

## *Halostagnicola bangensis* sp. nov., an alkaliphilic haloarchaeon from a soda lake

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An extremely haloalkaliphilic archaeon, strain T26<sup>T</sup>, belonging to the genus *Halostagnicola*, was isolated from sediment of the soda lake Bange in the region of Tibet, China. Phylogenetic analysis based on 16S rRNA gene sequence similarities showed that strain T26<sup>T</sup> was closely related to *Halostagnicola alkaliphila* 167-74<sup>T</sup> (98.4%), *Halostagnicola larsenii* XH-48<sup>T</sup> (97.5%) and *Halostagnicola kamekurae* 194-10<sup>T</sup> (96.8%). Strain T26<sup>T</sup> grew optimally in media containing 25% (w/v) salts, at pH 9.0 and 37 °C in aerobic conditions. Mg<sup>2+</sup> was not required for growth. The cells were motile, pleomorphic and Gram-stain-variable. Colonies of this strain were pink pigmented. Hypotonic treatment caused cell lysis. The polar lipids of the isolate consisted of C<sub>20</sub>C<sub>20</sub> and C<sub>20</sub>C<sub>25</sub> derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and minor phospholipids components. Glycolipids were not detected, in contrast to the two neutrophilic species of this genus. The genomic DNA G+C content of strain T26<sup>T</sup> was 60.1 mol% and DNA–DNA hybridization showed a relatedness of 19 and 17% with *Halostagnicola alkaliphila* CECT 7631<sup>T</sup> and *Halostagnicola larsenii* CECT 7116<sup>T</sup>, respectively. The comparison of 16S rRNA gene sequences, detailed phenotypic characterization, polar lipid profile and DNA–DNA hybridization studies revealed that strain T26<sup>T</sup> belongs to the genus *Halostagnicola*, and represents a novel species for which the name *Halostagnicola bangensis* sp. nov. is proposed. The type strain is T26<sup>T</sup> (=CECT 8219<sup>T</sup>=IBRC-M 10759<sup>T</sup>=JCM 18750<sup>T</sup>).

The haloarchaea constitute a large group of extremely halophilic aerobic archaea that are placed in the family *Halobacteriaceae* within the order *Halobacteriales* and are recognized as the most abundant micro-organisms in hypersaline environments (Grant *et al.*, 2001; Ventosa, 2006). Members of the family *Halobacteriaceae* have been isolated from various hypersaline environments and at the time of writing, the family *Halobacteriaceae* comprises 47 genera with 179 species (Parte, 2014) (<http://www.bacterio.net/halobacteriaceae.html>) that display a wide variety of physiological characteristics, including ranges of salinity, temperature and pH that facilitate growth. The genus *Halostagnicola* was established by Castillo *et al.* (2006) to accommodate a strain isolated from sediment of Lake Xilinhote, a saline lake in Inner Mongolia, China. This organism is pleomorphic, neutrophilic and requires at least 2.5 M (15%, w/v) NaCl, but not MgCl<sub>2</sub>, for growth; it exhibits optimal growth at

3.4 M (20%, w/v) NaCl. Polar lipid composition includes phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, derived from both C<sub>20</sub>C<sub>20</sub> and C<sub>20</sub>C<sub>25</sub> archaeol and two unidentified glycolipids. The genomic DNA G+C content was 61 mol%. On the basis of 16S rRNA gene sequence analysis, hybridization data, phenotypic properties and polar lipid composition this genus currently comprises three species, *Halostagnicola larsenii* (Castillo *et al.*, 2006), *Halostagnicola kamekurae* (Nagaoka *et al.*, 2010) and *Halostagnicola alkaliphila* (Nagaoka *et al.*, 2011), isolated from a saline lake, from solar salt, and from rock salt, respectively. In this study we describe the taxonomic properties of an aerobic, extremely haloalkaliphilic archaeon, designated strain T26<sup>T</sup>. We propose that strain T26<sup>T</sup> represents a novel species of the genus *Halostagnicola*.

