Haloferax alexandrinus sp. nov., an extremely halophilic canthaxanthin-producing archaeon from a solar saltern in Alexandria (Egypt)

Dalal Asker^{1,2} and Yoshiyuki Ohta²

¹ Food Science and Technology, Faculty of Agriculture, Alexandria University, Egypt

² Faculty of Applied Biological Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima 739-8528, Japan

Author for correspondence: Yoshiyuki Ohta. Tel: +81 824 24 7923. Fax: +81 824 24 7923.
e-mail: kohta@hiroshima-u.ac.jp

An extremely halophilic red micro-organism designated strain TM^T was isolated from a solar saltern in Alexandria, Egypt. The micro-organism stains Gram-negative, is very pleomorphic, non-motile and strictly aerobic and requires at least 10 g NaCl l⁻¹ for growth. The growth optimum is 250 g NaCl l⁻¹. Growth is also observed over a wide range of MgSO₄ concentrations (10–40 g l⁻¹). Aerobic reduction of nitrate without gas production was detected. Cells grew aerobically in a minimal salts medium containing ammonium chloride and glucose. Strain TM^T produced acid from fructose, glucose, rhamnose, maltose and glycerol. The G+C content of the DNA was 59.5±0.3 mol%. On the basis of polar lipid analysis, the isolate belonged to the genus *Haloferax*. Analysis of the 16S rDNA sequence showed the highest similarity (>99%) to be to the type strain *Haloferax volcanii*. Although the spectrum of antibiotic susceptibility was similar to that of validly described species of the genus *Haloferax*, the strain could be distinguished from them by its different response to josamycin and rifampicin. Strain TM^T is unique within the genus *Haloferax* in producing canthaxanthin. Comparative analysis of phenotypic properties and DNA–DNA hybridization between strain TM^T and *Haloferax* species supported the conclusion that TM^T is a novel species within this genus, for which the name *Haloferax alexandrinus* sp. nov. is proposed. The type strain is TM^T (= JCM 10717^T = IFO 16590^T).

Keywords: extremely halophilic bacteria, *Haloferax alexandrinus* sp. nov., Archaea, carotenoids, canthaxanthin

INTRODUCTION

Extremely halophilic archaea are chemo-organo-trophic organisms that satisfy some of their energy requirements with light. These archaea are classified in one order, *Halobacteriales*, and one family, *Halobacteriaceae* (Grant & Larsen, 1989). Recently, 16S rDNA sequencing, DNA–DNA hybridization, polar lipid analysis and other studies have recognized 15 genera. The currently recognized genera are *Halobacterium*, *Haloarcula*, *Haloferax*, *Halococcus*, *Natronobacterium*, *Natronococcus* (Grant & Larsen, 1990; Tindall *et al.*, 1984; Tindall, 1992; Torreblanca *et al.*, 1986),

Halorubrum (McGenity & Grant, 1995), *Halobaculum* (Oren *et al.*, 1995), *Natrialba* (Kamekura & Dyall-Smith, 1995), *Natronomonas* (Kamekura *et al.*, 1997), *Halogeometricum* (Montalvo-Rodríguez *et al.*, 1998), *Natrinema* (McGenity *et al.*, 1998), *Haloterrigena* (Ventosa *et al.*, 1999), *Natronorubrum* (Xu *et al.*, 1999) and *Halorhabdus* (Wainø *et al.*, 2000).

Members of the family *Halobacteriaceae* are characterized by red-coloured cells, the colour mainly being due to the presence of C₅₀-carotenoids (bacterioruberins) as the major carotenoids (Kushwaha *et al.*, 1974; Rønnekleiv & Liaaen-Jensen, 1992, 1995). Some members of the genera *Halobacterium* and *Haloarcula* have been reported to partially produce C₄₀-carotenoids and ketocarotenoids such as β-carotene, lycopene, 3-hydroxy echinenone and *trans*-astaxanthin as the minor carotenoids (Kelly *et al.*, 1970; Kushwaha

Abbreviation: S-DGD-1, sulfated diglycosyl diether.

The GenBank/EMBL/DBJ accession number for the 16S rDNA sequence of *Haloferax alexandrinus* strain TM^T (= JCM 10717^T = IFO 16590^T) is AB037474.

et al., 1972, 1974, 1982; Kushwaha & Kates, 1973; Calo *et al.*, 1995). Recently, the biotechnological potential of these members of the *Archaea* has increased because of their unique features, which facilitate many industrial procedures. For example, no sterilization procedures are required, because of the extremely high NaCl concentration used in the growth medium; this is useful for preventing contamination by other organisms. In addition, no cell-disrupting devices are required, as cells lyse spontaneously in fresh water, and these micro-organisms are able to utilize single carbon sources such as sugars, acetate or succinate for growth (Rodríguez-Valera *et al.*, 1980; Kauri *et al.*, 1990; Calo *et al.*, 1995; Asker & Ohta, 1999). In summer at El-Mallahet, a solar saltern near Alexandria City in Egypt, the temperature ranges from 37 to 40 °C. As a result, the concentration of the total dissolved salts increases to saturation at pH 7.2. It is noteworthy that under these extreme conditions the surface of the saltern is characterized by a reddish purple colour corresponding to the growth of red halophilic members of the *Archaea*; other organisms cannot survive there. In a previous work, an attempt was made to find a new biological source of canthaxanthin by isolating 31 red, extremely halophilic microbes from this saltern. Of the strains isolated, strain TM^T produced the highest levels of carotenoids [2.06 mg (g dry cells)⁻¹], including β -carotene, 3-hydroxy echinenone, bacterioruberins, and a remarkable amount of canthaxanthin [700 μ g (g dry cells)⁻¹] (Asker & Ohta, 1999); however, among the *Archaea*, production of canthaxanthin has not been reported previously. To the authors' knowledge, no other ecological studies on the halobacteria of this saltern have been performed, although this saltern may be a rich source diverse halophilic bacteria.

