , , , , , , , , , ,	albumin
proteins	gelating casein	gelatine casein	alpha keratin
	albumine callegon	albumin callease	aipiia-kerauii
peptones	albumins,conagen,	albumm,conagen,	
(casein, meat)	alpha-keratin	alpha-keratin	+
starch	(w)	(w)	-
maltose			-
	++	+	
	(w)+	(w)+	
	(w)	(w)	
Salinity range	0.75-3.5 (1.5-2.0)	1.0-4.0 (2.0)	1.0-4.5
(opt.).			(2.0 - 2.5)
M Na <sup>+</sup> (at pH			
0.5)			
pH range (opt)	8 2 10 55 (0 5)	8 1 10 42 (0 5)	8 5 10 25 (0 5)
of 2 M No <sup>+</sup>	0.2-10.33 (9.3)	0.1-10.42 (9.3)	0.3-10.23 (9.3)
at 2 M Na	40	45	45
Max.	48	45	45
temperature			
(°C)			
(at 2.0 M Na $^+$			
and pH 9.5)			
Predominant	i-C17:0, i-C17:1ω9c,	i-C17:0, i-	iC17:0,
polar	i-C19:1ω9c,	C17:109c,	iC17:1ω9c
lipid fatty	C18:1@9. C16:0	i-C19:109c, C16:0	
acids	,		
Respiratory	110-8	110-8	110-8
linoquinono	00-0	0Q-0	00-0
Conomo eine	2.1	2.0	2.0
Genome size	5.1	5.0	2.9
(MDp)		· · · ·	<pre></pre>
G + C, %	61.5	62.5	60.0
(genomic)			
Habitat	Hypersaline soda lakes	3	

Features common for all 3 strains: inability to utilize urea and nitrate as the N-source; ability to utilize sulfate as the sulfur source; positive tests for cytochrome oxidase and catalase and negative for lipase (with Tween80); absence of tryptophanase gene *trnA*, indicative of the inability to produce indole from tryptophane.

<sup>\*</sup> this work; w, weak growth; partial, incomplete prey cells lysis.

the new isolates as extremely salt-tolerant obligate alkaliphiles: the salinity ranged from 0.75 to 1.0 to 3.5–4.0 M total Na<sup>+</sup> (optimum at 2–2.5 M) and the pH from 8.1 to 10.5 with an optimum at 9.5. The temperature range (optimum) for both strains determined at pH 9.5 and 2 M total Na<sup>+</sup> were 20–48 and 35 °C, respectively. A phenotypic comparison of AB-CW1, AB-CW4 and *N. proteinivora* BSker1<sup>T</sup> is given in Table 1.

#### Functional genome analysis

In the functional genomic analysis, we focused on the genetic repertoire potentially responsible for the predatory phenotype of the new isolates and haloalkaliphilic adaptation. According to what is known from other specialized predatory bacteria (Bratanis et al., 2020), two major sets of extracellular, and, to a lesser extent, membrane-bound proteins, which might be involved in predation, were encoded by both genomes. The first set includes murein-specific glycosyl hydrolases and di-peptidases which, acting together, can hydrolyze the cell wall.

#### Table 2

Extracellular peptidoglycan hydrolases, peptidases and cell-invasion factors encoded in the genomes of Natronospira strains AB-CW1 and AB-CW4.

