**Natronospira proteinivora** gen. nov., sp. nov, an extremely salt-tolerant, alkaliphilic gammaproteobacterium from hypersaline soda lakes

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**Abstract**

Brine samples from Kulunda Steppe soda lakes (Altai, Russia) were inoculated into a hypersaline alkaline mineral medium with β-keratin (chicken feather) as a substrate. The micro-organisms dominating the enrichment culture were isolated by limiting serial dilution on the same medium with casein as a substrate. The cells of strain BSker1ᵀ were motile, curved rods. The strain was an obligately aerobic heterotroph utilizing proteins and peptides as growth substrates. The isolate was an obligate alkaliphile with a pH range for growth from pH 8.5 to 10.25 (optimum at pH 9.5), and it was extremely salt tolerant, growing with between 1 and 4.5 M total Na⁺ (optimally at 2–2.5 M). BSker1ᵀ had a unique composition of polar lipid fatty acids, dominated by two C₁₇ species. The membrane polar lipids included multiple unidentified phospholipids and two aminolipids. According to phylogenetic analysis of the 16S rRNA gene sequence, the isolate forms a novel branch within the family *Ectothiorhodospiraceae* (class *Gammaproteobacteria*) with the highest sequence similarity to the members of this family being 91%. On the basis of distinct phenotypic and genotypic properties, strain BSker1ᵀ (=JCM 31341ᵀ=UNIQEM U1008ᵀ) is proposed to be classified as a representative of a novel genus and species, *Natronospira proteinivora* gen. nov., sp. nov.

Hypersaline soda lakes with salt concentrations reaching saturation are a rare example of saline alkaline lakes, with brines dominated by sodium carbonates resulting in molar values of soluble alkalinity and pH up to 11. Despite these double extreme conditions, this type of salt lakes can be very productive due to the presence of haloalkaliphilic cyanobacteria and unicellular algae [1–3]. They harbour dense microbial populations dominated by natronophilic (soda-loving) prokaryotes, the identities of which are only recently being uncovered [4–6]. Apart from the fundamental interest, soda lake prokaryotes have attracted significant attention as a potent resource of halo-alkali-stable exo-enzymes, especially hydrolases [7–9].

Given the domination of cyanobacteria (whose biomass is protein-rich) as primary producers in soda lakes, it is surprising that relatively little data are still available on the identity of soda lake prokaryotes utilizing proteins as a growth substrate. To our knowledge, there is only a single report specifically addressing this aspect, characterizing the anaerobic, protein-utilizing, haloalkaliphilic bacterium *Proteinivorax tanatarense* from a Siberian soda lake [10]. The other studies on soda lake proteolytic micro-organisms mostly relate to finding alkali-stable proteases [11–15]. Among those aerobic, proteolytic prokaryotes, the only example of extremely salt-tolerant alkaliphiles belongs to the haloarchaea, while protein-utilizing, alkaliphilic bacteria which can grow with up to saturating salt conditions have not yet been reported.

Here, we describe properties of an extremely salt-tolerant and alkaliphilic, protein-utilizing gammaproteobacterium, enriched with keratin from hypersaline lakes, and propose to assign it to a new genus and novel species, *Natronospira proteinivora* gen. nov., sp. nov.

The source of the isolate was brines from four hypersaline soda lakes (mixed in equal proportion) in the south Kulunda Steppe (Altai, Russia) obtained in July 2016. The salinity ranged from 150 to 400 g l⁻¹, the pH from 9.7 to 10.4 and carbonate alkalinity from 1.2 to 5 M.

The extremely salt-tolerant bacteria utilizing recalcitrant proteins for growth were enriched under aerobic conditions using defatted chicken feathers. The mineral base medium...
containing 4 M total Na⁺ (2 M Na⁺ as sodium carbonates and 2 M NaCl at pH 9.5 and 1 g K₂HPO₄ l⁻¹) was supplemented with 1 ml l⁻¹ each of trace of metal solution and vitamin mix [16] and 1 mM MgCl₂. The incubations were performed at 37 °C in closed serum bottles with 10 % liquid placed on a shaker at 180 r.p.m. The enrichment development was monitored by the extent of feather degradation and by microscopy. After 1 month, the culture was serially diluted in the same medium but with casein, instead of keratin, as the substrate, and the maximum positive dilutions were plated onto a solid medium prepared by 1:1 mixing of the liquid medium and 4 % agarose at 50 °C. To compensate for the lowering of salinity, solid NaCl was added directly to the mixture before pouring the plates. The plates were incubated at 37 °C for 2 weeks, and the dominant colony type was picked and inoculated into the liquid medium with casein and repeatedly purified by plating. This, eventually, resulted in isolation of strain BSker1ᵀ. The purity of strain BSker1ᵀ was checked microscopically (Zeiss Axioplan Imaging 2 microscope) and by 16S rRNA gene sequencing. For the total cell electron microscopy, cells were centrifuged and resuspended in 3 M NaCl and 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. The cells of BSker1ᵀ were motile, vibrio-shaped and varying in length depending on the growth phase and growth conditions. The electron microscopy indicated the presence of two different types of appendages: a single thick and long appendage, apparently a flagellum; and multiple thin filaments making a network, most probably of the pili type (Fig. 1). The positive alkali test (3 % KOH) showed the Gram-negative type of the cell.