In the present work, strain TM^T was further characterized. On the basis of the 16S rRNA gene sequence, the polar lipid composition, physiological analysis and DNA–DNA hybridization, the creation of a new species for the genus *Haloferax* appears to be justified.

METHODS

Strains and culture conditions. The reference strains used were obtained from the Japan Collection of Microorganisms (JCM) and are listed in Table 3. These strains were grown at 37 °C in a complex medium containing (in g l⁻¹ unless otherwise indicated): Bacto Casamino acids (Difco), 5; Bacto yeast extract (Difco), 5; sodium glutamate, 1; trisodium citrate, 3; MgSO₄·7H₂O, 20; KCl, 2; NaCl, 200; FeCl₂·4H₂O, 36 mg l⁻¹ and MnCl₂·4H₂O, 0.36 mg l⁻¹. The pH was adjusted to 7.2 with 1 M NaOH.

Strain TM^T was grown in a standard growth medium (Asker & Ohta, 1999) containing (in g l⁻¹): yeast extract, 10; Casamino acids, 7.5; NaCl, 250; MgSO₄·7H₂O, 40; KCl, 2; trisodium citrate, 3; and trace-elements solution, 10 ml. The trace-elements solution contained the following (in mg 100⁻¹ ml): FeCl₂·4H₂O, 2.3; CaCl₂·7H₂O, 7; MnSO₄·H₂O, 0.3; ZnSO₄, 0.44; CuSO₄·5H₂O, 0.050. The pH was adjusted to 7.2 with 1 M NaOH. The culture was incubated on a shaker at 37 °C for 7 d.

Morphological, biochemical and physiological characterization. The phenotypic tests were performed with strain TM^T in accordance with the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). All tests were carried out using the optimal growth conditions defined in our previous work (Asker & Ohta, 1999). In brief, the micro-organism was identified on the basis of colony morphology by streaking on a standard growth agar medium, incubated at 37 °C for 7 d. Motility was studied by using the 'hanging drop' technique and the deep-agar method. Anaerobic growth was tested in the standard growth medium in the presence of 5 g l⁻¹ nitrate, L-arginine. HCl or DMSO in completely filled stoppered tubes. Controls without additives were included, and all incubations were performed in the dark. *Haloferax denitrificans* was used as a positive control for the formation of nitrite and gas from nitrate, and *N. pellirubrum* JCM 10476^T served as a positive control for the anaerobic growth on arginine. The Voges–Proskauer test was performed; in addition, tryptophan deaminase and the utilization of citrate were tested for by using API-20 E (bioMérieux). Arginine dihydrolase, lysine and ornithine decarboxylase were tested for by using the method of Skerman (1967), modified by the addition of the following (in g l⁻¹): NaCl, 250; MgSO₄·7H₂O, 40; KCl, 2; and trisodium citrate, 3. Phosphatase activity was tested for by adding 1% (w/v) aqueous phenolphthalein diphosphate solution to the standard growth medium; β -galactosidase activity was detected using ONPG. Variation of pigmentation at different salt concentrations was determined on standard growth agar media at NaCl concentrations of 100, 150, 200 and 250 g l⁻¹. Hydrolysis of Tween 80 and gelatin was tested as described by Gutierrez & Gonzalez (1972). Gram staining was carried out as described by Dussault (1955). Other bacteriological tests were carried out as described by Gibbons (1957). The production of acids from different sugars was tested for by using the API 50 CHE test (bioMérieux) in the standard growth medium except that the yeast extract was omitted, the concentration of Casamino acids was reduced to 5 g l⁻¹, and 0.18 g phenol red l⁻¹ was added. The final pH was adjusted to 7.2, and incubation was done at 37 °C for 2–4 d.

To test for the ability to grow on single carbon sources, a chemically defined medium was used. This medium was prepared by omitting the yeast extract and the Casamino acids from the standard growth medium and adding the carbon sources being tested, as follows (in g l⁻¹): glycerol, 0.5; sodium succinate, 4.5, or glucose, 10; supplemented by 0.27 g l⁻¹ NH₄Cl. The production of poly β -hydroxy butyrate was examined by growing the cells in the chemically defined medium, in which the NH₄Cl content was reduced to 0.005% (w/v). Furthermore, 1% (w/v) glucose was added. The presence of poly β -hydroxy butyrate was tested according to Gerhardt *et al.* (1981). Tolerance for high concentrations of the Mg²⁺ cation was determined at 123.2, 197.2 and 394.4 g l⁻¹ and up to the saturation of MgSO₄·7H₂O in the standard growth medium, containing 150 or 250 g NaCl l⁻¹.