AB-CW1		AB-CW4				
Locus MEA5	44+	Enzyme	Signal	<b>Locus</b> WP_30672+	Enzyme	Signal
Peptic	doglyca	n degradation/invasion systems				
4211		peptidoglycan N-acetylglucosamine deacetylase CE4	Sec/SPII	6803	M23 peptidoglycan DD- Zn-endopeptidase + LysM*	Sec/SPII
4212		peptidoglycan glucosaminidase GH73	membrane	6804	DedA (toxin:H+ efflux pump)	membrane
4221-		Tol-Pal system: TolABQR-Pal-YgbC-YbgF	membrane	7004	peptidoglycan glucosaminidase GH73	membrane
4226	6	(self-protection from colicins)	Sec/SPI/	7005	peptidoglycan N-acetylglucosamine deacetylase CE4	Sec/SPII
4573		lyzozyme/peptidoglycan lyase GH23 + LysM	Sec/SPII	7173–7174	TonB/TolB lipoprotein colicin translocation system (killing)	Sec/SPII
4776		lyzozyme/peptidoglycan lyase GH23	Sec/SPI	7175	lyzozyme/peptidoglycan lyase GH23	Sec/SPII
4778		LysM peptidoglycan-binding protein	Sec/SPII	7177	exodeoxyribonuclease VII large subunit	-
4831		M23 peptidoglycan DD- Zn-endopeptidase	membrane	7178	M23 peptidoglycan DD- Zn-endopeptidase	Sec/SPII
4967		M23 peptidoglycan DD- Zn-endopeptidase	Sec/SPI	7180	M48 intramembrane glutamic Zn-endopeptidase	membrane
4968		S41 oligo-endopeptidase	Sec/SPI	7206	lyzozyme/peptidoglycan lyase GH23 + LysM	Sec/SPI
5115		ArnA lipoprotein (antibiotic resistance)	globular	7239	S11 D-alanyl-D-alanine carboxypeptidase (murein recycling)	Sec/SPI
5116		lyzozyme/peptidoglycan lyase GH23	Sec/SPI	7447	lyzozyme/peptidoglycan lyase GH23 + LysM	Sec/SPII
5118		porin: IgA1 protease OMP protein autotransporter	Sec/SPI	7941	lyzozyme/peptidoglycan lyase GH23	Sec/SPI
5119		S9 prolyl endopeptidase	Sec/SPI	7943	LysM peptidoglycan-binding protein	Sec/SPII
5142		lyzozyme/peptidoglycan lyase GH23	Sec/SPII	7999	M23 peptidoglycan DD- Zn-endopeptidase	Sec/SPII
5223		M23 peptidoglycan DD- Zn-endopeptidase	Sec/SPI	8357	S41 oligo-endopeptidase	Sec/SPI
5224		annydro-N-acetyimuramic acid kinase AnmK (murein recycling)	_	8358	M23 peptidogiycan Zn DD- –endopeptidase	Sec/SPI
5381		S9C prolyl oligopeptidase	Sec/SPI	8602	muropeptide:H <sup>+</sup> symporter AmpG	membrane
5382		antibiotic biosynthesis monooxygenase	-	8603	CotH/Fibronectin III (putative invasion factor)	Sec/SPII
5383		lytic murein transglycosylase/lysozyme GH103	Sec/SPI	8605	S8 endopeptidse (excreted)	globular
5384		proline iminopeptidase (exo) S33 (C-term. membrane)	Sec/SPI	8607	lytic murein transglycosylase/lysozyme GH103	Sec/SPI
5385		OMP lipoprotein	Sec/SPI/ SPII	8608-8609	Type II toxin/antitoxin system BrnTA	_
5389-	5390	2x TonB (interact with colicin transporter CirAB)	Sec/SPI	8767	OmpA outer membrane protein/porin (colicin export)	Sec/SPI Sec/
5391		M50 Zn-peptidase	Sec/SPI	8768	PilO (type IV secretion: promote cell adhesion)	SPI
5413		M23 pentidoglycan Zn DD-endopentidase	Sec/SPI	8769	lyzozyme/nentidoglycan lyzose GH23 + LysM	Sec/SPI
5435		S9 serine endopentidase	Sec/SPI	9151	pentidoglycan DD- Zn-endopentidase M23	Sec/SPI
5440		lyzozyme/peptidoglycan lyase GH23 + LysM	Sec/SPI	9318	CBM9/glycoprotein-N-acetylglucosamine-aminidase	Sec/SPI
5441		M24 Xaa-Pro Mn-dipeptidase	_	9393	LysM peptidoglycan-binding domain	Sec/SPII
5922		S1-C chymotrypsin-like serine protease	Sec/SPII		J. I.I. 0.J. 0.	
5926		LysM peptidoglycan-binding protein	Sec/SPII			
6346		peptidoglycan N-acetylglucosamine deacetylase CE4	Sec/SPI			
6349		lyzozyme/peptidoglycan lyase GH23	Sec/SPII			
6518-	6519	Lpp20 lipoproteins (putative invasive colonization)	Sec/SPII			
6520		lyzozyme/peptidoglycan lyase GH23 + LysM	Sec/SPII			
6521		M12B Zn-endopeptidase: invasin homologue; promote invasive behavior of the enteropathogens	Sec/SPI			
Extrac	cellular	peptidases/proteases				
4299		M14 Zn-carboxypeptidase	Sec/SPI	6884	S41 carboxypeptidase	Sec/SPI
4300		M48B Zn-endoprotease	Sec/SPI	6909	M15B/C/D Zn-carboxy/di-peptidase (endolysin)	Sec/SPI
4312		M15B/C Zn-carboxypeptidase (endolysin)	Sec/SPI	6922	M48 Zn-protease	Sec/SPI
4336		S41 carboxypeptidase	Sec/SPI	6923	M14 Zn-carboxypeptidase	Sec/SPI
4427		M12B Zn-protease (putative caseinase/gelatinase)	Sec/SPII	7052	C40 di-peptidase	Sec/SPI/SPII
4440		domain	Sec/SPI	7055	M56 Zn-endopeptidase (antibiotic resistance)	membrane
4478		C40 di-peptidase	Sec/SPI/ SPII	7099	Zn-protease M12B(putative caseainase/gelatinase)	Sec/SPI/SPII
4491		M14C Zn-carboxypeptidase (putative murein cycling)	membrane	7121	haemolysin III:killing toxin (pore-forming) (1)	membrane
4669–	4670	Fic-family invasion factor	_	7148	S8 endopeptidase	Sec/SPI
4671		S9 dipeptidyl peptidase	Sec/SPI	7201	S9 endopeptidase	Sec/SPI
4745		S8 endopeptidase	Sec/SPI	7362	M48 Zn-protease	Sec/SPII
4879-	4881	3 x S8 endopeptidases	Sec/SPI	7449	S49 endopeptidase	membrane
4935		M3 Zn-oligopeptidase	Sec/SPII	7511	SIC cnymotrypsin carboxypeptidase	Sec/SPII
5043		SyA prolyl oligopeptidase	Sec/SPI	7597	NIZOA Zn-aminopeptidase	Sec/SPI
5074		w14C Zn-carboxypeptidase (putative murein cycling)	Sec/SPI	/058	59A prolyl oligopeptidase	Sec/SPI
5119		protyt oligopeptidase S9C	Sec/SPI	/0// 7010	so enuopeptidase (extracellular)	globular
5128	E104	sy endopeptidase	Sec/SPI	7818	58 endopeptidase (extracellular)	globular
5133-	0104	so endopeptidases	Sec/SPI	/049 7074 7076	a s Se endopentidação	Sec /SDI
5155		M2A 7p olicopoptidase	Sec/SPII	/8/4-/8/6 7077	o x oo endopeptidases	Sec/SPI
5103		mon zii-oligopeptidase	Sec/SPII	/ 3/ / 8027	57 chuopephiase S1C chumotruncin carboxumontidaca (artracallular)	bec/ SPI
2300		M12B Zn-endoprotesse	Sec/SPI	8102	S1C chymotrypsin carboxypeptidase (extracellular)	Sec /SDI
2233		mize zu-chaopioicase	SPII	0103	anchor)	3CC/ 3P1
5474		S11/13 D-alanyl-D-alanine carboxypeptidase	Sec/SPI	8186	M14C Zn-carboxypeptidase (putative murein cycling)	Sec/SPI
5570		M28A Zn-aminopeptidase	Sec/SPI	8237	S9C prolyl oligopeptidase	Sec/SPII