Strain T26<sup>T</sup> was isolated from sediment of the soda lake Bange (GPS position; 31° 43' 39.0576 N 89° 28' 51.9378 E) in the region of Tibet, China. The sampling was carried out in August 2004 during development of an European project (Multigenome Access Technology for Industrial Catalysts; QLK3-CT-2002-01972) in cooperation with the Chinese Government, which permitted the isolation of novel strain T26<sup>T</sup> from a soda lake in Inner Mongolia, China.

Abbreviation: HPTLC, high performance thin layer chromatography.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain T26<sup>T</sup> is HF544345.

Two supplementary figures are available with the online Supplementary Material.

The physical parameters of this lake at the time of sampling were as follows: 27.5 °C, altitude 4560 m, pH 10.5 and conductivity 25.8 mS cm<sup>-1</sup>. Isolation of the haloalkaliphilic archaeon was performed using serial dilutions from a pre-enrichment culture of the sediment which were spread on modified alkaline solid medium (Duckworth *et al.*, 1996) (g l<sup>-1</sup>): yeast extract 5.0, Casamino acids 2.5, sodium glutamate 0.5, KH<sub>2</sub>PO<sub>4</sub> 4.0, MgSO<sub>4</sub> · 7H<sub>2</sub>O 0.8, NaCl 157.0, Na<sub>2</sub>CO<sub>3</sub> 21.0, NaHCO<sub>3</sub> 17.0, and agar 20.0. This solution was made by dissolving NaCl, Na<sub>2</sub>CO<sub>3</sub> and NaHCO<sub>3</sub> in 700 ml of distilled water and sterilized separately; this was then added to the rest of the medium components to complete the final volume of 1 litre. The final pH of the medium was adjusted automatically to pH 9.0–9.5. Plates sealed in plastic bags were incubated at 37 °C. After 30 days of incubation, representative colonies were transferred to the same medium. A pure culture of strain T26<sup>T</sup> was obtained by repeated streaking, the colonies of strain T26<sup>T</sup> showed a pink pigmentation. The strain was maintained on the same medium in slant tubes. Cryotubes were prepared for long-time preservation using liquid culture medium and glycerol (40%, v/v) and stored at –80 °C.

Cell morphology and motility was examined in liquid medium after 15 days of growth by optical and phase-contrast microscopy (BX41; Olympus). Strain T26<sup>T</sup> showed motile pleomorphic cells, with coccoid to bacillary shape. The size of single cells was 1.0–1.2 × 2.0–4.0 µm and cells grew separated without forming clusters (Fig. S1, available in the online Supplementary Material).

