Natronotalea proteinilytica gen. nov., sp. nov. and Longimonas haloalkaliphila sp. nov., extremely haloalkaliphilic members of the phylum Rhodothermaeota from hypersaline alkaline lakes

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Abstract

Two proteolytic bacterial strains, BSker2T and BSker3T, were enriched from sediments of hypersaline alkaline lakes in Kulduna Steppe (Altai, Russia) with chicken feathers as substrate, followed by pure culture isolation on hypersaline alkaline media with casein. The cells were non-motile, filamentous, flexible rods. The isolates were obligately aerobic heterotrophs utilizing proteins and peptides as growth substrates. Both were obligate alkaliphiles, but differed in their pH optimum for growth: pH 9.5–9.8 for BSker2T and pH 8.5–8.8 for BSker3T. The salt range for growth of both isolates was between 2 and 4.5 M total Na+ with an optimum at 2.5–3 M. No organic osmolytes were detected in cells of BSker2T, but they accumulated high intracellular concentrations of K+. The polar lipid fatty acids were dominated by unsaturated C16 and C18 species. The 16S rRNA gene phylogeny indicated that both strains belong to the recently proposed phylum Rhodothermaeota. BSker2T forms a novel genus-level branch, while BSker3T represents a novel species-level member in the genus Longimonas. On the basis of distinct phenotypic and genotypic properties, strain BSker2T (=JCM 31342T=UNIQEM U1009T) is proposed to be classified as a representative of a novel genus and species, Natronotalea proteinilytica gen. nov., sp. nov., and strain BSker3T (=JCM 31343T=UNIQEM U1010T) as a representative of a novel species, Longimonas haloalkaliphila sp. nov.

Hypersaline lakes characterized by highly alkaline salt-saturated brines with pH from 9 to 11 can harbour diverse and dense haloalkaliphilic prokaryotic communities [1–4], which have recently been subjected to intensive fundamental and application-oriented studies [5–7]. One of the least studied aspects in this area concerns the identity of aerobic prokaryotes capable of utilizing insoluble proteinaceous substrates for growth at extremely high salt and pH conditions. Our recent focused research in this direction allowed us to identify the first aerobic, extremely salt-tolerant and obligately alkaliphilic gammaproteobacterium from hypersaline soda brines in south-eastern Siberia. For this organism specialized in utilization of proteins as growth substrates, we suggested the novel genus and species Natrononaspe proteinivora [8]. Here we describe properties of a second group of extremely haloalkalotolerant, protein-utilizing bacteria enriched from sediments of hypersaline alkaline lakes that represent a new genus and two novel species in the phylum Rhodothermaeota (forming a deep lineage within the phylum Bacteroidetes) [9].

Surface sediments from two types of hypersaline alkaline lakes in Kulduna Steppe (Altai, Russia) were used as the inoculum for enrichment cultures: (1) from typical soda lakes with extremely high alkalinity, Tanatar-1 and Tanatar-2 (July 2016, salinity=300–400 g l−1, pH=9.7–10.2, total carbonate alkalinity=3.4–3.5 M), and (2) from Stamp Lake with low alkalinity (July 2015, salinity=325 g l−1, pH=9.1, total carbonate alkalinity=0.15 M).

The protein-utilizing bacteria were enriched under aerobic conditions using defatted chicken feathers with β-keratin as a growth substrate. A mineral base medium containing 4 M total Na+ with extremely high alkalinity, Tanatar-1 and Tanatar-2 (July 2016, salinity=300–400 g l−1, pH=9.8) was used for the Tanatar sample, while the Stamp lake sediments were inoculated into 4 M NaCl-based medium adjusted to pH 9 with 1 M Na2CO3. Both media also included 1 g K2HPO4 l−1 and 5 g KCl l−1. After sterilization, the media were supplemented with 1 ml trace metal solution and vitamin mix l−1 [10] and 1 mM MgCl2. Defatted chicken feathers were added as substrate at approximately 2 g l−1. Before inoculation, the sediments were resuspended...
1:10 in the basic medium and the suspension was allowed to stand for 20 min, resulting in precipitation of the course fractions. A 1 ml aliquot from the top fraction containing mostly colloidal sediments was then used to inoculate 40 ml cultures in 200 ml closed serum bottles placed on a rotary shaker at 37°C and at 200 r.p.m. The development of the enrichment culture was monitored by the extent of feather degradation and by microscopy. After 20–30 days, the cultures were serially diluted in the same medium but with casein as substrate, and the maximal positive dilutions were plated onto a solid medium prepared by 1:1 mixing of the liquid medium and a 4% solution of extensively washed agar at 50°C. To compensate for the lower salinity, sterile, solid NaCl was added directly to the mixture before pouring the plates. After 1–3 weeks of incubation in closed plastic bags at 37°C, the dominant colony types were transferred to the respective liquid media with casein and purified by repeated plating. This, eventually, resulted in isolation of two bacterial strains: BSker2^T from the Tanatar lakes and BSker3^T from Stamp Lake. The purity was checked microscopically (Zeiss Axioplan Imaging 2 microscope) and by 16S rRNA gene sequencing.