(continued on next page)

#### Table 2 (continued)

AB-CW1			AB-CW4		
<b>Locus</b> MEA544+	Enzyme	Signal	<b>Locus</b> WP_30672+	Enzyme	Signal
5639	S9C acyl-aminopeptidase	Sec/SPI	8282	M3A Zn-oligopeptidase	Sec/SPII
	(cleave off <i>N</i> -acetylglucosamine residue from glycoprotein)		8442	beta-aspartyl dipeptidase T2 (works on glycoproteins)	Sec/SPII
5642	P1/S58 L-aminopeptidase (putative peptide antibiotic synthesis)	Sec/SPI	8444	S8A subtilase endopeptidase	Sec/SPI
5649	M19 Zn-dipeptidase	Sec/SPI	8473	M19 Zn-dipeptidase	Sec/SPI
5678	beta-aspartyl dipeptidase T2 (works on glycoproteins)	Sec/SPII	8479	P1/S58 aminopeptidase (putative peptide antibiotic synthesis)	Sec/SPI
5778	S9A prolyl oligopeptidase	Sec/SPI	8509	S9A prolyl oligopeptidase	Sec/SPI
5853	S9A prolyl oligopeptidase	Sec/SPI	8520	S9A prolyl oligopeptidase	Sec/SPII
5856	M28 Zn-aminopeptidase	Sec/SPII	8550	M48 Zn-endoprotease	Sec/SPI
5866	S41 endopeptidase	Sec/SPII	8564	S9A prolyl oligopeptidase	Sec/SPI
5921	integrin (cell-cell adhesion)	Sec/SPI	8584	M12B Zn-endoprotease (membrane anchored)	Sec/SPI
5922	S1-C chymotrypsin-like serine protease	Sec/SPII	8659	M14B Zn-carboxypeptidase	Sec/SPI
6067	S8 endopeptidase	Sec/SPI	8660	S8A fibrinolytic endopeptidase + fibronectin III domain	Sec/SPI
6095	M1 Zn-aminopeptidase	Sec/SPII	8734	M13 Zn-endopeptidase	Sec/SPII
6124	S8 endopeptidase (membrane-anchored at C-terminal)	Sec/SPI	8750-8751	S8A subtilase-like endopeptidases	Sec/SPI
6133–6134	S8 endopeptidases (membrane-anchored at C-terminal)	Sec/SPI	8756	S9C acyl-aminopeptidase	Sec/SPII
				(cleave off N-acetylglucosamine residue from glycoprotein)	
6213	S1C chymotrypsin-like endoprotease	Sec/SPI	8837	S8A subtilase endopeptidase	Sec/SPI
6362	S9 endopeptidase	Sec/SPI	8870	Xaa-Pro Mn-aminopeptidase M24B	TAT/SPI
6382	S1-C chymotrypsin protease (globular, external)	_	8954	S1C chymotrypsin-like carboxypeptidase	Sec/SPI
6571	Xaa-Pro Mn-aminopeptidase M24B	TAT/SPI	8996	M1 Zn-aminopeptidase	Sec/SPII
6664	S1-C chymotrypsin protease	Sec/SPI	9065	S8A subtilase endopeptidase	Sec/SPI
6705	S41A carboxypeptidase	Sec/SPI	9107-9108	S8A subtilase endopeptidases	Sec/SPI
6911–6912	M10 Zn-endopeptidases (virulence invasive factor)	Sec/SPI	9201	S9C di-peptidase	Sec/SPII
			9312	M56 Zn-peptidase (antibiotic resistance)	membrane
			9323	M48 Zn-endoprotease	Sec/SPII
			9328	S10 carboxypeptidase	Sec/SPI
			9343	S1C chymotrypsin-like carboxypeptidase	Sec/SPI
			9349	S41A carboxypeptidase	Sec/SPI
			9358	M28 Zn-aminopeptidase	Sec/SPII
			9361	S9B di-peptidase 4	Sec/SPI
			9385	S9B di-peptidase 4	Sec/SPII

