***Natrialba hulunbeirensis* sp. nov. and *Natrialba chahannaoensis* sp. nov., novel haloalkaliphilic archaea from soda lakes in Inner Mongolia Autonomous Region, China**

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Two haloalkaliphilic archaeal strains, X21^T and C112^T, were isolated from soda lakes in Inner Mongolia Autonomous Region, China. Their morphology, physiology, biochemical features, polar lipid composition and 16S rRNA genes were characterized in order to elucidate their taxonomy. According to these data, strains X21^T and C112^T belong to the genus *Natrialba*, although there are clear differences with respect to their physiology and polar lipid composition between the two strains and the type species, *Natrialba asiatica*. On the basis of low DNA–DNA hybridizations, these two strains should be considered as new species of genus *Natrialba*. The names *Natrialba hulunbeirensis* sp. nov. (type strain X21^T = AS 1.1986^T = JCM 10989^T) and *Natrialba chahannaoensis* sp. nov. (type strain C112^T = AS 1.1977^T = JCM 10990^T) are proposed.

Keywords: *Natrialba hulunbeirensis*, *Natrialba chahannaoensis*, archaea, haloalkaliphiles

INTRODUCTION

The family *Halobacteriaceae* was proposed by Gibbons (1974) to accommodate the rods and cocci that required high concentrations (more than 12%, w/v) of sodium chloride for growth, and included two genera, *Halobacterium* and *Halococcus*. The isolation of haloalkaliphilic and pleomorphic halobacteria necessitated the extension of this scheme to include six genera: *Halobacterium*, *Halococcus*, *Haloarcula*, *Haloferax*, *Natronobacterium* and *Natronococcus* (Grant & Larsen, 1989; Torreblanca *et al.*, 1986). Currently, members of the aerobic, extremely halophilic archaea are classified in 15 genera: *Halobacterium*, *Halococcus*, *Haloarcula*, *Haloferax*, *Halorubrum*, *Halobaculum*, *Natrialba*, *Natronomonas*, *Natronobacterium*, *Natronococcus*, *Halogeometricum*, *Natrinema*, *Haloterrigena*, *Natronorubrum* and *Halorhabdus* (McGenity & Grant, 1995; Oren *et al.*, 1995; Kamekura & Dyall-

Smith, 1995; Kamekura *et al.*, 1997; Montalvo-Rodríguez *et al.*, 1998; McGenity *et al.*, 1998; Ventosa *et al.*, 1999; Xu *et al.*, 1999; Wainø *et al.*, 2000). Most halobacteria are neutrophilic, but some of these genera include species that are haloalkaliphilic, requiring not only high NaCl concentrations but also high pH and low Mg²⁺ concentrations for growth.

The phenotypic characteristics of haloalkaliphilic archaea that permit their differentiation are comparatively very limited, so it is relatively difficult to classify them on the basis of their phenotypic features alone. In addition, their phospholipid composition is highly conserved and glycolipids are absent from the majority of strains. Thus, chemotaxonomy on the basis of lipid composition is difficult. Therefore, in combination with other phenotypic features such as morphology, physiology and biochemistry, the phylogenetic inference of 16S rRNA sequences and DNA–DNA hybridization play important roles in the classification of haloalkaliphilic archaea. In 1997, the ICSB Subcommittee on the Taxonomy of Halobacteria proposed minimum standards for the classification of halobacterial archaea and suggested that the classification of halobacteria should be polyphasic and consistent with the phylogenetic analysis of 16S rRNA

Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate methyl ester; S₂-DGD-1, 2,6-HSO₃-Manp-α(1→2)-GlcP-α(1→1)-sn-glyceroldiether.

The GenBank accession numbers for the 16S rDNA sequences of strains X21^T and C112^T are respectively AF262026 and AJ004806.

gene sequences (Oren *et al.*, 1997). Recently, great progress has been made in the classification of haloalkaliphilic archaea: Kamekura *et al.* (1997) classified some species previously included in the genus *Natronobacterium* into three different genera: *Natronomonas*, *Natrialba* and *Halorubrum*. Additionally, Xu *et al.* (1999) proposed a new genus, *Natronorubrum*, with two new haloalkaliphilic species, *Natronorubrum bangense* and *Natronorubrum tibetense*.

In this study, we describe two novel soda lake isolates, X21^T and C112^T. We have determined the complete 16S rDNA sequences of these isolates, as well as their polar lipid compositions. In addition, we have performed DNA–DNA hybridization experiments with other members of the genus *Natrialba* in order to determine the taxonomic positions of strains X21^T and C112^T.

