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Natronolimnobius baerhuensis gen. nov., sp. nov. and *Natronolimnobius innermongolicus* sp. nov., novel haloalkaliphilic archaea isolated from soda lakes in Inner Mongolia, China

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Abstract Three novel isolates of haloalkaliphilic archaea, strains IHC-005^T, IHC-010, and N-1311^T, from soda lakes in Inner Mongolia, China, were characterized to elucidate their taxonomic positions. The three strains were aerobic, Gram-negative chemoorganotrophs growing optimally at 37–45°C, pH 9.0–9.5, and 15–20% NaCl. Cells of strains IHC-005^T/IHC-010 were motile rods, while those of strain N-1311^T were non-motile pleomorphic flats or cocci. The three strains contained diphytanyl and phytanyl-sesterterpanyl diether derivatives of phosphatidylglycerol and phosphatidylglycerophosphate methyl ester. No glycolipids were detected. On phylogenetic analysis of 16S rRNA gene sequences, they formed an independent cluster in the Natro group of the family *Halobacteriaceae*. Comparison of their morphological, physiological, and biochemical properties, DNA G+C content and 16S rRNA gene sequences, and DNA-DNA hybridization study support the view that strains IHC-005^T/IHC-010 and strain N-1311^T represent separate species. Therefore, we propose *Natronolimnobius baerhuensis* gen. nov., sp. nov. for strains IHC-005^T (=CGMCC 1.3597^T =JCM 12253^T)/IHC-010 (=CGMCC 1.3598=JCM 12254) and *Natronolimnobius innermongolicus* sp. nov. for N-1311^T (=CGMCC 1.2124^T =JCM 12255^T).

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Introduction

Members of the family *Halobacteriaceae* are aerobic or faculatively anaerobic, red-colored due to the presence of cartenoid pigments (except for a few species), chemoorganotrophic archaea requiring more than 1.5 M NaCl for growth (Gibbons 1974; Grant et al. 2001). They are ubiquitous in hypersaline environments such as salt lakes, salterns, salted foods, salt mines, and soda lakes. Formerly, the taxonomy of the family *Halobacteriaceae* had relied on their morphological, physiological, and biochemical properties (Gibbons 1974); however, the present taxonomic scheme of the *Halobacteriaceae* genera has been based on 16S rRNA sequence analyses and polar lipid composition since the mid-1980s (Ross et al. 1985; Torreblanca et al. 1986; Grant and Larsen 1989; Kamekura et al. 1997; Grant et al. 2001). The family as a whole forms a relatively tight phylogenetic cluster; however, it includes quite diverse members recognizing 18 genera and nearly 50 species to date. Among the family, haloalkaliphilic members constitute a distinctive phenotypic group that requires alkaline pH and low Mg^{2+} content for growth and lacks glycolipids in most strains; nonetheless, they are polyphyletic, as indicated by 16S rRNA gene sequence comparisons. Currently, 11 species in six genera of the haloalkaliphilic *Halobacteriaceae* are known, i.e., *Natronobacterium gregoryi*, *Natronococcus amylolyticus*, *Ncc. occultus*, *Natronorubrum bangense*, *Nrr. tibetense*, *Natronomonas pharaonis*, *Halorubrum tibetense*, *Hrr. vacuolatum*, *Natrialba magadii*, *Nab. chahannaoensis*, *Nab. hulunbeirensis*, and *Halobiforma nitratireducens* (Soliman and Trüper 1982; Tindall et al. 1984; Mwatha et al. 1993; Kanai et al. 1995; Kamekura et al. 1997; Xu et al. 1999, 2001; Xin et al. 2001; Hezayen et al. 2002;

Fan et al. 2004). All species, except for *Hrr. tibetense*, *Hrr. vacuolatum*, and *Nmn. pharaonis*, are accommodated in the Natro group as defined by 16S rRNA gene sequence analysis (McGenity et al. 1998). In addition to the known species, a number of partially characterized haloalkaliphilic archaea are described elsewhere (Morth and Tindall 1985; Zvyaginteseva and Tarasov 1987; Wang and Tang 1989; Upasani and Desai 1990; Duckworth et al. 1996; Tian et al. 1997; Tindall et al. 1980; Wang et al. 2000; Ochsenreiter et al. 2002).

Recently, we isolated two new haloalkaliphilic archaea from a soda lake in Inner Mongolia Autonomous Region, China. Preliminary phylogenetic analysis based on the 16S rRNA gene sequences revealed that they are not closely related to the hitherto known *Halobacteriaceae* genera, but showed high similarities to an uncharacterized isolate from another soda lake in Inner Mongolia that has been maintained in our laboratory. In this paper, we describe characteristics of these three haloalkaliphilic archaeal strains and propose a new genus and two new species in the family *Halobacteriaceae*.