Susceptibility to antibiotics was tested by spreading 100 μ l exponential-phase cultures (3–4 d) on the standard growth agar medium plate and applying antibiotic discs (Becton Dickinson microbiology system: chloramphenicol, 30 μ g; erythromycin, 15 μ g; neomycin, 30 μ g; josamycin, 30 μ g; rifampicin, 5 μ g; novobiocin, 30 μ g; bacitracin, 10 μ g and tetracycline, 30 μ g). Zones of inhibition were recorded after incubation at 37 °C for 6 d. Novobiocin, bacitracin, rifampicin and josamycin were further tested in broth at the

Table 1. Primers used in the amplification and sequencing of 16S rDNA of strain TM^T

Primer name*	Sequence (5' → 3')	Position†	Reference
Forward			
F1‡	attccggttgatcctgccgg	1–20	Kamekura & Seno (1992)
F2‡	tccgacagtgaggacgaaa	688–707	Kamekura & Seno (1992)
TMF3	cgcagcaggcgcaaacctt	329–348	This study
TMF4	gcaacgagcgagaccg	1039–1055	This study
TMF5	gagaggaggtgcatggccg	983–1001	This study
Reverse			
R1‡	cccgggtatctaattccggtt	720–739	Kamekura & Seno (1992)
R2‡	aggaggtgatccagccgcag	1453–1472	Kamekura & Seno (1992)
TMR3	gtattaccgcgcggtg	457–474	This study
TMR4	gcgacggcggtgtgtgc	1340–1357	This study

* All of the primers listed above were used to sequence the complete 16S rDNA in this study.

† Numbers correspond to positions in the sequence with GenBank accession number D11107.

‡ Two sets of forward and reverse primers (F1 + R1 and F2 + R2) were used to amplify two PCR products of approximately 740 bp (1–739) and 784 bp (688–1472), respectively, of strain TM^T 16S rDNA.

following concentrations: 5, 10, 20, 30, 40, 50, 60, 100, 150, 180 and 200 µg ml⁻¹.

Phase-contrast microscopy and scanning electron microscopy. Micrographs were prepared from exponential-phase cells grown in the standard growth medium. Drops of the culture were mixed on a microscope slide with an equal volume of melted 2% (w/v) agarose containing 25% (w/v) NaCl, and covered with a cover-slip. For examination with scanning electron microscopy, 4 µl culture was treated with 2% (w/v) glutaraldehyde solution and transferred to plastic slides. These specimens were subsequently dehydrated through an ethanol gradient of 30, 50, 75 and 95%, then subjected to an overnight dehydration in 100% ethanol. Drying was accomplished by using an Hitachi HCP-2 Critical Point Dryer. The specimens were placed on self-adhesive conducting aluminium tape and gold-coated using an Hitachi E-1030 Sputter Ion. The specimen was examined using a JEOL JSM-5800LV electron microscope at 20 kV. Micrographs were recorded at magnifications of 3500. Images were scanned and electronically enhanced using the ADOBE PHOTO DELUXE and POWERPOINT editing programs.

Lipid analysis. Core lipids were isolated and analysed by using TLC as described by Minnikin *et al.* (1975) and Ross *et al.* (1981). Freeze-dried cells (100 mg) were hydrolysed in methanol/toluene/concentrated H₂SO₄ (3:3:0.1, by vol.) at 55 °C for 15–16 h; this was followed by extraction with 1 ml hexane. The hexane extract was concentrated in a vacuum, then chromatographed on silica gel 60 F₂₅₄ glass-packed thin-layer plates (20 × 20 cm; Merck), using petroleum ether/diethyl ether (85:15, v/v) as the developing solvent. The lipids were visualized by spraying with 10% (w/v) ethanolic dodecamolybdophosphoric acid, followed by heating at 160 °C for 15 min. Polar lipids were extracted from 1 g freeze-dried cells with methanol/chloroform/saline (2:1:0.8, by vol.) as described by Kates (1986). The polar lipids were separated using TLC on silica gel plates (10 × 20 cm; Merck), using single development as described by Torreblanca *et al.* (1986) and Oren *et al.* (1996, 1999). The following two different solvent systems were used: chloroform/methanol/acetic acid/water (85:22.5:10:4 or 80:12:

15:4, by vol.) (Montalvo-Rodríguez *et al.*, 1998). Glycolipid spots were detected by spraying the plates with 0.5% α-naphthol in 50% (v/v) methanol and then 50% (w/v) H₂SO₄ in ethanol, before heating them at 150 °C (Torreblanca *et al.*, 1986), or by spraying them with 0.1% CeSO₄ in 1 M H₂SO₄, followed by heating at 150 °C for 5 min (i.e. a general lipid stain, allowing differentiation of glycolipids from other lipids by colour) (Kates, 1972). Ammonium molybdate/sulfuric acid reagent was used for the detection of phospholipids.