METHODS

Bacterial strains and culture conditions. Strain X21^T was isolated by enrichment from a sediment sample (pH 10) collected from Chahannao soda lake in the Inner Mongolia Autonomous Region of China, whereas strain C112^T was isolated from a sediment sample (pH 9.5) of an unnamed soda lake in the Hulunbeir prefecture of the Inner Mongolia Autonomous Region, China. The medium and methods for enrichment and isolation were described previously (Tindall *et al.*, 1980). In addition, the following strains of the genus *Natrialba* were used in this study: *Natrialba magadii* NCIMB 2190^T and *Natrialba asiatica* strains JCM 9576^T and JCM 9577. These strains were cultivated aerobically at 37 °C as described previously (Tindall, 1992). The growth medium for the following studies contained (l⁻¹): 7.5 g Casamino acids (Difco), 10 g yeast extract (Difco), 3.0 g trisodium citrate, 0.3 g MgSO₄ · 7H₂O, 2.0 g KCl, traces of Fe²⁺ and Mn²⁺, 200 g NaCl and 8.0 g Na₂CO₃.

Phenotypic characterization. Phenotypic tests on strains X21^T and C112^T were carried out in accordance with the recommended standard methods for halobacteria (Oren *et al.*, 1997). Cell motility and shape were examined by phase-contrast microscopy without fixation and by Gram staining with acetic acid fixation. The optimal conditions of growth, reduction of nitrate and utilization of different carbon sources were determined as described previously (Grant & Tindall, 1980; Mwatha & Grant, 1993). Tests for catalase and oxidase activities and hydrolysis of starch, gelatin, casein and Tween 80 were performed as described previously (Gonzalez *et al.*, 1978). Antibiotic susceptibility and H₂S production was tested as described previously (Colwell *et al.*, 1979). Growth was monitored by turbidity at 660 nm.

Lipid composition. Total lipids were extracted and analysed on silica-gel plates (Kieselgel 60 F₂₅₄; Merck) by one- and two-dimensional TLC (Ross *et al.*, 1981; Ross, 1982). The core lipids were analysed by TLC, as described by Ross *et al.* (1981).

DNA base composition and DNA–DNA hybridization. Genomic DNA was prepared and purified as described previously (Zhou *et al.*, 1994). The G+C content of the DNA was determined by the thermal denaturation method (Marmur & Doty, 1962). DNA–DNA hybridization was carried out as described by Tindall *et al.* (1984) with a minor modification: DNA fragments were labelled with

[α-³²P]dCTP using a nick-translation kit (Boehringer Mannheim).

16S rRNA gene sequence and phylogenetic analysis. The methods used for DNA preparation, PCR amplification of 16S rRNA genes and gene sequencing were described previously (Zhou *et al.*, 1994).

Multiple sequence alignments were performed using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). Phylogenetic analysis of multiple sequence alignments was performed with TREECON W version 1.3b (Van de Peer & De Wachter, 1994). Phylogenetic tree construction was carried out by the neighbour-joining method with Kimura's two-parameter calculation model in TREECON W version 1.3b.

RESULTS

Morphology

The two strains X21^T and C112^T were Gram-negative, rod-shaped and non-motile. Cells were 0.4–0.6 × 1–2.5 µm and lysed in distilled water. On agar plates, both strains formed red, circular and smooth colonies, 0.5 mm in diameter for strain X21^T and 2.0 mm in diameter for strain C112^T after 1 week at 37 °C.

Physiological characterization

Growth of both strains occurred in the pH range 8.5–10.5, with optimum growth at pH 9.0. Strain X21^T grew in media containing between 12 and 30 % (w/v) NaCl, with an optimum at 20 % (w/v) NaCl. Strain C112^T grew in media containing between 10 and 30 % (w/v) NaCl, with an optimum at 15 % (w/v) NaCl. Growth of both strains occurred in the temperature range 20–55 °C, with respective optima at 50 and 45 °C for strains X21^T and C112^T.

