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Transfer of *Natrialba asiatica* B1T to *Natrialba taiwanensis* sp. nov. and description of *Natrialba aegyptiaca* sp. nov., a novel extremely halophilic, aerobic, non-pigmented member of the *Archaea* from Egypt that produces extracellular poly(glutamic acid)

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A novel extremely halophilic member of the *Archaea*, strain 40^T, was isolated from Egypt (Aswan). This isolate requires at least 1·6 M sodium chloride for growth and exhibits optimal growth between 37 and 42 °C. Determination of the entire 16S rRNA gene sequence revealed the highest similarity to the type strain of *Natrialba asiatica* (> 99%). Polar lipid analysis indicated that strain 40^T and *Natrialba asiatica* have essentially identical compositions, indicating that the former is a member of genus *Natrialba*. However, physiological and biochemical data provided evidence that *Natrialba asiatica* strains B1T and 172P1^T, as well as strain 40^T, are sufficiently different to be divided in three different species. The G+C content of strain 40^T was 61·5±0·6 mol%. In addition, DNA–DNA hybridization data supported the placement of the isolate in a new species in the genus *Natrialba*, *Natrialba aegyptiaca* sp. nov., and indicated that *Natrialba asiatica* strain B1T should also be placed in a separate species, *Natrialba taiwanensis* sp. nov. Morphological studies of strain 40^T indicated clearly that this isolate appears in three completely different cell shapes (cocci, rods, tetrads) under different conditions of growth, including different sodium chloride concentrations and different growth temperatures. Another interesting property of strain 40^T is the ability to produce an extracellular polymer, which was found to be composed predominantly of glutamic acid (85% w/w), representing poly(glutamic acid), carbohydrates (12·5% w/w) and unidentified compounds (2·5% w/w). Among the *Archaea*, production of an extracellular polysaccharide has been described for some members of the genera *Haloferax* and *Haloarcula.*

Keywords: *Natrialba aegyptiaca* sp. nov., *Natrialba asiatica*, extremely halophilic archaeon, exopolymer, poly(glutamic acid)

INTRODUCTION

The halobacteria comprise a well-defined, monophyletic group of aerobic or facultatively anaerobic micro-organisms that require high salt concentrations for growth and are recognized as part of the archaebacteria or *Archaea* (Woese *et al.*, 1990). The taxonomy of these aerobic, halophilic micro-organisms has developed slowly since the first authentic pure cultures were described by Klebahn (1919). The methods in use for the taxonomy of this group of organisms reflect developments in the taxonomy of other groups of organisms. Fifteen genera of the family *Halobacteriaceae* have been described: *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Halorubrum*, *Halogeometricum*, *Haloterrigenia*, *Halobaculum*, *Halo-*

The GenBank accession number for the complete 16S rDNA sequence of strain 40^T is AF251941.

rhabdus, *Natrialba*, *Natrinema*, *Natronobacterium*, *Natronococcus*, *Natronorubrum* and *Natronomonas* (Grant & Larsen, 1989; Tindall, 1992; 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). The current divisions are based largely on 16S rDNA gene sequences and chemotaxonomic criteria, particularly polar lipid composition, but there is no strict correlation between the two. *Natrialba asiatica* gen. nov., sp. nov. was described as a representative of a new genus within the family *Halobacteriaceae* (Kamekura & Dyall-Smith, 1995). Two strains of this species (B1T and $172P1^T$) were isolated from hypersaline habitats (solar salt and beach sand). The major features that distinguish between *Natrialba asiatica* and the other species of neutrophilic, extremely halophilic archaea are the white (pale-yellow) colour of the colonies and the presence of the glycolipids 2,3-di-*O*-phytanyl- or phytanyl-sesterterpenyl-1-*O*-[2,6-$(SO_4)_2$-α-mannopyranosyl-(1 → 2)-α-glucopyranosyl]-*sn*-glycerol (S_2-DGD-1) (Kamekura & Dyall-Smith, 1995).

Extremely halophilic archaea are biotechnologically important organisms, particularly because they grow under high salt concentrations and because simple carbon sources such as sugars, starch, acetate or butyrate are sometimes utilized for growth (Lillo & Rodríguez-Valera, 1990; Hezayen *et al.*, 2000). Under these conditions, very few organisms, if any, can develop at growth rates anywhere near as high as those exhibited by these organisms. Therefore, the requirement for enclosed sterile conditions, usually needed to suppress contaminants overgrowing the fermentation, is greatly reduced and extremely simple production systems can be developed, such as open ponds similar to those used for sewage treatment. We isolated several extremely halophilic archaea from hypersaline soils (Aswan, Egypt). These isolates were screened with respect to the formation of extracellular polymers occurring as slime and one isolate, strain 40^T, was selected for further study. Strain 40^T formed mucoid colonies and produced large amounts of an exopolymer. Among the members of the family *Halobacteriaceae*, some members of the genus *Haloferax*, including *Haloferax mediterranei*, *Haloferax gibbonsii* and *Haloferax denitrificans*, and *Haloarcula* spp. have been reported to produce extracellular polysaccharide material (Antón *et al.*, 1988; Paramonov *et al.*, 1998; Parolis *et al.*, 1999; Nicolaus *et al.*, 1999).

