

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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DOI

[10.1099/ijsem.0.002272](https://doi.org/10.1099/ijsem.0.002272)

Publication date

2017

Document Version

Accepted author manuscript

Published in

International Journal of Systematic and Evolutionary Microbiology

Citation (APA)

Sorokin, D. Y., Khijniak, T. V., Galinski, E. A., & Kublanov, I. V. (2017). *Natronotalea proteinilytica* gen. nov., sp. nov. and *Longimonas haloalkaliphila* sp. nov., extremely haloalkaliphilic members of the phylum Rhodothermaeota from hypersaline alkaline lakes. *International Journal of Systematic and Evolutionary Microbiology*, 67(10), 4161-4167. Article 002272. <https://doi.org/10.1099/ijsem.0.002272>

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--Manuscript Draft--

Manuscript Number:	IJSEM-D-17-00584R2
Full Title:	Natronotalea proteinilytica gen. nov., sp. nov, and Longimonas haloalkaliphila sp. nov., extremely haloalkaliphilic members of the phylum Rhodothermaeota from hypersaline alkaline lakes
Article Type:	Taxonomic Description
Section/Category:	New taxa - Bacteroidetes
Keywords:	proteolytic keratin hypersaline lake haloalkaliphilic Rhodothermaeota
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Manuscript Region of Origin:	RUSSIAN FEDERATION
Abstract:	Two proteolytic bacterial strains, BSkер2T and BSkер3T, were enriched from sediments of hypersaline alkaline lakes in Kulunda Steppe (Altai, Russia) with chicken feathers as substrate, followed by pure culture isolation on hypersaline alkaline media with casein. The cells are nonmotile filamentous flexible rods. The isolates are obligate aerobic heterotrophs utilising proteins and peptides as growth substrates. Both are obligate alkaliphiles, but differed in their pH optimum: 9.5-9.8 for BSkер2T and 8.5-9 for BSkер3T. The salt range for growth of both isolates is between 2 and 4.5 M total Na ⁺ with an optimum at 2.5-3 M. No organic osmolytes were detected in cells of BSkер2T, but it 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. BSkер2T forms a novel genus-level branch, while BSkер3T represents a novel species-level member in the genus Longimonas. On the basis of distinct phenotypic and genotypic properties, strain BSkер2T (JCM 31342T=UNIQEM U1009T) is proposed to be classified as a new genus and species Natronotalea proteinilytica and strain BSkер3T (JCM 31343T=UNIQEM U10110T) as a new species Longimonas haloalkaliphila.

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

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24 Running title: *Natronotalea proteinilytica* gen. nov., sp. nov., and *Longimonas*
25 *haloalkaliphila* sp. nov.
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29 The 16S-rRNA gene sequence of strain BSk_{er}2^T and BSk_{er}3^T are deposited in the GenBank
30 under the numbers KU720569 and KU72070.
31

32 Two proteolytic bacterial strains, BSk₂^T and BSk₃^T, were enriched from sediments
33 of hypersaline alkaline lakes in Kulunda Steppe (Altai, Russia) with chicken feathers as
34 substrate, followed by pure culture isolation on hypersaline alkaline media with casein.
35 The cells are nonmotile filamentous flexible rods. The isolates are obligate aerobic
36 heterotrophs utilising proteins and peptides as growth substrates. Both are obligate
37 alkaliphiles, but differed in their pH optimum: 9.5-9.8 for BSk₂^T and 8.5-9 for
38 BSk₃^T. The salt range for growth of both isolates is between 2 and 4.5 M total Na⁺
39 with an optimum at 2.5-3 M. No organic osmolytes were detected in cells of BSk₂^T, but
40 it accumulated high intracellular concentrations of K⁺. The polar lipid fatty acids were
41 dominated by unsaturated C₁₆ and C₁₈ species. The 16S rRNA gene phylogeny indicated
42 that both strains belong to the recently proposed phylum *Rhodothermaeota*. BSk₂^T
43 forms a novel genus-level branch, while BSk₃^T represents a novel species-level
44 member in the genus *Longimonas*. On the basis of distinct phenotypic and genotypic
45 properties, strain BSk₂^T (JCM 31342^T=UNIQEM U1009^T) is proposed to be classified
46 as a new genus and species *Natronotalea proteinilytica* and strain BSk₃^T (JCM
47 31343^T=UNIQEM U10110^T) as a new species *Longimonas haloalkaliphila*.

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

67
68 Surface sediments from two types of hypersaline alkaline lakes in Kulunda Steppe (Altai,
69 Russia) were used as the inoculum for enrichment cultures: (1) from typical soda lakes with
70 extremely high alkalinity Tanatar-1 and Tanatar-2 (July 2016, salinity=300-400 g l⁻¹, pH=9.7-
71 10.2, total carbonate alkalinity=3.4-3.5 M) and (2) from Stamp Lake with low alkalinity (July
72 2015, salinity=325 g l⁻¹, pH=9.1, total carbonate alkalinity=0.15 M).

73
74 The protein-utilizing bacteria were enriched under aerobic conditions using defatted chicken
75 feathers with β -keratin as a growth substrate. The mineral base medium containing 4 M total
76 Na⁺ (2 M Na⁺ as sodium carbonates + 2 M NaCl) at pH 9.8 was used for the Tanatar sample,
77 while the Stamp lake sediments were inoculated into 4 M NaCl-based medium adjusted to pH
78 9 with 1 M Na₂CO₃. Both media also included 1 g l⁻¹ K₂HPO₄ and 5 g/l KCl. After