Materials and methods

Source of strains

A water sample (pH 9.3) was collected from Baerhu Soda Lake in Inner Mongolia Autonomous Region of China, in August 1999. The sample was incubated in an enrichment medium composed of (per liter): 2.0 g yeast extract (Difco), 2.0 g casamino acids (Difco), 1.0 g sodium glutamate, 3.0 g trisodium citrate, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaSO_4 \cdot 2H_2O$, 1.0 g KCl, 200.0 g NaCl, and 8.0 g Na_2CO_3, pH 9.5, at 37°C for 1 week; the grown culture was spread on agar plates of the same medium (20.0 g agar per liter) and incubated at 37°C. Alternatively, the sample was directly spread on the agar plates and cultivated in the same way. Separated reddish colonies were repeatedly transferred onto agar plates. Finally, six purified strains were obtained from the isolation procedure described above. Preliminary determination of partial 16S rRNA gene sequences revealed that one strain out of the six strains was assigned to the genus *Natronorubrum*, three to the genus *Natrialba*, and the remaining two strains to a hitherto unknown *Halobacteriaceae* genus (data not shown). Accordingly, the two strains, designated as IHC-005^T and IHC-010, were further characterized in this study. On the other hand, a strain labeled as N-1311^T (=CGMCC 1.2124^T), which had been isolated from another soda lake in Inner Mongolia and maintained at the laboratory, was also studied. These three strains were routinely cultivated in JCM medium No. 167 composed of (per liter): 1.0 g KH_2PO_4, 1.0 g KCl, 1.0 g NH_4Cl, 0.24 g $MgSO_4 \cdot 7H_2O$, 0.17 g $CaSO_4 \cdot 2H_2O$, 200.0 g NaCl, 0.1 mg $ZnSO_4 \cdot 7H_2O$, 0.03 mg $MnCl_2 \cdot 4H_2O$, 0.3 mg H_3BO_3, 0.2 mg $CoCl_2 \cdot 6H_2O$, 0.01 mg $CuCl_2$-$2H_2O$, 0.02 mg $NiCl_2 \cdot 6H_2O$, 0.03 mg $Na_2MoO_4 \cdot 2H_2O$, 1.0 g sodium glutamate, 5.0 g yeast extract (Difco), 5.0 g casamino acids (Difco), and 8.0 g Na_2CO_3, pH 9.5, at 37°C.

Characterization of strains

Phenotypic characterization was carried out in accordance with the recommended minimal standards for the family *Halobacteriaceae* (Oren et al. 1997). Cell morphologies were observed in liquid medium, using a phase contrast microscope, and colony morphology on agar plates examined with a stereomicroscope. Growth temperature and pH, utilization of various substances as carbon and energy sources, lipid analyses, DNA G+C content, 16S rRNA gene sequences, and DNA–DNA relatedness were determined as described by Xin et al. (2001).

Results and discussion

Morphology

Cells of strains IHC-005^T/IHC-010 in liquid cultures were mostly rods (ca. 0.5×3–5 μm), while those of strain N-1311^T were pleomorphic, including boards (ca. 0.6×3–6 μm), disks, and cocci (0.5–1.5 μm). The former two strains were motile, whereas the latter was non-motile. Strains IHC-005^T and N-1311^T lysed in 0.5 M or lower NaCl concentration. The three strains had no gas vacuoles in the cells. They stained as Gram-negative according to the method of Dussault (1955). Colonies on agar plates were bright red, translucent, circular with entire edge, raised or convex, and 0.3–0.5 mm in diameter for strains IHC-005^T/ IHC-010, 0.6–0.7 mm in diameter for strain N-1311^T.

Physiological and biochemical properties

As shown in Table 1, strains IHC-005^T and N-1311^T were mesophilic, alkaliphilic, and extremely halophilic. At optimal growth conditions (37°C, pH 9.0, 20% NaCl for IHC-005^T, and 45°C, pH 8.9, 20% NaCl for N-1311^T), the two strains proliferated with the doubling time of 3.8 and 3.3 h, respectively. Certain biochemical and physiological properties, including differential ones between strains IHC-005^T/IHC-010 and strain N-1311^T, are given in Table 1. As common features of the three strains, they were strictly aerobic and never showed growth in an anaerobic medium containing nitrate, arginine, DMSO, or trimethylamine N-oxide; they were oxidase- and catalase-positive and produced indole. They were sensitive to novobiocin, bacitracin, anisomycin, aphidicolin, erthromycin, and rifampicin, but not penicillin, ampicillin, chloramphenicol, and neomycin (50 μg/ml each).