In this study, we describe the taxonomic characterization of strain 40^T and the analysis of the extracellular polymer it produces. We found that this strain was a member of the genus *Natrialba*, but it is sufficiently different from the only described neutrophilic species, *Natrialba asiatica*, to justify classification in a new species. Furthermore, we obtained evidence that *Natrialba asiatica* strain B1T should be classified in a new species.

METHODS

Strains. Strain 40^T (= DSM 13077^T = JCM 11194^T) was isolated from a soil sample collected from the surface of a hypersaline soil close to Aswan city (Egypt). The two *Natrialba asiatica* strains, B1T (= DSM 12281 = JCM 9577) and $172P1^T$ (= DSM 12278^T = JCM 9576^T), were obtained from the DSMZ (Braunschweig, Germany).

Soil analysis. In order to obtain data on the habitat of the isolate, the soil sample was subjected to chemical analysis. pH was determined by soil/distilled water extraction (1:5 w/v) with a pH-meter fitted with a glass electrode. K^+ and Na^+ ions were determined by using a Corning 400 flame photometer (Black *et al.*, 1965). Ca^{2+}, Mg^{2+}, CO_3^{2-}, HCO_3^- and Cl^- ions were determined according to the method of Chapman & Pratt (1961). Phosphate and sulfate ions were determined according to the method of Vogel (1978) and the organic matter was determined according to Elwakeel & Riley (1956). The total dissolved salts were determined by drying the filtered soil/distilled water extract (1:5 w/v) from 20 g soil and the percentage salt content was obtained.

Media and growth conditions. Strain 40^T was isolated on S-G medium (Sehgal & Gibbons, 1960), which contained the following (g l^{-1}): NaCl, 250; $MgSO_4 . 7H_2O$, 20; KCl, 2; trisodium citrate, 3; yeast extract, 10 (Difco); and Casamino acids, 7·5. The pH was adjusted to 7·2 with 1 M NaOH. The organism was grown and maintained on proteose peptone-salt medium, which contained (g l^{-1}): NaCl, 250; KCl, 2; $MgSO_4 . 7H_2O$, 5; proteose peptone, 7·5; and yeast extract, 5. The pH was adjusted to 7·2 with 1 M NaOH. To prepare agar plates, media were solidified with 20 g agar l^{-1}. Media were sterilized by autoclaving. Basal medium contained the following (g l^{-1}): NaCl, 225; KCl, 2; $MgSO_4 . 7H_2O$, 5; and yeast extract, 1. Basal medium was used to test the different activities of strain 40^T and the other two neutrophilic *Natrialba* strains.

Light-microscope examination. Gram staining was performed by using acetic acid-fixed samples, as described by Dussault (1955). Wet mounts were prepared for motility determination from various growth stages from both broth and solid media and were examined with an Ortholux II microscope (Leitz).

Physiological and biochemical analysis. Several authors have established that biochemical tests depend on the culture media utilized rather than on the substrate under test (Gibbons, 1957; Norberg & v. Hofsten, 1969; Sehgal & Gibbons, 1960; Tomlinson & Hochstein, 1972). In order to obtain comparable results, we carried out the biochemical tests for the three strains, strain 40^T, *Natrialba asiatica* $172P1^T$ and *Natrialba asiatica* B1T, in parallel on the same media under the same conditions.

Catalase and oxidase activities were determined according to Gerhardt *et al.* (1994). Hydrolysis of starch, gelatin, casein and lipids, urease activity, nitrate reduction, indole formation from tryptone, hydrogen sulfide production, citrate utilization and Voges–Proskauer and methyl red reactions were assessed according to Gerhardt *et al.* (1994) and Harrigan & McCance (1966) using the test substrate added to the basal medium mentioned above and incubating the tests at 37 °C. The utilization of sugars and other compounds as carbon sources and acid production from sugars was assessed on basal medium supplemented with 1 % (w/v) of the tested substrate and the basal medium as a control. Growth was monitored by measuring the OD_{600} using an Ultrospec 2000 photometer (Pharmacia Biotech); a

decrease in the pH to a value of less than 6·0 was considered as evidence of acid production (Oren *et al.*, 1995).

Antibiotic susceptibility. The susceptibility of the strains to antibiotics was determined on surface-seeded maintenance medium with susceptibility disks obtained from Oxoid except for that containing anisomycin, which was prepared in our laboratory. The following antibiotics were used (amounts per disc in parentheses): penicillin G (5 μg), chloramphenicol (10 μg), tetracycline (30 μg), streptomycin (25 μg), nitrofurantoin (300 μg), bacitracin (10 U), novobiocin (5 μg), fucidic acid (10 μg), anisomycin (20 μg), trimethoprim (2·5 μg), rifampicin (30 μg), nystatin (100 U), nalidixic acid (30 μg) and erythromycin (15 μg).

Bacterioruberin analysis. Pigmentation was determined following growth on S-G medium and also on the basal medium containing 1 % (w/v) sodium acetate both on agar plates and in liquid medium. Bacterioruberins were extracted from cells that were grown on the agar basal medium containing 1 % (w/v) sodium acetate with methanol/acetone (1:1 v/v) as described by Gochnauer *et al.* (1972). Bacterioruberin extracts were analysed by wavelength scan using an Ultrospec 2000 photometer (Pharmacia Biotech) in the wavelength range of 200–700 nm.

