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Characterization of two novel haloalkaliphilic archaea *Natronorubrum bangense* gen. nov., sp. nov. and *Natronorubrum tibetense* gen. nov., sp. nov.

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Two haloalkaliphilic archaea were isolated from a soda lake in Tibet. The two strains, designated A33^T and GA33^T, were Gram-negative, pleomorphic, flat, non-motile and strictly aerobic. Growth required at least 12% NaCl. Growth was between pH 8·0 and pH 11 with an optimum at pH 9·0–9·5. Cells were chemo-organotrophic. Polar lipids were C_{20}–C_{25} derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate. The nucleotide sequences of the 16S rRNA genes from the two strains were obtained by the analysis of the cloned rDNAs. On 16S rRNA phylogenetic trees, the two strains formed a monophyletic cluster. They differed from their closest neighbours, *Halobacterium trapanicum* and *Natrialba asiatica*, in polar lipid composition, as well as physiological and phenotypic characteristics. DNA–DNA hybridization indicated that the two strains belonged to different species of the same genus. The results indicated that the strains A33^T and GA33^T should be classified in a new genus *Natronorubrum* gen. nov. as *Natronorubrum bangense* sp. nov. (strain A33^T) and *Natronorubrum tibetense* sp. nov. (strain GA33^T).

Keywords: *Natronorubrum bangense* gen. nov., sp. nov., *Natronorubrum tibetense* gen. nov., sp. nov., haloalkaliphilic archaea

INTRODUCTION

Halobacteria are among the most halophilic organisms known and require at least 1·5 M NaCl for their growth. They contain one order, *Halobacteriales* and one family, *Halobacteriaceae* (Grant & Larsen, 1989). Before the 1970s, halobacterial taxonomy was mainly based on standard biochemical tests and morphology (Gibbons, 1974). At the end of the 1970s, however, the situation changed dramatically. 16S rRNA–DNA hybridization studies demonstrated that the halobacteria should be classified into nine clades of two groups (Ross & Grant, 1985). The polar lipid compositions of these isolates had proven particularly useful in the classification of halobacteria (Ross & Grant, 1985; Torreblanca *et al.*, 1986). Polar lipid analysis and 16S rRNA–DNA hybridization studies not only produced groupings of six genera of halobacteria (*Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Natronobacterium* and *Natronococcus*) but also induced a further reclassification of some uncertain halobacterial species (Grant & Larsen, 1989; Ross & Grant, 1985). With the recent availability of more complete 16S rRNA sequences for many halobacteria, it became clear that there was even more diversity at the genus level within the family *Halobacteriaceae* (Kamekura & Dyall-Smith, 1995; McGenity & Grant, 1995). However, the use of phylogenetic parameters alone to describe taxa was considered not very accurate, and the combination of phylogenetic and phenotypic features (polyphasic taxanomy) is now necessary for the delineation of taxa (Oren *et al.*, 1997).

In this paper, the complete 16S rDNA sequences from two haloalkaliphilic archaea, strains A33^T and GA33^T, are reported. The polar lipids of these isolates were also analysed, and the results of these biochemical tests were compared with those of other members of

Abbreviations: PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are listed in the Methods.

haloalkaliphilic archaea to determine the taxonomic positions for the strains A33^T and GA33^T.

METHODS

Strains and culture conditions. Strains A33^T and GA33^T were isolated from sediment (pH 10, 18 °C) of the Bange salt-alkaline lake in Tibet. The isolation medium and the method for isolation was used as described previously (Tindall *et al.*, 1980). These strains were grown aerobically at 37 °C in a complex medium of the following composition (g l^{-1}) casamino acids (Difco), 7·5; yeast extract (Difco), 10; trisodium citrate, 3·0; $MgSO_4 . 7H_2O$, 0·3; KCl, 2·0; Fe^{2+} and Mn^{2+}, trace; NaCl, 200; Na_2CO_3, 8·0.