79 sterilization, the media were supplemented with 1 ml l⁻¹ of trace metal solution and vitamin
80 mix [10] and 1 mM MgCl₂. Defatted chicken feathers were added as substrate at
81 approximately 2 g l⁻¹. Before inoculation, the sediments were resuspended 1:10 in the basic
82 medium and the suspension was allowed to stand for 20 min., resulting in precipitation of the
83 coarse fractions. 1 ml from the top fraction containing mostly colloidal sediments was then
84 used to inoculate 40 ml cultures in 200 ml closed serum bottles placed on a rotary shaker at
85 37°C and at 200 rpm. The development of the enrichment culture was monitored by the extent
86 of feather degradation and by microscopy. After 20-30 days, the cultures were serially diluted
87 in the same medium but with casein as substrate and the maximal positive dilutions were
88 plated onto a solid medium prepared by 1:1 mixing of the liquid medium and a 4% solution of
89 extensively washed agar at 50°C. To compensate for the lower salinity, sterile solid NaCl was
90 added directly to the mixture before pouring the plates. After 1-3 weeks of incubation in
91 closed plastic bags at 37°C the dominant colony types were transferred to the respective
92 liquid media with casein and purified by repeated plating. This, eventually, resulted in
93 isolation of two bacterial strains: BSk_{er}2^T from the Tanatar lakes and BSk_{er}3^T from the Stamp
94 Lake. The purity was checked microscopically (Zeiss Axioplan Imaging 2 microscope,
95 Göttingen, Germany) and by 16S rRNA gene sequencing.

96 On casein agar the colonies of both strains were flat and spreading, orange-red in
97 colour and formed a clear zone of casein hydrolysis (**Suppl. data, fig.S3**). The pigment
98 extracted from the cells with acetone/MeOH had an absorbance maximum at 480 nm and two
99 shoulders at around 450 and 510 nm (**Suppl. data, fig.S1**). Exponentially growing cells of
100 both isolates were long flexible nonmotile rods. In the stationary phase the BSk_{er}3^T cells
101 elongated up to 100 µm and formed coiled aggregates (**Fig. 1**). The cells were apparently
102 covered with a thick EPS matrix since even high-speed centrifugation did not allow to obtain
103 a compact cell pellet. The KOH test proved a Gram-negative type of cell wall.

104 The membrane polar lipids were extracted from the freeze-dried cells and their
105 composition was analyzed by TLC at the DSMZ Identification Service according to [11-12].
106 The fatty acid methyl esters were analyzed by GC-MS according to [13-14]. Respiratory
107 lipoquinones were extracted from the lyophilized cells by cold acetone, separated by TLC
108 [15] and subsequently eluted and further analyzed by tandem mass spectrometry (LCG
109 Advantage Max) in combination with HPLC-MS.

110 The polar lipid analysis of cell membranes of strain BSk_{er}2^T showed the presence of
111 two glycolipids and four unidentified phospholipid species (**Supplementary fig. S2**). The
112 respiratory quinone analysis identified a single menaquinon species MK-7 in cells of
113 BSk_{er}2^T. In their PLFA profiles, the novel isolates were similar to the two extremely
114 halophilic closest relatives from *Rhodothermaeota*, *Longimonas halophila* and *Saliniseta*
115 *longa*. But there was variability in the abundance of other C₁₅-C₁₇ components, both between
116 the two BSk_{er} strains and between them and the nearest relatives (**Supplementary table S1**).

117 Organic compatible solutes were analysed in BSk_{er}2^T cells grown at 4 M total Na⁺,
118 either at pH 8.6 (NaCl base) or pH 10 (sodium carbonate base), using HPLC and ¹H-NMR
119 after extraction according to a modified Bligh and Dyer method [16-17]. The polar fraction
120 was analyzed on a Nucleosil 100-3 aminopropyl phase HPLC column (Macherey & Nagel,
121 Düren, Germany) using acetonitrile/water (80:20, v/v) as mobile phase at a flow rate of 1
122 mL/min [18]. Compounds were monitored using a combination of refractive index and UV
123 detector. Amino-reactive compounds were analyzed by gradient HPLC with pre-column
124 Fmoc-ADAM derivatization as described previously [19]. No known organic osmolytes
125 were detectable, neither on the aminopropyl phase column (for neutral and zwitterionic
126 solutes) nor with Fmoc derivatization (for amino reactive solutes). The latter revealed that
127 glutamate was the dominant amino acid at a concentration of 0.45 and 0.33 mmol (g protein)⁻¹
128 for the chloride and the soda sample, respectively. Both values are within the expected range.

129 For *E.coli* cells a regular glutamate value of 0.15 mmol (g protein)⁻¹ and a transient
130 accumulation to 0.68 mmol (g protein)⁻¹ upon salt stress has been reported [20]. For NMR
131 analysis, the dry cells were extracted with 1 mL chloroform/methanol/water (10:5:4, by vol.)
132 followed by phase separation according to [18]. The polar fraction was evaporated overnight
133 and the dry residue was dissolved in 1 mL D₂O as lock signal. The sample was further
134 supplemented with the internal standard benzene-1,2,4,5-tetracarboxylate sodium salt to give
135 a final concentration of 10 mM. ¹H NMR spectra were recorded on a Bruker Avance 300 DPX
136 spectrometer. The soda sample (lower protein content and lower osmolarity) revealed no
137 distinct resonances apart from the internal standard. The chloride sample displayed a number
138 of peaks, none of which could be related to any known compatible solutes. In relation to the
139 internal standard, the strongest signals between 1 to 4 ppm represented presumptive
140 concentrations of unknown compounds of no more than 0.25 mmol (g protein)⁻¹, while for the
141 model halophilic organism *Halomonas elongata* grown at 3.42 M NaCl an ectoine content of
142 approx. 7 mmol (g protein)⁻¹ was recorded [21]. This value is almost 30x higher than what
143 we observed here, suggesting that the "salt-out" osmotic strategy is not used by the novel
144 extreme haloalkaliphile.