Detection of diether lipids. Diether lipids were released from 100 mg freeze-dried cells using comparatively mild hydrolytic methods, which did not lead to significant cleavage of hydroxylated isoprenoid ether lipids (B. J. Tindall, unpublished data). The ether lipids were analysed by silica gel TLC (Macherey-Nagel, art. no. 818 135) using double-development in hexane/*tert*-butylmethylether (4:1 v/v) (detection of diether lipids) or in hexane/*tert*-butylmethylether/acetic acid (25:25:1 by vol.) for the detection of di- and tetraether lipids. Lipid material was visualized with dodecamolybdophosphoric acid.

Extraction of respiratory lipoquinones and polar lipids. Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cell material by the two-stage method described by Tindall (1990a, b). Respiratory quinones were extracted using methanol/hexane (2:1 v/v) (Tindall, 1990a, b) and the polar lipids were extracted by adjusting the remaining methanol/0·3 % aqueous NaCl phase (containing the cell debris) to give a chloroform/methanol/0·3 % aqueous NaCl mixture (1:2:0·8, by vol.). The polar-lipid extraction solvent was stirred overnight and the cell debris was pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0·3 % aqueous NaCl mixture to a ratio of 1:1:0·9 (by vol.).

Analysis of polar lipids. Polar lipids were separated by two-dimensional silica gel TLC (Macherey-Nagel art. no. 818 135). The first direction was developed in chloroform/methanol/water (65:25:4 by vol.) and the second in chloroform/methanol/acetic acid/water (80:12:15:4 by vol.). Total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin reagent (free amino groups), periodate/Schiff reagent (α-glycols), Dragendorff reagent (quaternary nitrogen) and anisaldehyde/sulfuric acid (glycolipids).

PCR amplification of the 16S rRNA gene and DNA sequencing. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of PCR products were carried out as described previously (Rainey & Stackebrandt, 1993; Rainey *et al.*, 1992). Two independent PCRs were applied, resulting in two PCR products comprising positions 1–1390 and 357–1525 of the 16S rDNA sequence. The following primers were used: first PCR, 5′-TCCGGTTGATCCTGCC-3′ and 5′-CGGTGTGTGCAAGGAGC-3′; second PCR, 5′-ACGGGGCGCAGCAGGC-3′ and 5′-AAGGAGGTGATCCAGCC-3′. Purified PCR products were sequenced using the Taq Dye-Deoxy Terminator Cycle Sequencing kit (Applied Biosystems) as directed in the manufacturer's protocol, employing the following sequencing primers: 5′-TACGGGAGGCAGCAG-3′ and 5′-GCAACGAGCGCAACCC-3′. DNA sequencing was performed according to Sanger *et al.* (1977). Sequencing reactions were electrophoresed using an Applied Biosystems 373A DNA sequencer. The 16S rDNA sequences were aligned manually. The global phylogenetic position of the new isolate, strain 40^T, was investigated using 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 the ae2 editor (Maidak *et al.*, 1997). Similarity values were calculated based on the aligned 16S rDNA sequences from the following organisms: *Natrialba asiatica* strains B1T (= JCM 9577) (accession no. D14124) and $172P1^T$ (= JCM 9576^T) (D14123) and *Natrialba magadii* NCIMB 2190^T (X72495). Similarity values quoted between any two organisms are their binary similarity values.

G + C content of the DNA and DNA–DNA hybridization. Cell suspensions were passed through a French press and the DNA was subsequently purified on a hydroxyapatite column according to the method of Cashion *et al.* (1977). The purified DNA was hydrolysed with P1 nuclease and dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989) to determine the G + C content. The deoxyribonucleosides were analysed by HPLC (LKB) on a Nucleosil 100-5 C18 (250 × 4 mm) column fitted with a guard column (20 × 4 mm) of the same material. The deoxyribonucleosides were eluted with 0·6 M ammonium dihydrogen orthophosphate/acetonitrile (40:3 v/v, pH 4·4) as solvent, at a flow rate of 0·7 ml min^{-1} and a column temperature of 26 °C (Tamaoka & Komagata, 1984).

DNA–DNA hybridization was carried out according to the method of De Ley *et al.* (1970), with small modifications, using a Gilford System 2600 spectrophotometer equipped with a Gilford 2527-R thermoprogrammer and plotter. The DNA concentration was 45 μg ml^{-1}. Due to the high G + C content of the DNA, 10 % (v/v) DMSO was added to 2·5 × SSC buffer to depress the melting point (T_m) of the DNA (Escara & Hutton, 1980). The resulting buffer was 2 × SSC plus 10 % (v/v) DMSO. The mean T_m was determined from the DNA melting curves and the optimal temperature of renaturation (TOR) was calculated according to the equation $TOR = T_m - 25$. The optimal temperature of renaturation was determined to be 67 °C.

DNA–DNA hybridization values were calculated by regression analysis of the renaturation rates over the time period 10–30 min after initiating the reaction by using the computer program TRANSFER.BAS (Jahnke, 1992). The DNA–DNA hybridization values were calculated from the formula $H = (4 \times V_M - V_A - V_B)/(2 \times \sqrt{(V_A \times V_B)})$, where H is the percentage degree of binding or duplexing; V_M is the renaturation rate of the mixture of DNAs A and B; V_A is the renaturation rate of the DNA from organism A and V_B is the renaturation rate of the DNA from organism B.