The growth was monitored by OD_{600}. Cell mobility and shape were examined by microscopy either without fixation under phase-contrast or with acetic acid fixation.

Biochemical characterization. The biochemical tests on strains A33^T and GA33^T were carried out in accordance with standard methods (Colwell *et al.*, 1979; Holding & Collee, 1971; Vreeland, 1993).

Lipid composition. The cellular lipids were extracted and analysed on silica gel plates (Kieselgel 60 F_{254}; 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. DNA was isolated and purified as described previously (Zhou *et al.*, 1994). The G+C content was determined by thermal denaturation. DNA–DNA hybridization was carried out as described by Tindall *et al.* (1984) with a minor modification: DNA fragments were labelled with [α-^{32}P]dCTP using the Boehringer Mannheim nick translation kit.

16S rRNA gene sequence. The methods used for DNA preparation, PCR amplification of the 16S rRNA gene and gene sequencing were described previously (Xu *et al.*, 1995; Zhou *et al.*, 1994).

Phylogenetic analysis of 16S rRNA sequences. The raw sequence dataset included nearly complete sequences for 40 halobacteria. The multiple sequence alignments were performed using CLUSTAL W version 1.7 (Thompson *et al.*, 1994). The resulting aligned dataset contained information on 1451 nucleotide positions for 40 halobacteria. The phylogenetic analysis for this multiple sequence alignment was done with the TREECON for Windows version 1.2 (Van de Peer & De Wachter, 1994) and the program package PHYLIP version 3.572 (Felsenstein, 1993). The phylogenetic tree was mainly constructed by the neighbour-joining method with the Kimura two-parameter calculation model in TREECON for Windows version 1.2. As references, other phylogenetic trees were also constructed with other methods in PHYLIP version 3.5 such as the parsimony and maximum-likelihood methods.

Nucleotide sequence accession numbers. The accession numbers for the 16S rRNA sequence for strains A33^T and GA33^T are Y14028 and AB005656, respectively. The 16S rRNA gene sequences of the test strains have the following GenBank/EMBL/DDBJ accession numbers (in parentheses): *Halobacterium cutirubrum* CCM 2088 (K02971); *Halobacterium halobium* ATCC 29341 (M11583); *Halobacterium salinarum* DSM 671 (M38280); *Halobacterium* sp.Y12 F.R.Valera (D14127); *Haloarcula marismortui* 1 ATCC 43049^T (X61688); *Haloarcula marismortui* 2 ATCC 43049^T (X61689); *Haloarcula sinaiiensis* 1 ATCC 33800 (D14129); *Haloarcula sinaiiensis* 2 ATCC 33800 (D14130); '*Haloarcula aidinensis*' AS 1.2042 (AB000563); *Haloarcula vallismortis* 1 ATCC 29715^T (U17593); *Haloarcula vallismortis* 2 ATCC 29715^T (D50851); *Haloarcula mukohataei* JCM 9738^T (D50850); *Haloarcula argentinensis* JCM 9737^T (D50849); *Haloferax volcanii* ATCC 29605 (K00421); *Haloferax mediterranei* ATCC 33500^T (D11107); *Haloferax gibbonsii* ATCC 33959^T (D13378); *Haloferax denitrificans* ATCC 35960^T (D14128); *Halorubrum saccharovorum* NCIMB 2081^T (U17364); *Halorubrum lacusprofundi* ACAM 34^T (U17365); *Halorubrum sodomense* ATCC 33755^T (D13379); *Halorubrum trapanicum* NRC 34021 (X82168); *Halorubrum* sp. Ch2 ACM 3911 (L00922) ; *Halorubrum vacuolatum* JCM 9060 (D87972); *Halobaculum gomorrense* DSM 9297^T (L37444); *Halococcus morrhuae* 1 ATCC 17082 (X00662); *Halococcus morrhuae* 2 NRC 16008 (D11106); *Halobacterium trapanicum* JCM 8979 (D63786); *Natrialba asiatica* JCM 9576^T (D14123); *Natrialba* B1T JCM 9577 (D14124); *Halobacterium trapanicum* NCIMB 767 (D14125); L-11 F.J.Post (D14126); *Natronobacterium* SSL1 ATCC 43988 (D88256); *Natronobacterium magadii* NCIMB 2190^T (X72495); *Natronobacterium gregoryi* NCIMB 2189^T (D87970); *Natronomonas pharaonis* JCM 8858 (D87971); '*Natronobacterium innermongoliae*' AS 1.1985 (AF009601) (Tian *et al.*, 1997); *Natronococcus occultus* NCIMB 2192^T (Z28378); *Natronococcus amylolyticus* Ah-36^T (D43628). The tree was rooted by using the 16S rDNA sequence of *Methanospirillum hungatei* DSM 864 (M60880) as the outgroup.