Whole-cell protein profiles. Colonies were taken from standard growth agar plates, and whole-cell proteins were extracted by boiling in SDS-PAGE sample buffer for 15 min. Five microlitres of the solution mixture was separated by SDS-PAGE, according to the procedure described by Laemmli (1970), using an ATTO AE-7300 compact PAGE unit at a constant current of 20 mA per gel for approximately 1 h. Proteins were stained with 0.5% Coomassie brilliant blue and destained in an aqueous solution of 10% (v/v) acetic acid and 25% (v/v) methanol. High-molecular-mass and low-molecular-mass markers (Amersham Pharmacia Biotech) were applied. The gel was scanned and analysed using Scanning Gel (version 1.1, PDQUEST MAC, Diversity Database; pdi). All separated band weights were calculated from those data.

Plasmid analysis. A rapid procedure for the detection of large and small plasmids was performed (Kado & Liu, 1981).

DNA base composition and 16S rDNA sequence analysis. Determination of the G+C content of the DNA was performed by using HPLC (Katayama-Fujimura *et al.*, 1984). Exponential-phase cells (72 h) grown in the standard growth medium at 37 °C on a rotary shaker were lysed in distilled water, and total DNA was extracted and purified using the method of Marmur (1961). The purified DNA was dissolved in distilled water (1 mg ml⁻¹) and then was heated at 100 °C for 15 min before being cooled rapidly in an ice bath. To the denatured DNA (10 µg) was added 10 µl nuclease P1 solution (2 U ml⁻¹ 40 mM sodium-acetate buffer containing 2 × 10⁻⁴ M ZnCl₂, pH 5.3) before incubation at

50 °C for 1 h. Standard solution and the hydrolysate were subjected separately to HPLC. The 16S rDNA was amplified using the PCR method of Embley (1991), modified by McGenity & Grant (1993). Four primers (forward, F1 and F2; reverse, R1 and R2) (Table 1) were designed from very conserved regions of 16S rDNA of halophilic archaea (Hui & Dennis, 1985; Kamekura & Seno, 1992). These primers are numbered according to *Haloferax mediterranei* 16S rDNA (accession no. D11107), and were applied in two independent PCR reactions, resulting in two PCR products comprising positions 1–740 and 688–1472 of the 16S rDNA sequence. Purified PCR products were sequenced automatically using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) as directed by the manufacturer's protocol, employing nine primers (Table 1). Sequencing reactions were electrophoresed using the Applied Biosystems 377 ABI PRISM DNA Sequencer (Applied Biosystems). A total of 1472 bases were identified during these analyses. The phylogenetic position of the resulting sequence of strain TM^T was determined using the EMBL database and FASTA (Pearson & Lipman, 1988). Similarity values were calculated from the alignment of the strain TM^T sequence with sequences from the following organisms: *Haloferax volcanii* ATCC 29605^T (accession no. K00421^T), *Haloferax mediterranei* ATCC 33500^T (D11107), *Haloferax denitrificans* ATCC 35960^T (D14128) and *Haloferax gibbonsii* ATCC 33959^T (D13378), using CLUSTAL W (version 1.8) multiple sequence alignments (Thompson *et al.*, 1994). Alignment gap base positions were not taken into consideration for the calculation. Each 1467-base sequence of all five strains was used for the calculation. The evolutionary distance matrix was used to determine the similarity (Kimura, 1980). The programs used for this analysis can all be found in the PHYLIP program (version 3.5.1) (Felsenstein, 1993).

Preparation of labelled DNA, and DNA–DNA hybridization.

The DNA of strain TM^T was nick-translated with [α -³²P]-dCTP by using a nick-translation kit (Boehringer Mannheim). The labelled DNA was purified with Quick Spin Columns, Sephadex G-25 (Boehringer Mannheim) and ethanol precipitation. The mean specific activity obtained with this procedure was 3×10^8 d.p.m. (μ g DNA)⁻¹. The labelled DNA was denatured prior to hybridization, by heating at 100 °C (block incubator; ASTEC) for 5 min, and then placed on ice. DNA–DNA hybridization was performed by using the competition procedure of the membrane method (Johnson, 1994). Competitor DNAs were sonicated at 50 W for two 15 s time intervals. Membrane filters (HAHY; Millipore) containing reference DNA (25 mg cm⁻²) were placed in 100 ml screw-cap vials that contained the labelled, sheared, denatured DNA and the denatured, sheared competitor DNAs. The ratio of the concentrations of competitor to labelled DNA was at least 150:1. The final volume and concentration were adjusted to 5 ml, $2 \times$ SSC and 30 % formamide. Hybridization was performed for 18 h in a hybridization incubator (HB-100; TAITEC) at 56 °C, which is within the temperature limits for the method (De Ley & Tjittgat, 1970). After hybridization, the filters were washed in $2 \times$ SSC at 56 °C. The radioactivity bound to the filters was measured in a liquid-scintillation counter (Beckman Instruments), and the percentage homology was calculated according to Johnson (1994). At least three independent determinations were performed.

Fig. 1. Morphology of strain TM^T. (a) Scanning electron micrograph, (b) phase-contrast photomicrograph and (c) light-microscope photomicrograph, showing the pleomorphic forms including cocci, ovals, triangles, squares and rods. Bars, 5 μ m.