Polymer isolation and analysis. Cells were grown on solidified maintenance medium, harvested after 3 d from agar plates and suspended in distilled water. Cells were sedimented by ultracentrifugation (60 min, 40000 ***g***) and the polymer in the supernatant was precipitated by addition of 1 vol. ethanol. The polymer was collected, resuspended in water, dialysed against water and then lyophilized to obtain the polymer as a fine powder. The polymer sample was subjected to analysis of the presence of carbohydrates (Anthrone reaction; Gerhardt *et al*., 1994). In order to detect amino acids, the polymer was hydrolysed in 6 M HCl (1 mg/100 ml) for 12 h, lyophilized and dissolved in an equal volume of distilled water. The hydrolysed polymer sample was then subjected to HPLC analysis after *o*-phthaldialdehyde (OPA) derivatization as follows: 460 µl hydrolysed sample was mixed with 200 µl 0·5 M borate buffer, pH 9·5, and 100 µl OPA reagent. The mixture was incubated at room temperature for 200 s. Fifty microlitres 0·75 M HCl was added to adjust the pH to 7·0–7·5, which stopped the reaction. One hundred microlitres of the OPA-derivatized sample was mixed with 400 µl start eluent (26:74 methanol/50 mM sodium acetate). A 20 µl aliquot was injected onto a reverse-phase column (0·46 × 12·5 cm, RP18 Techsphere ODS-2; Kontron Instruments), equilibrated with starting eluent. OPA–amino acids were eluted with a methanol/50 mM sodium acetate gradient, in which the methanol content increased linearly from 26 to 75% (v/v), at 40 °C and a flow rate of 1·0 ml min^{-1}. OPA–amino acids were monitored fluorimetrically at 330/450 nm (excitation/emission) by using a model 1046A fluorescence detector (Hewlett Packard). Calibration was done with chromatographically pure amino acids (collection AS-10 from Serva Feinbiochemica).

RESULTS AND DISCUSSION

The new aerobic, extremely halophilic member of the *Archaea*, strain 40^T, was isolated from hypersaline soil close to Aswan (Egypt). Habitat (soil) analysis provided evidence that this micro-organism was adapted to low concentrations of K^+ ions and required a high total salt concentration for growth (Table 1). The isolate was identified primarily by its mucoid colony morphology on agar plates, which indicated the presence of an extracellular polymer.

Table 1. Chemical analysis of the soil sample from which strain 40^T was isolated

Abbreviations: OM, organic matter; TDS, total dissolved salt; ND, not detected.

Ion	Concentration (% w/w)
Na^+	5·60
K^+	0·04
Ca^{2+}	1·35
Mg^{2+}	0·17
CO_3^{2-}	0·14
HCO_3^-	0·15
Cl^-	10·7
SO_4^{2-}	2·24
TDS	28·53
OM	1·70
PO_4^{3-}	ND

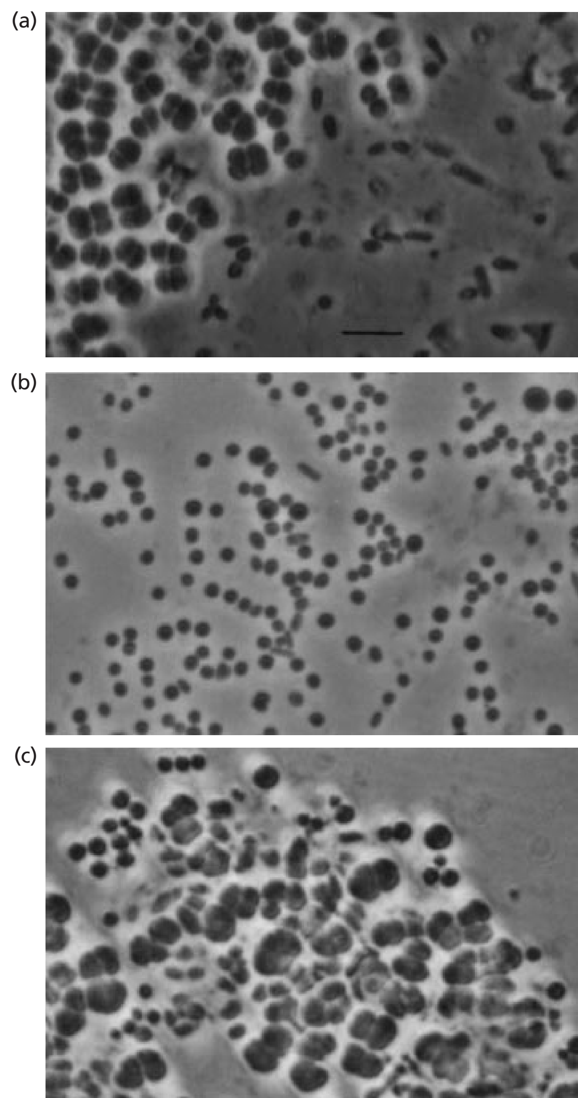

Fig. 1. Phase-contrast micrographs of strain 40^T grown in different media at various conditions to demonstrate cell shape differentiation. (a) Cells were cultivated in the maintenance medium for 2 d; (b) cells were cultivated at 38 °C for 4 d in the basal agar medium plus 1% (w/v) sodium acetate (or sodium gluconate, which results in comparable cell shapes) as carbon source; (c) cells after 7 d incubation from agar maintenance medium at 60 °C. Bar, 5 µm.