On casein agar, the colonies of both strains were flat and spreading, orange–red in colour and formed a clear zone of casein hydrolysis (Fig. S1, available in the online Supplementary Material). The pigment extracted from the cells with acetone/methanol had an absorbance maximum at 480 nm and two shoulders at around 450 and 510 nm (Fig. S2). Exponentially growing cells of both isolates were long, flexible, non-motile rods. In the stationary phase, the BSker3^T cells elongated up to 100 µm and formed coiled aggregates (Fig. 1). The cells were apparently covered with a thick extracellular polymeric substance (EPS) matrix since even high-speed centrifugation did not allow us to obtain a compact cell pellet. The KOH test proved a Gram-negative type of cell wall.

Membrane polar lipids were extracted from freeze-dried cells and their composition was analysed by TLC at the DSMZ Identification Service according to the methods of Tindall [11, 12]. The fatty acid methyl esters were analysed by gas chromatography-mass spectrometry (MS) according to the methods of Labrenz et al. [13] and Strömpl et al. [14]. Respiratory lipoquinones were extracted from the lyophilized cells by cold acetone, separated by TLC [15], and subsequently eluted and further analysed by tandem MS (LCG Advantage Max) in combination with HPLC-MS. The polar lipid analysis of cell membranes of strain BSker2^T showed the presence of two glycolipids and four unidentified phospholipid species (Fig. S3). The respiratory quinone analysis identified the single menaquinone species MK-7 in cells of BSker2^T. In their phospholipid fatty acid (PLFA) profiles, the novel isolates were similar in the major PLFAs to their two extremely halophilic closest relatives from the phylum Rhodothermaeota, Longimonas halophila and Salisaeta longa. However, there was variability in the abundance of other C_{15–C_{17}} components, both between the two BSker strains and between them and the nearest relatives (Table S1).

Organic compatible solutes were analysed in BSker2^T cells grown at 4 M total Na^+, either at pH 8.6 (NaCl base) or pH 10 (sodium carbonate base), using HPLC and ^1^H nuclear magnetic resonance (NMR) after extraction according to a modified method of Bligh and Dyer [16, 17]. The polar fraction was analysed on a Nucleosil 100–3 aminopropyl phase HPLC column (Macherey and Nagel) using acetonitrile/water (80:20, v/v) as the mobile phase at a flow rate of

![Cell morphology of strains BSker2^T (a) and BSker3^T (b, c) grown with casein at 4 M total Na^+ and 37°C; phase-contrast microphotographs. (a and b), cells from exponential and stationary growth phases, respectively; (c), complex aggregation of extremely elongated cells of BSker3^T in late stationary growth phase. Bars, 10 µm.](image-url)
1 ml min⁻¹ [18]. Compounds were monitored using a combination of refractive index and UV detector. Amino-reactive compounds were analysed by gradient HPLC with pre-column FMOC-ADAM derivatization as described previously [19]. No known organic osmolytes were detectable, neither on the aminopropyl phase column (for neutral and zwitterionic solutes) nor with FMOC derivatization (for amino reactive solutes). The latter revealed that glutamate was the dominant amino acid at a concentration of 0.45 and 0.33 mmol (g protein)⁻¹ for the chloride and the soda sample, respectively. Both values are within the expected range. For Escherichia coli cells, a regular glutamate value of 0.15 mmol (g protein)⁻¹ and a transient accumulation to 0.68 mmol (g protein)⁻¹ upon salt stress have been reported [20].