Table 1 Certain physiological and biochemical properties of strains IHC-005^T/IHC-010 and strain N-1311^T

Characteristic	IHC005^T/IHC-010	N1311^T
Growth temperature (°C)		
Range	30–46[a]	19–54
Optimum	37[a]	38–43
Growth pH		
Range	7–10[a]	7.5–10
Optimum	9[a]	9.5
NaCl requirement (%)		
Minimum	15[a]	10
Optimum	20–25[a]	15–20
Reduction of nitrate	–	+[b]
H_2S formation from:		
Sulfur	–	+
Thiosulfate	+	–
Hydrolysis of:[c]		
Tween 80	+	–
Gelatin	–	+
Utilization of carbon sources:[d]		
Fructose	+	–
Lactose	+	–
Maltose	+	–
Mannose	+	–
Rhamnose	+	–
Sorbitol	–	+
Sucrose	d	–
D-xylose	+	–
Citric acid	–	+
L-lactic acid	w	+
L-malic acid	w	+
Propionic acid	–	+
Succinic acid	d	–
L-ornitine	–	w

[a]Data for strain IHC-005^T
[b]No nitrogen gas was formed
[c]No strains hydrolyzed starch or casein
[d]All strains utilized arabinose, galactose, glucose, glycerol, rhaffinose, acetic acid, fumaric acid, and pyruvinic acid, but not D-ribose, starch, mannitol, glycine, L-alanine, L-arginine, L-asparaginic acid, L-glutamic acid, or L-lysine

Polar lipid analysis

Two-dimensional TLC of the polar lipid fraction revealed that the three strains had diphytanyl (C20:C20) and phytanyl-sesterterpanyl (C20:C25) moieties of phosphatidylglycerol, and phosphatidylglycerophosphate methyl ester as major polar lipid components. In addition, lipid fractions of strains IHC-005^T/IHC-010 contained small amounts of an unidentified phospholipid different from PL1, PL2, PL3, or PL4 (Morth and Tindall, 1985). No glycolipids were detected from the three strains.

Phylogenetic analysis

Almost entire 16S rRNA gene sequences were determined for the three strains in this study (1,435 bases for IHC-005^T and IHC-010, and 1,437 bases for N-1311^T). No ambiguous base were found in the 16S rRNA gene sequences of strains IHC-005^T and IHC 010. As for strain N-1311^T, only four ambiguous bases were found in its sequence. The 16S rRNA gene sequences were compared with those of the Natro groups and *Halobacterium salinarum* as root organisms to construct a phylogenetic tree. Eliminating gaps, uncertain bases, and unalignable regions (positions 999–1007, 1032–1042, and 1439–1452), 1,335 positions of the sequences were compared to calculate the evolutionary distances and construct a phylogenetic tree. In the phylogenetic tree as shown in Fig. 1, strains IHC-005^T, IHC-010, and N1311^T were clustered with an additional strain, 82M4, from the Lake Magadi (Duckworth et al. 1996). This cluster was supported by a relatively high bootstrap value (92%) and a maximum-likelihood analysis, using the fastDNAmal program (Olsen et al. 1994). The