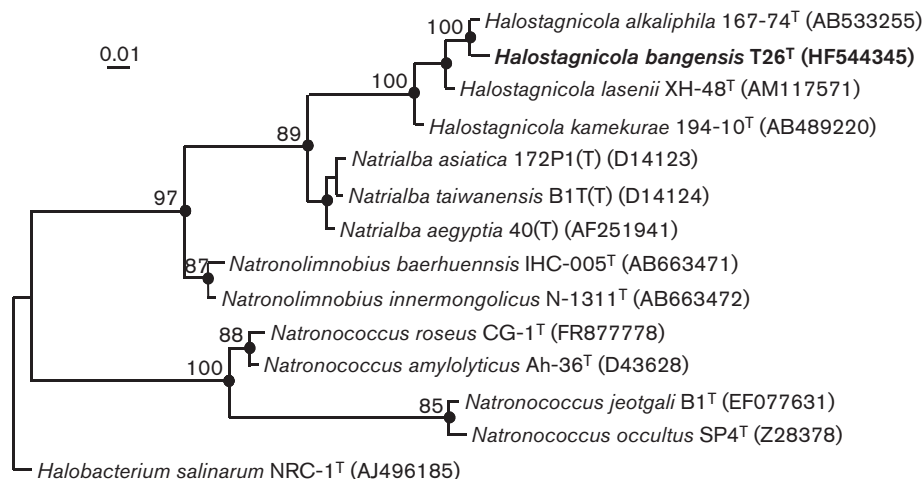
Genomic DNA of strain T26<sup>T</sup> was obtained by the method of Marmur (1961). The 16S rRNA gene of strain T26<sup>T</sup> was amplified by PCR (Sambrook & Russell, 2001) using universal primers as described previously (DeLong, 1992; Arahal *et al.*, 1996). The PCR product was sequenced by StabVida (Oeiras, Portugal), using the Sanger method. The DNA sequencing reaction was carried out with the BigDye terminator kit version 3.1 from Applied Biosystems. The sequencing reaction was purified by gel filtration and resolved in an ABI 3730XL sequencer. The nucleotide sequence of the 16S rRNA gene of strain T26<sup>T</sup> (1428 bp) was aligned using ChromasPro software, version 1.7.6 and the ARB software package, version 5.5 (Ludwig *et al.*, 2004). The sequence similarity comparison was analysed by comparing the 16S rRNA gene sequence of strain T26<sup>T</sup> with known sequences from the EzTaxon-e database (<http://www.ezbiocloud.net/eztaxon>; Kim *et al.*, 2012). The phylogenetic analysis based on complete 16S rRNA gene sequences showed that strain T26<sup>T</sup> was closely related to *Hst. alkaliphila* 167-74<sup>T</sup> (98.4%), *Hst. larsenii* XH-48<sup>T</sup> (97.5%) and *Hst. kamekurae* 194-10<sup>T</sup> (96.8%). Lower similarities were obtained with the type strains of species of other haloarchaeal genera.

The phylogenetic study was performed by reconstructing phylogenetic trees using the evolutionary clustering algorithms, neighbour-joining, maximum-parsimony and maximum-likelihood (Saitou & Nei, 1987) methods in the ARB program package, version 5.5 (Ludwig *et al.*, 2004).

Maximum-likelihood analysis was performed with RAxML (Randomized Axelerated Maximum-likelihood) 7.0.4 using the General Time Reversible (GTR + C) model of nucleotide substitution (Stamatakis *et al.*, 2005). Base-frequency filters were applied in the sequence-comparison analysis and the effects on the results were evaluated. To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed (Felsenstein, 1985). The phylogenetic tree (Fig. 1) reconstructed by maximum-parsimony confirmed that strain T26<sup>T</sup> was related to *Hst. alkaliphila* 167-74<sup>T</sup>, *Hst. larsenii* XH-48<sup>T</sup> and *Hst. kamekurae* 194-10<sup>T</sup> and was within the cluster constituted by the three recognized species of the genus *Halostagnicola*. Topologies of phylogenetic trees inferred using the neighbour-joining and maximum-likelihood algorithms were highly similar to that of the tree reconstructed by maximum-parsimony (Fig. 1).

Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). The following strains were used as reference for comparative purposes: *Hst. larsenii* CECT 7116<sup>T</sup>, *Hst. kamekurae* CECT 7536<sup>T</sup> and *Hst. alkaliphila* CECT 7631<sup>T</sup>. The optimal growth range in the presence of NaCl was determined using the modified alkaline medium containing various concentrations of NaCl (5–30%, w/v) at intervals of 5%. Magnesium range was tested using MgCl<sub>2</sub> (0–10%) at intervals of 1% (w/v). Growth experiments at high magnesium concentrations are not required for alkaliphilic strains due to the limited solubility of magnesium at high pH values (Oren *et al.*, 1997). For the determination of the pH range for growth, strain T26<sup>T</sup> was assayed from pH 5.0 to 11.0 at intervals of 0.5 units. The pH range for growth was determined in liquid medium by adding MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5), CHES (pH 8.9–9.5) or CAPS (10.0–11.0) at a concentration of 50 mM to the isolation medium (Scorpio, 2000). The temperature range for growth of strain T26<sup>T</sup> was determined by incubating at 4, 10, 20, 37 and 45 °C in modified alkaline medium with optimal NaCl and Mg<sup>2+</sup> concentrations. Strain T26<sup>T</sup> was capable of growing over a wide range of NaCl concentrations, from 15 to 30% (w/v). Mg<sup>2+</sup> was not required for growth.

