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Characterization of a novel halophilic archaeon, *Halobiforma haloterrestris* gen. nov., sp. nov., and transfer of *Natronobacterium nitratireducens* to *Halobiforma nitratireducens* comb. nov.

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Strain 135^T, a novel red-pigmented, aerobic, extremely halophilic member of the *Archaea* showing rod, coccus and slightly pleomorphic morphology, was isolated from hypersaline soil close to Aswan (Egypt). This organism is neutrophilic, motile and requires at least 2·2 M NaCl, but no $MgCl_2$, for growth and exhibits optimal growth at 42 °C. Polar lipid analysis revealed the presence of sulfated triglycosyl diether and triglycosyl diether as the sole glycolipids as well as the absence of the glycerol diether analogue of phosphatidyl glycerosulfate. C20:C20 and C20:C25 core lipids are present in almost equal proportions. The G+C content of the DNA is 66·9 mol%. 16S rDNA analysis revealed that strain 135^T was a member of the phyletic group defined by the family *Halobacteriaceae*, but there was a low degree of similarity to other members of this family. Highest similarity values of 96·4 and 93·8–94·3% were obtained to the 16S rDNA of *Natronobacterium nitratireducens* and *Natronobacterium gregoryi*, *Natronococcus occultus* and *Natronococcus amylolyticus*. Strain 135^T is able to accumulate polyhydroxybutyrate as intracellular reserve material. On the basis of the data presented, strain 135^T should be placed in a new genus, *Halobiforma* gen. nov. as *Halobiforma haloterrestris* sp. nov. The type strain is strain 135^T (= DSM 13078^T = JCM 11627^T). Moreover, the transfer of *Natronobacterium nitratireducens* to *Halobiforma nitratireducens* comb. nov. is proposed.

Keywords: *Halobiforma haloterrestris*, *nitratireducens*, halophilic, archaea, polyhydroxybutyrate

INTRODUCTION

The aerobic, extremely halophilic archaea that belong to the family *Halobacteriaceae*, order *Halobacteriales*, class *Haloarchaea*, are chemo-organotrophic organisms that use amino acids or carbohydrates to grow and need at least 1·5 M NaCl for growth; most exhibit optimal growth at 3·5–4·5 M NaCl (Grant *et al*., 2001). Except for some members of the genus *Natrialba*, which are non-pigmented, all members of the family *Halobacteriaceae* are red-pigmented due to the presence of carotenoids (Kamekura & Dyall-Smith, 1995; Hezayen *et al*., 2001; Grant *et al*., 2001). Before the 1970s, halobacterial taxonomy was based mainly on standard biochemical tests and morphology (Gibbons, 1974). The current taxonomy is based largely on 16S rDNA sequences and chemotaxonomic criteria, particularly polar lipid composition, which has induced a further reclassification of some uncertain halophilic species and the creation of new genera (Grant & Larsen, 1989; Ross & Grant, 1985; Kamekura & Dyall-Smith, 1995; Kamekura *et al*., 1997). The availability of complete 16S rDNA sequences of many haloarchaea has resulted in the recognition of even more diversity at the genus level within the family

Abbreviations: PHB, poly-β-hydroxybutyrate; VPMR, Voges–Proskauer methyl red.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain 135^T is AF333760.

Halobacteriaceae (Kamekura & Dyall-Smith, 1995; McGenity & Grant, 1995). This has resulted in a dramatic increase in the number of these halophilic taxa. Fifteen genera of the family *Halobacteriaceae* have been described: *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Halorubrum*, *Halogeometricum*, *Haloterrigena*, *Halobaculum*, *Halorhabdus*, *Natrialba*, *Natrinema*, *Natronobacterium*, *Natronococcus*, *Natronorubrum* and *Natronomonas* (Tindall *et al*., 1984; Torreblanca *et al*., 1986; Kamekura & Dyall-Smith, 1995; McGenity & Grant, 1995; Oren *et al*., 1995; Kamekura *et al*., 1997; McGenity *et al*., 1998; Montalvo-Rodríguez *et al*., 1998; Xu *et al*., 1999; Ventosa *et al*., 1999; Wainø *et al*., 2000; Grant *et al*., 2001). However, differences in the evolutionary rates in various groups of organisms prevent the use of phylogenetic parameters alone in delineating taxa; consequently, the use of 16S rDNA sequences alone to describe taxa is not enough and the combination of genotypic and phenotypic characteristics (polyphasic taxonomy) constitutes a practical solution to the delineation of taxa (Oren *et al*., 1997).

In this paper, 1457 bp of the 16S rDNA sequence from the halophilic organism strain 135^T is reported as well as polar lipid analysis and physiological and biochemical characteristics. Based on the data presented, we found that strain 135^T is not identical to any of the present species and is sufficiently different from them to justify classification as a novel species within a new genus. In addition, our findings also support the removal of *Natronobacterium nitratireducens* (Xin *et al*., 2001) from the genus *Natronobacterium* and its placement in the same genus as the novel isolate, as *Halobiforma nitratireducens* comb. nov.