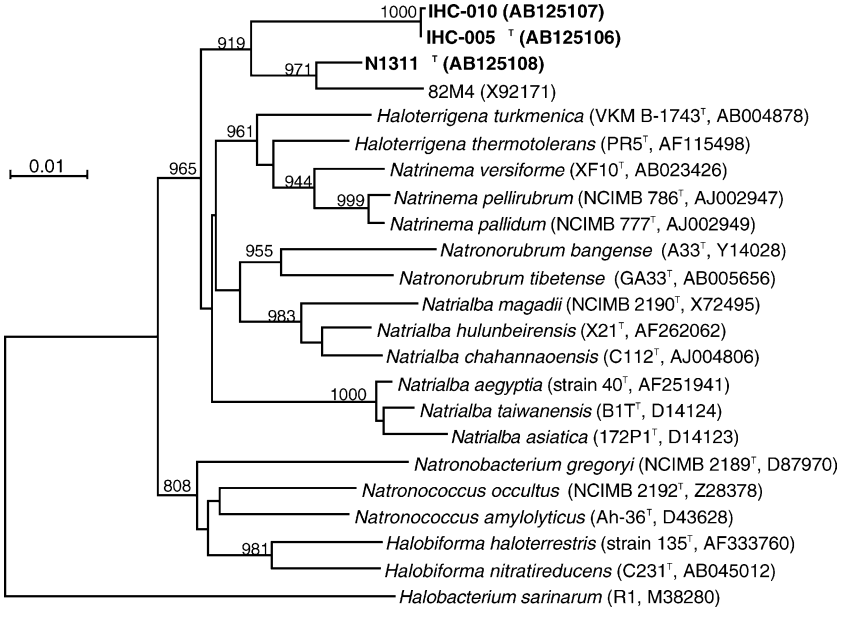

Fig. 1 16S rRNA gene sequence-based phylogenetic tree showing the position of strains IHC-005^T, IHC-010, and N-1311^T in the Natro group of the family *Halobacteriaceae*. The tree was constructed by the neighbor-joining method. *Numbers* indicate the bootstrap scores of 1,000 trials; values greater than 80% are shown. *Bar* denotes evolutionary distance

cluster was distantly placed from members of the other genera/clusters of the Natro group with 93.3–95.8% sequence similarities. The cluster can be subdivided into two subclusters, IHC-005^T/IHC-010 and N1311^T/82M4, with 94.9–96.9% sequence similarities.

DNA G + C content and DNA–DNA relatedness

The G + C content of total DNA of strains IHC-005^T, IHC-010, and N1311^T were 59.2, 59.6, and 63.1 mol%, respectively. The DNA–DNA hybridization values between strains IHC-005^T and IHC-010 were very high (96–99%), while the two strains showed low DNA–DNA hybridization values (18–21%) to strain N-1311^T.

Identity of the genus

The three strains IHC-005^T, IHC-010, and N-1311^T were aerobic, extremely halophilic (requiring at least 1.5 M NaCl), red-colored, having diether core lipids, and high G + C content of DNA. These features illustrate that they are members of the family *Halobacteriaceae*. Furthermore, the 16S rDNA sequences comparison revealed that they belong to the Natro group (McGenity et al. 1998), where most of haloalkaliphilic members exist. In accordance with the known haloalkaliphilic members of this family, the three strains grow at high pH and low Mg^{2+} content and contain almost equal amounts of C20:C20 and C20:C25 diether lipids but not glycolipids. On the 16S rRNA gene sequence-based phylogenetic analysis, the three strains as well as an uncharacterized strain 82M4 (Duckworth et al. 1996) formed an independent cluster. The cluster seemed to be stable and was significantly distant from other clusters (genera), indicating that the three strains should represent a new genus. They can be divided into two subclusters (strains IHC-005^T/IHC-010 and strains N-1311^T/82M4), and the difference between the two subclusters (94.9–96.9% similarity) is comparable to that of certain different genera, e.g., *Natrinema* and *Haloterrigena* (96.7–98.1%) and *Natronorubrum* and *Nab. magadii* group (94.3–96.8%). However, we do not intend to separate them into two genera at the moment. Their taxonomic status should be argued when more related strains are isolated and studied and/or any taxonomic criteria other than the 16S rRNA gene sequence analysis to distinguish the *Halobacteriaceae* genera are established. Thus, we now concluded that the three strains represent a new genus in the family *Halobacteriaceae*.

Identity of the species

Among the three strains studied, strain IHC-005^T and IHC-010 share almost identical taxonomic properties. Slight difference was observed in utilization of L-lactic acid and 16S rRNA gene sequences (0.07% difference). The DNA–DNA hybridization values between the two strains were nearly 100%, supporting that both strains belong to a single species. Strain N-1311^T is easily discerned from the two strains, IHC-005^T/IHC-010, e.g., cell morphology (pleomorphic shapes vs rod shapes), physiological and biochemical properties as shown in Table 1, lipid composition (i.e., presence or absence of an unidentified phospholipid), the DNA G + C content values (3.5–3.9% difference), 16S rRNA gene sequences (96.9% similarity), and the DNA–DNA relatedness (ca. 20%). Therefore, strain N-1311^T should represent a separate species from strains IHC-005^T/IHC-010. Phylogenetically, strain N-1311^T may be closer to another haloalkaliphilic archaeon 82M4 (98.1% 16S rRNA gene sequence similarity), and there may be a possibility that both strains should be included in a same species. Lacking any other taxonomic information on 82M4, however, we cannot draw a conclusion concerning the relationship between the two strains at present.

