Natronorubrum sediminis sp. nov., an archaeon isolated from a saline lake

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Two novel haloalkaliphilic archaea, strains CG-6^T and CG-4, were isolated from sediment of the hypersaline Lake Chagannor in Inner Mongolia, China. Cells of the two strains were pleomorphic, non-motile and strictly aerobic. They required at least 2.5 M NaCl for growth, with optimum growth at 3.4 M NaCl. They grew at pH 8.0–11.0, with optimum growth at pH 9.0. Hypotonic treatment with less than 1.5 M NaCl caused cell lysis. The two strains had similar polar lipid compositions, possessing C₂₀C₂₀ and C₂₀C₂₅ derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester. No glycolipids were detected. Comparison of 16S rRNA gene sequences and morphological features placed them in the genus *Natronorubrum*. 16S rRNA gene sequence similarities to strains of recognized species of the genus *Natronorubrum* were 96.2–93.8%. Detailed phenotypic characterization and DNA–DNA hybridization studies revealed that the two strains belong to a novel species in the genus *Natronorubrum*, for which the name *Natronorubrum sediminis* sp. nov. is proposed; the type strain is CG-6^T (=CECT 7487^T =CGMCC 1.8981^T =JCM 15982^T).

Extremely halophilic archaea have been isolated from various hypersaline environments such as hypersaline lakes, solar salterns, saline soils and salt mines. They are classified within the family *Halobacteriaceae* (Grant *et al.*, 2001; Ventosa, 2006) and members of this family are ubiquitous in these hypersaline environments. They are usually pink- to red-pigmented due to the presence of carotenoids (Kamekura & Dyll-Smith, 1995; Hezayen *et al.*, 2001; Grant *et al.*, 2001). At the time of writing, the aerobic, extremely halophilic archaea were classified in 27 genera (Grant *et al.*, 2001; Ventosa, 2006; Castillo *et al.*, 2006a, b; Savage *et al.*, 2007, 2008; Burns *et al.*, 2007; Gutiérrez *et al.*, 2007; Bardavid *et al.*, 2007).

In 1999, Xu *et al.* (1999) described the genus *Natronorubrum* to accommodate two novel haloalkaliphilic species, *Natronorubrum bangense* and *Natronorubrum tibetense*, which were isolated from sediment of Bange salt-alkaline lake in Tibet, China. On the basis of 16S rRNA gene sequence analysis, phenotypic properties and polar lipid composition, two more species, *Natronorubrum aibiense* (Cui *et al.*, 2006) and *Natronorubrum sulfidifaciens*

(Cui *et al.*, 2007), were included in the genus. Recently, Oren *et al.* (2009) emended the descriptions of nine genera of the family *Halobacteriaceae*, including the genus *Natronorubrum*. The main features of the species of *Natronorubrum* are as follows: cells are pleomorphic, flat, triangular, square or disc-shaped and stain Gram-negative; cells lyse in distilled water; they are extremely halophilic, with growth occurring in media containing 2.1–5.2 M NaCl; they are neutrophilic or alkaliphilic with growth up to pH 11; they metabolize sugars, in some cases with formation of acids; the major polar lipids are C₂₀C₂₀ and C₂₀C₂₅ glycerol diether derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester; and they have DNA G + C contents of 59.9–61.2 mol%.

In this study, the taxonomic features of two strains, CG-6^T and CG-4, isolated from sediment of the hypersaline Lake Chagannor, located in Inner Mongolia Autonomous Region, China, were investigated. On the basis of the data presented in this work, strains CG-6^T and CG-4 represent a novel species of the genus *Natronorubrum*.

The two haloalkaliphilic strains (CG-6^T and CG-4) were isolated from a sediment sample from Lake Chagannor (43° 21.583' N 113° 08.193' E). At the time of sampling, the water of the lake had a temperature of 17.1 °C, the pH was 9.5, the conductivity was 21.3 mS cm⁻¹ and the salinity was 16%. Approximately 0.5 g sediment sample was resuspended in alkaline saline medium and incubated

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CG-6^T and CG-4 are FN376859 and FN376860, respectively.