145 To analyze the intracellular potassium, the freeze-dried cells of BSk_{er}2^T were
146 extracted according to a modified Bligh and Dyer protocol [16-17]. The water-soluble
147 fraction was subjected to cation analysis by isocratic HPLC with conductivity detection
148 (conductoMonitor III, Thermo Scientific, Waltham MA, USA) on a Metrosept Cation C4-
149 100/4.0 column (Methrom, Herisau, Switzerland) using an eluent of 1.7 mM nitric acid and
150 0.7 mM dipicolinic acid at a flow rate of 0.9 ml min⁻¹. Potassium content was corrected for
151 the proportion originating from intercellular medium and related to protein content
152 determined by the bicinchoninic assay (Uptima, Montlucon, France). The estimated specific
153 potassium content was 305 and 115 mg (mg cell protein)⁻¹ in the cells grown in NaCl base

154 and in soda base, respectively. The first value is close to what is usually found in haloarchaea
155 [22] , while the much lower content in the soda-grown cells might be explained by two times
156 less osmotic pressure of this weak electrolyte in comparison with the strongly electrolytic
157 NaCl [2]. In conjunction with extreme halophily, this is an indication that strain BSk_{er}2^T
158 employs the "salt-in" osmoprotection mechanism, which is also found in an extremely
159 halophilic member of the *Rhodothermaceae* - *Salinibacter ruber* [23].

160
161 The 16S rRNA gene sequence-based phylogenetic analysis was performed in Mega 6 package
162 [24] using Maximum Likelihood algorithm. The results demonstrated that BSk_{er}2^T forms a
163 novel genus lineage within the family *Rhodothermaceae*, phylum *Rhodothermaeota*, with a
164 maximum pairwise sequence similarity of 92 % to its validly characterized halophilic
165 members *Longimonas halophila* [25], *Salisaeta longa* [26] and *Longibacter salinarum* [27].
166 On the other hand, BSk_{er}3^T apparently represents a novel species in the genus *Longimonas*
167 with 97% sequence similarity to the extremely halophilic *L. halophila* (**Fig. 2**). The
168 phenotypic comparison of the BSk_{er} isolates with the two closest relatives is given in **Table**
169 **1**. Interestingly, despite a significant phylogenetic distance, the unusual cell morphology and
170 some other important characteristics (such as substrate profile, extreme salt tolerance, the type
171 of lipoquinones) were common among the soda lake isolates and the three halophilic genera
172 mentioned above. The G + C content in the genomic DNA was analyzed by the DSMZ
173 Identification Service using the HPLC method [28]. The determined values for BSk_{er}2^T and
174 BSk_{er}3^T were 55.9 and 58.2 mol%, respectively.

175 The BSk_{er} strains are obligately aerobic organoheterotrophs which grow best with
176 various proteins and peptides, including the following: casein, gelatin, filter-sterilized bovine
177 serum albumin and haemoglobin; various peptones and yeast extract. Heat-sterilized alpha-
178 keratine (fine powdered fraction), soya protein, lactalbumin and bovine collagen were only
179 utilized by strain BSk_{er}2^T. The protease activity, qualitatively tested in strain BSk_{er}2^T by the

180 agar-diffusion approach, was cell-associated (**Supplementary Fig. S3**). In addition, BSker2^T
181 was able to utilize amylose (**Supplementary Fig. S4**) in the presence of low (100 mg l⁻¹)
182 background concentration of casein hydrolysate. Polymeric substrates tested but not utilized
183 included amylopectin, birch wood xylan, amorphous forms of cellulose and chitin and
184 emulsified olive oil. Among the monomeric substrates tested were sugar hexoses and
185 pentoses, sugar alcohols and C₂-C₆ organic acids. Both strains grew (again only in the
186 presence of a minimum of 100 mg l⁻¹ of casein hydrolysate) with glycerol and maltose. In
187 addition, BSker2^T also utilized cellobiose. Anaerobic fermentative growth with maltose and
188 peptone was not observed.

189 With respect to its salt demand, both BSker2^T and BSker3^T can be qualified as
190 extreme halophiles with their total Na⁺ range for growth between 2 and 4.5 M (optimum
191 around 3 M) (**Fig. 3a**). In contrast to the extremely halophilic relatives, the BSker strains were
192 not dependent on high Mg concentrations. On the other hand, the strains also differed from
193 most of soda lake bacterial isolates by obligate growth dependence on the presence of high Cl⁻
194 concentrations (minimum 0.5 M). The latter might be related to its usage as a counter anion
195 for intracellular potassium accumulation.

196 At optimal salinity, the pH range for growth with casein was substantially different for
197 the two strains. The soda lake isolate BSker2^T had a profile typical for obligate natronophiles
198 with the pH range from 8.2 to 10.2 (optimum around 9.5), while BSker 3^T was only
199 moderately alkaliphilic with an optimum at pH 8.5-9 (**Fig. 3b**).

200

201 In conclusion, , the two aerobic bacterial isolates from hypersaline alkaline lakes represent the
202 first examples of extremely halophilic and alkaliphilic bacteria specialized in utilization of
203 proteinaceous compounds and with an apparent usage of the "salt-in" osmoprotection
204 strategy. With this combination of properties, they are clearly different from their nearest

205 phylogenetic relatives and are proposed to be classified as a novel genus and species
206 *Natronotalea proteinilytica* (strain Bsker2^T) and *Longimonas haloalkaliphila* sp. nov.
207 (Bsker3^T).

208
209 **DESCRIPTION OF NATRONOTALEA GEN. NOV.**

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

213
214 Extremely haloalkaliphilic protein-utilizing aerobic member of the family *Rhodothermaceae*,
215 phylum *Rhodothermaeota*, found in hypersaline alkaline lakes. The type species is
216 *Natronotalea proteinilytica*.