\*LysM – peptidoglycan-binding domain similar to CBM50.

Particularly those are lysozyme-like peptidoglycan lyase from the GH23 family (with or without LysM/CBM50 carbohydrate-binding domain), glucosaminidase from the GH73 family and peptidoglycan-active [Zn]-dipeptidases from the M23 family (Table 2). Furthermore, those often also included cell invasion factors, such as M10 and M12 family end-poeptidases, Fic family, haemolysin III, which might be part of the predatory system. Interestingly, however, the genome of *N. proteinivora* also features a similar genetic potential, albeit less abundantly represented, even though this species does not possess the predatory potential. Apparently, in case of *N. proteinivora* the encoded hydrolases are likely involved in the internal murein recycle.

A second set of encoded proteins potentially important for a predatory life style includes multiple copies of extracellular peptidases/proteases among which the most abundant are the serine families S8, S9 and S41 and metallopeptidases (mostly Zn-dependent) from the families M12, M14 and M48. Practically all those excreted hydrolases have Sec/ SPI/SPII signal peptide (except only for a single case with the TAT signal), indicating that they do not stop in the periplasm but are crossing the outer membrane and, thus, are capable of direct interaction with the extracellular polymers and the whole (prey) cells (Table 2).

With respect to the haloalkaliphilic adaptation, the genomes of both strains encode the biosynthesis pathway for compatible solutes ectoine and hydroxy-ectoine in a single operon *ectABCD*; two multisubunit Na<sup>+</sup>: H<sup>+</sup> antiporters (*mnhEFGABCD1D2D3/mrpEFGBCD1D2D3*); two mono-subunit Na<sup>+</sup>:H<sup>+</sup> antiporters (*nhaC* and *CPA1*); a K<sup>+</sup>:H<sup>+</sup> antiporter *CPA2* and a K<sup>+</sup>:H<sup>+</sup> symporter *trkAH*, as has been observed for *N. proteinivora* (Sorokin and Merkel, 2022).

Overall, strains AB-CW1 and AB-CW4 represent the first example of extremely salt-tolerant natronophilic bacteria from a soda lake habitat with a potent predatory potential. On the basis of distant phylogenomics and unique phenotypic properties, the novel isolates are proposed to form two novel species in the genus *Natronospira*, whose genus diagnosis also needs to be amended. Moreover, phylogenomic analysis also suggested that this genus is to be reclassified into a separate family *Natronospiraceae* fam. nov., and order *Natronospirales* ord. nov. The new species and the higher taxa protologues are presented in Table 3.

#### Amended description of the genus Natronospira Sorokin et al. 2017

In addition to the properties reported earlier (Sorokin et al., 2017; Sorokin and Merkel, 2022), the major polar phospholipids in the members of the genus were identified as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Some of the *Natronospira* members have the ability to predate on bacterial cells.

#### **Funding information**

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

#### CRediT authorship contribution statement

Dimitry Y. Sorokin: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. Alexander Y. Merkel: Writing – original draft, Methodology, Investigation. Tatyana V. Kalganova: Writing – original draft, Methodology. Nicole J. Bale: genus is Natronospira.

Natronospiraceae. A member of the

class Gammaproteobacteria. The type

dominated by phosphatidylcholine

minor component. The polar lipid

i17:109c and i19:109c with a less

aerobic organoheterotrophs using mostly proteins and peptides for

growth. Also have the capacity to

Obligately alkaliphilic with a pH range for growth from 8.1 to 10.40

Extremely salt tolerant with the salt

lipoquinone is UQ-8. Strictly

predate on bacterial cells.

and an optimum at pH 9.5.

range (in the form of sodium

Russia).