RESULTS AND DISCUSSION

The two isolates stained Gram-negative, and were strictly aerobic, non-motile, flat and pleomorphic (triangular, square, disk-shaped and other polygonal shapes), as determined by phase-contrast microscopy. Cells lysed in distilled water. Both strains were catalase- and oxidase-positive and were able to use various kinds of sugars (glucose, sucrose, fructose, maltose and lactose). On agar plates, both strains formed red-coloured colonies about 2 mm in diameter after one week of culture. At 45 °C, strain A33^T grew optimally in the presence of 22·5% NaCl (w/v) at pH 9·5, whereas strain GA33^T grew optimally in the presence of 20% NaCl at pH 9·0.

Alkaliphily, G+C content and archaeal core lipid composition indicated that both strains were members of the haloalkaliphilic archaea, but biochemical tests together with morphological details distinguished the two strains from the other species of haloalkaliphilic archaea (Table 1).

The polar lipid composition showed that the two strains had, in common with other haloalkaliphilic archaea, a characteristic simple pattern. All haloalkaliphilic archaea characterized to date have C_{20}–C_{25} diether core lipids of phosphatidylglycerol (PG) and phosphatidylglycerol phosphate (PGP) together with minor amounts of unidentified phospholipids. Therefore, the members of the haloalkaliphilic archaea could be distinguished from non-haloalkaliphilic archaea

Table 1. Comparisons of phenotypic features of some members of haloalkaliphilic archaea

ND, Not determined.

Characteristic	*Natronorubrum bangense* A33^T	*Natronorubrum tibetense* GA33^T	*Natronobacterium gregoryi* NCMB 2198^T	*Natronomonas pharaonis* DSM 2160	*Natronobacterium magadii* NCMB 2190^T
Cell shape	Pleomorphic	Pleomorphic	Long rod	Rod	Rod
Gram stain	–	–	–	–	–
Motility	–	–	–	+	+
Colour	Red	Red	Red	Red	Orange-red
Optimum NaCl (%)	22·5	20	17·5	20	20
Salt range (%)	12–25	12–30	12–30	12–30	12–30
Temperature (°C)	45	45	37–40	45	37–40
Optimum pH	9·5	9·0	9·5	8·5–9·0	9·5
pH range	8·0–11·0	8·5–11·0	8·5–11·0	8·0–11·0	8·0–11·5
H_2S from $Na_2S_2O_3$	–	–	+	+	+
Nitrate reduction	–	–	–	+	–
Catalase & oxidase	+	+	+	+	+
Indole formation	+	+	ND	+	ND
Starch hydrolysis	–	–	–	–	–
Casein hydrolysis	–	–	–	–	+
Tween hydrolysis:					
Tween 40	–	+	ND	ND	ND
Tween 60	–	+	ND	ND	ND
Tween 80	–	–	ND	ND	ND
Gelatin liquefaction	–	+	–	+	–
Sensitivity to erythromycin	+	+	+	–	+
Growth stimulated by:					
Acetate	+	+	+	–	+
Glucose	+	+	+	–	–
Fructose	+	+	+	–	–
Sucrose	+	+	+	–	–
Mannitol	–	–	+	–	–
G+C content (mol%)	59·9	60·1	65·9	64·3	63·0
Polar lipids:					
PG and PGP	+	+	+	+	+
Phospholipid1	+	+	+	+	–
Phospholipid 2	–	–	–	–	–
Phospholipid 3	+	+	+	–	+