RESULTS

Morphological, biochemical, cultural and physiological characteristics of strain TM^T

Colonies of strain TM^T on standard growth agar medium were circular, convex, entire, translucent, smooth and red. The colonies appeared as a very small points within 2–3 d, and the size increased up to 0.5–1 mm in diameter after 6 d incubation. The pigment intensity of the colonies was affected by the salt concentration: at 250 g NaCl l⁻¹, the colonies were orange-red, but reducing the concentration to 150 g NaCl l⁻¹ increased the pigment intensity to deep red. Gas vacuoles were never observed. The cells stained negative with Gram staining and were pleomorphic (irregular cocci, short and long rods, squares, triangles and ovals) when grown on the standard growth medium at the optimum growth conditions (Fig. 1).

Table 2. Characteristics that distinguish strain TM^T from the validly described species within the genus *Haloferax*

Strains: 1, TM^T; 2, *Haloferax volcanii* NCBM 2012^T; 3, *Haloferax denitrificans* ATCC 35960^T; 4, *Haloferax mediterranei* ATCC 33500^T; 5, *Haloferax gibbonsii* ATCC 33959^T. Data on the validly described species of the genus *Haloferax* were obtained from Calo *et al.* (1995), Grant & Larsen (1990), Juez *et al.* (1986), Mullakhanbhai & Larsen (1975), Nieto *et al.* (1993), Oren *et al.* (1995), Rodríguez-Valera *et al.* (1980, 1983), Rønnekleiv & Liaaen-Jensen (1995), Tindall *et al.* (1989), Tindall (1992), Tomlinson *et al.* (1986), Torreblanca *et al.* (1986). +, Positive; −, negative; ±, varied; ND, not determined.

Characteristic	1	2	3	4	5
Gas vacuoles (production)	−	−	−	+	−
Anaerobic growth in the presence of nitrate	−	−	+	+	−
Starch hydrolysis	−	−	−	+	−
Tween 80 hydrolysis	+	−	−	+	+
Gelatin hydrolysis	+	−	+	+	+
Casein hydrolysis	−	−	−	+	+
Gas produced from nitrate	−	−	+	+	−
H ₂ S produced from cysteine	−	+	−	−	−
Mg ²⁺ requirements for growth (g l ^{−1})	10–40	4.9–9.9	ND	4.9–9.9	2.5–4.9
NaCl range for growth (g l ^{−1})	100–320	−	88–263	76–270	100–320
Optimum NaCl for growth (g l ^{−1})	250	146	110–180	160	200–250
Cell stability (NaCl, g l ^{−1})	100	30	88	30	30–40
Acid produced from:					
Mannose	−	ND	−	ND	+
Galactose	−	+	+	ND	+
Lactose	−	+	−	+	ND
Resistant to antibiotics (µg ml ^{−1})					
Josamycin (180)	+	+	−	−	−
Rifampicin (100)	+	−	−	−	−
Carotenoid composition†	1, 3, 4, 5	2, 5	5	3, 5	5

* Only sparse growth occurs at 233 g l^{−1} (Mullakhanbhai & Larsen, 1975).

† The carotenoid composition is as follows: 1, β-carotene; 2, lycopene; 3, 3-hydroxy-echinenone; 4, canthaxanthin; 5, bacterioruberins.

Cell dimensions varied from 1.1 to 1.5 µm × 1.6–2.0 µm; rod-shaped cells were 1.1–1.5 × 3.5–4 µm in size. This strain was not motile. Upon gradual dilution of the culture in 250 g NaCl l^{−1} with water, the cells changed in shape from pleomorphic forms to spheres, and the spheres then underwent lysis below 100 g NaCl l^{−1}. Unlike *Haloferax volcanii*, cells did not become sphaeroplasts in the presence of low concentrations of magnesium and calcium (Cohen *et al.*, 1983). It was a strict aerobe and was unable to grow anaerobically by using alternative electron acceptors such as nitrate or DMSO, or by fermenting L-arginine. However, the aerobic reduction of nitrate and nitrite without gas production was detected. The Voges–Proskauer test was negative. Catalase and oxidase activity tests were positive. The cells hydrolysed gelatin and Tween 80 but not starch or casein. Indole was formed from tryptone. H₂S was produced from sodium thiosulfate. The arginine dihydroxylase was negative; in the absence of this enzyme, the mechanism supporting anaerobic growth on arginine cannot operate (Hartmann *et al.*, 1980). The strain was positive for β-galactosidase, phosphatase and tryptophan deaminase activities. Tests for lysine decarboxylase and ornithine decarboxylase were negative. The urease test was

negative. Poly β-hydroxybutyrate was not detected. In the standard growth medium, a high growth rate was observed at 250 g NaCl l^{−1} and at 123.2 g MgSO₄ l^{−1}. At the same concentration of NaCl, the strain tolerated elevated concentrations of MgSO₄ up to 394.4 g l^{−1}, but the lag phase was extended for several days. However, with the reduction in NaCl concentration to 125 g l^{−1} in the same medium, strain TM^T could tolerate up to 493 g MgSO₄ l^{−1}. Amino acids and yeast extract in the complex medium supported growth. Growth was not supported by polypeptone, tryptone or casein. The strain was able to grow in chemically defined media containing ammonium chloride and single carbon sources such as glucose, glycerol and succinate. However, the addition of a mixture of biotin (1 µg ml^{−1}) and thiamine (8 µg ml^{−1}) to the chemically defined medium (with succinate and glycerol as carbon sources) could replace yeast extract and Casamino acids (complex medium) in supporting growth of the culture. Acid production from a variety of sugars (fructose, glucose, rhamnose, maltose, L-arabinose, D-xylose, ribose, sucrose, N-acetyl gluconate and glycerol) was observed. Lactose, galactose, mannose, citrate and starch were not utilized. Strain TM^T was susceptible to 30 µg novobiocin ml^{−1} and 30 µg bac-