Cell shape, motility and Gram staining

Starting from a single colony for inoculation, we observed three different cell shapes of strain 40^T, depending on the growth stage and the cultivation conditions: (i) non-motile coccoid cells, (ii) motile rods and pleomorphic rods (short and long rods, oval cells and coccobacilli) and (iii) non-motile tetrads (Fig. 1). The presence and the ratio of the different cell shapes changed at different growth stages on maintenance

medium. All shapes were represented after 2 d of incubation on maintenance medium but, after 4 d of incubation, the tetrads began to separate and further incubation for 2 d resulted in a mixture of only rods and cocci. Differences in cell shape were also obtained when cells were cultivated on basal agar medium plus either 1 % (w/v) sodium acetate or gluconate as carbon source (Fig. 1). After 2 d of incubation, the tetrads began to lose their ability to aggregate and, after 4 d of incubation, only individual cocci and coccobacilli were present. Recultivation of these coccoid and coccobacilli cells on the maintenance medium showed that the three different shapes appeared again. When the organism was cultivated under suboptimal conditions, i.e. high temperature or low NaCl concentration, strongly pleomorphic shapes were produced (Fig. 1). Interestingly, when cells of different shapes were suspended in distilled water, the tetrads and individual cocci did not lyse and all shapes appeared again after recultivation in isolation or maintenance media. The cell shape of the other two *Natrialba* strains is coccobacilli and rods (Kamekura & Dyall-Smith, 1995). All cells of strain 40^T stained Gram-negative, as did both of the *Natrialba asiatica* strains.

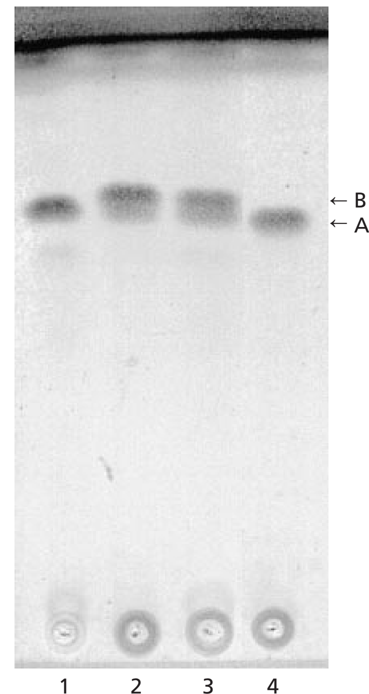

Fig. 2. Thin-layer chromatogram of diether lipids. The solvent system was hexane/*tert*-butylmethylether (4:1 v/v) with double development. Ethers are indicated as: A, Phytanyl-phytanyl diether (C20:C20); B, sesterpanyl-phytanyl diether (C25:C20). Lanes: 1 and 4, diether lipids from *Halobacterium salinarum* (standard, C20:C20); 2, diether lipids from *Natronomonas pharaonis* (standard, C20:C20 and C25:C20); 3, diether lipids from strain 40^T.

16S rDNA gene sequence

The complete 16S rDNA sequence of strain 40^T was determined. The sequence was compared with 16S rDNA gene sequences from representative members of the *Archaea* (Maidak *et al.*, 1996). The greatest degree of similarity (99·2 %) was obtained with the 16S rDNA gene sequence from *Natrialba asiatica* B1T. However, the *Natrialba asiatica* strains 172P1^T and B1T shared only 98·5 % similarity with respect to their 16S rDNA gene sequences, similar to the value obtained between strain 40^T and *Natrialba asiatica* 172P1^T (98·7 %). The similarity values of all strains to *Natrialba magadii* were lower (< 96 %). No significant problems were observed in reading the 16S rDNA sequence. Such problems are usually encountered when mixed cultures or single strains with significant interoperon heterogeneity are sequenced. Thus, we conclude, in addition to observations based on cultivation of the strain, that strain 40^T is a pure culture, showing morphological variation not previously recorded in members of the family *Halobacteriaceae*. Due to the high degree of 16S rDNA sequence similarity between strain 40^T and the two *Natrialba asiatica* strains, no attempt was made to represent the data in the form of a dendrogram. At such high levels of sequence similarity, the resolution of the 16S rDNA data is insufficient to allow one to assign strains unambiguously to different taxa.

In order to investigate whether the three strains belonged to a single species or to several species in the same genus, it was necessary to carry out additional work. Data such as the chemical composition, may be used to examine whether strains belong within the same genus, while biochemical and physiological tests may be used to examine the taxonomic status of strains at the genus and species level. DNA–DNA hybridization experiments may be used to determine whether strains within the same genus belong to the same species.

Chemotaxonomic characterization

TLC analysis of acid hydrolysis products from strain 40^T clearly indicated the presence of diether-linked isoprenoid lipids and no evidence for the presence of fatty acids was obtained. In addition, it was possible to show that strain 40^T produced phytanyl-phytanyl (C20:C20) and sesterpanyl-phytanyl (C25:C20) diethers (Fig. 2).