For NMR analysis, the dry cells were extracted with 1 ml chloroform/methanol/water (10:5:4, by vol.) followed by phase separation according to the method of Galinski and Herzog [18]. The polar fraction was evaporated overnight, and the dry residue was dissolved in 1 ml D₂O as lock signal. The sample was further supplemented with the internal standard benzene-1,2,4,5-tetracarboxylate sodium salt to give a final concentration of 10 mM. ¹H NMR spectra were recorded on a Bruker Avance 300 DPX spectrometer. The soda sample (lower protein content and lower osmolarity) revealed no distinct resonances apart from the internal standard. The chloride sample displayed a number of peaks, none of which could be related to any known compatible solutes. In relation to the internal standard, the strongest signals between 1 and 4 p.p.m. represented presumptive concentrations of unknown compounds of no more than 0.25 mmol (g protein)⁻¹; while for the model halophilic organism Halomonas elongata grown at 3.42 M NaCl, an

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**Fig. 2.** Maximum-likelihood 16S rRNA gene sequence based phylogenetic tree showing the positions of strains BSker²ᵀ and BSker³ᵀ (in bold type) within the phylum Rhodothermaeota. Branch lengths (see bar) 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. In total, 1305 positions were used in the alignment of 24 sequences. Numbers at nodes indicate bootstrap values of 1000 repetitions. A representative of the phylum Bacteroidetes, Marivirga tractuosa DSM 4126ᵀ (Genbank accession CP002349.1), was used as an outgroup.
To analyse the intracellular potassium, freeze-dried cells of BSker2 were extracted according to a modified protocol of Bligh and Dyer [16, 17]. The water-soluble fraction was subjected to cation analysis by isocratic HPLC with conductimetric detection (Thermo Scientific conductoMonitor III) on a Metrosept Cation C4-100/4.0 column (Methrom) using an eluent of 1.7 mM nitric acid and 0.7 mM dipicolinic acid at a flow rate of 0.9 ml min⁻¹. The estimated specific potassium content was 305 and 115 mg (mg cell protein)⁻¹ in the cells grown in NaCl base and in soda base, respectively. The first value is close to what is usually found in halarchaea [22], while the much lower content in the soda-grown cells might be explained by two times lower osmotic pressure of this weak electrolyte in comparison with the strongly electrolytic NaCl [2]. In conjunction with extreme halophily, this is an indication that strain BSker2 employs the 'salt-in' osmoprotection mechanism, which is also found in an extremely halophilic member of the phylum *Rhodothermaeota* – *Salinibacter ruber* [23].

The 16S rRNA gene sequence based phylogenetic analysis was performed in the MEGA 6 package [24] using the maximum-likelihood algorithm. The results demonstrated that BSker2 forms a novel genus lineage within the family *Rhodothermaeaceae*, phylum *Rhodothermaeota*, with a maximum pairwise sequence similarity of 92% to the characterized halophilic members of the genus with validly published names, *Longimonas halophila* [25], *Salisaeta longa* [26] and *Longibacter salinarum* [27]. On the other hand, BSker3 apparently represents a novel species in the genus *Longimonas* with 97% sequence similarity to the extremely halophilic *Longimonas halophila* (Fig. 2). The phenotypic comparison of the BSker isolates with the three closest phylogenetic relatives is given in Table 1. Interestingly, despite a significant phylogenetic distance, the unusual cell morphology and some other important characteristics (such as substrate profile, extreme salt tolerance, the type of lipoquinones) were common among the soda lake isolates and the three halophilic genera mentioned above. The G+C content of the genomic DNA was analysed by the DSMZ Identification Service using the HPLC method [28]. The values determined for BSker2 and BSker3 were 55.9 and 58.2 mol%, respectively.

The BSker strains were obligately aerobic organoheterotrophs which grew best with various proteins and peptides including casein, gelatin, filter-sterilized bovine serum...
With respect to their salt demand, both BSker2 and BSker3 were not observed. Anaerobic fermentative growth with maltose and peptone maltose. In addition, BSker2 was tested qualitatively in strain BSker2 T. At optimal salinity, the pH range for growth with casein hydrolysate. Polymeric substrates tested but not utilized included amylopectin, birch wood xylan, amorphous forms of cellulose and chitin, and emulsified olive oil. Among the monomeric substrates tested were sugar hexoses and pentoses, sugar alcohols and C2–C6 organic acids. Both strains grew (again only in the presence of a minimum of 100 mg casein hydrolysate l–1) with glycerol and maltose. In addition, BSker2T also utilized cellobiose. Anaerobic fermentative growth with maltose and peptone was not observed.

With respect to their salt demand, both BSker2T and BSker3T can be qualified as extreme halophiles with their total Na+ range for growth between 2 and 4.5 M (optimum around 3 M) (Fig. 3a). In contrast to their extremely halophilic relatives, the BSker strains were not dependent on high Mg concentrations. On the other hand, the strains also differed from most of the soda lake bacterial isolates by obligate growth dependence on the presence of high Cl– concentrations (minimum 0.5 M). The latter might be related to its usage as a counter anion for intracellular potassium accumulation.