METHODS

Archaeal strain. Strain 135^T (= DSM 13078^T = JCM 11627^T) was isolated from a soil sample that was collected from the surface of a hypersaline soil (Aswan, Egypt), using S–G medium [containing (l^{-1}): 250 g NaCl, 2 g KCl, 20 g $MgSO_4$. $7H_2O$, 10 g Casamino acids, 7·5 g yeast extract, 3 g sodium citrate; Sehgal & Gibbons, 1960]. Soil particles were spread on the surface of S–G agar plates and were then incubated in sealed plastic bags at 40 °C. After 2 weeks incubation, several pigmented colonies appeared on the agar plates. A representative red colony for strain 135^T was transferred to S–G broth medium and was grown at 40 °C. To ensure the selection of a pure colony, serial dilutions were made from liquid growth and spread on agar plates. After 2 weeks incubation at 40 °C, a single colony was picked and was used for further study.

Media and growth conditions. The isolation medium S–G was also used for physiological characterization as well as cell maintenance. Salt-minerals medium was used to test the utilization of different organic substrates, either nitrogenous or non-nitrogenous, as nitrogen and/or carbon and energy sources and it contained (l^{-1}): 250 g NaCl, 2 g KCl, 5 g $MgSO_4$. $7H_2O$, 2 g NH_4Cl, supplemented with 1 % (w/v) of the tested substrate. The basal medium is the salt-minerals medium supplemented with 0·1 % (w/v) yeast extract and was used for biochemical characterization as mentioned previously (Hezayen *et al*., 2001). Unless indicated otherwise, the pH was adjusted to 7·5 with 1 M NaOH and media were sterilized by autoclaving. Agar plates were prepared by adding 2 % (w/v) agar before autoclaving.

Light microscopic examination. Gram staining was performed by using acetic-acid-fixed samples as described by Dussault (1955). Wet mounts were prepared to detect motility, cell shape and cell dimensions from various growth stages from both broth and solid S–G medium at different NaCl concentrations and were examined with an Ortholux II microscope (Leitz).

Physiological and biochemical characterization. The growth response to NaCl was examined in liquid and solid S–G medium by using serial NaCl concentrations ranging from 50 to 320 g l^{-1} and to pH by testing growth at pH 5–10. The growth response to temperature was examined by testing growth in liquid and in S–G agar at up to 60 °C. The requirement for Mg^{2+} for growth was tested qualitatively by growing the strain in S–G liquid medium with and without $MgSO_4$. $7H_2O$. Growth was monitored in liquid cultivations by Klett photometer. Catalase and oxidase activities were tested according to Gerhardt *et al*. (1994). Hydrolysis of starch, gelatin, casein and lipids, esterase and urease activities, nitrate reduction, indole formation from tryptone, hydrogen sulfide production, citrate utilization and Voges–Proskauer and methyl red (VPMR) reactions were performed according to Gerhardt *et al*. (1994) as mentioned previously (Hezayen *et al*., 2001). Production of acid from sugars was tested as described previously (Hezayen *et al*., 2001). Utilization of *n*-butyric acid, xylose, arabinose, fructose, glucose, galactose, sucrose, maltose, lactose, starch, mannitol, sorbitol, citrate, gluconate, acetate, pyruvate, glycerine, bacto-peptone, gelatin, nutrient broth, glycine, proteose peptone, Casamino acids and tryptone as sole nitrogen and/or carbon and energy sources was performed in the salt-minerals medium supplemented with 1 % (w/v) substrate. In the case of non-nitrogenous compounds, 0·2 % (w/v) NH_4Cl was supplied as the sole nitrogen source and the test was also performed in the basal medium. In the case of sugars, the test medium was buffered with 20 mM PIPES. Growth was monitored with a Klett photometer.

Antibiotic susceptibility. The susceptibility of the strain to antibiotics was determined as described previously (Hezayen *et al*., 2001). The following antibiotics were tested (μg per disc unless indicated otherwise): penicillin G (5), chloramphenicol (10), tetracycline (30), streptomycin (25), nitrofurantoin (300), bacitracin (10 U), novobiocin (5), fusidic acid (10), anisomycin (20), trimethoprim (2·5), rifampicin (30), nystatin (100 U), nalidixic acid (30) and erythromycin (15).

Carotenoid extraction. Carotenoids were extracted with methanol/acetone (1:1, v/v) as described by Gochnauer *et al*. (1972) from cells that were grown on agar plates and in S–G broth. Carotenoids were also tested in the water lysate from cells grown in S–G broth and the absorption spectrum was determined by wavelength scan using an Ultrospec 2000 photometer (Pharmacia Biotech) in the wavelength range 300–700 nm.

Polymer isolation and analysis. Intracellular accumulation of the polymer poly-*β*-hydroxybutyrate (PHB) was tested from cells grown for 8 days in S–G broth and in the basal medium supplemented with 1 % (v/v) butyric acid or 1 % (w/v) proteose peptone; the polymer was analysed and isolated as described previously in detail (Hezayen *et al*., 2000).

Detection of diether lipids. Diether lipids were released from 100 mg freeze-dried cells using comparatively mild hydrolytic methods that did not lead to significant cleavage of hydroxylated isoprenoid ether lipids, as described previously (Hezayen *et al*., 2001).

Extraction of respiratory lipoquinones and polar lipids. Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cell material using the two-stage method of Tindall (1990a, b).

Analysis of polar lipids. Polar lipids were separated by two-dimensional silica gel TLC. Total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids) (see Hezayen *et al*., 2001).