Fig. 2. SDS-PAGE gel of whole-cell proteins. Lanes: 1, high-molecular-mass marker; 2, strain *TM*^T; 3, *Haloferax volcanii* JCM 8879^T; 4, *Haloferax denitrificans* JCM 8864^T; 5, *Haloferax mediterranei* JCM 8866^T; 6, *Haloferax gibbonsii* JCM 8863^T; 7, *Halorubrum saccharovororum* JCM 8865^T; 8, *Natrinema pellirubrum* JCM 10476^T; 9, *Haloarcula vallismortis* JCM 8877^T; 10, low-molecular-mass marker. The high-molecular-mass marker contained the following (approx. molecular masses in kDa): myosin (212); α_2 -macroglobulin (170); β -galactosidase (116), transferrin (76) and glutamic dehydrogenase (53). The low-molecular-mass marker contained the following (kDa): phosphorylase b (94), BSA (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20) and α -lactalbumin (14).

tracin ml⁻¹, but was resistant to 10–20 μ g bacitracin ml⁻¹, 30 μ g chloramphenicol, 15 μ g erythromycin, 30 μ g neomycin, 180 μ g josamycin, 100 μ g rifampicin and 30 μ g tetracycline. Furthermore, the susceptibility to antibiotics in comparison with that of four reference strains was tested: *N. pellirubrum* JCM 10476^T, *Haloferax mediterranei* and *Halorubrum saccharovororum* JCM 8865^T were susceptible to 5 μ g rifampicin; only *Haloferax mediterranei* is susceptible to 15 μ g erythromycin. Table 2 shows the comparisons of the phenotypic features of strain *TM*^T and those of the type strains of *Haloferax* species.

Lipid analysis

Strain *TM*^T appeared to contain the diphytanyl diether moieties (C₂₀–C₂₀) as core diether lipids, tetraether lipids being absent. The polar lipids detected were phosphatidylglycerol, phosphatidylglycerophosphate-methyl ester, sulfated diglycosyl diether (S-DGD-1) and diglycosyl diether. On the TLC, S-DGD-1 of strain *TM*^T runs similarly to S-DGD-1 from species of *Haloferax* (S-DGD-1 is the glycolipid marker of *Haloferax*). The sulfated tetraglycosyl diether of *N. pellirubrum* JCM 10476^T, the triglycosyl diether of *Haloarcula vallismortis* JCM 8877^T and the S-DGD-3 of *Halorubrum saccharovororum* JCM 8865^T were not detected. Moreover, strain *TM*^T does not contain phosphatidylglycerosulfate, which is present in *Halorubrum saccharovororum* JCM 8865^T, *N. pellirubrum* JCM 10476^T and *Haloarcula vallismortis* JCM 8877^T.

Whole-cell protein profiles

Similarities between strain *TM*^T and *Haloferax* species were also observed in the whole-cell protein profiles, as determined by SDS gel electrophoresis (Fig. 2). On the other hand, these protein profiles clearly differentiated strain *TM*^T from members of the genera *Natrinema*, *Halorubrum* and *Haloarcula*. Moreover, among the *Haloferax* species, strain *TM*^T could be differentiated by a distinct 66·51 kDa band of polypeptide. A quantitative analysis of the relatedness of the protein patterns indicated that strain *TM*^T was closely related to species of *Haloferax*.

Characterization of a plasmid in strain *TM*^T

Two small plasmids with of 3 and 2 kbp were identified in cells of strain *TM*^T after DNA extraction and agarose gel electrophoresis.

DNA base composition and 16S rDNA sequence analysis

The G + C content of strain *TM*^T was 59·5 $\pm$ 0·3 mol %, as determined by HPLC (mean of three independent determinations). The G + C content is within the range

Table 3. Levels of DNA–DNA relatedness between strain *TM*^T and other halobacterial species

Values are means of three experiments, which did not differ by more than 5%. ^T, Type strain.