217
218 **DESCRIPTION OF NATRONOTALEA PROTEINILYTICA SP. NOV.**

219 *Natronotalea proteinilytica* (pro.te.i.ni.ly'ti.ca N.L. neut. n. *proteinum*, protein; N.L. fem. adj.
220 *lytica* (from Gr. fem. adj. *lytikê*), dissolving; N.L. fem. adj. *proteinilytica* dissolving proteins)

221
222 Cells have the Gram-negative type of cell wall, long flexible rods, 0.5 x 5-15 µm, nonmotile,
223 forming EPS. The colonies are flat, spreading up to 5 mm, orange-red. The cell pigment has
224 an absorbance maximum at 480 nm. The polar lipids include 4 unidentified phospho- and two
225 glyco- lipids. The respiratory quinones are represented by MK-7. The polar lipid fatty acids
226 are dominated by unsaturated 16:1ω7c and 18:1ω7c. It is a strictly aerobic organoheterotroph
227 utilizing various proteins and peptides for growth. It can also grow, but less actively, with
228 amylose, maltose, cellobiose and glycerol. It is obligately alkaliphilic, with a pH range for
229 growth from 8.2 to 10.2 (optimum at 9.5-9.8). It is a chloride-dependent extreme halophile
230 which requires a Na⁺ range for growth from 2 to 4.5 M (optimum at 2.5-3 M). The upper
231 temperature limit for growth (at optimal pH and salinity) is 48°C. The G + C content of the
232 genomic DNA in the type strain is 55.9 mol% (HPLC). The type strain BSk2^T (JCM
233 31342^T=UNIQEM U1009^T) was isolated from sediments of hypersaline soda lakes in
234 Kulunda Steppe (Altai, Russia). The 16S rRNA gene sequence accession number of the type
235 strain in GenBank is KU720569.

236
237
238
239
240 **DESCRIPTION OF LONGIMONAS HALOALKALIPHILA SP. NOV.**

241 *Longimonas haloalkaliphila* (Gr. n. *hals halos*, salt; N.L. n. *alkali*, soda ash (from Arabic *al-*
 242 *qalyi*, the ashes of saltwort); N.L. adj. *philus* (from Gr. adj. *philos -ê -on*), friend, loving; N.L.
 243 fem. adj. *haloalkaliphila*, salt and alkali-loving)
 244

245 Cells have the Gram-negative type of cell wall, nonmotile, long, flexible rods, 0.5-0.6 x 8-30
 246 µm in exponential growth phase, and up to 100 µm long in aggregates in aged cultures. The
 247 colonies are flat, spreading up to 8 mm, orange-red. The cell pigment has an absorbance
 248 maximum at 480 nm. The polar lipid fatty acids are dominated by unsaturated 16:1ω7c and
 249 18:1ω7c. It is a strictly aerobic organoheterotroph utilizing a limited number of proteinaceous
 250 and peptide substrates for growth. Less active growth was observed with maltose and
 251 glycerol. It is obligately but only moderately alkaliphilic, with a pH range for growth from 7.8
 252 to 9.3 (optimum at 8.5-8.8). It is a chloride-dependent extreme halophile which requires a
 253 Na⁺ range for growth from 2 to 4.5 M (optimum at 2.5-3 M). The upper temperature limit for
 254 growth (at optimal pH and salinity) is 50°C. The G + C content of the genomic DNA in the
 255 type strain is 58.2 mol% (HPLC). The type strain BSk_{er3}^T (JCM 31343^T=UNIQEM U1010^T)
 256 was isolated from sediments of a hypersaline alkaline lake in Kulunda Steppe (Altai, Russia).
 257 The 16S rRNA gene sequence accession number of the type strain in GenBank is KU720570.
 258

259 **Funding information**

260 This work was supported by the Russian Science Foundation (grant 16-14-00121).
 261

262 **Conflict of interest:**

263 The authors declare that there is no conflict of interests.
 264
 265

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331 performance liquid chromatography. *FEMS Microbiol Lett* 1984; 25: 125–128.
- 332
- 333
- 334

335 **Table 1.** Comparative properties of the BSk_{er} strains and their closest halophilic relatives from the
 336 phylum *Rhodothermaeota*: *Longimonas halophila* [25], *Salisaeta longa* [26] and *Longibacter*
 337 *salinarum* [27]. nd - no data

Property	BSker2^T	BSker3^T	<i>Longimonas halophila</i>	<i>Salisaeta longa</i>	<i>Longibacter salinarum</i>
Cell morphology	Long flexible rod (0.5 x 5-15 µm)	Long flexible rod (0.5-0.6 x 8-30 µm; >100 µm in old cultures)	Long rod (0.4-0.6 x 5-9 µm)	Long flexible rod (0.8 x 15-30 µm)	Long rod (0.3-0.4 x 6-12 µm)
Pigmentation	Red-orange	Red-orange	Red	Red	Red
Relation to oxygen	Obligate aerobe	Obligate aerobe	Facultative anaerobe (fermentation)	Obligate aerobe	
Growth substrates	Proteins, peptides, starch, maltose, cellobiose, glycerol	Proteins, peptides, maltose, glycerol	Glucose, sucrose, maltose, fructose, ribose	Glycerol, glucose, maltose	Glycerol, sucrose, mannitol, strach*
Salinity range (opt.), M Na ⁺	2-4.5 (2.5-3.0)	2-4.8 (2.5-3.0)	0.7-4.3 (1.0-1.4)	1.6-4.1 (2.5)	0.3-3.3 (1.3-2.0)
High Mg demand	no	no	yes	yes	no
pH range (opt.)	8.2-10.2 (9.5-9.8)	7.6-9.3 (8.5-8.8)	6.5-8.5 (7.5-8.0)	6.5-8.5	6.5-8.5 (7.5-8.0)
Max. growth T (°C)	46	48	50	50	50
Dominant PLFA (in order of dominance)	16:1ω7c, 18:1ω7c i17:0, i16:0; 16:0	16:1ω7c, 18:1ω7c ai17:0, i16:0, i17:0, i17:1ω9c	16:1ω7c, i16:0, 18:1ω7c; i15:0, 16:0, ai17:0	16:1ω7c, 16:0; i15:0; i16:0, ai17:0	i17:1ω9c, 16:1ω8c, i15:0, i17:0; ai17:0
Predominant lipoquinone	MK-7	nd	MK-7	nd	MK-7
G + C, mol%	55.9	58.2	61.5	62.9	58.1
Habitat	Hypersaline soda lakes (s-w Siberia)	Hypersaline alkaline lake (s-w Siberia)	Solar saltern (China)	Dead Sea (Israel)	Solar saltern (China)

338 *since this organism did not utilize maltose, its capability to grow with starch is questionable
 339

340 **Legends to the figures**

341

342 **Fig. 1** Cell morphology of strain BSk_{er}2^T (**a**) and BSk_{er}3^T (**b-c**) grown with casein at 4 M
343 total Na⁺ and 37°C, phase contrast microphotographs. (a and b), cells from exponential and
344 stationary growth phase, respectively; (c), complex aggregation of extremely elongated cells
345 of BSk_{er}3^T in late stationary growth phase.