Isolation of genomic DNA and 16S rDNA amplification and sequencing. DNA was extracted from cells lysed in distilled water by phenol/chloroform extraction followed by ethanol precipitation, modified according to Mak & Ho (1992). The gene encoding 16S rRNA was amplified by PCR with the forward primer 5′-GCCGGAGGTCATTGCTAGTGGA-GTC-3′ (corresponding to *Halobacterium cutirubrum* NCIMB 763 positions 16–40) and the reverse primer 5′-AGGAGGTGATCCAGCCGCAGATTCC-3′ (corresponding to *Hbt. cutirubrum* NCIMB 763 positions 1472–1448). PCR was performed for 30 cycles with denaturation for 30 s at 95 °C, annealing for 1 min at 40 °C and polymerization for 3 min at 72 °C. The amplified 16S rDNA was cloned into vector pGEM-T Easy (Promega) and recombinant plasmids were propagated in *Escherichia coli* XL-1 Blue (Stratagene). The sequence of the 16S rDNA was determined by the chain-termination method using an automatic LI-COR model 4000L sequencer (MWG-Biotech). Standard universal and reversal sequencing primers as well as synthetic oligonucleotides were used as primers, and the 'primer-hopping' strategy was employed. Phylogenetic analysis was performed as outlined previously (Rehm, 2001). Initial investigations on the global 16S rDNA sequence-based position of the novel isolate used the ARB database (Ludwig & Strunk, 1996). On the basis of this work, the 16S rDNA sequences were compared with the existing 16S rDNA database for members of the phyletic group defined as members of the family *Halobacteriaceae*. A greater degree of resolution among closely related taxa was achieved using sequences aligned using CLUSTAL X. Similarity values were calculated based on the aligned 16S rDNA sequences from the following micro-organisms: *Nbt. nitratireducens* C231^T (AB045012), *Natronobacterium gregoryi* NCIMB 2189^T (D87970), *Natrononococcus occultus* NCIMB 2192^T (Z88378), *Natrialba magadii* NCIMB 2190^T (X72495), *Natrialba asiatica* 172P^T (D14123), *Halococcus* sp. JCM 8979 (D63786), strain 135^T (= DSM 13078^T) and *Natronococcus amylolyticus* Ah-36^T (D43628). Similarity values quoted between any two organisms are their binary similarity values. 16S rDNA-based dendrograms were generated using the following programs from PHYLIP: DNAPARS, DNAML, FITCH, KITCH and NEIGHBOR.

G+C content of the DNA. This was determined according to Mesbah *et al*. (1989) and Tamaoka & Komagata (1984) as described previously (Hezayen *et al*., 2001).

RESULTS AND DISCUSSION

Strain 135^T was isolated from hypersaline soil close to Aswan, Egypt. On the basis of its pigmentation, NaCl-dependent growth, antibiotic susceptibility, chemical composition and 16S rDNA sequence, strain 135^T was identified as a member of the family *Halobacteriaceae*. The 16S rDNA similarity values and G+C content of strain 135^T indicate that it represents a novel species (see Fig. 4). However, it is also possible to distinguish this organism from all other genera described within the family *Halobacteriaceae* by evaluating the phenotypic data (see Figs 1–3).

Morphology, physiology and biochemistry

Colonies of strain 135^T formed on S–G agar plates were circular, flat or slightly elevated, entire, small and measured around 1 mm in diameter, opaque, glossy and red-pigmented.

Strain 135^T was capable of growing over a wide range of NaCl concentrations ranging from 13–14 % (w/v) to saturation in liquid S–G medium. The strain grew optimally in the presence of 20 % (w/v) NaCl, as has been shown for most extremely halophilic archaea, which grow optimally in the range 20–26 % (w/v) NaCl (Grant *et al*., 2001). NaCl could be partially replaced by KCl, showing growth at 6 % (w/v) NaCl in the presence of 12 % (w/v) KCl, but no growth was observed at 3 % (w/v) NaCl in the presence of 12 % (w/v) KCl. Addition of Mg^{2+} was not required for growth.

Cells of strain 135^T showed completely different cell shapes when grown in liquid and solid media (Figs 1 and 2). Cells from liquid cultivations lysed in water as well as in 1 % (w/v) bacto-peptone, even in the presence of 25 % (w/v) NaCl, like rod-shaped halophilic archaea, while cells from agar plates (cocci) did not lyse, like the coccus-shaped halophilic archaea (Kamekura *et al*., 1988). Among the members of the family *Halobacteriaceae*, only the alkaliphilic species *Nbt. gregoryi* has been briefly described to appear as rods in liquid cultivations and as spherical cells on solid medium (Tindall *et al*., 1984). Cells stained Gram-negative. When a liquid medium was inoculated with cells grown on solid medium, the rod shapes appeared and, when solid medium was inoculated with cells from liquid medium, the coccus-shaped cells appeared.