Both strains were catalase- and oxidase-positive and strictly aerobic. Both strains reduced nitrate to nitrite anaerobically and produced H₂S from cysteine but not from Na₂S₂O₃. They were sensitive to some antibiotics (such as erythromycin, rifampicin and bacitracin) but insensitive to tetracycline hydrochloride. Both strains hydrolysed gelatin and did not hydrolyse Tween 80. Strain X21^T hydrolysed Tween 40 but did not hydrolyse starch or casein. Strain C112^T hydrolysed starch and casein but did not hydrolyse Tween 40. Both strains utilized some sugars (fructose and maltose) and amino acids (asparagine, arginine and proline) as sole carbon sources for growth. They were unable to use sucrose, lactose, mannitol, serine, threonine or isoleucine. In addition, strain C112^T utilized glucose, acetate and lysine as sole carbon sources for growth, but strain X21^T did not. Acid production by both strains was observed from fructose and maltose, but not from glucose.

Polar lipids

Two-dimensional TLC revealed that the major polar lipids present in both strains were diphytanyl moieties (C₂₀–C₂₀) and phytanyl–sesterterpenyl moieties

Fig. 1. Phylogenetic tree of *Natrialba* and related genera from their 16S rDNA sequences. The tree was constructed by the neighbour-joining method and Kimura's two-parameter calculation model. Numbers represent confidence levels from 100 replicate bootstrap sampling. Bar, 0.05 expected changes per site.

(C₂₀–C₂₅) of phosphatidylglycerol (PG) and phosphatidylglycerophosphate methyl ester (PGP-Me). Glycolipids were not present. In addition, a minor amount of an unidentified polar lipid was present in strain X21^T.

16S rRNA gene sequence analysis

The almost complete sequences of the 16S rDNA from strains X21^T and C112^T, with respective lengths of 1472 and 1474 bp, were determined and compared to the sequences of members of the genus *Natrialba* and other closely related halobacteria. Positions with any gaps and alignment uncertainty were omitted from the analysis. A total of 1376 unambiguous nucleotides were used for computing evolutionary distance. The phylogenetic tree (Fig. 1) indicated that the two strains were closely related to the species of the genus *Natrialba* and constituted a subgroup with the two described haloalkaliphilic species (*Natrialba magadii* NCIMB 2190^T and '*Natrialba wudunaoensis*' Y21). The sequence similarities of strain X21^T were 98.6% to strain C112^T, 97.3% to *Natrialba magadii* NCIMB 2190^T, 98.9% to '*Natrialba wudunaoensis*' Y21, 94.7% to *Natrialba asiatica* JCM 9576^T and 95.5% to *Natrialba asiatica* JCM 9577. The sequence similarities of strain C112^T were 96.8% to *Natrialba magadii* NCIMB 2190^T, 99.2% to '*Natrialba wudunaoensis*' Y21, 95.4% to *Natrialba asiatica* JCM 9576^T and 96% to *Natrialba asiatica* JCM 9577. Strains X21^T and C112^T respectively had 95.4 and 95.5% sequence similarity to *Natronorubrum tibetense* GA33^T, 95.5 and 95.4% sequence similarity to *Haloterrigena thermo-*

Table 1. DNA–DNA hybridization between strains X21^T and C112^T and other type strains of species of the genus *Natrialba*

Strain	X21 ^T	C112 ^T
Strain X21 ^T	100	–
Strain C112 ^T	29	100
<i>Natrialba magadii</i> NCIMB 2190 ^T	36	13
<i>Natrialba asiatica</i> JCM 9576 ^T	18	3
' <i>Natrialba wudunaoensis</i> ' Y21	26	37

tolerans PR5^T and 95.2 and 95.0% sequence similarity to *Natrinema pallidum* NCIMB 777^T. Strains X21^T and C112^T had less than 92% sequence similarity to members of the other genera of aerobic extremely halophilic archaea. The phylogenetic placement of the two strains (Fig. 1) indicated that strains X21^T and C112^T were members of the genus *Natrialba*.

DNA–DNA hybridization and G + C content of the DNA

The DNA–DNA relatedness of the two strains (X21^T and C112^T) to other described species of the genus *Natrialba* is shown in Table 1. DNA–DNA hybridization indicated that both strains (X21^T and C112^T) had low DNA relatedness to the other described species of *Natrialba* (3–37%) and, since the DNA hybridization was lower than 70% in all cases, strains X21^T and C112^T thus represent two distinct genospecies.

The G + C contents of the genomic DNAs from strains X21^T and C112^T were respectively 64.3 and 63.7 mol%, as determined by the thermal denaturation method.