346

347 **Fig. 2.** Maximum Likelihood 16S rRNA gene sequence-based phylogenetic tree showing
348 position of strains BSk_{er}2^T and BSk_{er}2^T (in bold) within the phylum *Rhodothermaeota*.
349 Branch lengths (see scale) correspond to the number of substitutions per site with corrections,
350 associated with the model (GTR, G + I, 4 categories). All positions with less than 95% site
351 coverage were eliminated. Totally 1305 positions were used in the alignment of 24 sequences.
352 Numbers at nodes indicate bootstrap values of 1000 repetitions. Strains BSk_{er}2^T and BSk_{er}3^T
353 are in bold. A representative of *Bacteroidetes* phylum, *Marivirga tractuosa* DSM4126
354 (Genbank accession CP002349.1) was used as an outgroup.

355

356

357 **Fig. 3.** Influence of pH at 3 M total Na⁺ (**a**) and Na⁺ at pH 9 (BSk_{er}3^T) - 9.5 (BSk_{er}2^T) (**b**) on
358 growth with casein at 37°C. Incubation time: 42-55 h BSk_{er}2^T and 96 h for BSk_{er}3^T.

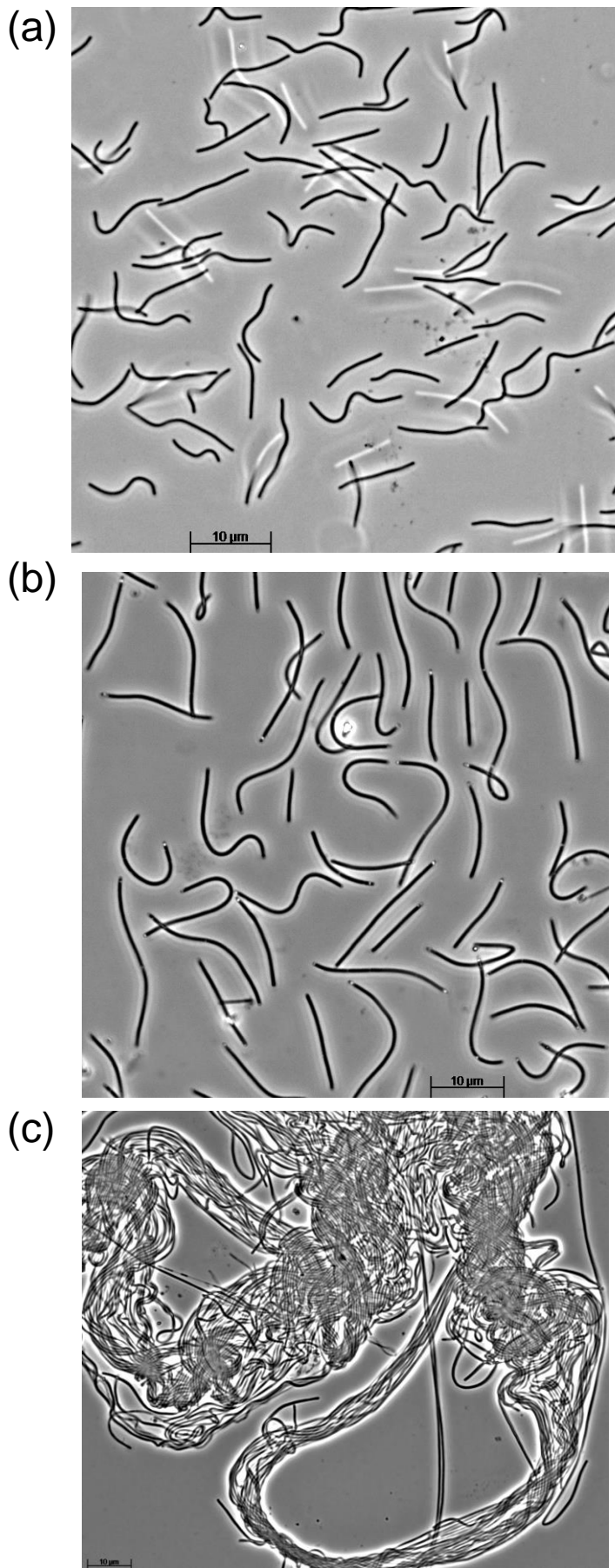


Fig.1

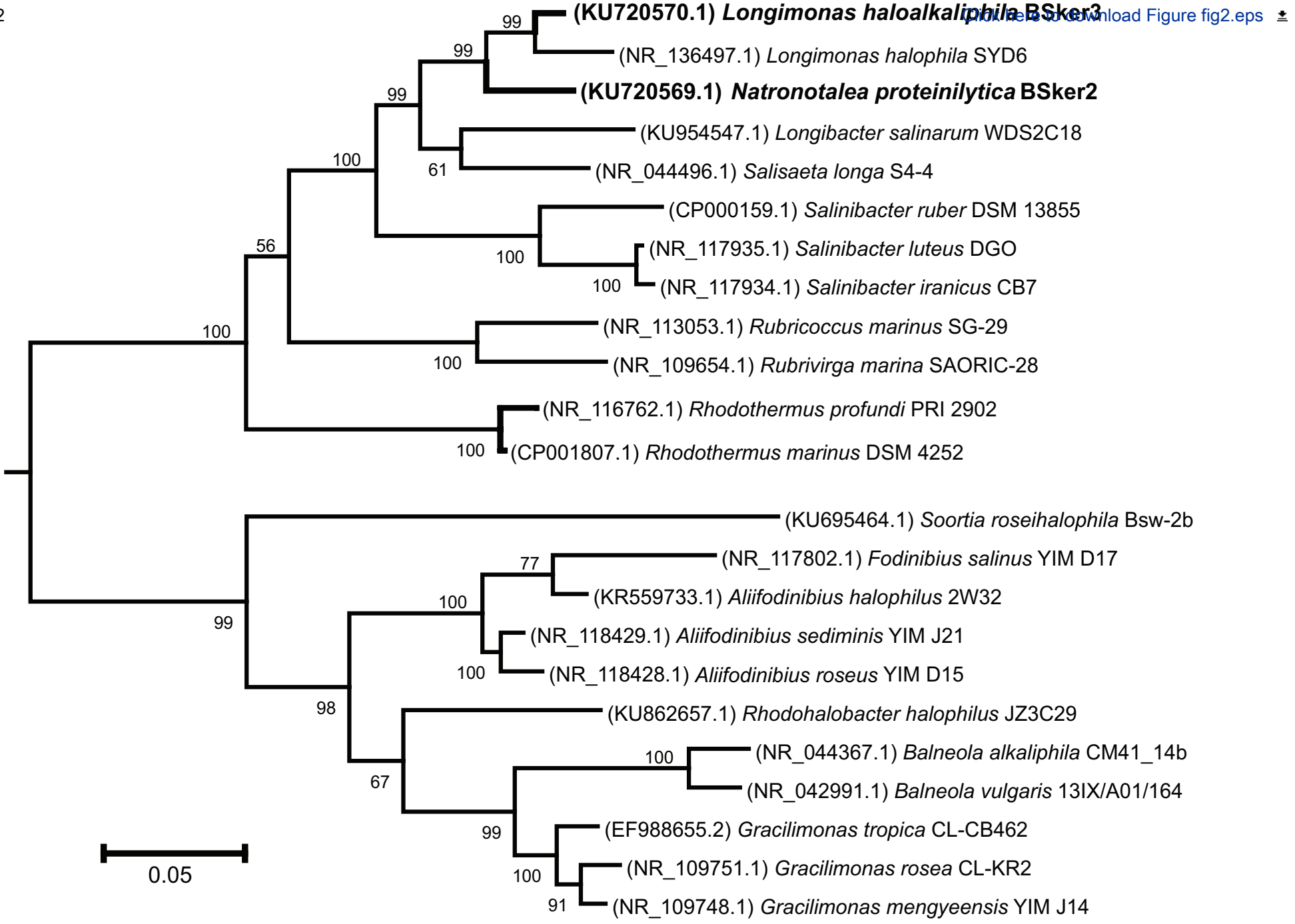


Fig.2

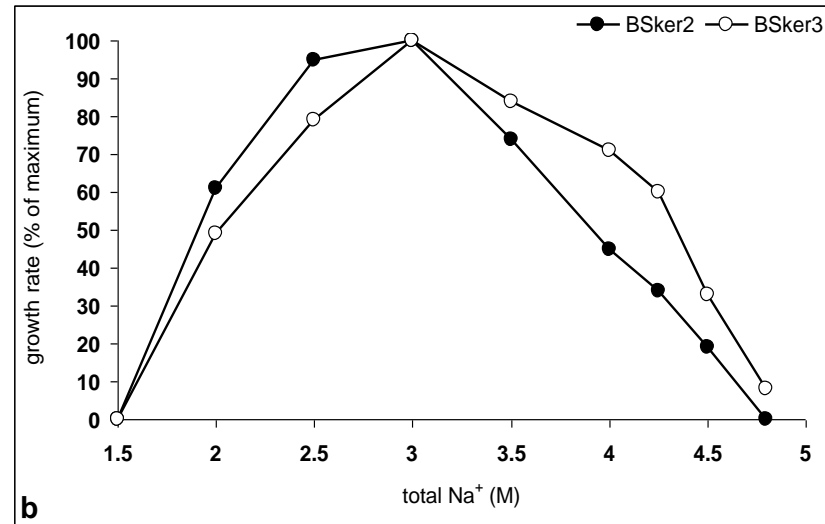
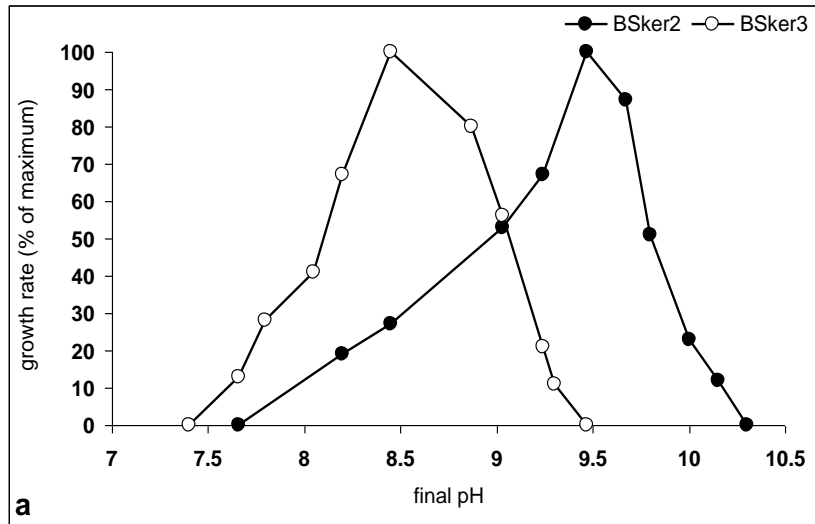


Fig.3

Supplementary data to:

Journal: International Journal of Systematic and Evolutionary Microbiology

***Natronotalea proteinilytica* gen. nov., sp. nov, and *Natronotalea halophila* sp. nov., extremely salt-tolerant alkaliphilic members of the phylum *Rhodothermaeota* from hypersaline soda lakes**

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Content:

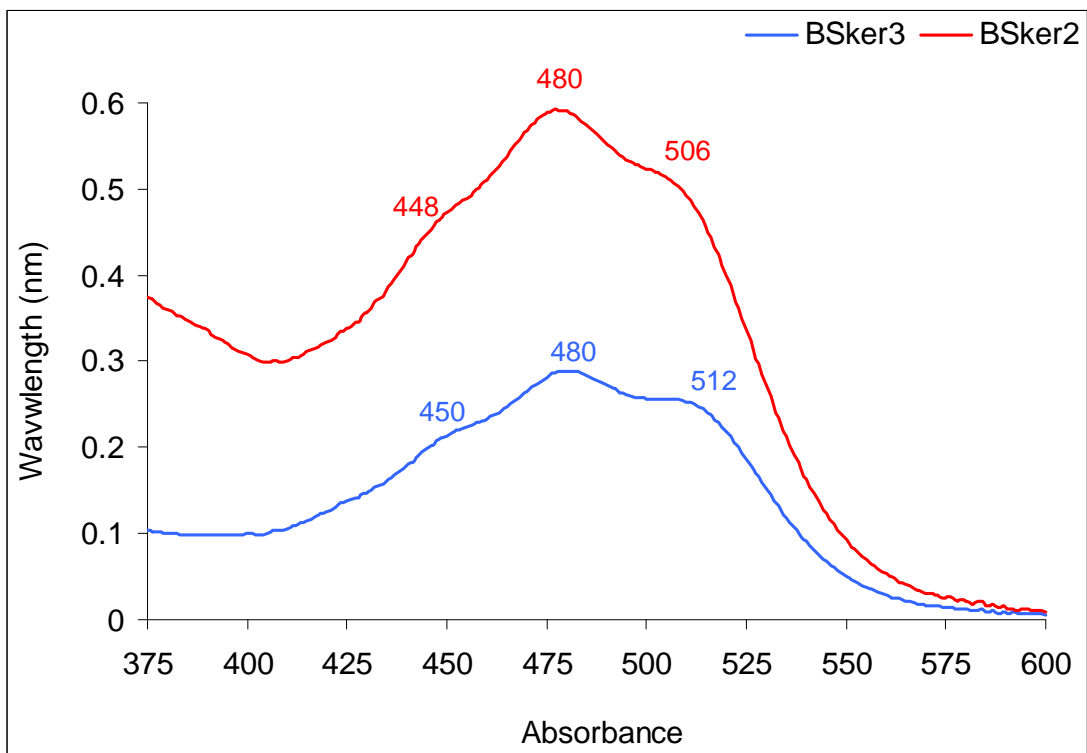
Fig.S1 - Absorption spectra of carotenoids extracted with acetone/MeOH (1:1) from the cells of extremely haloalkaliphilic proteolytic bacteria from hypersaline alkaline lakes

Fig.S2 - Polar lipid profile (2D TLC) of extremely haloalkaliphilic proteolytic bacterium BSk2 from hypersaline alkaline lakes (DSMZ Identification Service). PL – phospholipid, GL - glycolipid.

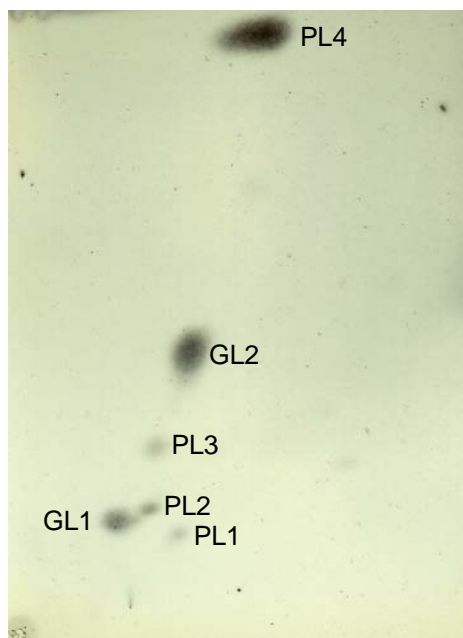
Table S1. Comparative composition of PLFA of BSk2 strains.

Fig.S3 - Proteolytic activity in BSk2 strains on casein

Fig.S4 - Alpha-amylase activity in BSk2 strains.



Supplementary Fig.S1. Absorption spectra of carotenoids extracted with acetone/MeOH (1:1) from the cells of extremely haloalkaliphilic proteolytic bacteria from hypersaline alkaline lakes



Supplementary Fig.S2. Polar lipid profile (2D TLC) of extremely haloalkaliphilic proteolytic bacterium BSk2 from hypersaline alkaline lakes (DSMZ Identification Service). PL – phospholipid, GL - glycolipid

Supplementary table S1. Comparative composition of PLFA in BSkер strains and related type species within the *Rhodothermaeota*. Compounds above 5% are in bold. BSkер strains were grown with casein at 4 M total Na⁺, pH 9.5 (BSker2) or pH 9 (BSker3), 37°C until late exponential growth phase.

Compound	BSker2	BSker3	<i>Longimonas halophila</i> ^a	<i>Salisaeta longa</i> ^b
12:0		0.8		
14:0	1.2	1.9	0.9	2.4
i15:0	2.7	2.5	9.4	10.9
ai15:0	0.7			
15:0			0.6	2.3
i16:0	10.9	9.9	13.1	8.5
16:0	6.9	7.7	7.1	22.1
16:1 ω7c	21.9	25.8	23.9	27.9
OH16:0	1.1			
i17:1ω9c	3.0	6.8	1.4	1.7
ai17:1ω9c		0.5		
i17:0	11.7	7.4	1.6	3.1
ai17:0		9.8	6.2	5.1
17:1ω6c	1.1			
3-OH i17:0	5.3	4.8	2.7	2.9
i18:1ω9c	3.8			
18:1ω9c		5.0		
18:1ω7c	18.2	14.4	11.5	1.2
18:0	1.6	2.7		

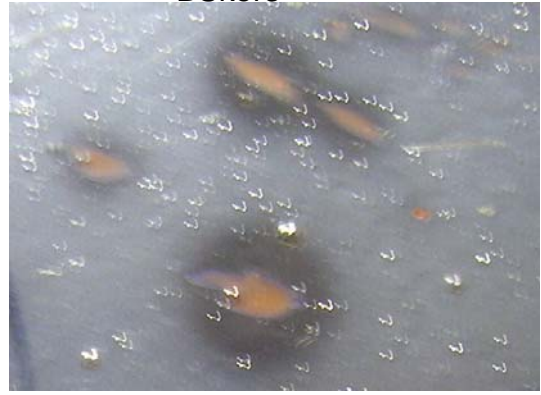
Xia J, Zhou YX, Zhao LH, Chen GJ, Du ZJ. *Longimonas halophila* gen. nov., sp. nov., isolated from a marine solar saltern. *Int J Syst Evol Microbiol* 2015; 65: 2272-2276.