The optimum growth temperature in S–G medium containing 20 % (w/v) NaCl was 42 °C and the maximum temperature at which growth was observed was 58 °C. Although the isolate is, based on 16S rDNA similarity, related to the alkaliphilic species *Nbt. gregoryi*, *Nbt. nitratireducens*, *Ncc. occultus* and *Ncc. amylolyticus*, it exhibited neutrophilic growth characteristics over a pH range of 6–9·2 at 42 °C in the presence of 20 % (w/v) NaCl, showing best growth at pH 7·5; this clearly separates the novel isolate from the former species. The isolate was tested for its ability to use different substrates for growth (see above): none of the tested non-nitrogenous compounds could serve as the sole carbon source in the presence of NH_4Cl as the sole nitrogen source but, when the test medium was supplemented with 0·1 % (w/v) yeast extract, only glycerol, sucrose, arabinose, glucose, maltose, acetate, pyruvate and *n*-butyric acid were used for weak

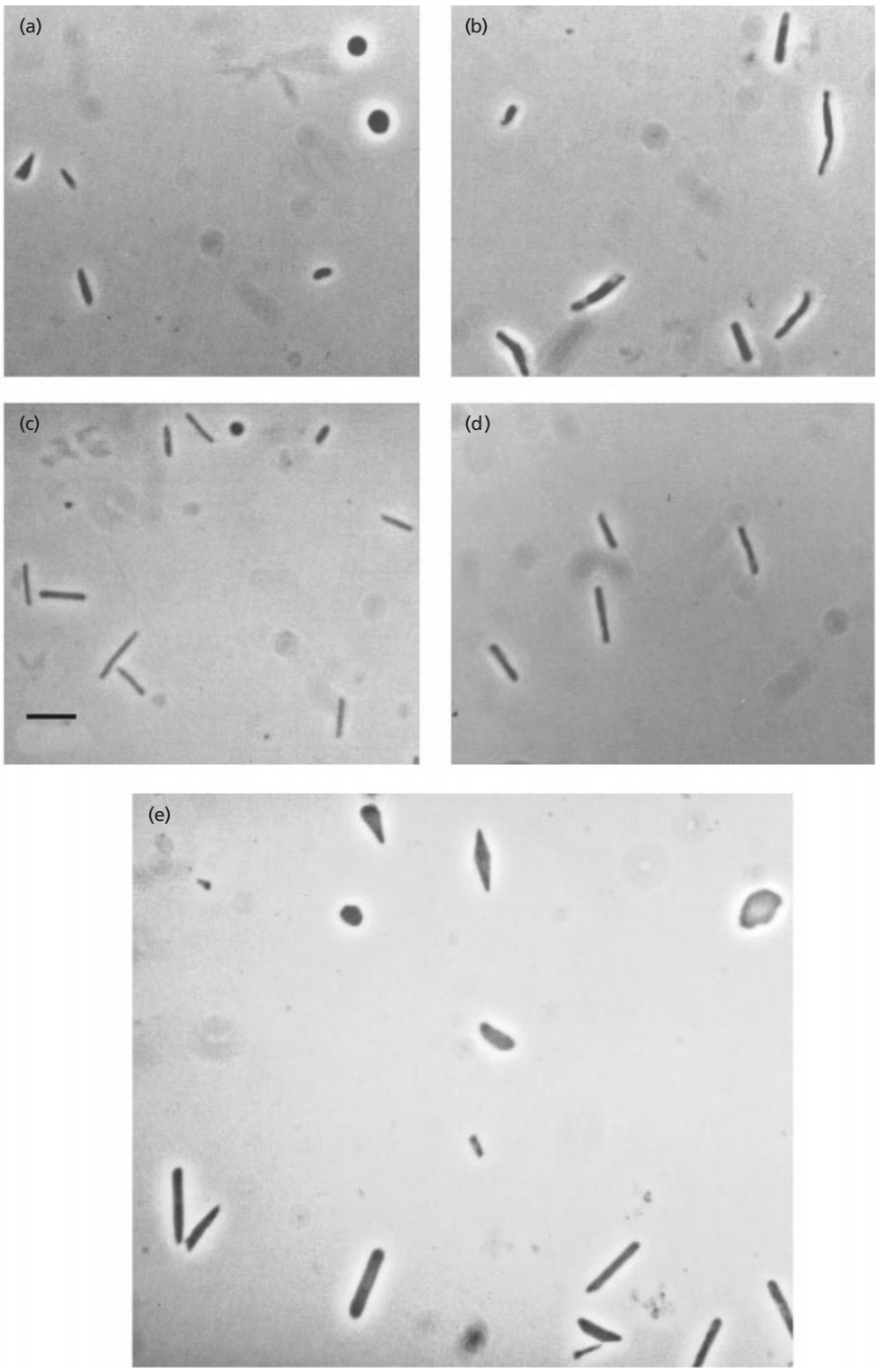

Fig. 1. Phase-contrast micrographs of strain 135^T grown in liquid S–G medium containing 25 % (w/v) NaCl (a–d) or 14 % (w/v) NaCl (e) to demonstrate cell shape differentiation. Bar, 5 μm.

growth. Among the nitrogenous compounds tested, neither bacto-peptone nor nutrient broth (Difco) could serve for growth in liquid culture because of the presence of bile acids, which mediate cell lysis, while gelatin and glycine resulted in weak growth. However, proteose peptone, yeast extract, tryptone and Casamino acids served as good carbon as well as nitrogen sources, mediating good growth. The VPMR