Draft

3.0

GCF 030848495

100637-102177

AB-CW4<sup>T</sup>

JCM 335397; UQM 41580

carbonates) from 1.0 to 4.0 M of

total Na<sup>+</sup> (optimum at 2.0 M). The

upper temperature limit for growth

(at optimal pH and salinity) is 45 °C. The G + C content of the genomic DNA is 62.5 % (genome). The type strain, AB-CW4 (JCM 335397 = UQM 41580), was isolated from a mix sample of aerobic surface sediments and brines of hypersaline soda lakes in Kulunda Steppe (Altai,

abundant 16:0. The only respiratory

fatty acids are dominated by i17.0

and phosphatidyl-ethanolamine

with phosphatidylglycerol as a

#### Table 3

Description of Natronospirales ord. nov., Natronospiraceae fam. nov., Natronospira elongata sp. nov., and Natronospira bacteriovora sp. nov

member of the order Natronospirales,

class Gammaproteobacteria. The type

genus is Natronospira.

Parameter	Order: Natronospirales	Family: Natronospiraceae	Species: Natronospira elongata	Species: Natronospira bacteriovora
Order name	Natronospirales			
Family name		Natronospiraceae		
Species name			Natronospira elongata	Natronospira bacteriovora
Status	ord. nov.	fam. nov.	sp.nov.	sp.nov.
Description of	Na.tro.no.spi.ra'les (N.L. fem. n.	Na.tro.no.spi.ra.ce'ae (N.L. fem. n.	Natronospira elongata (e.lon.ga'ta. L. fem.	Natronospira bacteriovora (bac.te.
a new taxon	Natronospira, a bacterial genus;	Natronospira, a bacterial genus;	part. adj. elongata, elongated).	rio.vo'ra. Gr. neut. n. bakterion, a
	-ales, ending to denote an order; N.	<ul> <li>–aceae, ending to denote a family;</li> </ul>	Cells are Gram-negative, from vibrio to	small rod; L. press. part. vorans,
	L. fem. pl. n. Natronospirales, the	N.L. fem. pl. n. Natronospiraceae, the	long loose spirilla, 0.25–0.3 x 1–20 μm,	devouring; N.L fam. adj.
	Natronospira order).	Natronospira family).	motile by a single polar flagellum. The	bactriovorans, devouring bacteria).
	The order encompasses extremely	The family includes extremely salt-	colonies are yellowish, up to 4 mm, flat	Cells are Gram-negative, from
	salt-tolerant and obligately	tolerant and obligately alkaliphilic	and round. The polar phospholipids are	vibrio to small spirilla, 0.25 x 1–3
	alkaliphilic aerobic heterotrophic	aerobic heterotrophic bacteria	dominated by phosphatidylcholine and	μm, motile by a single polar
	bacteria utilizing mostly various	utilizing mostly various proteins for	phosphatidylethanolamine with	flagellum. The colonies are
	proteins for growth. Currently	growth. Currently it includes a	phosphatidylglycerol as a minor	yellowish, up to 3 mm, flat and
	include a single family	single (type) genus Natronospira. A	component. The polar lipid fatty acids are	round. The polar phospholipids are

dominated by *i*17:0, *i*17:1ω9c, 18:1ω9,

i19:109c and 16:0. The only respiratory

lipoquinone is UQ-8. Strictly aerobic

proteins and peptides for growth. Also

cells. Obligately alkaliphilic with a pH

range for growth from 8.2 to 10.55 and

tolerant with the salt range (in the form of sodium carbonates) from 0.75 to 3.5  $\rm M$ 

of total Na<sup>+</sup> (optimum at 2-2.5 M). The

upper temperature limit for growth (at

optimal pH and salinity) is 48 °C. The G + C content of the genomic DNA is 61.5

% (genome). The type strain, AB-CW1

isolated from a mix sample of aerobic

hypersaline soda lakes in Kulunda Steppe

(JCM 335396 = UQM 41579), was

surface sediments and brines of

(Altai, Russia).

AB-CW1<sup>T</sup>

Draft

3.1

Russia

GCA\_034931365

50901-52441

JCM 335396; UQM 41579

an optimum at pH 9.5. Extremely salt

have the capacity to predate on bacterial

organoheterotrophs using mostly

Type strain Culture collection numbers Genome status GenBank genome assembly Genome size (Mbp) 16S-rRNA gene locus in the genome Country of origine Region Source of isolation Latitude Longitude Sampling date pH of the sample Salinity of the sample Number of strains in study Information regarding to Nagoya protocol

Altai region, Aerobic sediments and brines from hypersaline soda lakes
51°39' N; 49°10' N; 48°14' N 79°48' E; 46°39' E; 46°35' E

E; 46°35' E July 2022 10.2-10.8

25-43 %

1

Not applicable

Writing – review & editing, Writing – original draft, Methodology, Investigation. Jaap Sininghe Damsté: Writing – review & editing.

#### Data availability

Data will be made available on request.

#### Acknowledgements

We thank M. Koenen for experimental assistance with lipid analysis, D. Dorhout and M. Verweij for analytical support.

#### Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.syapm.2024.126519.