Source of unlabelled DNA	DNA hybridization (%) with ³² P-labelled DNA from <i>TM</i> ^T
Strain <i>TM</i> ^T (= JCM 10717 ^T = IFO 16590 ^T)	100
<i>Haloferax volcanii</i> JCM 8879 ^T (= ATCC 29605 ^T)	49
<i>Haloferax denitrificans</i> JCM 8864 ^T (= ATCC 35960 ^T)	27
<i>Haloferax mediterranei</i> JCM 8866 ^T (= ATCC 33500 ^T)	21
<i>Haloferax gibbonsii</i> JCM 8863 ^T (= ATCC 33959 ^T)	36
<i>Halorubrum saccharovororum</i> JCM 8865 ^T (= ATCC 29252 ^T)	0
<i>Natrinema pellirubrum</i> JCM 10476 ^T (= NCIMB 786 ^T)	0
<i>Haloarcula vallismortis</i> JCM 8877 ^T (= ATCC 29715 ^T)	0

reported for *Haloferax* species (59.5–66, Table 2). The complete 16S rDNA sequence of strain TM^T was determined. The sequence was compared with the published 16S rDNA sequences of representative members of the *Archaea*. The sequence showed a high degree of similarity (99.7%) to the sequence of *Haloferax volcanii* (K00421), demonstrating that the two strains are indeed closely related but not identical. Strain TM^T was related to *Haloferax denitrificans* (99.3% similarity), *Haloferax gibbonsii* (99.2% similarity) and *Haloferax mediterranei* (98.2% similarity). It is noteworthy to mention that the similarity values between four distinguished species of the genus *Haloferax* are 98.3–99.5%, when the alignment gap base positions are not taken into consideration for the calculation. Because of the high degree of 16S rDNA sequence similarity between strain TM^T and *Haloferax volcanii* as well as other members of this genus, no attempt was made to reconstruct a phylogenetic tree.

DNA–DNA hybridization studies

Data on the DNA–DNA hybridization between the reference strains and strain TM^T are shown in Table 3. The relatedness obtained between DNAs from the reference strains and the ³²P-labelled DNA from strain TM^T showed that there was a low level of hybridization among them, the values ranging from 49 to 0%. Thus, the DNA–DNA hybridization confirmed that strain TM^T is differentiated at the species level from the other representatives of the genus *Haloferax*.

DISCUSSION

The presence of the glycerol diether moieties and the absence of glycerol ester lipid indicate that strain TM^T is an archaeon (Ross *et al.*, 1981; Torreblanca *et al.*, 1986). The high salt requirement (at least 100 g NaCl l⁻¹) for growth, the lysis at low salinity, the resistance to chloramphenicol, erythromycin, neomycin, josamycin, rifampicin and tetracycline (antibiotics which inhibit the growth of halophilic eubacteria) and the susceptibility to novobiocin and bacitracin are all characteristics of the family *Halobacteriaceae* (Tindall, 1992). Strain TM^T is characterized by the lack of phosphatidylglycerol sulfate and the presence of S-DGD-1 and C₂₀–C₂₀ in the absence of C₂₀–C₂₅ core diether lipids. These features of strain TM^T are consistent with those of the genus *Haloferax* (Tindall, 1992; Tindall *et al.*, 1989; Torreblanca *et al.*, 1986; Kamekura & Dyall-Smith, 1995). Electrophoresis of the whole-cell protein profile has been used to differentiate between taxa of the *Halobacteriales* (Hesselberg & Vreeland, 1995; McGenity *et al.*, 1998). The electrophoresis demonstrated that strain TM^T is related to the genus *Haloferax*. On the basis of its 16S rDNA sequence, strain TM^T clearly demonstrated its affiliation with representatives of the genus *Haloferax*. The greatest similarity percentages were obtained with 16S rDNA sequence from *Haloferax volcanii* and *Haloferax denitrificans* (99.7 and 99.3%, respectively). In

the same manner, *Haloferax volcanii* and *Haloferax denitrificans* shared 99.5% similarity. Hezayen *et al.* (2001) have reported a high degree of similarity (< 99%) between the 16S rDNA sequences of the two species. Therefore, it was necessary to carry out DNA–DNA hybridization and phenotypic analysis to investigate whether strain TM^T belongs to *Haloferax volcanii* or to a new species.

At the level of phenotypic properties (Table 1), strain TM^T exhibits the typical pleomorphic flattened shape of *Haloferax* species (Mullakhanbhai & Larsen, 1975; Rodríguez-Valera *et al.*, 1983; Juez *et al.*, 1986), but displays a distinctly higher requirement for MgSO₄. Two members of this genus (*Haloferax mediterranei* and *Haloferax denitrificans*) might be differentiated from strain TM^T: *Haloferax mediterranei* contains gas vacuoles and is capable of anaerobic growth in the presence of nitrate with gas production; *Haloferax denitrificans* is not gas-vacuolated, but produces gas and grows anaerobically with nitrate. Strain TM^T neither contains gas vacuoles nor is capable of anaerobic growth in the presence of nitrate. Strain TM^T differed from *Haloferax gibbonsii* by its inability to produce acid from galactose or mannose and by its ability to hydrolyse casein. No growth of strain TM^T was observed at pH values above 7.5. In contrast, *Haloferax gibbonsii* has the ability to grow at pH values above 8. In addition, *Haloferax gibbonsii* is motile by means of a polar flagellum. The most important features distinguishing strain TM^T from *Haloferax volcanii* were its extreme requirement for NaCl and its good growth at saturating salt concentrations (303 g l⁻¹); in contrast, *Haloferax volcanii* has a moderate salt requirement and is strongly inhibited at that concentration. To maintain cell stability, *Haloferax volcanii* requires a lower salinity (30 g NaCl l⁻¹) than strain TM^T (Mullakhanbhai & Larsen, 1975). The above features clearly emphasize the great environmental differences between the isolation source of strain TM^T (a solar saltern) and *Haloferax volcanii* (the Dead Sea). Moreover, strain TM^T differed from *Haloferax volcanii* by its lipolytic and gelatinase activities, by its inability to produce H₂S from cysteine, and by its ability to utilize galactose and lactose. Although the spectrum of antibiotic susceptibility was similar to those of other *Haloferax* spp., the strain was much more resistant to josamycin than were *Haloferax mediterranei*, *Haloferax denitrificans* and *Haloferax gibbonsii*: whilst strain TM^T was able to grow at 180 µg josamycin ml⁻¹, the minimal inhibitory concentration of this antibiotic against these other *Haloferax* species was 31.2 µg ml⁻¹ (Nieto *et al.*, 1993). Strain TM^T was also resistant to 100 µg rifampicin ml⁻¹. In contrast, other *Haloferax* species were sensitive to 15–31.2 µg rifampicin ml⁻¹ (Torreblanca *et al.*, 1986; Nieto *et al.*, 1993). We found that this antibiotic has a strong inhibitory effect on *Haloferax mediterranei* (5 µg ml⁻¹). The different responses of the genus *Haloferax* to some antibiotics could be used for taxonomic purpose (Nieto *et al.*, 1993). Thus, it could be said that strain TM^T