The membrane polar lipids were extracted from the freeze-dried cells and their composition was analysed by TLC by the DSMZ Identification Service according to the methods of Tindall [17, 18]. The fatty acid methyl esters were analysed by gas chromatography mass spectrometry according to Labrenz et al. [19] and Strömpl et al. [20] Respiratory lipiquinones were extracted from lyophilized cells by cold acetone and first separated by TLC [21] and then eluted and further analysed by tandem mass spectrometry (LCG Advantage Max) in combination with HPLC mass spectrometry. The polar lipid analysis demonstrated the presence of five phospholipids, two aminolipids and two undefined lipid species (Fig. S1, available in the online Supplementary Material). The respiratory quinones were represented by a single ubiquinone species, Q-8. The polar lipid fatty acid (PLFA) profile of strain BSker1ᵀ was quite unique and significantly different from those of the closest phylogenetic relatives (see below) with the domination of two C₁₇ species, i₁₇:0 and i₁₇:1ω₉c, making up 84.5 % of the total (Table S1).

The 16S rRNA gene sequence analysis was performed using the MEGA 6 package [22]. The results indicated that the isolate forms a novel lineage within the family Ectothiorhodospiraceae (class Gammaproteobacteria) with a maximum sequence similarity of 91 % to characterized members of this family (Fig. 2). This family contains multiple genera of extremely salt-tolerant, halophilic and haloalkaliphilic bacteria from hypersaline habitats with variable types of metabolism, including organoheterotrophs, chemo-lithoautotrophs and anoxygenic phototrophs [23]. The G+C content of genomic DNA of strain BSker1ᵀ, analysed by the DSMZ Identification Service using the HPLC method [24], was 60.1 mol%.

Strain BSker1ᵀ was an obligately aerobic organoheterotroph utilizing various proteins and peptides as the growth substrate, including the following: heat-sterilized α-keratin (powdered, fine fraction), casein, lactalbumin, gelatin, soy protein, bovine collagen, filter-sterilized bovine serum albumin and haemoglobin, various peptones and yeast extract. The protease activity was mostly associated with the cells, and a minor part was also found in the culture supernatant in the fraction above 30 kDa (Fig. S2).

Strain BSker1ᵀ was an extremely salt-tolerant and obligately alkaliphilic bacterium. At pH 9.5, it grew with casein between 1 and 4.5 M total Na⁺ with an optimum of 2–2.5 M total Na⁺ (Fig. 3a), and the growth was independent of the presence of Cl⁻. At optimal salinity, the pH range for growth with casein was between pH 8.5 and 10.25, with an optimum at pH 9.5 (Fig. 3b). The phenotypic comparison of BSker1ᵀ with its nearest phylogenetic relatives from the family Ectothiorhodospiraceae is given in Table 1.

Overall, strain BSker1ᵀ represents a first example of an extremely salt-tolerant, aerobic, alkaliphilic bacterium.
isolated from soda lakes and specializing in utilization of insoluble proteins for growth. On the basis of the unique phylogeny and phenotypic properties, the novel isolate is proposed to represent a novel genus and species, *Natronospira proteinivora* gen. nov., sp. nov.

**DESCRIPTION OF NATRONOSPIRA GEN. NOV.**

*Natronospira* (Na.tr.o.no.spi’ra Gr. n. natron arbitrarily derived from the Arabic n. natrun or natron soda; L. fem. n. spira a coil; N.L. fem. n. *Natronospira* a soda-loving spirillum).
Extremely natronophilic, protein-utilizing, aerobic member of the family *Ectothiorhodospiraceae* found in hypersaline soda lakes. The type species is *Natronospira proteinivora*.