Analysis of the polar lipids of strain 40^T and the two strains of *Natrialba asiatica* indicated that the same polar lipids were present. In addition to the diether analogues of phosphatidyl glycerol and methyl-phosphatidyl glycerophosphate, both of which occur in all taxa of the family *Halobacteriaceae* examined to date (Tindall, 1992), a single, major glycolipid was detected. The R_f of this compound in both solvent systems indicated that either it was a glycolipid containing four or more sugar units or it contained a shorter sugar backbone, but was further derivatized by the presence of additional functional groups, such as

Table 2. Growth characteristics of *Natrialba aegyptiaca* 40T, *Natrialba asiatica* 172 P1T and *Natrialba taiwanensis* B1TT

Characters are scored as: −, negative; ±, weak; +, normal; + +, good; + + +, very good. Acid production from sugars is scored as: +, final pH less than 6·0; ±, final pH more than 6·0; −, final pH equal to or greater than the original pH. All three strains gave weak reactions in the methyl red test and were positive for growth on 12% KCl and 6% NaCl and on 12% KCl and 3% NaCl, aerobic growth, catalase and oxidase activity, production of acid from xylose, arabinose and sucrose, reduction of nitrate, indole formation, H_2S production from cystine, ammonia production from arginine, urease activity, utilization of NH_4Cl as sole nitrogen source in the presence of starch or glucose as carbon source and susceptibility to fucidic acid (10 µg per disk), bacitracin (10 U), nitrofurantoin (300 µg), novobiocin (5 µg), anisomycin (20 µg), trimethoprim (2·5 µg) and rifampicin (30 µg). All three strains stained Gram-negative, did not require addition of Mg^{2+} and/or K^{+} and were negative for growth in stab cultures, spore formation, denitrification, acetylmethylcarbinol and susceptibility to nystatin (100 U per disk), tetracycline (30 µg), nalidixic acid (30 µg), erythromycin (15 µg), chloramphenicol (10 µg), streptomycin (25 µg) and penicillin G (5 U). diam., Diameter.

Characteristic	*Natrialba aegyptiaca* 40T	*Natrialba asiatica* 172P1T	*Natrialba taiwanensis* BIT T
Temperature for growth (°C):			
Optimum	37–42	30–40	35–40
Maximum	60	50	55
pH for growth:			
Optimum	7–8	6·6–7·0	7·5–7·8
Range	6–9	6–8	5–10
NaCl concentration for growth (M):			
Optimum	2·5–3·0	4·0	3·5
Maximum	Saturation	Saturation	Saturation
Minimum	1·6	2·0	2·0
Pigmentation	Mainly not pigmented*	Not pigmented	Not pigmented
Replacement of NaCl by 20% KCl/1% NaCl	+	−	+
Production of acid from:			
Glucose	+	±	±
Maltose	+	±	±
Lactose	±	−	−
Starch	+	−	+
Degradation of:			
Starch	+ + +	−	+
Casein	+	+	−
Gelatin	+	+	−
Lipase activity against egg yolk (lecithin)	+	+ +	−
Esterase activity against:			
Tween 20	+	+ +	+
Tween 40	+	+ +	+
Tween 80	+	+ +	+
Citrate utilization†	+	−	−
Lysis in distilled water	Only rods lyse	Cells lyse	Cells lyse
Cell form	Rods and cocci (cocci are mainly in tetrads)	Coccobacilli or rods	Coccobacilli or rods
Cell size	Rods, 0·5–1·2 × 1·5–6 µm; cocci, 0·5–2·0 µm diam.	0·5–0·7 × 1–3 µm	0·3–0·5 × 1–1·5 µm
Cell motility	Rods are motile	Motile	Motile
Agar colony	Pulvinate, viscous (1–2 mm diam.)	Convex, smooth (2–3 mm diam.)	Small, smooth (1–2 mm diam.)
Agar slope	Opaque, white to pale yellow	Translucent, white to pale yellow	Translucent, glistening to pale yellow

* Pigmented under certain conditions.

† On Christensen citrate agar (Christensen, 1949).

phosphate or sulfate. Phosphate could not be detected and treatment of the glycolipid to release sulfate indicated that only two sugars were present in the backbone (i.e. a diglycosyl diether lipid was detected). The R_f of the intact glycolipid suggested more than one sulfate was probably present and the results are consistent with the presence of the glycolipid S_2-DGD-1 (Kamekura & Dyall-Smith, 1995). The polar lipid compositions of all three organisms were essentially identical, placing them all in the genus *Natrialba*.

Biochemical and physiological characterization

On the basis of the 16S rDNA sequence similarity values and the chemical composition, together with the fact that strain 40T was not alkaliphilic, further comparison of this strain was made only with the two strains of *Natrialba asiatica*, no further comparison being made with *Natrialba magadii*. The biochemical and physiological characterization of strain 40T and the two *Natrialba asiatica* strains B1T and 172P1T is summarized in Table 2. Cells of strain 40T were mainly non-pigmented, but weak pigmentation was observed on agar plates after long incubation times (> 5 d) on both maintenance and isolation media, even under optimal conditions, or on agar plates containing basal medium plus 1% (w/v) sodium acetate as carbon source. Pigmentation was due to production of bacterioruberins, as determined by the absorption spectrum of methanol/acetone extracts, which showed the characteristic peaks at 494 and 528 nm and a shoulder at 470 nm. Weak growth was observed when

organic nitrogen sources, such as proteose peptone, yeast extract or Casamino acids, were replaced by an inorganic nitrogen source (e.g. NH_4Cl) together with starch or glucose as sole carbon source.

DNA G+C content

The G+C contents for strain 40^T and the two *Natrialba asiatica* strains B1T and $172P1^T$ were respectively $61·5 \pm 0·6$, $62·3 \pm 0·4$ and $62·3 \pm 0·1$ mol% (means of three independent determinations), which are in the range reported for *Natrialba* species (Kamekura & Dyall-Smith, 1995).