Gram staining was performed using acetic acid-fixed samples, as described by Dussault (1955). Anaerobic growth was tested in the presence of nitrate and L-arginine by adding to the alkaline medium 3% KNO<sub>3</sub> or 4% L-arginine, respectively, in filled stoppered tubes and plates of cultures incubated for 15 days at 37 °C in an anaerobic jar (Oren *et al.*, 1997). The reduction of nitrate was detected using sulfanilic acid and α-naphthylamine reagents (Smibert & Krieg, 1981). Tests for catalase and oxidase activities and for the hydrolysis of starch, gelatin, casein and Tween 80 were performed as described by Oren *et al.* (1997). Hydrogen sulfide formation was determined by monitoring the production of a black sulfide precipitate in the alkaline medium containing 0.5% (w/v) sodium thiosulfate. Indole production from tryptophan, and the utilization of sugars, alcohols, amino acids and organic acids were assessed as



**Fig. 1.** Maximum-parsimony phylogenetic tree based on the 16S rRNA gene sequence comparison, showing the relationships between strain T26<sup>T</sup> and members of the genus *Halostagnicola* and other related haloarchaea. The sequence data used were obtained from the GenBank database (accession numbers are given in parentheses). Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 80% bootstrap support. Filled circles indicate that the corresponding nodes were also obtained in the trees generated with the maximum-likelihood and neighbour-joining algorithms. Bar, 1% substitution per nucleotide position.

described by Gerhardt *et al.* (1994) and Barrow & Feltham (2003).

Antimicrobial sensitivity tests were performed by spreading the culture suspension on alkaline solid medium plates and applying discs impregnated with antimicrobial compounds following the technique of Bauer–Kirby (Bauer *et al.*, 1966). The following antimicrobial discs (Difco and Becton Dickinson) were tested: ampicillin (10 µg), bacitracin (10 UI), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), neomycin (30 UI), novobiocin (30 µg), gentamicin (10 UI), trimethoprim/sulfamethoxazole (1.25/23.75 µg), kanamycin (30 µg), penicillin G (10 U), rifampicin (5 µg), tetracycline (30 µg), rifampicin (5 µg) and streptomycin (10 µg). The results were scored according to the manufacturer's instructions. Discs containing anisomycin (50 µg) were prepared in our laboratory and applied in the same way. Strain T26<sup>T</sup> was sensitive to anisomycin (50 µg), bacitracin (10 UI), novobiocin (30 µg), rifampicin (5 µg), erythromycin (15 µg), streptomycin (10 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg), but resistant to ampicillin (10 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), neomycin (30 UI), gentamicin (10 UI), kanamycin (30 µg), penicillin G (10 UI) and tetracycline (30 µg). Phenotypic characteristics, pH range and optimum NaCl concentration for growth, hydrolysis of different compounds and utilization of several substrates is shown in the species description. There were no discrepancies in phenotypic test determined in our laboratory and those reported for the closely related species. The differential features of strain T26<sup>T</sup> from closely related species are highlighted in Table 1.

The genomic DNA G+C content was determined by thermal denaturation ( $T_m$ ) using the melting midpoint (Marmur & Doty, 1962) with the equation of Owen & Hill (1979). The genomic DNA G+C content of strain T26<sup>T</sup> was 60.1 mol%, which is close to the described value for the related species of the genus *Halostagnicola* (Table 1) and that reported for the type species *Hst. lasenii* XH-48<sup>T</sup> (61.0 mol%; Castillo *et al.*, 2006).