On the basis of the phenotypic and phylogenetic properties described above, we propose a new genus, *Natronolimnobius* gen. nov., to include the three strains. *Natronolimnobius baerhuensis* sp. nov., as the type species, is proposed for strains IHC-005^T/IHC-010 (type strain, IHC-005^T) and *Natronolimnobius innermongolicus* for N-1311^T . The three strains are deposited in the China General Microbiological Culture Collection Center, the Academy of Sciences, China and the Japan Collection of Microorganisms, RIKEN BioResource Center, Japan as follows: *Natronolimnobius baerhuensis* IHC-005^T (= CGMCC 1.3597^T = JCM 12253^T), IHC-010 (= CGMCC 1.3598 = JCM 12254) and *Natronolimnobius innermongolicus* N-1311^T (= CGMCC 1.2124^T = JCM 12255^T).

Description of *Natronolimnobius* gen. nov.

- *Natronolimnobius* (Na.tro.no.lim.no'bi.os. N. Gr. n. *natron*, arbitrarily derived from the Arabic n. *natrum*, soda; Gr. n. *limnos*, lake; Gr. masc. n. *bios*, life; N.L. masc. n. *Natronolimnobius*, organism living in a soda lake).
- Cells are rod-shaped or pleomorphic flat-shaped, Gram-negative, red-pigmented, and strictly aerobic and oxidase- and catalase-positive.
- Requires at least 1.5 M NaCl for growth.
- Cells lyse in less than 0.5 M NaCl.
- Grows at neutral to alkaline pH range (pH 7.5–10.0).
- Mesophilic or thermotolerant (up to 54°C), chemoorganotrophic.
- Sensitive to novobiocin, bacitracin, anisomycin, aphidicoline, erythromycin, and rifampin (50-μg/ml).
- Possess C20:C20 and C20:C25 diethers.
- No glycolipids are detected.
- The DNA G + C content ranges between 59 and 63 mol%.

– On the basis of 16S rRNA gene sequence phylogenetic analysis, it forms an independent cluster, with more than 96.9% sequence similarities, in the Natro group.
– The type species is *Natronolimnobius baerhuensis*.

Description of *Natronolimnobius baerhuensis* sp. nov.

– *Natronolimnobius baerhuensis* (bae.rhu.en'sis. N.L. masc. adj. *baerhuensis*, of Baerhu, pertaining to a soda lake where the type strain was isolated).
– Cells are Gram-negative, rod-shaped, and motile.
– Cells lyse in distilled water or in dilute medium containing less than 0.5 M NaCl.
– Colonies are bright red, translucent, circular, 0.3–0.5 mm in diameter, raised or convex.
– Grows between pH 7.0 and 10.0, with an optimum at pH 9, and between 30 and 46°C, with an optimum at 37°C.
– Requires at least 15% NaCl for growth with an optimum 20%, is chemoorganotrophic and aerobic.
– Forms sulfides from thiosulfate, but not sulfur.
– Produces indole.
– Hydrolyses Tween 80 but not starch, casein, or gelatin.
– Utilizes arabinose, fructose, galactose, glucose, glycerol, lactose, maltose, mannose, rhamnose, raffinose, xylose, acetate, fumarate, and pyruvate.
– Unknown phospholipid may be present.
– Sensitive to anisomycin, aphidicolin, bacitracin, erthromycin, novobiocin, rifampicin, but not ampicillin, chloramphenicol, neomycin, or penicillin.
– The DNA G+C content is 59–60%.
– The type strain is IHC-005^T (CGMCC 1.3597^T =JCM 12253^T).

Description of *Natronolimnobius innermongolicus* sp. nov.

– *Natronolimnobius innermongolicus* (in.ner.mon.go'li.cus. N.L. masc. adj. *innermongolicus*, of Inner Mongolia, China, pertaining to an autonomous region of China, where the type strain was isolated).
– Cells are Gram-negative, pleomorphic flat-shaped, and non-motile.
– Colonies are light red, translucent, circular, 0.6–0.7 mm in diameter, raised or convex.
– Grows between pH 7.5 and 10.0, with an optimum at pH 9.5, and between 19 and 54°C, with an optimum at 45°C.
– Requires at least 10% NaCl for growth with an optimum 15–20%.
– Is chemoorganotrophic and aerobic.
– Forms sulfides from sulfur, but not thiosulfate.
– Reduces nitrate to nitrite.
– Produces indole.
– Hydrolyzes gelatin but not starch, casein or Tween 80. Utilizes arabinose, galactose, glucose, glycerol, raffinose, sorbitol, acetate, citrate, fumarate, lactate, malate, propionate, and pyruvate.
– Sensitive to anisomycin, aphidicolin, bacitracin, erthromycin, novobiocin, rifampicin, but not ampicillin, chloramphenicol, neomycin, or penicillin.
– The DNA G+C content is 63%.
– The type strain is N-1311^T (=CGMCC 1.2124^T =JCM 12255^T).

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