DISCUSSION

Some decades ago, halobacterial taxonomy was based mainly on physiological and morphological features (Gibbons, 1974). In the 1980s, polar lipid composition and 16S rRNA–DNA hybridization proved particularly useful in the classification of halobacteria (Ross & Grant, 1985; Torreblanca *et al.*, 1986), defining two additional genera (*Haloarcula*, *Haloferax*), but also indicating the need for further reclassification of some halobacterial species of uncertain taxonomic standing (Grant & Larsen, 1989; Ross & Grant, 1985). In recent years, more and more complete 16S rRNA gene sequences have become available for the halobacteria. Phylogenetic analysis of 16S rRNA gene sequences has now shown considerable taxonomic diversity within the family *Halobacteriaceae* (Kamekura & Dyall-Smith, 1995; McGinity & Grant, 1995; Duckworth *et al.*, 1996) and, to date, 15 genera have been described, including a number of genera where representative strains are all haloalkaliphilic and others that include some haloalkaliphilic types.

Table 2. Major features of strains X21^T and C112^T and species of the genus *Natrialba*

Taxa are indicated as: 1, strain X21^T; 2, strain C112^T; 3, *Natrialba magadii* NCIMB 2190^T; 4, *Natrialba asiatica* JCM 9576^T; 5, *Natrialba asiatica* JCM 9577; 6, '*Natrialba wudunaoensis*' Y21. Cells of all strains are rods and stain Gram-negative. All strains are positive for catalase, oxidase and hydrolysis of gelatin. NR, Not reported.

Characteristic	1	2	3	4	5	6
Motility	—	—	+	+	+	—
Colony pigmentation	Red	Red	Orange-red	White	Pale-yellow	Red
NaCl concentration for growth (%):						
Optimum	20	15	20	24	20	18
Range	12–30	10–30	12–30	12–30	12–30	12–25
Optimum temperature (°C)	50	45	37–40	30–40	35–40	40
Optimum pH	9.0	9.0	9.5	6.6–7.0	7.5–7.8	9.0
H ₂ S production from cysteine	+	+	+	—	+	+
Nitrate reduction	+	+	—	+	+	+
Hydrolysis of:						
Starch	—	+	—	—	+	—
Casein	—	+	+	+	+	+
Utilization of:						
Glucose	—	+	—	—	+	—
Fructose	+	+	—	—	+	+
Maltose	+	+	—	—	+	+
Acetate	—	+	+	NR	NR	+
DNA G + C content (mol %)	64.3	63.7	63.0	60.3–63.1	60.3–63.1	65.4
Polar lipid composition	PG, PGP-Me	PG, PGP-Me	PG, PGP-Me	PG, PGP-Me, S ₂ -DGD-1	PG, PGP-Me, S ₂ -DGD-1	PG, PGP-Me

A number of haloalkaliphiles have been isolated from soda lakes in the Inner Mongolia Autonomous Region of China, including strain Y21, which has been shown to be a species of *Natrialba* (Wang *et al.*, 2000), the strains described here, X21^T and C112^T, and some incompletely characterized strains, such as X213, C212 and Y212 (Wang & Tang, 1989). We now report the full characterization of the two strains X21^T and C112^T, which show features typical of the archaeal genus *Natrialba* (Table 2). Our results show that both strains are typical members of the haloalkaliphilic archaea, growing optimally in the presence of 20% NaCl (w/v) at pH 9.0 and containing C₂₀–C₂₀/C₂₀–C₂₅ diether core lipids of PG and PGP-Me. The genus *Natrialba* contains non-alkaliphilic and alkaliphilic species and they have different polar lipid compositions. As shown in Table 2, there are two subgroups in the genus *Natrialba*; members of one subgroup (*Natrialba asiatica* JCM 9576^T and JCM 9577) contain a glycolipid (2,6-HSO₃-Manp-α(1 → 2)-Glc-α(1 → 1)-sn-glyceroldiether; S₂-DGD-1) in addition to phospholipids (PG and PGP-Me), and members of the other subgroup (*Natrialba magadii* NCIMB 2190^T and our two isolates) contain only PG and PGP-Me. This feature would imply that the genus *Natrialba* constitutes a heterogeneous group and it is not in accordance with the principles of polyphasic taxonomy (Grant & Larsen, 1989; Oren *et al.*, 1997). Future studies should clarify the classification of the entire group.