according to the major polar lipid composition (except *Halorubrum vacuolatum*). Different groups of halobacteria contain their own special polar lipid compositions. For example, besides the PG and PGP found in all halobacteria, TGD-1(tri-glycosyl diether), S-TGD-1(sulfated triglycosyl diether) and S-TeGD (sulfated tetraglycosyl diether) exist in *Halobacterium*; TGD-2 in *Haloarcula*; DGD-1(diglycosyl diether) and S-DGD-1 but no phosphatidylglycerosulphate (PGS) in *Haloferax*; S-DGD-3 and PGS but no DGD-1 in *Halorubrum*; S-DGD-1 but no PGS in *Halobaculum*; TGD-2 and S-DGD-1 but no PGS in *Halococcus*; S_2-DGD-1 in *Natrialba*; a specific uncharacterized glycolipid in the unnamed taxa, *Halobacterium trapanicum* (NCIMB 767); but in main polar lipid compositions, only PG and PGP are found in *Natronobacterium* and *Natronococcus*, so polar lipid compositions could be used as a taxonomic indicator to some extent in halobacterial archaea.

We now report the sequences of 16S rRNAs from two haloalkaliphilic archaea, strains A33^T and GA33^T, which contain 1475 and 1474 residues respectively. When compared with those of other halobacteria, the two strains showed reasonably high levels of similarity to their closest counterpart, *Halobacterium trapanicum* NCIMB 767 (93·3 and 92·7%, respectively), and also to *Natrialba asiatica* (91·1 and 92·1%, respectively), *Natronococcus amylolyticus* (90·4 and 91·7%, respectively), *Natronobacterium gregoryi* (89·8 and 90·6%, respectively), *Natronobacterium magadii* (88·7 and 90·2%, respectively) and to *Natronomonas pharaonis* (84·2 and 82·8%, respectively), in contrast with the 95·2% similarity between strains A33^T and GA33^T.