Analysis of the extracellular polymer

The new isolate, strain 40^T, was identified primarily on the basis of its ability to produce an extracellular polymer. This property was observed by mucoid colony morphology. Analysis of the extracellular polymer indicated that the polymer was composed of 85% (w/w) L-glutamic acid, 12·5% (w/w) carbohydrates and 2·5% (w/w) unidentified compounds.

DNA–DNA hybridization

DNA–DNA hybridizations were carried out between strain 40^T and the two *Natrialba asiatica* strains B1T and $172P1^T$ and between the two *Natrialba asiatica* strains, in order to examine further the species status of strains B1T and 40^T. The mean levels of DNA–DNA binding between strain 40^T and *Natrialba asiatica* strains B1T and $172P1^T$ were respectively 66·0% (standard deviation 1·8%) and 57·4% (standard deviation 1·8%). The two *Natrialba asiatica* strains showed 55·8% DNA–DNA binding (mean, standard deviation 1·8%). All values were based on four independent determinations.

The DNA–DNA binding values between strain 40^T and the two *Natrialba asiatica* strains $172P1^T$ and B1T indicated that strain 40^T represents a new species within the genus *Natrialba*. This contrasts with the results published by Kamekura & Dyall-Smith (1995), where a value of 86% DNA–DNA binding was reported between the two *Natrialba asiatica* strains. It should be noted that two different methods have been used to study the DNA–DNA hybridization, but we have not been able to account for the differences. Kamekura & Dyall-Smith (1995) did not give the DNA G+C contents of the two strains in their work, nor they give exact details of the hybridization and wash temperatures, making any attempt to reproduce their results speculative. It should be noted that the method used by Kamekura & Dyall-Smith (1995) was also used to study DNA–DNA hybridization of members of the genera *Haloferax*, *Haloarcula* and *Haloterrigena* (Gutiérrez *et al.*, 1989, 1990; Ventosa *et al.*, 1999), where close examination of the DNA–DNA binding values between different strains indicates that some of the reciprocal hybridization results are contradictory or that they contradict observed co-relation with 16S rDNA sequence data, and they have been questioned by other groups (Tindall, 1992; Xin *et al.*, 2000). During the course of our studies, hybridization work was carried out in parallel on a number of strains within the genera *Halobacterium* and *Halorubrum* that were known to be strains of the same species (B. J. Tindall and U. Steiner, unpublished). In both cases, hybridization values between strains of the same species gave values of > 80%. Based on a combination of the DNA–DNA hybridization results obtained in this work and the biochemical tests reported here, we propose that *Natrialba asiatica* strains B1T and $172P1^T$ be recognized as separate species. However, it should also be noted that there are clear differences between strain 40^T and the two *Natrialba asiatica* strains B1T and $172P1^T$ based on morphological, physiological and biochemical tests (Table 2; Kamekura & Dyall-Smith, 1995).

Taxonomic position of *Natrialba asiatica* strains and strain 40^T

The new isolate, strain 40^T, showed a number of morphological, physiological and biochemical differences from *Natrialba asiatica* strains, e.g. the formation of mucoid colonies due to production of extracellular polymer, salt requirement, temperature and pH tolerance, biochemical properties and pigmentation under certain conditions (Table 2; Kamekura & Dyall-Smith, 1995). Furthermore, the different cell shapes (rods, cocci and tetrads), pleomorphism and cell organization, depending on different environmental stimuli, as well as the predominance of cocci and coccobacilli on agar plates (of basal medium plus acetate as carbon and energy sources) were characteristic of strain 40^T. Variations in cell shape for the same species have been described for some members of the family *Halobacteriaceae* (Grant & Larsen, 1989; Montalvo-Rodríguez *et al.*, 1998; Wainø *et al.*, 2000), although the changes we observed in strain 40^T have not been reported previously.

It should be noted that some of the results obtained are different from those recorded by Kamekura & Dyall-Smith (1995), particularly the results for the production of acids from carbohydrates. Careful examination of the methods used by Kamekura & Dyall-Smith (1995) indicates that they were based on previously published methods. However, in the case of the production of acid from carbohydrates, it is not clear whether the method of Rodríguez-Valera *et al.* (1983) (quoted as Torreblanca *et al.*, 1986) or that of Franzmann *et al.* (1988) was used. In the case of Rodríguez-Valera *et al.* (1983), acid production from a concentration of 1% substrate was tested in the presence of 0·5% yeast extract, while Franzmann *et al.* (1988) used a concentration of 0·01%. Such differences in yeast extract concentration may influence the results, either by limiting the extent to which a strain may grow or by the production of amines during the

decarboxylation of amino acids present in the yeast extract. In selecting 0·1% yeast extract, we have followed methods outlined by Tindall (1992) and Oren *et al*. (1997). Overall, the differences observed above justify the inclusion of strain 40^T in the genus *Natrialba* as a new species.