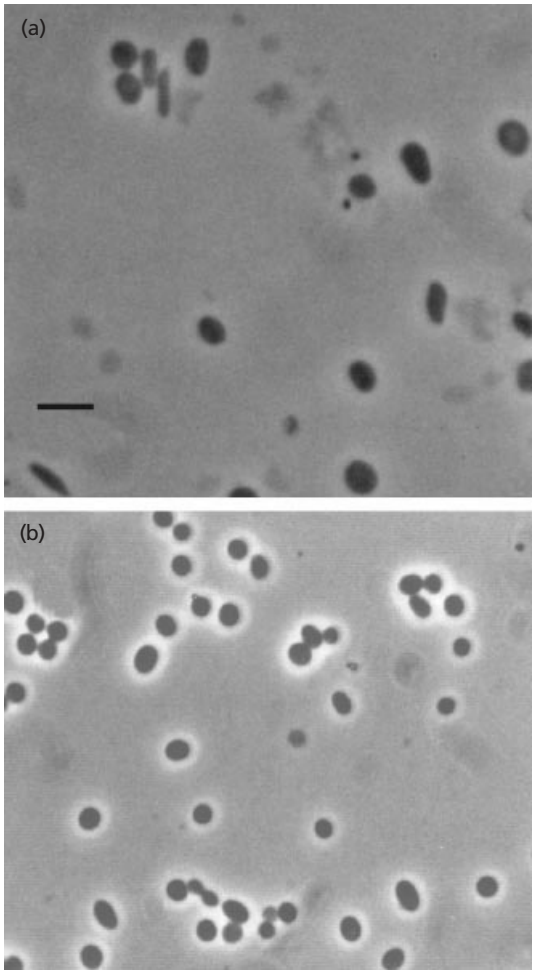

Fig. 2. Cells of strain 135^T cultivated on S–G agar at 25% (w/v) NaCl (a) or 13% (w/v) NaCl (b) to demonstrate cell shape differentiation. Bar, 5 μm.

test was negative. Nitrate reduction, denitrification, indole formation from tryptone, sulfide from cysteine and ammonia from arginine were positive. The strain was sensitive to fusidic acid, bacitracin, nitrofurantoin, novobiocin, anisomycin and rifampicin but was resistant to nystatin, tetracycline, nalidixic acid, trimethoprim, erythromycin, chloramphenicol, streptomycin and penicillin G. Strain 135^T was strictly aerobic and no anaerobic growth was observed, even in the presence of nitrate or arginine hydrochloride. Other phenotypic characteristics are listed in the species description below.

As shown for other extremely halophilic archaea (Fernandez-Castillo *et al*., 1986; Hezayen *et al*., 2000), strain 135^T was able to accumulate PHB up to 40% (w/v) of cellular dry weight. In contrast to the other extremely halophilic archaea, strain 135^T was able to accumulate PHB up to 15% (w/w) of cellular dry weight even when proteose peptone or Casamino acids and yeast extract were used instead of butyric acid.

Cells of strain 135^T were rich in carotenoids under all cultivation conditions, showing absorption maxima at wavelengths of 370, 390, 494 and 528 nm with a shoulder at 466–476 nm. These maxima correspond to those of bacterioruberin, which is a characteristic feature of extremely halophilic archaea (Gochnauer *et al*., 1972; Grant *et al*., 2001).

Chemotaxonomy: ether lipids, quinones and polar lipids

The ether lipids comprised only diether lipids, tetraether lipids being absent. The diether lipids present were the diphytanyl derivative C20:C20 and the phytanyl sesterterpanyl derivative C20:C25, in almost equal amounts. Other groups in which C20:C25 diethers are present produce either significant amounts of the C20:C25 diether or only traces to small amounts.

Menaquinones were the only respiratory lipoquinones present and comprised MK-8 and MK-8(VIII-H_2). Furthermore, a sulfated triglycosyl diether and a triglycosyl diether were produced as sole glycolipids; these are absent in the majority of neutrophilic and alkaliphilic members of the family *Halobacteriaceae* (Table 1).

Table 1. Distinction of genera belonging to the family *Halobacteriaceae* based on phenotypic properties

Data for reference taxa taken from Hezayen *et al*. (2001), Kamekura & Dyall-Smith (1995), McGenity & Grant (1995), Ihara *et al*. (1997), Montalvo-Rodríguez *et al*. (1998) and Tindall *et al*. (1984). Abbreviations: PGS, phosphatidyl glycerosulfate; S_2-DGD, *bis*-sulfated diglycosyl diether; TGD, triglycosyl diether; S-TGD, sulfated triglycosyl diether. All genera contain phosphatidyl glycerol and methylated phosphatidyl glycerophosphate.

Property	*Natrialba*	*Halobacterium*	Strain 135^T	*Haloarcula*	*Natronobacterium*	*Halogeometricum*
Mg^{2+} required for growth (mM)	0	5	0	3	< 10	5
PGS	−	+	−	+	−	−
S_2-DGD	+	−	−	−	−	−
S-TGD	−	+	+	−	−	−
TGD	−	+	+	+	−	−
G+C content (mol%)	60·3–63·1	66–70·9	66·9	62·7–68	61·2–64·6	59·1