At optimal salinity, the pH range for growth with casein was substantially different for the two strains. The soda lake isolate BSker2T had a profile typical for obligate nanorophiles with a pH range from pH 8.2 to 10.2 (optimum around pH 9.5), while BSker 3T was only moderately alkaliphilic with an optimum at pH 8.5–8.8 (Fig. 3b).

In conclusion, the two aerobic bacterial isolates from hypersaline alkaline lakes represent the first examples to our knowledge of extremely halophilic and alkaliphilic bacteria specialized in utilization of proteinaceous compounds and with an apparent usage of the ‘salt-in’ osmoregulation strategy. With this combination of properties, they are clearly different from their nearest phylogenetic relatives and are proposed to be classified as representatives of the novel genus and species Natronotalea proteinilytica gen. nov., sp. nov. (strain Bsker2T) and Longimonas haloalkaliphila sp. nov. (Bsker3T).

**DESCRIPTION OF NATRONOTALEA GEN. NOV.**

Natronotalea (Na.tro.no.ta’le.a Gr. n. natron arbitrarily derived from the Arabic n. natrun or natron soda; L. fem. n. talea a staff, stick – a long rod; N.L. fem. n. Natronotalea a soda-loving long rod).

Extremely haloalkaliphilic, protein-utilizing, aerobic member of the family Rhodothermaceae, phylum Rhodothermaeota, found in hypersaline alkaline lakes. The type species is Natronotalea proteinilytica.

**DESCRIPTION OF NATRONOTALEA PROTEINILYTICA SP. NOV.**

Natronotalea proteinilytica [pro.te.i.ni.ly’ti.ca N.L. neut. n. proteinum protein; N.L. fem. adj. lytica (from Gr. fem. adj. lytike) dissolving; N.L. fem. adj. proteinilytica dissolving proteins].

Cells have the Gram-negative type of cell wall and are long, flexible rods, 0.5×5–15 μm, non-motile and form EPS. Colonies are flat, spreading up to 5 mm, and orange–red. The cell pigment has an absorbance maximum at 480 nm. The polar lipids include four unidentified phospholipids and two glycolipids. The respiratory quinones are represented
by MK-7. The PLFAs are dominated by unsaturated 16:1ω7c and 18:1ω7c. Strictly aerobic organoheterotroph utilizing various proteins and peptides for growth. It can also grow, but less actively, with amylose, maltose, cellobiose and glycerol. Obligately alkaliphilic, with a pH range for growth from pH 8.2 to 10.2 (optimum at pH 9.5–9.8). Chloride-dependent, extreme halophile which requires a Na⁺ range for growth of from 2 to 4.5 M (optimum at 2.5–3 M). The upper temperature limit for growth (at optimal pH and salinity) is 46°C.

The type strain, BSker2^T (=JCM 31342^T=UNIQEM U1009^T), was isolated from sediments of a hypersaline alkaline lake in Kulunda Steppe (Altai, Russia). The G+C content of the genomic DNA of the type strain is 55.9 mol% (HPLC).

**DESCRIPTION OF LONGIMONAS HALOALKALIPHILA SP. NOV.**

*Longimonas haloalkaliphila* [Gr. n. *hals halos* salt; N.L. n. *alkali* soda ash (from Arabic *al-qalyi* the ashes of saltwort); N.L. adj. *philus* (from Gr. adj. *philos* -ε-ον friend, loving; N.L. fem. adj. *haloalkaliphila* salt and alkali-loving].

Cells have the Gram-negative type of cell wall and are non-motile, long, flexible rods, 0.5–0.6×8–30 μm in exponential growth phase, and up to 100 μm long in aggregates in aged cultures. Colonies are flat, spreading up to 8 mm, and orange-red. The cell pigment has an absorbance maximum at 480 nm. The PLFAs are dominated by unsaturated 16:1ω7c and 18:1ω7c. Strictly aerobic organoheterotroph utilizing a limited number of proteinaceous and peptide substrates for growth. Less active growth is observed with maltose and glycerol. Obligately but only moderately alkaliphilic, with a pH range for growth from pH 7.8 to 9.3 (optimum at pH 8.5–8.8). Chloride-dependent extreme halophile which requires a Na⁺ range for growth of from 2 to 4.8 M (optimum at 2.5–3 M). The upper temperature limit for growth (at optimal pH and salinity) is 48°C.

The type strain, BSker2^T (=JCM 31343^T=UNIQEM U1010^T), was isolated from sediments of a hypersaline alkaline lake in Kulunda Steppe (Altai, Russia). The G+C content of the genomic DNA of the type strain is 58.2 mol% (HPLC).


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