DNA–DNA hybridization studies between strain T26<sup>T</sup> and the type strains of the phylogenetically most closely related species of the genus *Halostagnicola* were performed by the competition procedure of Johnson (1994) as described by Ventosa *et al.* (1999). The DNA–DNA hybridization relatedness values between strain T26<sup>T</sup> and *Hst. alkaliphila* CECT 7631<sup>T</sup> and *Hst. lasenii* CECT 7116<sup>T</sup> were 19 and 17%, respectively. These levels of DNA–DNA hybridization were low enough for this strain to be classified as a representative of a genotypically distinct species within the genus *Halostagnicola*. This value was below the threshold value of 70% generally accepted for the definition of prokaryotic species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002).

The biomass of T26<sup>T</sup> for chemotaxonomic purposes was obtained after 20 days of aerobic incubation in alkaline liquid medium under optimal conditions: 25% NaCl, 37 °C and pH 9.0. The strains used for comparative purposes were cultured considering the optimal pH described for each strain and standardizing to the same conditions for all of cultures. Polar lipids were extracted with chloroform/methanol following the method for extraction of membrane polar lipids of halophilic archaea previously described by

**Table 1.** Differential features that distinguish strain T26<sup>T</sup> from other related species of the genus *Halostagnicola*

Strains: 1, T26<sup>T</sup>; 2, *Hst. alkaliphila* CECT 7631<sup>T</sup>; 3, *Hst. larsenii* CECT 7116<sup>T</sup>; 4, *Hst. kamekurae* CECT 7536<sup>T</sup>. All data are from this study, except the data of genomic DNA G+C content, that were obtained from the original descriptions. +, Positive; –, negative.

Characteristic	1	2	3	4
Colony pigmentation	White/pink	White/pink	Pink	White/pink
NaCl optimum range (%)	25.0–30.0	20.0–30.0	20.0–30.0	10.0–30.0
pH optimum	9.0–9.5	8.5–10.0	6.0–9.0	6.0–9.0
Mg <sup>2+</sup> requirement	–	+	+	–
Oxidase	+	+	+	–
Catalase	+	–	+	–
Citrate utilization	–	–	+	–
Starch hydrolysis	–	–	+	–
Gelatin hydrolysis	–	–	+	–
Indole production	–	–	–	+
Utilization of:				
D-Fructose	–	+	+	–
Lactose	+	–	+	+
D-Mannitol	+	–	+	+
Succinate	+	–	+	+
Glutamate	+	–	+	+
Acetate	+	–	+	–
Isolation source	Sediment of soda lake	Commercial rock salt	Saline lake	Solar salt
DNA G+C content (mol%)	60.1*	60.7†	61.0†	59.8†

\*Determined by the  $T_m$  method.

†Taken from Nagaoka *et al.* (2011), Castillo *et al.* (2006) and Nagaoka *et al.* (2010), respectively.

Corcelli & Lobasso (2006); the extracts were carefully dried under a flush of nitrogen before weighing and then dissolved in chloroform to get a concentration of 10 mg lipid ml<sup>-1</sup> dissolved in CHCl<sub>3</sub>. The total lipid extracts were analysed by high performance thin layer chromatography (HPTLC), using HPTLC silica gel 60 plates crystal back (Merck 10 × 20 cm, art. 5626; Merck); the plates were eluted in the solvent system chloroform/methanol/90 % acetic acid (65 : 4 : 35, by vol.) (Angelini *et al.*, 2012; Corral *et al.*, 2013). To detect all polar lipids, the plate was sprayed with sulfuric acid 5 % in water and charred by brief heating at 160 °C (Kates, 1986). The glycolipids appeared as purple spots and the rest of polar lipids as brown spots after prolonged heating. Alternatively, spraying the plate with a solution of primuline and detecting the lipids upon excitation by UV light (336 nm) was used (Fuchs *et al.*, 2007). The following stainings were performed in order to identify the chemical nature of the lipids present in the TLC bands: molybdenum-blue spray reagent (Sigma) for phospholipids (Kates, 1986); azure-A/sulfuric acid for sulfatides and sulfoglycolipids (Kean, 1968); ninhydrin in acetone/lutidine (9 : 1) for free amino groups. The thin layer chromatogram of the polar lipids (Fig. S2) suggested that the major polar lipids of strain T26<sup>T</sup> consist of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester derived from both C<sub>20</sub>C<sub>20</sub> and C<sub>20</sub>C<sub>25</sub> archaeol. A minor amount of biphosphatidylglycerol and phospholipidic components were evidenced. Glycolipids were not detected, in contrast to the two neutrophilic species