A thin-layer chromatogram of polar lipids extracted from strains CG-6^T and CG-4 and related strains is available as supplementary material with the online version of this paper.

aerobically at 37 °C; strains CG-6^T and CG-4 were isolated after 7 days of incubation and plated on alkaline saline solid medium until pure. The alkaline isolation medium contained the following (g l⁻¹): peptone (Difco), 5.0; meat extract (Difco), 3.0; KH₂PO₄, 3.9; MgSO₄·7H₂O, 0.78; NaCl, 157.0; Na₂CO₃, 21.4; and NaHCO₃, 17.0. NaCl, Na₂CO₃ and NaHCO₃ were autoclaved separately and added to the medium prior to incubation (Duckworth *et al.*, 1996). Solid medium contained 2.0 % (w/v) agar; strains were maintained on this solid medium.

Phenotypic characterization was carried out in accordance with the recommended minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). For the determination of cellular morphology and motility, a sample from an exponentially grown liquid culture was examined by phase-contrast light microscopy. The morphology of the colonies, their pigmentation and their size were observed on the isolation and maintenance solid medium (with different salt concentrations) after 10 days of incubation. Growth at different concentrations of salts was determined on isolation medium supplemented with 0, 0.5, 1, 3, 5, 7, 10, 15, 20, 25 or 30 % (w/v) total salts. The pH range for growth was determined on the medium at pH 5.0–11.0 (at 0.5 pH unit intervals), achieved using appropriate biological buffers (Na₂HPO₄/Na₂HPO₄ for pH below 8.0, Na₂CO₃/NaHCO₃ for pH 8.0–10.0 and Na₂HPO₄/NaOH for pH higher than 10.0), as described previously (Gomori, 1955). The pH was readjusted after sterilization and growth was assessed using optical density at 600 nm. The temperature range for growth was determined at 15–55 °C (at intervals of 5.0 °C). Catalase activity was assayed by adding 3 % H₂O₂ to culture plates. The oxidase reaction was performed on filter paper moistened with a 1 % (w/v) aqueous solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (Barrow & Feltham, 1993). Anaerobic growth was tested in the presence of 5 % nitrate and 3 % L-arginine in filled stoppered tubes (Oren *et al.*, 1997). To determine the utilization of different organic substrates as carbon and energy or as carbon, nitrogen and energy sources, a medium containing yeast extract at 0.05 % (w/v) supplemented with 1 % (w/v) of the tested substrate was used (Torreblanca *et al.*, 1986). Susceptibility to antibiotics was determined on agar medium plates by using antibiotic discs containing ampicillin (10 µg), bacitracin (10 U), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), nalidixic acid (30 µg), neomycin (10 µg), novobiocin (30 µg), penicillin G (10 U), rifampicin (30 µg), streptomycin (10 µg) and tetracycline (30 µg). Strains CG-6^T and CG-4 were oxidase- and catalase-positive. Other phenotypic characteristics are summarized in Table 1 and the species description. The results of the utilization of different substrates and antibiotic susceptibility are included in the species description.

Polar lipids of strain CG-6^T and CG-4 were extracted with chloroform/methanol as described previously (Kamekura, 1993). TLC was carried out using Merck HPTLC silica gel

Table 1. Differentiation of *Natronorubrum sediminis* sp. nov. from other species of the genus

Strains: 1, *Nrr. sediminis* sp. nov. strains CG-6^T and CG-4; 2, *Nrr. sulfidifaciens* JCM 14089^T (data from Cui *et al.*, 2007 and this study); 3, *Nrr. tibetense* AS 1.2123^T (Xu *et al.*, 1999 and this study); 4, *Nrr. bangense* AS 1.1984^T (Xu *et al.*, 1999); 5, *Nrr. aibiense* JCM 13488^T (Cui *et al.*, 2006).