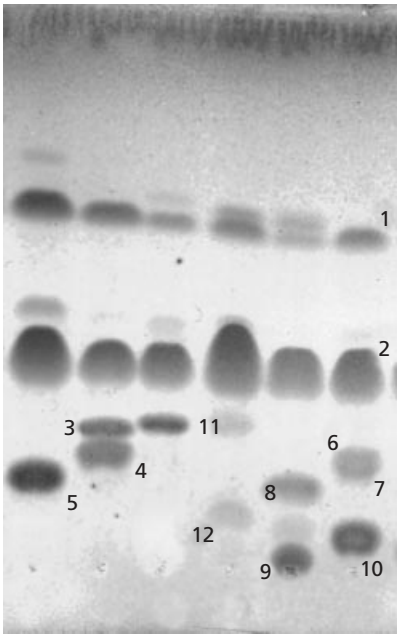

Fig. 3. Thin-layer chromatogram of the total polar lipids of *Haloferax mediterranei* DSM 1411 (a), *Haloarcula vallismortis* DSM 3756^T (b), *Halogeometricum borinquense* DSM 11551^T (c), strain 135^T (d), *Natrinema pellirubrum* NCIMB 786^T (e) and *Halobacterium salinarum* NRC 34001 (f). Plates were subjected to double development in the solvent chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Total lipids were visualized using dodecamolybdophosphoric acid and specific spray reagent used to detect functional groups. The lipids were identified as follows (all compounds are the diether derivatives): 1, phosphatidyl glycerol; 2, methylated phosphatidyl glycerophosphate; 3, triglycosyl diether; 4 and 7, phosphatidyl glycerosulfate; 5 and 8, sulfated diglycosyl diether; 6, triglycosyl diether; 9, sulfated glycolipid; 10, sulfated triglycosyl diether; 11, triglycosyl diether; 12, sulfated glycolipid.

The polar lipid analysis revealed the presence of phosphatidyl glycerol and methylated phosphatidyl glycerol phosphate, whereas no phosphatidyl glycerosulfate was detected (Fig. 3). Nevertheless, the presence of diether lipids, MK-8 and MK-8(VIII-H_2) coupled with the presence of polar lipids are characteristic features of the monophyletic group defined by the family *Halobacteriaceae* (Table 1).

Within the group to which strain 135^T belongs, which can be defined both chemically and by 16S rDNA sequence analysis, there are a number of further subgroups that can be distinguished by their physiology (neutrophile or alkaliphile), other phenotypic characteristics and their chemical composition. Within this group, strain 135^T is unique at present in producing both a triglycosyl diether lipid and its sulfated derivative. Thus, strain 135^T may be distinguished unambiguously from all other known members of this subgroup and from other members of the family *Halobacteriaceae*.

G+C content of the DNA

The G+C content of the DNA of strain 135^T was 66·9 mol% (mean of three independent determinations), which differs significantly from the value of about 63·5 mol% for the most closely related *Natronococcus* species.

Inference of evolutionary relationships based on the 16S rDNA sequence

Approximately 99% (1457 bp) of the 16S rDNA sequence was determined. Initial comparison of the sequence with members of the family *Halobacteriaceae* revealed that strain 135^T grouped consistently with the species *Nbt. gregoryi*, *Nbt. nitratireducens*, *Ncc. occultus* and *Ncc. amylolyticus* (Fig. 4). Therefore, the novel strain was compared with these three described species and the 16S rDNA sequences of *Nab. magadii*, *Nab. asiatica* and *Halococcus* sp. were used as outgroup sequences to polarize the data. All of the analysis methods consistently grouped strain 135^T together with *Nbt. nitratireducens*. Irrespective of which method of analysis was used, the branching order between the pairs strain 135^T/*Nbt. nitratireducens*, *Ncc. occultus*/*Ncc. amylolyticus* and *Nbt. gregoryi* could not be resolved unambiguously (Fig. 4). Based on these data alone, it is not possible to conclude whether the pair strain 135^T/*Nbt. nitratireducens* belongs to a monophyletic group together with *Natronobacterium*.

Although bootstrap values for the pair strain 135^T/*Nbt. nitratireducens* were high, binary similarity values showed a similarity value of 96·4%. This may be attributed to the comparatively long internal edge that connects this group to the rest of the sequences. All distance matrix methods showed that *Nbt. gregoryi* did not form a unique cluster with strain 135^T/*Nbt. nitratireducens*; however, it should be noted that both the bootstrap values and visual inspection of the rather short internal edges linking the three 'clusters' *Nbt. gregoryi*, *Ncc. occultus*/*Ncc. amylolyticus* and strain 135^T/*Nbt. nitratireducens* showed that no significance should be attached to this branching order. DNAML gave a similar topology to the distance matrix methods, whereas the topology of the DNAPARS dendrogram indicated that *Nbt. gregoryi* and strain 135^T/*Nbt. nitratireducens* formed a group to the exclusion of members of the genus *Natronococcus* (Fig. 4). Each of these methods makes different assumptions about the way the dataset is analysed (Felsenstein, 2001), and it is evident that the interpretation of the 'correct' topology is dependent on knowing which of the assumptions hold true in this particular case. Most methods employed also make inherent assumptions that sequence evolution is always divergent and that parallelism and convergent evolution do not play a role (see Sneath, 1989). Indeed, such effects are usually only detectable when one compares independently evolving datasets (see Sneath, 1974). However, it should also be noted that the rather short internal edges are indicative of the fact that additions to the