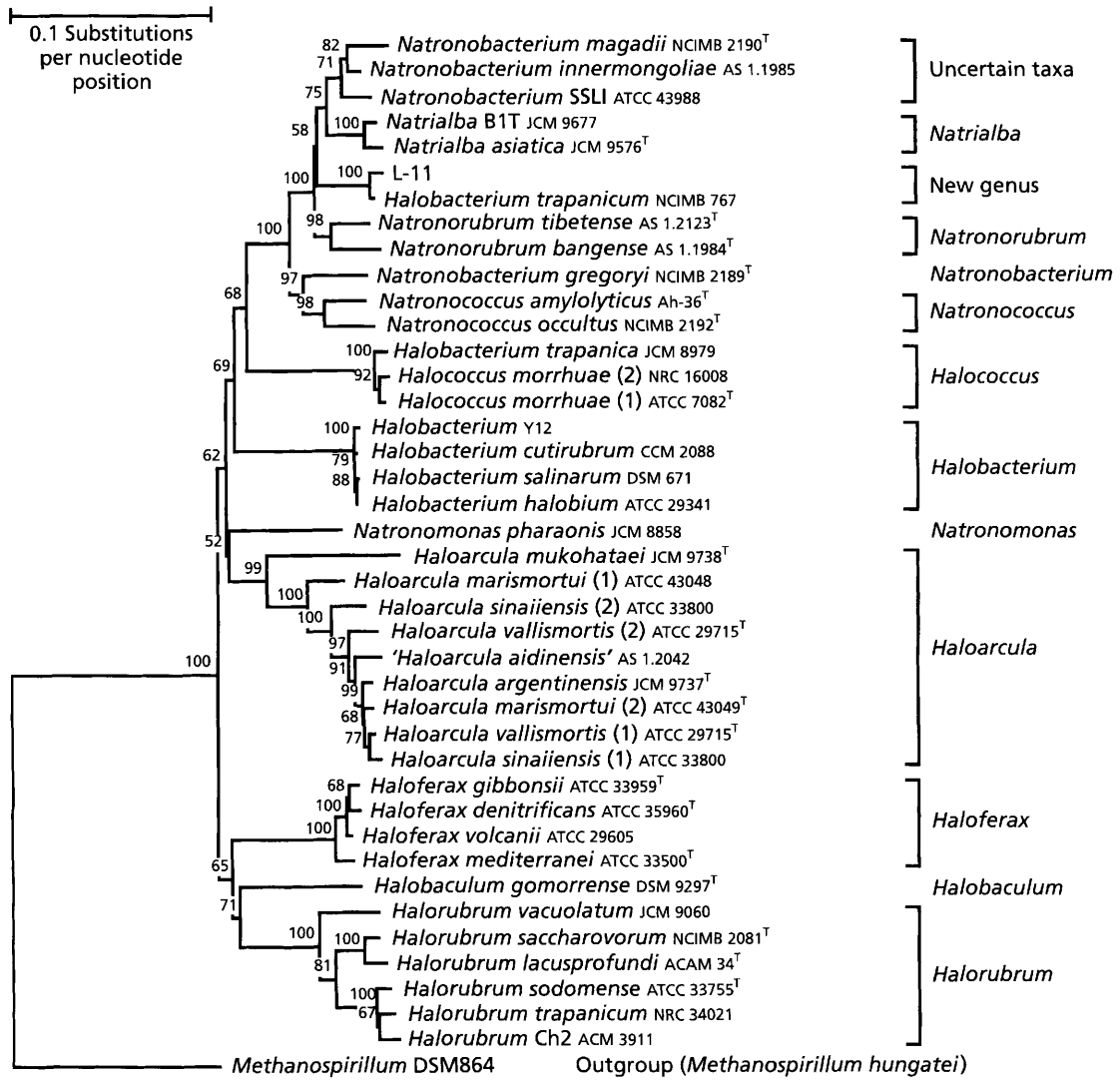

Fig. 1. The phylogenetic tree of halobacteria from their 16S rRNA sequences. The tree was constructed by the neighbour-joining method and Kimura-two parameter calculation model. The numerals represent the confidence level from 100 replicate bootstrap sampling.

Phylogenetic analysis also showed the monophyletic position of the two strains in halobacterial archaea (Fig. 1). Among halobacterial archaea, the classification of the non-alkaliphilic halobacteria (the clade with core diether lipids of C_{20}–C_{20}) is comparatively straightforward (except for *Halorubrum vacuolatum*), and groups delineated by their polar lipid contents are consistent with the rRNA groups. However, the situation for the clade with core diether lipids of C_{20}–C_{25} is unclear. This clade contains the non-alkaliphilic and alkaliphilic halobacteria, and was divided into five groups or genera by Kamekura *et al.* (1997) on the basis of 16S rRNA sequences: *Natronomonas*, *Natronobacterium*, *Natrialba*, *Halococcus* and an unnamed new genus. The members in some of these groups contain both the non-alkaliphilic and alkaliphilic halobacteria as well as different polar lipid compositions, which apparently does not agree with the principle of polyphasic taxonomy (Grant & Larsen, 1989; Oren *et al.*, 1997), the polar lipid composition being one of the important indicators in halobacterial taxonomy (Grant & Larsen, 1989).