#### References

- Albers, S.-V., Meyer, B.H., 2011. The archaeal cell envelope. Nat. Rev. Microbiol. 9, 414–426.
- Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. Syst. Biol. 55, 539–552.
- Bale, N.J., Rijpstra, W.I.C., Oshkin, I.Y., Belova, S.E., Dedysh, S.N., Sinninghe Damsté, J. S., 2019. Fatty acid and hopanoid adaption to cold in the methanotroph *Methylovulum psychrotolerans*. Front. Microbiol. 10, 589
- Bale, N.J., Ding, S., Hopmans, E.C., Villanueva, L., Boschman, R.C., 2021. Lipidomics of environmental microbial communities. I: Visualization of specific niches using untargeted analysis of high-resolution mass spectrometry data. Front. Microbiol. 12, 659302.
- Bratanis, E., Andersson, T., Lood, R., Bukowska-Faniband, E., 2020. Biotechnological potential of *Bdellovibrio* and like organisms and their secreted enzymes. Front. Microbiol. 11, 662.
- Capella-Gutiérrez, S., Silla-Martínez, J.M., Gabaldón, T., 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics (oxford, England) 25 (15), 1972–1973.
- Chaumeil, P.A., Mussig, A.J., Hugenholtz, P., Parks, D.H., 2022. GTDB-Tk v2: memory friendly classification with the genome taxonomy database. Bioinformatics (oxford, England) 38 (23), 5315–5316.
- de Masi, L.G., Sturey, C.D., Lieberman, J.A., Donnenberg, M.S., 2013. The type 2 secretion and type 4 pilus systems of *Escherichia coli*. In: *Escherichia coli*. Pathotypes and Principles of Pathogenesis, Second Edition, Donnenberg, M.S. (ed.). Amsterdam et al., Elsevier, pp. 387-416.
- Fujinami, S., Fujisawa, M., 2010. Industrial applications of alkaliphiles and their enzymes – past, present and future. Environ. Technol. 31, 845–856.
- Gao, S., Paez-Espino, D., Li, J., Ai, H., Liang, J., Luo, Z., Zheng, J., Chen, H., Shu, W., Huang, L., 2022. Patterns and ecological drivers of viral communities in acid mine drainage sediments across Southern China. Nat. Comm. 13, 2389.
- Grant, B.D., Jones, B.E., 2016. Bacteria, archaea and viruses of soda lakes. In: Soda lakes of East Africa, M. Schagerl (ed); Springer International Publishing, Switzerland, pp. 97-147.
- Kalyaanamoorthy, S., Minh, B.Q., Wong, T., von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589.
- Keshri, J., Yousuf, B., Mishra, A., Jha, B., 2015. The abundance of functional genes, *cbbL*, *nifH*, *amoA* and *apsA*, and bacterial community structure of intertidal soil from Arabian Sea. Microbiol. Res. 175, 57–66.
- Kevbrin, V., Boltyanskaya, Y., Zhilina, T., Kolganova, T., Lavrentjeva, E., Kuznetsov, B., 2013. Proteinivorax tanatarense gen. nov., sp. nov., an anaerobic, haloalkaliphilic, proteolytic bacterium isolated from a decaying algal bloom, and proposal of Proteinivoraceae fam. nov. Extremophiles 17, 747–756.
- Kim, D., Park, S., Chun, J., 2021. Introducing EzAAI: a pipeline for high throughput calculations of prokaryotic average amino acid identity. J. Microbiol. (seoul, Korea) 59, 476–480.
- Krienitz, L., Schagerl, M., 2016. In: Tiny and Tough. Microphytes in East African Soda Lakes. Springer International Publishing, Switzerland, pp. 149–177.

Mandaković, D., Cintolesi, Á., Maldonado, J., Mendoza, S.N., Aite, M., Gaete, A., Saitua, F., Allende, M., Cambiazo, V., Siegel, A., Maass, A., González, M., Latorre, M., 2020. Genome-scale metabolic models of *Microbacterium* species isolated from a high altitude desert environment. Sci. Rep. 10, 5560.

- Minh, B.Q., Nguyen, M.A., von Haeseler, A., 2013. Ultrafast approximation for phylogenetic bootstrap. Mol. Biol. Evol. 30, 1188–1195.
- Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., von Haeseler, A., Lanfear, R., 2020. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol. Biol. Evol. 37, 1530–1534.
- Oduor, O.S., Schagerl, M., 2007. Phytoplankton primary productivity characteristics in response to photosynthetically active radiation in three Kenyan Rift valley salinealkaline lakes. J. Plankton Res. 29, 1041–1050.
- Parks, D.H., Chuvochina, M., Waite, D.W., Rinke, C., Skarshewski, A., Chaumeil, P.A., Hugenholtz, P., 2018. A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. Nat. Biotechnol. 36, 996–1004.
- Pfennig, N., Lippert, K.D., 1966. Über das Vitamin B12-Bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. 55, 245–256.
- Pritchard, L., Glover, R.H., Humphris, S., Elphinstone, J.G., Toth, I.K., 2016. Genomics and taxonomy in diagnostics for food security: soft-rotting enterobacterial plant pathogens. Anal. Methods. 8, 12–24.
- Rawlings, N.D., Barrett1, A.J., Thomas, P.D., Huang, X., Bateman, A., Finn, R.D., 2018. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. Nucleic Acids Res. 46, D624-D632.