Characteristic	1	2	3	4	5
Cell shape*	PL	PL	PL	PL	R
Motility	–	+	–	–	+
Optimum NaCl concentration (% w/v)	20	18	20	22.5	18
H ₂ S formation	–	+	–	–	–
Nitrate reduction	+	+	–	–	+
Indole production	–	+	+	+	+
Tween 80 hydrolysis	+	–	–	–	–
Gelatin hydrolysis	–	–	+	–	–
Utilization of:					
D-Glucose	+	–	+	+	+
D-Galactose	–	–	–	–	+
D-Fructose	+	–	+	+	+
D-Xylose	+	+	–	–	–
DNA G + C content (mol%)	61.9–62.5	60.9	60.1	59.9	61.2

*PL, Pleomorphic; R, rod.

†Weak growth in our study.

60 plates in the solvent system chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.). Glycolipids were determined as purple spots by spraying with 0.5 % α -naphthol in methanol/water (1:1) and then with sulfuric acid/ethanol (1:1), followed by heating at 160 °C. The polar lipids of strains CG-6^T and CG-4 were C₂₀C₂₀ and C₂₀C₂₅ derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, which are the major phospholipids found in members of the genus *Natronorubrum* (Oren *et al.*, 2009). No glycolipids were detected (Supplementary Fig. S1, available in IJSEM Online).

Chromosomal DNA from strains CG-6^T and CG-4 was isolated and purified according to the method described by Marmur (1961). Genomic DNA G + C contents were determined from the mid-point value (*T*_m) of each of the thermal denaturation profiles (Marmur & Doty, 1962) by using the equation of Owen & Hill (1979). The genomic DNA G + C contents of strains CG-6^T and CG-4 were 62.5 and 61.9 mol%, respectively. These values are quite close to those of species of the genus *Natronorubrum* (59.9–61.2 mol%; Table 1). The 16S rRNA genes of strains CG-6^T and CG-4 were amplified by PCR using three universal primer sets as described previously (López-García *et al.*, 2001; Arahal *et al.*, 1996). Almost-complete 16S rRNA gene sequences of strains CG-6^T (1371 bp) and CG-4 (1371 bp) were determined. The ARB software package (Ludwig *et al.*, 2004) was used for 16S rRNA gene sequence analysis.

Following the recommendations of Ludwig *et al.* (1998), alternative treeing methods (maximum-parsimony, neighbour-joining and maximum-likelihood) were used (Saitou & Nei, 1987). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). 16S rRNA gene sequence data showed that strains CG-6^T and CG-4 shared a high degree of similarity (99.8%); they were closely related to *Nrr. sulfidifaciens* JCM 14089^T (similarities of 96.2 and 96.1%, respectively), *Nrr. tibetense* AS 1.2123^T (95.5 and 95.4%), *Nrr. bangense* AS 1.1984^T (95.1 and 95.1) and *Nrr. aibiense* JCM 13488^T (93.9 and 93.8%). Lower similarities were obtained with the type strains of species of the genera *Haloterrigena* and *Natrinema*. The maximum-parsimony phylogenetic tree (Fig. 1) showed that both strains were within the cluster made up of the type strains of *Natronorubrum* species. Trees constructed according to the neighbour-joining and maximum-likelihood methods resulted in highly similar tree topologies, so only the maximum-parsimony results are shown.

DNA–DNA hybridization experiments were carried out to determine the genotypic relatedness between the two isolates. These studies were carried out by following the competition procedure of Johnson (1994). Competitor DNAs were sonicated (Braun Melsungen) at 50 W for two periods of 15 s. Membrane filters 0.5 cm in diameter (HAHY; Millipore) and containing reference DNA (25 µg cm⁻²) were placed in 5 ml screw-capped vials (Greiner) containing labelled, sheared, denatured DNA and denatured, sheared competitor DNA. The ratio of the concentration of the competitor DNA to that of the labelled DNA was at least 150:1. The final volume was 140 µl and the solvent in which the determinations were carried out was a mixture of 2 × SSC and 30% formamide. The optimal hybridization temperatures were 57.2 and 57.0 °C, which are within the limits of validity for the filter method (De Ley & Tijtgat, 1970). The vials were shaken gently for 18 h in a water bath (Grant Instruments); these procedures were done in triplicate. After hybridization, the filters were washed in 2 × SSC at the optimal renaturation temperatures given above. The radioactivity bound to the filters was measured in a liquid scintillation counter