In our analysis, the two strains show the closest relationship with *Halobacterium trapanicum* NCIMB 767 in the phylogenetic tree (Fig. 1), but they can not be classified in the same genus due to the different polar lipid compositions, different morphology and optimum salinity for growth (Ihara *et al.*, 1997). The results of the phylogenetic trees constructed by the parsimony and maximum-likelihood methods also supported this conclusion. That is, the two strains should not be classified with either *Halobacterium trapanicum* NCIMB 767 or *Natrialba* spp.; they ought to be placed in a new group. The DNA–DNA hybridization value (35%), the low level of sequence

similarity of 16S rDNAs (95·2%) and the phenotypic differences (Table 1) between strains A33^T and GA33^T indicate that the two strains should belong to different species of the same genus. Thus, the two haloalkaliphilic archaea are classified into a new genus, *Natronorubrum*, and two novel species, *Natronorubrum tibetense* and *Natronorubrum bangense*, respectively.

Description of *Natronorubrum* gen. nov.

Natronorubrum [Na.tro.no.ru'brum. Gr. n. *natron*, derived from arabic *natrun*, soda (sodium carbonate); L. neut.adj. *rubrum* red; M.L. neut. n. *Natronorubrum*, the red of soda].

Gram-negative, pleomorphic flat-shaped (triangular, square, disk and other polygonal shapes) in favourable conditions. Colonies are pigmented red. Cells are non-motile, strictly aerobic, oxidase- and catalase-positive. Growth requires at least 12% NaCl; cells are lysed in distilled water. Growth is between pH 8·0 and pH 11·0, with an optimum at pH 9·0–9·5. Growth occurs in the temperature range 25–55 °C with an optimal temperature of 45 °C. Chemo-organotrophic, many sugars (glucose, fructose, sucrose, maltose and lactose) are utilized, sometimes with acid production. Polar lipids are C_{20}–C_{25} derivatives of PG and PGP. Type species is *Natronorubrum bangense*.

Description of *Natronorubrum bangense* sp. nov.

Natronorubrum bangense (ban.gen'se. M.L. neut. adj. *bangense*, from Bange, China).

Cells are pleomorphic, flat-shaped and uniformly stain Gram-negative. Growth occurs in media containing 12–25% NaCl, with an optimum at 22·5% NaCl. Growth is between pH 8·0 and pH 11·0, with an optimum at pH 9·5. Amino acids are utilized. Growth is stimulated by many sugars (glucose, fructose, maltose, sucrose and lactose). Indole is formed from tryptophan. Starch and casein are not hydrolysed. H_2S is not produced from thiosulfate. Gelatin is not liquefied. Tween 40, 60 and 80 are not hydrolysed. The polar lipids are C_{20}–C_{25} derivatives of PG, PGP and two uncharacterized minor phospholipids. The G+C content of the DNA is 59·9 mol%. Type strain is A33^T, deposited as AS 1.1984. (AS; Academia Sinica, China General Microbiological Culture Collection Centre, Zhong-guancun, Beijing 100080, China).

Description of *Natronorubrum tibetense* sp.nov.

Natronorubrum tibetense (ti.bet.en'se. M.L. neut. adj. *tibetense* from/of Tibet).

Cells are pleomorphic, flat-shaped and uniformly stain Gram-negative. Growth occurs in media containing 12–30% NaCl with an optimum at 20% NaCl. Growth is between pH 8·5 and pH 11·0, with an optimum at pH 9·0. Amino acids are utilized. The growth is stimulated by many sugars (glucose, fructose, maltose, sucrose and lactose). Indole is formed from tryptophan. Starch and casein are not hydrolysed. H_2S is not produced from thiosulfate. Gelatin is liquefied. Tween 40 and 60 are hydrolysed but Tween 80 is not. The polar lipids are C_{20}–C_{25} derivatives of PG, PGP and two uncharacterized minor phospholipids. The G+C content of the DNA is 60·1 mol%. Type strain is GA33^T, deposited as AS 1.2123

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