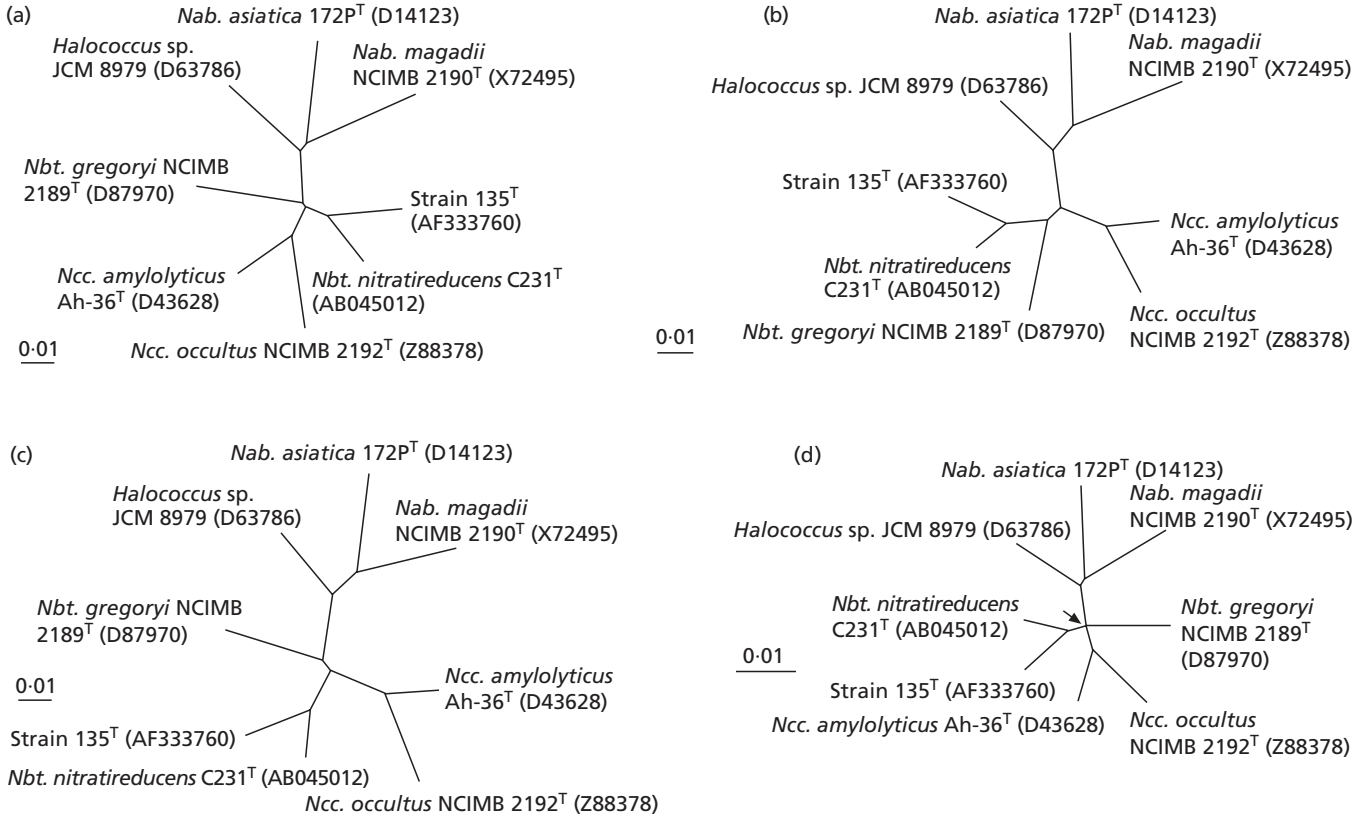

Fig. 4. (a)–(c) 16S rDNA sequence-based dendrograms estimated using the programs NEIGHBOR (a), DNAPARS (b) (this version now incorporates branch lengths) and DNAML (c) in PHYLIP version 3.6. (d) 16S rDNA sequence-based dendrogram in which the branches that cannot be unambiguously determined have been collapsed (node indicated by arrow). All trees are unrooted, with the organisms *Nab. magadii*, *Nab. asiatica* and *Halococcus* sp. serving as the outgroup.

dataset may reduce the resolution further, particularly if new sequences are added that join the cluster in the region of the internal edges where the groups *Nbt. gregoryi*, strain 135^T/*Nbt. nitratireducens* and members of the genus *Natronococcus* join. Based on the data presented, it is only possible to conclude that each of the groups *Nbt. gregoryi*/strain 135^T/*Nbt. nitratireducens* and members of the genus *Natronococcus* form distinct phyletic groups. While the use of different inference methods indicates that these three groups also comprise a larger phyletic group, it is not possible to determine whether the group *Nbt. gregoryi*/strain 135^T/*Nbt. nitratireducens* or the group strain 135^T/*Nbt. nitratireducens*/*Ncc. occultus*/*Ncc. amylolyticus* is monophyletic to the exclusion of the others. The problem of resolving such branching orders is becoming increasingly apparent in phyletic groups where there are numerous short internal edges. Thus, it is impossible to determine whether each of the three phyletic groups recovered by the methods used should be divided into one, two or three genera. Such problems are to be expected in a potentially continuous dataset and indicate limitations in the use of the 16S rDNA sequence data as the sole (and primary) dataset for detecting evolutionary relationships (Fig. 4). Other datasets that also indicate evolutionary relationships need therefore to be taken into consideration, and there are several advantages in using datasets that are not potentially continuous in nature, but which are discontinuous (Tindall, 2000a).