(Beckman Instruments) and relatedness was calculated according to Johnson (1994). At least two independent determinations were carried out for each experiment and the reported results are mean values. DNA–DNA hybridization values between strains CG-6^T and CG-4 were 100 and 98% (the latter being for the reverse hybridization). These data clearly support the conclusion that the two strains are members of the same species (Stackebrandt *et al.*, 2002).

On the basis of phenotypic characteristics (Table 1), polar lipid profiles, DNA G+C contents and 16S rRNA gene sequence analyses, strains CG-6^T and CG-4 are considered to represent a novel species of the genus *Natronorubrum*, for which the name *Natronorubrum sediminis* sp. nov. is proposed.

Description of *Natronorubrum sediminis* sp. nov.

Natronorubrum sediminis (se.di'mi.nis. L. n. *sedimen*, -inis sediment; L. gen. n. *sediminis* of a sediment).

Cells are Gram-negative, non-motile, pleomorphic rods, 4.0–6.0 × 0.8–1.0 µm. Colonies are circular, smooth, entire, of shining aspect, pink-pigmented and 1.0–4.0 mm in diameter after 10 days incubation at 37 °C on plates containing 20% (w/v) total salts. Extremely halophilic; the cells lyse in water. Growth occurs in 2.5–5.0 M NaCl and is optimal in 3.4 M NaCl. MgCl₂ is not required for growth. The pH and temperature ranges for growth are pH 8.0–11.0 (optimum at pH 9.0) and 25–50 °C (optimum at 37 °C). Chemo-organotrophic, aerobic, and oxidase- and catalase-positive. Indole is not produced from tryptophan. Methyl red, Voges–Proskauer and Simmons' citrate tests are negative. Does not grow anaerobically in the presence of nitrate or L-arginine. Starch, gelatin, DNA, aesculin and casein are not hydrolysed. Urea and Tween 80 are hydrolysed. Produces arginine dihydrolase and lysine decarboxylase, but not ornithine decarboxylase. Nitrate is reduced to nitrite; gas is not produced from nitrite. H₂S is not produced from Na₂S₂O₃. Utilizes D-glucose, D-fructose, D-xylose, D-arabinose, D-mannose and L-asparagine. No growth is observed on glycerol, maltose, trehalose, starch, propionate, fumarate, acetate, L-lysine, D-mannitol, D-sorbitol, lactose, D-galactose, raffinose, D-ribose, malate,

Fig. 1. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequence comparisons showing the relationships between *Natronorubrum sediminis* sp. nov. strains CG-6^T and CG-4 and strains of species of the genus *Natronorubrum* and other related haloarchaea. Bootstrap values >80% (based on 1000 replications) are shown. GenBank/EMBL accession numbers are given in parentheses. Bar, 0.01% sequence divergence.

succinate, glutamate, isoleucine, L-serine or glycine. Sensitive to novobiocin (30 µg). Resistant to ampicillin (10 µg), bacitracin (10 U), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), nalidixic acid (30 µg), neomycin (10 µg), penicillin G (10 U), rifampicin (30 µg), streptomycin (10 µg) and tetracycline (30 µg). The polar lipids are C₂₀C₂₀ and C₂₀C₂₅ derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester. No glycolipids are detected. The DNA G+C content is 61.9–62.5 mol% (T_m).

The type strain is CG-6^T (=CECT 7487^T =CGMCC 1.8981^T =JCM 15982^T), isolated from sediment of the saline Lake Chagannor in Inner Mongolia, China. The DNA G+C content of the type strain is 62.5 mol%.

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