of this genus. Phosphatidylglycerol sulfate was not detected. This lipid profile of T26<sup>T</sup> possesses all major phospholipids present in the three type strains described within the genus *Halostagnicola*. Small differences from the most closely related species, *Hst. alkaliphila*, were noted in the presence of minor phospholipids. In both cases phosphatidylglycerol was present with different chain length as evidenced by the double spot shown in the chromatogram; this is as expected for haloarchaea that thrive in alkaliphilic conditions.

In conclusion, the data from the polyphasic approach that included 16S rRNA gene sequences, the DNA–DNA hybridization data, the polar lipids and the phenotypic characteristics clearly indicated that strain T26<sup>T</sup> represents a novel species of the genus *Halostagnicola* for which the name *Halostagnicola bangensis* sp. nov. is proposed.

### Description of *Halostagnicola bangensis* sp. nov.

*Halostagnicola bangensis* (bang.en'sis. N.L. fem. adj. *bangensis* pertaining to Bange, Tibetan soda lake in China, from which the strain was isolated).

Cells are motile and pleomorphic, with coccoid to bacilar shapes. The size of the single cells is 1.0–1.2 × 2.0–4.0 μm and they grow separately without forming groups. Gram-stain-variable; in young cultures most cells are Gram-stain-negative, while few cells are observed as Gram-stain-positive. Colonies on solid medium after incubation at 37 °C for 30 days are circular, regular edged, smooth, convex and

1–2 mm in diameter. In the first 2 weeks the colonies appear white and gradually turn pink upon incubation for a further 2 weeks. Hypotonic treatment with less than 10 % NaCl provokes lysis. No gas vesicles inside the cells. Growth occurs in alkaline medium at 20–30 % (w/v) NaCl (optimum 25 %, w/v), at 30–45 °C (optimum 37 °C) and at pH 9.0–10.0 (optimum range pH 9.0–9.5) in aerobic conditions.  $Mg^{2+}$  is not required for growth. Cells are chemo-organotrophic and strictly aerobic; oxidase and catalase activities are positive. Anaerobic growth does not occur with nitrate or arginine. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not produced. Hydrogen sulfide is not produced from sodium thiosulfate or cysteine. Indole is not produced from tryptophan. Nitrate is reduced to nitrite, but nitrite is not reduced further and no gas is formed. Methyl red and Voges–Proskauer tests are negative, citrate is not utilized. Tween 80, starch, DNA, gelatin and casein are not hydrolysed. Tests for urease and phosphatase activities are negative. The following substrates are utilized for growth as sole source of carbon and energy: L-arabinose, D-glucose, maltose, sucrose, D-mannose, raffinose, trehalose, glycerol, sorbitol, mannitol, acetate, glutamate, lactate, malate, pyruvate, succinate and propionate. D-Galactose, fructose, lactose, sorbose and rhamnose are not used as sole source of carbon and energy. The following compounds are used as a sole source of carbon, nitrogen and energy: L-serine, threonine, glycine, asparagine and L-lysine. Isoleucine is not used as sole source of carbon, nitrogen and energy. The polar lipid pattern consists of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester derived from both  $C_{20}C_{20}$  and  $C_{20}C_{25}$  archaeol as major lipids. A minor amount of biphosphatidyl glycerol and minor phospholipidic components are evidenced. Glycolipids are not present.

The type strain is T26<sup>T</sup> (=CECT 8219<sup>T</sup>=IBRC-M 10759<sup>T</sup>=JCM 18750<sup>T</sup>), isolated from sediment of the soda saline lake Bange located in Tibet, China. The genomic DNA G + C content of the type strain is 60.1 mol% ( $T_m$ ).

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