Vaisman N, Oren A. *Salisaeta longa* gen. nov., sp. nov., a red, halophilic member of the *Bacteroidetes*. *Int J Syst Evol Microbiol* 2009; 59: 2571-2574.

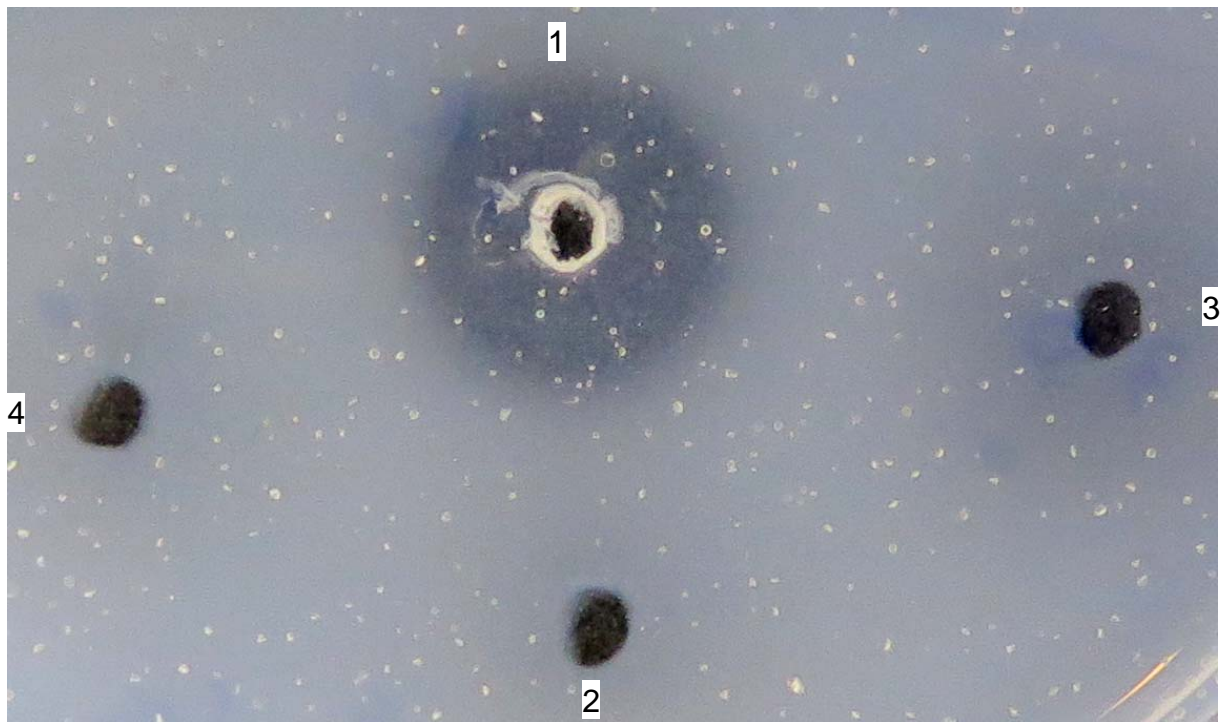
BSker2



BSker3



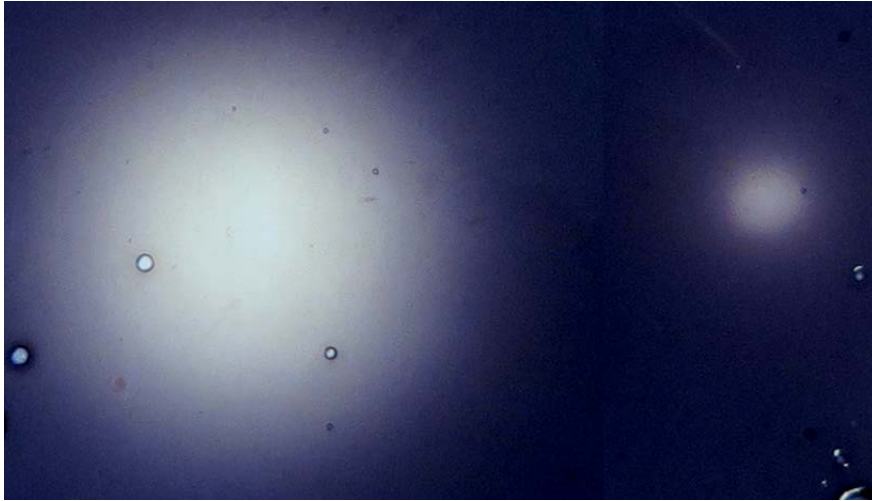
Colonies of extremely haloalkaliphilic proteolytic strains on casein agar at 4 M total Na^+ and pH 9.2. The halos around the colonies indicate the zone of casein hydrolysis



Qualitative measurement of proteolytic activity in cell fractions of strain BSk2 by the agar diffusion approach (4 M total Na^+ , pH 9.5; 37°C, 48 h).

- 1 – cells lyzate;
- 2 – culture supernatant;
- 3 – supernatant fraction x10 concentrated > 30 kDa;
- 4 – supernatant x10 concentrated 10-30kDa

Supplementary fig.S3. Proteolytic activity of extremely haloalkaliphilic isolates from hypersaline alkaline lakes



Supplementary fig.S4. Detection of alpha-amylase activity BSk2 isolates
Single colony was grown on agar medium (4 M Na⁺, pH 9.1) containing 0.1 g/L casein hydrolysate and 1 g/L soluble starch. After incubation for 5 days at 37°C, The plate was flooded with 50 mM I₂ solution. Left – BSk2; right – BSk3