The 16S rDNA sequences of the two strains (X21^T and C112^T) exhibited high levels of similarity to that of their closest counterpart, '*Natrialba wudunaoensis*' Y21 (98.9 and 99.2%, respectively), and also to that of *Natrialba magadii* NCIMB 2190^T (97.3 and 96.8%). Although the two strains possessed a close phylogenetic relationship to the members of the genus

Natrialba, the DNA–DNA relatedness was lower than 70% with previously described members of this genus, indicating that the two strains are genetically distinct from species described previously and should be classified as new species of the genus *Natrialba*. With regard to the taxonomic position of the two new strains and *Natrialba magadii* NCIMB 2190^T, although they have a relatively close relationship to *Natrialba asiatica* JCM 9576^T (Fig. 1), it could be argued that these alkaliphilic halobacteria could be placed in a different genus because they have different polar lipid compositions and phenotypic characters from the type species of the genus, *Natrialba asiatica*.

On the basis of the data described above, the two haloalkaliphilic archaea, strains X21^T and C112^T, represent new members of the genus *Natrialba*, for which we propose the names *Natrialba hulunbeirensis* sp. nov. (strain X21^T) and *Natrialba chahannaoensis* sp. nov. (strain C112^T).

Description of *Natrialba hulunbeirensis* sp. nov.

Natrialba hulunbeirensis (hu.lun.bei.ren'sis. N.L. adj. *hulunbeirensis* of Hulunbeir, relating to the isolation of the organism from a soda lake of Hulunbeir prefecture, China).

Cells are rod-shaped, 0.4–0.6 × 1–2.5 µm, non-motile, uniformly stained Gram-negative, strictly aerobic and lysed in distilled water. Colonies are red, circular, smooth and 0.5 mm in diameter after 1 week of incubation. Growth occurs in media containing between 12 and 30% NaCl; optimum at 20% NaCl. The pH range for growth is 8.5–10.5, with an optimum at pH 9.0. The temperature range for growth is between 20 and 55 °C, with an optimum at 50 °C. Oxidase and catalase are positive. The following substrates are

utilized for growth: asparagine, arginine, proline, fructose and maltose. Serine, threonine, isoleucine, lysine, acetate, glucose, sucrose, lactose and mannitol are not utilized for growth. Starch, casein and Tween 80 are not hydrolysed. Gelatin is liquefied. Tween 40 is hydrolysed. H_2S is produced from cysteine but not from thiosulfate. Nitrate is reduced. Sensitive to erythromycin, bacitracin and rifampicin but not to tetracycline. The polar lipids are C_{20} – C_{20} and C_{20} – C_{25} derivatives of PG, PGP-Me and an uncharacterized minor polar lipid. The G + C content of the DNA is 64.3 mol % (T_m). Isolated from a soda lake in China.

The type strain is strain X21^T, deposited in the Academia Sinica, China General Microbiological Culture Collection as AS 1.1986^T and as JCM 10989^T.

Description of *Natrialba chahannaoensis* sp. nov.

Natrialba chahannaoensis (cha.han.nao.en'sis. N.L. adj. *chahannaoensis* of Chahannao, referring to its isolation from Chahannao soda lake, China).

Cells are rod-shaped, 0.4–0.6 × 1–2.5 µm, non-motile, uniformly stained Gram-negative, strictly aerobic and lysed in distilled water. Colonies are red, circular, smooth and 2.0 mm in diameter after 1 week of incubation. Growth occurs in media containing between 10 and 30 % NaCl; optimum at 15 % NaCl. The pH range for growth is 8.5–10.5, with an optimum at pH 9.0. The temperature range for growth is between 20 and 55 °C, with an optimum at 45 °C. Oxidase and catalase are positive. The following substrates are utilized for growth: asparagine, arginine, proline, lysine, glucose, fructose, maltose and acetate. Serine, threonine, isoleucine, sucrose, lactose and mannitol are not utilized for growth. Starch, casein and gelatin are hydrolysed. Tweens 40 and 80 are not hydrolysed. H_2S is produced from cysteine but not from thiosulfate. Nitrate is reduced. Sensitive to erythromycin, bacitracin and rifampicin but not to tetracycline. The polar lipids are C_{20} – C_{20} and C_{20} – C_{25} derivatives of PG and PGP-Me. The G + C content of the DNA is 63.7 mol % (T_m). Isolated from a soda lake in China.

The type strain is strain C112^T (= AS 1.1977^T = JCM 10990^T).

ACKNOWLEDGEMENTS

We thank M. Kamekura for his kind advice. This work was partially supported by a grant from the National Natural Science Foundation of China and a grant from the Chinese Academy of Sciences.

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