Rinke, C., Chuvochina, M., Mussig, A.J., Chaumeil, P.-A., Davín, A.A., Waite, D.W., Whitman, W.B., Parks, D.H., Hugenholtz, P., 2021. A standardized archaeal taxonomy for the Genome Taxonomy Database. Nat. Microbiol. 6, 946–959.

Samylina, O.S., Sapozhnikov, F.V., Gainova, O.Y., Ryabova, A.V., Nikitin, M.A., Sorokin, D.Y., 2014. Algo-bacterial phototrophic communities of soda lakes in Kulunda Steppe (Altai, Russia). Microbiology (english Translation) 83, 849–860.

- Sarethy, I.P., Saxen, Y., Kapoor, A., Sharma, M., Sharma, S.K., 2011. Alkaliphilic bacteria: applications in industrial biotechnology. J. Ind. Microbiol. Biotechnol. 38, 769–790.
- Sorokin, D.Y., Merkel A.Y., 2022. Natronospira In: Bergey's Manual of Systematics of Archaea and Bacteria, Online. Whitaman W.B. (Ed.) John Wiley & Sons, Inc., doi: 10.1002/9781118960608.gbm01977.
- Sorokin, D.Y., Berben, T., Melton, E.D., Overmars, L., Vavourakis, C., Muyzer, G., 2014. Microbial diversity and biogeochemical cycling in soda lakes. Extremophiles 18, 791–809.
- Sorokin, D.Y., Banciu, H.A., Muyzer, G., 2015. Functional microbiology of soda lakes. Curr. Opin. Microbiol. 25, 88–96.
- Sorokin, D.Y., Khijniak, T.V., Galinski, E.A., Kublanov, I.V., 2017a. Natronotalea proteinilytica gen. nov., sp. nov, and Longimonas haloalkaliphilia sp. nov., extremely salt-tolerant alkaliphilic members of the phylum Rhodothermaeota isolated from hypersaline soda lakes. Int. J. Syst. Evol. Microbiol. 67, 4161–4167.
- Sorokin, D.Y., Kublanov, I.V., Khijniak, T.V., 2017b. Natronospira proteinivora gen. nov., sp. nov., an extremely salt tolerant alkaliphilic protein-utilizing gammaproteobacterium from hypersaline soda lakes. Int. J. Syst. Evol. Microbiol. 67, 2604–2608.
- Sorokin, D.Y., Mosier, D., Zorz, J.K., Dong, X., Strous, M., 2020. Wenzhouxiangella strain AB-CW3, a proteolytic bacterium from hypersaline soda lakes that preys on cells of Gram-positive bacteria. Front. Microbiol. 11, 597686.
- Sorokin, D.Y., 2017. Anaerobic haloalkaliphiles. In: Encyclopedia Life Science. John Wiley&Sons, Ltd:Chichester. doi: 10.1002/9780470015902.a0027654.
- Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E.P., Zaslavsky, L., Lomsadze, A., Pruitt, K.D., Borodovsky, M., Ostell, J., 2016. NCBI prokaryotic genome annotation pipeline. Nucleic Acids Res. 44, 6614–6624.
- Uma, G., Babu, M.M., Gnana, V.S., Selvaraj, P., Nisha, J., Citarasu, T., 2020. Nature and bioprospecting of haloalkaliphilics: a review. World J. Microbiol. Biotechnol. 36, 66.
- Valenzuela-Encinas, C., Neria-González, I., Alcántara-Hernández, R.J., Estrada-Alvarado, I., Zavala-Díaz de la Serna, F.J., Dendooven, L., Marsch, R., 2009. Changes in the bacterial populations of the highly alkaline saline soil of the former lake Texcoco (Mexico) following flooding. Extremophiles 13, 609–621.
- Vavourakis, C.D., Andrei, A.S., Mehrshad, M., Ghai, R., Sorokin, D.Y., Muyzer, G., 2018. A metagenomics roadmap to the uncultured genome diversity in hypersaline soda lake sediments. Microbiome 6 (1), 168.
- Vavourakis, C.D., Mehrshad, M., Balkema, C., van Hall, R., Andrei, A.Ş., Ghai, R., Sorokin, D.Y., Muyzer, G., 2019. Metagenomes and metatranscriptomes shed new light on the microbial-mediated sulfur cycle in a Siberian soda lake. BMC Biol. 17, 69.
- Wick, R.R., Judd, L.M., Gorrie, C.L., Holt, K.E., 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Computational Biology 13.
- Zheng, J., Ge, Q., Yan, Y., Zhang, X., Huang, L., Yin, Y., 2023. dbCAN3: automated carbohydrate-active enzyme and substrate annotation. Nucleic Acids Res. 51, W115–W121.

*Natronospira bacteriovora* sp. nov., and *Natronospira elongata* sp. nov., extremely salt-tolerant soda lake predatory proteolytics and proposal to classify the genus *Natronospira* into *Natronospiraceae* fam. nov., and *Natronospirales* ord. nov., within the class *Gammaproteobacteria*.