differed from the distinguished species of the genus *Haloferax* by its different response to the antibiotics listed above. Strain TM^T differed from *Haloferax volcanii* and other *Haloferax* spp. by its ability to produce bicyclic β -carotene as well as canthaxanthin (Table 1). Strain TM^T was screened with respect to the production of canthaxanthin – a feature which made it unique within the genus *Haloferax* and among other members of the *Archaea*. The low DNA–DNA hybridization values between strain TM^T and *Haloferax volcanii* (49%) proved that strain TM^T and *Haloferax volcanii* are not identical and belong to different species. In addition, the DNA–DNA hybridization values between strain TM^T and the other *Haloferax* spp. indicated that strain TM^T represents a new species within the genus *Haloferax*. Accordingly, on the basis of chemotaxonomy, phenotypic characteristics and low DNA–DNA hybridization with the type strains of *Haloferax* species, we suggest that strain TM^T is sufficiently different from the currently recognized species in the genus *Haloferax* to warrant designation as a new species, namely *Haloferax alexandrinus* strain TM^T.

Description of *Haloferax alexandrinus* sp. nov.

Haloferax alexandrinus (al.ex.and.ri'nus. L. masc. adj. *alexandrinus* pertaining to Alexandria, a city in Egypt, where the strain was isolated).

Cells stain Gram-negative, and are chemo-organotrophic, strictly aerobic, non-motile and pleomorphic (1.1–1.5 $\times$ 1.6–2.0 μ m; rod-shaped cells 1.1–1.5 $\times$ 3.5–4 μ m). Cells occur mostly singly and exhibit a range of morphological types, including triangular forms, square forms, irregular cocci and rod-shaped cells. Gas vacuoles are not formed. Colonies on standard agar growth medium are 0.5–1 mm in diameter, circular, convex, entire, translucent, smooth and red. Incapable of anaerobic growth with nitrate, DMSO or L-arginine. Extremely halophilic, growth occurring at NaCl concentrations between 100 g l⁻¹ and saturation. Optimum growth occurs at 250 g NaCl l⁻¹ with a high requirement of MgSO₄ (40 g l⁻¹). Tolerates the elevated concentrations of MgSO₄ up to 409 g l⁻¹. Cells lyse in water. The temperature range is 20–55 °C in the presence of 250 g NaCl l⁻¹. The pH range is 5.5–7.5, the optimum being pH 7.2 (at 37 °C). Cells grow aerobically in a chemically defined medium containing ammonium chloride and a single carbon source such as glucose, glycerol or succinate. Acidification of the unbuffered medium occurs when growth occurs in the presence of sugars or glycerol. Reduction of nitrate occurs under aerobic conditions. Aerobic reduction of nitrate and nitrite without gas production was detected. Tween 80 and gelatin are hydrolysed, and sulfide is produced from thiosulfate. Does not hydrolyse urea, starch or casein. Catalase and oxidase tests are positive. Arginine dihydroxylase and urease tests are negative. Tests for β -galactosidase and phosphatase are positive. Lysine decarboxylase and or-

nithine decarboxylase tests are negative. The strain can utilize various complex carbon and nitrogen sources such as amino acids and yeast extract. The strain is unable to utilize peptone, casein or starch. The major isoprenoid neutral lipids are the carotenoids (bacterioruberins, β -carotene and 3-hydroxy echinenone), including a large amount of canthaxanthin [700 μ g (g dry cells)⁻¹]. Acid hydrolysis of whole cells releases a single diether component identical to 2,3-di-*O*-phytanyl-*sn*-glycerol. The major polar lipids are the diether analogues of phosphatidylglycerol, phosphatidylglycerolphosphate-methyl ester, a diglycosyl glycerol diether and sulfate diglycosyl diether. The G+C content of the DNA is 59.5 $\pm$ 0.3 mol%. The type strain is TM^T (= JCM 10717^T = IFO 16590^T).

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