The data presented here illustrate the dilemma between the wish to have species that are easy to differentiate and biological reality. Whereas Colwell *et al*. (1995) write that 'a bacterial species is generally considered to be a collection of strains that show a high degree of overall similarity and differ considerably from related strain groups with respect to many independent characteristics', this is in contradiction to Cowan (1962), who viewed species as potentially overlapping circles. While we are clearly able to distinguish between the three strains studied in this work, we certainly have not been able to show that there are 'considerable differences'. The fact that the strains can be differentiated from one another, but the differences are small, lends support to the view expressed by Cowan (1962), that species are not always easy to differentiate, and different taxonomists may draw different conclusions from the same data. This is probably a more acute problem when organisms share a high degree of genetic and hence also phenotypic similarity. However, based on a combination of the DNA–DNA hybridization results, 16S rDNA nucleotide sequence comparisons, chemotaxonomic data and morphological, physiological and biochemical differences, we propose that strain 40^T be placed within the genus *Natrialba* in a new species, *Natrialba aegyptiaca* sp. nov., and that *Natrialba asiatica* strain B1T be transferred to a new species, *Natrialba taiwanensis* sp. nov.

In view of the fact that we consider *Natrialba asiatica* strains B1T and $172P1^T$ to be different species, the species description of *Natrialba asiatica* must be emended in order to accommodate these changes.

Emended description of *Natrialba asiatica*

Cells are non-pigmented rods or coccobacilli, 0·5–0·7 × 1–3 µm, Gram-negative and motile. The optimum temperature range for growth is 30–40 °C, with a maximum at 50 °C. The pH range for growth is pH 6–8, with an optimum in the range pH 6·6–7. The NaCl range for growth is from 2·0 M to saturation, with an optimum at 4·0 M. NaCl may be replaced to a large extent by KCl (Table 2). Aerobic, catalase and oxidase-positive. Acid produced from xylose, arabinose, sucrose, glucose (weak) and maltose (weak) but not from lactose or starch. Casein and gelatin are hydrolysed, but not starch. Strongly lipolytic on Tweens 20, 40 and 80, as well as using egg yolk (lecithin) as substrate.

Isolated from beach sand in Japan. The type strain is strain $172P1^T$, deposited in the DSMZ as DSM 12278^T and in the Japan Collection of Microorganisms, Wako, Saitama, Japan, as JCM 9576^T.

Description of *Natrialba aegyptiaca* sp. nov.

Natrialba aegyptiaca (ae.gyp′ti.a.ca. L. adj. *aegyptiaca* from Egypt, referring to the geographical region from which this organism was isolated).

Generally non-pigmented, but slightly pigmented under certain conditions, especially when the agar basal medium plus 1% (w/v) sodium acetate is used as carbon and energy sources or after long periods on isolation and maintenance media. Cells are either rods, 0·5–1·2 × 1·5–6 µm, or cocci and tetrads, individual cells measuring 0·5–2·0 µm in diameter; cells are pleomorphic, especially under suboptimal conditions. Cocci and coccobacilli dominate when acetate or gluconate is added to the basal medium as carbon and energy source. Gram-negative; rods are motile. The optimal temperature for growth is 37–42 °C, with a maximum at 60 °C. The pH range for growth is pH 6–9, with an optimum in the range pH 7–8. The NaCl range for growth is 1·6 M to saturation, with an optimum in the range 2·5–3·0 M. NaCl may be replaced to a large extent by KCl (Table 2). Aerobic, catalase- and oxidase-positive. Acid is produced from xylose, arabinose, glucose, sucrose, maltose, lactose (weak) and starch. Strongly amylolytic; casein and gelatin are also hydrolysed. Tweens 20, 40 and 80 as well as egg yolk (lecithin) are hydrolysed. Produces an exopolymer, which is composed of L-glutamic acid (85% w/w), carbohydrates (12·5% w/w) and unidentified compounds (2·5% w/w).

Isolated from a salt soil close to Aswan, Egypt. The type strain is strain 40^T, which has been deposited in the DSMZ as DSM 13077^T and in the Japan Collection of Microorganisms, Wako, Saitama, Japan, as JCM 11194^T.

Description of *Natrialba taiwanensis* sp. nov.

Natrialba taiwanensis (tai.wan.en′sis. N.L. gen. n. *taiwanensis* of Taiwan, referring to the isolation of the organism from solar salts produced in Taiwan).

Non-pigmented rods or coccobacilli, 0·3–0·5 × 1–1·5 µm. Cells are Gram-negative and motile. The optimum temperature for growth is 35–40 °C, with a maximum at 55 °C. The pH range for growth is pH 5–10, with an optimum in the range pH 7·5–7·8. The NaCl range for growth is 2·0 M to saturation, with an optimum at 3·5 M. NaCl may be replaced to a large extent by KCl (Table 2). Aerobic, catalase- and oxidase-positive. Acid is produced from xylose, arabinose, glucose (weak), sucrose, maltose (weak) and starch but not from lactose. Amylolytic, but casein and gelatin are not hydrolysed. Tweens 20, 40 and 80 are hydrolysed but not egg yolk (lecithin). This species was isolated by Kamekura & Dyall-Smith (1995) and was classified initially as *Natrialba asiatica* strain B1T. Classification of this strain as a new species is based on DNA–DNA hybridization values as well as on morphological, physiological and biochemical differences between *Natrialba asiatica* $172P1^T$, [*Natrialba asiatica*] B1T and *Natrialba aegyptiaca* strain 40^T (see Table 2).

Isolated from solar salts produced in Taiwan. The type strain is strain B1T^{T}, which has been deposited in the DSMZ as DSM 12281^{T} and the JCM as JCM 9577^{T}.

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