Unravelling the evolutionary and taxonomic status of strain 135^T

In the past two decades, increasing attention has been given to the use of 16S rDNA sequence data. However, the value of other datasets, such as chemical data, has been underestimated (Tindall, 1992). It is interesting to note that the presence of isoprenoid ether-linked lipids, first described in members of the halobacteria, still serves as a unique chemical marker since Tornabene & Langworthy (1979) provided evidence of their presence in methanogens, as well as drawing on published data on members of the genera *Halobacterium* (Faure *et al.*, 1963; Kates *et al.*, 1963), *Sulfolobus* (Langworthy, 1977b), *Caldariella* (De Rosa *et al.*, 1974) and *Thermoplasma* (Langworthy, 1977a). Equally, there is excellent correlation between the presence of diether-linked isoprenoid ether lipids, the presence of MK-8, MK-8(VIII-H_2), as well as the presence of phosphatidyl glycerol and methylated phosphatidyl glycerophosphate as the delineation of a unique group

within the *Archaea* based on 16S rDNA sequence analysis – the order *Halobacteriales*. In addition, there is evidence to show that the distribution of diether-linked isoprenoid lipids in alkaliphilic halobacteria correlates excellently with the 16S rDNA data, in contrast to their physiological properties as alkaliphiles. Thus, members of the genera *Natronobacterium*, *Natronococcus* and *Natronorubrum* produce C20:C20 and C20:C25 diethers in which neither predominates, a feature also found in the neutrophilic members of the group to which members of these genera belong. In contrast, in *Natronomonas pharaonis*, the C20:C25 diether predominates, while, in alkaliphiles close to members of the genus *Halorubrum*, the C20:C20 diether predominates (Tindall, 1985). Strain 135^T, like other members of this phyletic group, also produces C20:C20 and C20:C25 diethers. As pointed out by Tindall (2000b), *Nbt. gregoryi* is unique within the order *Halobacteriales* in producing a series of methylated menaquinones, and this features serves to distinguish it from other taxa. Members of the genus *Natronococcus* are consistently coccoid, never having been shown to produce rod-shaped cells, unlike strain 135^T, *Nbt. gregoryi* and *Nbt. nitratireducens*. *Nbt. gregoryi* is unique within the present group in that it does not produce a glycoprotein, a feature reported for *Natronococcus* species and *Nbt. nitratireducens*. Thus, the data presented indicate that strain 135^T and *Nbt. nitratireducens* should not be placed in the genus *Natronobacterium* or *Natronococcus*.

Taxonomy

Strain 135^T shares a high degree of 16S rDNA sequence similarity with *Nbt. nitratireducens*, and creating a new genus for strain 135^T implies that *Nbt. nitratireducens* also cannot remain within this genus. In view of the close 16S rDNA sequence similarity and the need for more detailed studies on the inter-relationship of genotypic and phenotypic properties in determining their relevance to the evolution and taxonomy of this group, we propose that these two strains be placed in a new genus. Thus, we propose the placement of these two organisms in a new genus, *Halobiforma* gen. nov., for which the designated type species is *Halobiforma haloterrestris* sp. nov. The abbreviation *Hbf.* will be suggested for the new genus.

Description of *Halobiforma* Hezayen, Tindall, Steinbüchel & Rehm gen. nov.

Halobiforma (Ha.lo.bi.for′ma. Gr. n. *hals*, *halos* salt; L. prefix *bi* two; L. n. *forma* form; N.L. n. *Halobiforma* the halophile with two different shapes).

Cells are pigmented and are rods, pleomorphic and cocci. Cells are Gram-negative and are motile. May be alkaliphilic or neutrophilic. Major diethers present are C20:C20 and C20:C25. Major polar lipids are phospholipids; glycolipids may be present in some species. When present, the glycolipids are a triglycosyl diether and its sulfated derivative. Phosphatidyl glycerosulfate is not present. The genus constitutes a group that shares more than 96% 16S rDNA sequence similarity. The type species is *Halobiforma haloterrestris*.

Description of *Halobiforma haloterrestris* Hezayen, Tindall, Steinbüchel & Rehm sp. nov.

Halobiforma haloterrestris (ha.lo.ter.res′tris. Gr. n. *hals*, *halos* salt; L. adj. *terrestris* belonging to the soil; N.L. adj. *haloterrestris* from salty soil).

Cells are pigmented and are rods that measure 0·5–1·5 × 2–8 μm, pleomorphic and cocci that measure 1·25–2·0 μm in diameter. Cells are Gram-negative and are motile. Only rods lyse in distilled water or 1% (w/v) bacto-peptone. The optimum temperature for growth is 42 °C. The pH range for growth is pH 6–9·2, with an optimum at pH 7·5. The NaCl range for growth is 2·2 M to saturation, with an optimum of 3·4 M NaCl. NaCl may be replaced to a large extent by KCl. Aerobic, catalase- and oxidase-positive. Acid is produced from xylose, arabinose, glucose, sucrose and maltose, but not from lactose, fructose, galactose or starch or urea. Casein and gelatin are hydrolysed, but not starch. Tweens 20, 40 and 80 are also hydrolysed, but not egg yolk (lecithin). NH_4 cannot serve as a nitrogen source, while organic nitrogen compounds such as proteose peptone, Casamino acids and tryptone can serve as nitrogen, carbon and energy sources. The strain accumulates PHB. The G + C content of the DNA is 66·9 mol%. Isolated from a salt soil close to Aswan (Egypt).

The type strain is strain 135^T (= DSM 13078^T = JCM 11627^T).

Description of *Halobiforma nitratireducens* (Xin *et al.* 2001) comb. nov.

Basonym: *Natronobacterium nitratireducens* Xin *et al.* 2001.

The description is identical to that published for *Nbt. nitratireducens* by Xin *et al.* (2001). The type strain is strain C231^T (= AS 1.1980^T = JCM 10879^T).

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