**DESCRIPTION OF *NATRONOSPIRA PROTEINIVORA* SP. NOV.**

*Natronospira proteinivora* (pro.te.i.ni*vo*ra N.L. neut. n. *proteinum* protein; L. fem. suff. *vora* devouring; N.L. fem. adj. *proteinivora* devouring proteins).

Cells are Gram-reaction-negative, from short spirilla to long filaments, 0.35–0.4/2–30 µm and motile by a single thick flagellum. The cells also have multiple thin filamentous appendages. The colonies are yellowish, up to 3 mm in diameter, flat and round. The polar lipids include several unidentified phospho- and aminolipids. The respiratory quinones are represented by ubiquinone Q-8. The polar lipid fatty acids are dominated by i17 : 0 and i17 : 1\(^{9}\)c.

Strictly aerobic organoheterotroph utilizing various proteins and peptides for growth. Obligately alkaliphilic with a pH range for growth of pH 8.5 to 10.25 and an optimum of pH 9.5. Chloride-independent, extreme natronophile, growing within the range of 1 to 4.5 M total Na\(^+\) with optimum growth at 2–2.5 M. The upper temperature limit for growth (at optimal pH and salinity) is 45 °C.

The type strain, BSker1\(^T\) (=JCM 31341\(^T\)=UNIQEM U1008\(^T\)), was isolated from brines of hypersaline soda lakes in Kulunda Steppe (Altai, Russia). The G+C content of the genomic DNA of the type strain is 60.1 mol% (HPLC).

**Table 1. Comparative properties of strain BSker1\(^T\) and its closest phylogenetic relatives from the family *Ectothiorhodospiraceae***

<table>
<thead>
<tr>
<th>Property</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Motile curved rod</td>
<td>Motile spirillum</td>
<td>Motile spirillum</td>
<td>Non-motile curved rod</td>
<td>Motile rod</td>
</tr>
<tr>
<td>Relationship to oxygen</td>
<td>Obligate aerobe</td>
<td>Obligate aerobe</td>
<td>Obligate anaerobe</td>
<td>Obligate aerobe</td>
<td>Facultative aerobe (can grow by nitrate reduction to nitrite)</td>
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<tr>
<td>Metabolism</td>
<td>Organoheterotroph</td>
<td>Organoheterotroph</td>
<td>Photoautotroph/phototrophetroph</td>
<td>Organoheterotroph</td>
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<td>Substrates</td>
<td>Proteins, peptides</td>
<td>Organic acids, amino acids, sugars</td>
<td>Inorganic sulfur, organic acids</td>
<td>Pyruvate, glycerol</td>
<td>Sugars, amino acids</td>
</tr>
<tr>
<td>Protease activity</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>NR</td>
<td>–</td>
</tr>
<tr>
<td>Salinity range for growth (opt.)</td>
<td>1-4.5 (2-2.5)</td>
<td>0.3–3.4 (1)</td>
<td>0.5–5 (2.5)</td>
<td>1.6–4.1 (2.5)</td>
<td>0.5–4 (1.5–1.8)</td>
</tr>
<tr>
<td>(M Na(^+))</td>
<td>8.5–10.25 (9.5)</td>
<td>(9-10)</td>
<td>(8.5–9)</td>
<td>6–9 (7.5–8)</td>
<td>6–10(^*) (8)</td>
</tr>
<tr>
<td>Predominant PLFAs</td>
<td>i17 : 0, i17 : 1(^{9})c</td>
<td>18 : L(^{9})c, 16 : 0</td>
<td>18 : L(^{9})c, 16 : 0</td>
<td>18 : L7(^{7})c, 16 : 0</td>
<td>18 : L(^{7})c, 19(^{9})c</td>
</tr>
<tr>
<td>Predominant lipoquinone</td>
<td>Q-8</td>
<td>NR</td>
<td>MK-8</td>
<td>NR</td>
<td>Q-8</td>
</tr>
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<td>DNA G+C content (mol%)</td>
<td>60.1</td>
<td>66.2</td>
<td>66.5–69.7</td>
<td>62.7</td>
<td>61.9</td>
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<tr>
<td>Habitat</td>
<td>Hypersaline soda lakes</td>
<td>Soda lakes</td>
<td>Salt lake</td>
<td>Sea solar saltern</td>
<td>Saline alkaline lake</td>
</tr>
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</table>

\(^*\)The final pH was not measured.
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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

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