Dimitry Y. Sorokin, Alexander Y. Merkel, Tatyana V. Kolganova, Nicole J. Bale and Jaap S. Sinninghe-Damsté

# **Supplementary Information**

 Table S1. General features of Natronospira genomes.

**Table S2**. Secretion systems type II and the pili type IV in genomes of strains AB-CW1 and AB-CW4.

**Table S3**. Comparative composition of PLFA in members of the genus *Natronospira* grown at pH 9.5, 37°C until late exponential growth phase. The AB-CW strains very grown on peptone at 3 M total Na<sup>+</sup> and *Natronospira proteinivora* BSker1<sup>T</sup> – at 4 M total Na<sup>+</sup> with casein. The major compounds are in bold.

**Figure S1.** Phylogenetic placement of strains AB-CW1 and AB-CW4 within the genus *Natronospira* based on 16S rRNA gene sequences. Bootstrap consensus tree is shown with values placed at the nodes. Bar, 0.1 change per position.

Table S1.

	N. elongata	N. bacteriovora	N. proteinivora
	AB-CW1 <sup>T</sup>	AB-CW4 <sup>T</sup>	BSker1 <sup>T</sup>
Total length, Mb	3.1	3	2.9
number of contigs	102	15	4
GC, %	61.5	62.5	60
N50	83.5 kb	372 kb	1.6 Mb
Genome coverage	1000.0x	630.0x	512.0x
Completeness, %	95.43	97.18	97.99
Contamination, %	0.42	0.04	0.02
Genes (total)	2792	2,725	2,650
Genes (protein coding)	2729	2,660	2,578
Genes (RNA)	51	52	51
Pseudo genes (total)	12	13	21
Complete rRNAs	1, 1, 1 (5S, 16S, 23S)	1, 1, 1 (5S, 16S, 23S)	1, 1, 1 (5S, 16S, 23S)
tRNAs	44	45	44
GenBank	GCA_034931365.1	GCA_030848495.1	GCF_024170465.1

# Table S2.

AB-CW1			AB-CW4			
Locus: MEA544+	Gene	Protein	Locus: WP_30672+	Gene	Protein	
		Type IV secretion system (pili for cell	adhesion, e	extracellu	lar DNA uptake)	
4695	pilE	type IV pilin	7304	fimT	pseudopilin	
4696	pilC	type IV pilin (surface virulence activator)	7305	pilV	pilus assembly protein	
4697	pilX	pilus assembly protein	7306	pilW	pilus assembly protein	
4698	pilW	pilus assembly protein	7307	pilX	pilus assembly protein	
4699	pilV	pilus assembly protein	7308	pilC	type IV pilin (surface virulence activator)	
4670; 4746	fimT	pseudopilin	7309	pilE	type IV pilin	
4822	fimA	fimbrial pilin with lectin domain	7989	pilA	type IV pilin assembly protein	
4823	pilB	type IV-A pilus assembly ATPase	7990	fimA	fimbrial pilin with lectin domain	
4824	pilA	type IV pilin assembly protein	7991	pilB	type IV-A pilus assembly ATPase	
5262-5263	pilU	type IV-A pilus ATPase (twitching motility)	9113-9114	pilU	type IV-A pilus ATPase (twitching motility)	
5906	pilT	type IV pilus twitching motility protein	9114	pilT	type IV pilus twitching motility protein	
		Type II secretion (pili for toxin and e	extracellula	r protein	s translocation)	
4924	gspG		7730	gspM		
4925	gspF		7731	gspL		
4926	gspE		7732	gspN		
4927	gspF		7733	gspK		
5264	gspL		7734	gspL		
5265	gspK		7735	gspJ		
5266	gspJ	type II secretion system proteins	7736	gspH	type II secretion system proteins	
5267	gspI		7737	gspG		
5268	gspH		8375	gspF		
5269	gspG		8376	gspG		
6182	gspI		8378	gspI		
6183	gspO		8379	gspH		
6184	gspP		8380	gspO		
6185	lamG	lectin associated with metalloprotease	8381	gspP		
			8382	lamG	lectin associated with metalloprotease	

Supplementary Table S3.

PLFA (>0.5%)	AB-CW1	AB-CW4	Bsker1 <sup>T</sup>
3-OH C11:0	0.8		
i3-OH C11:0			1.1
C14:0	2.4		
iC15:0	3.3	2.8	0.7
C16:0	12.4	5.1	5.6
iC16:0		1.9	0.6
C16:1 ω9	0.7	0.8	
iC17:0	23.6	28.7	48.1
ai C17:0	1.8	0.9	0.8
iC17:1 ω9	22.1	34.3	36.4
C18:0	5.5	2.6	1.8
iC18:0		1.4	1.8
C18:1 w7			1.8
C18:1 ω9	7.0		1.6
C18:1 w11	1.5	1.9	
iC19:0		0.7	
iC19:1 ω9	14.0	17.3	
i19:0 + C20:1	0.9		



Fig. S1.