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

Dalal Asker1,2 and Yoshiyuki Ohta2

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 TMT 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 MgSO\(_4\) 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 TMT 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 TMT is unique within the genus Haloferax in producing canthaxanthin. Comparative analysis of phenotypic properties and DNA–DNA hybridization between strain TMT and Haloferax species supported the conclusion that TMT is a novel species within this genus, for which the name Haloferax alexandrinus sp. nov. is proposed. The type strain is TMT (= 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, Halobacterales, 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).

Abbreviation: S-DGD-1, sulfated diglycosyl diether.
The GenBank/EMBL/DDJB accession number for the 16S rDNA sequence of Haloferax alexandrinus strain TMT (= JCM 10717\(^T\) = IFO 16590\(^T\)) is AB037474.

Halarubrum (McGenity & Grant, 1995), Halobaculum (Oren et al., 1995), Natrrialba (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 C\(_{10}\)-carotenoids (bacterioretinins) as the major carotenoids (Kushwaha et al., 1974; Ronnekleiv & Liaaen-Jensen, 1992, 1995). Some members of the genera Halobacterium and Haloarcula have been reported to partially produce C\(_{10}\)-carotenoids and ketocarotenoids such as  \( \beta \)-carotene, lycopene, 3-hydroxy echinonone and trans-astaxanthin as the minor carotenoids (Kelly et al., 1970; Kushwaha, 1974; Ronnekleiv & Liaaen-Jensen, 1992, 1995). Some members of the family Halobacteriaceae are characterized by red-coloured cells, the colour mainly being due to the presence of C\(_{10}\)-carotenoids (bacterioretinins) as the major carotenoids (Kushwaha et al., 1974; Ronnekleiv & Liaaen-Jensen, 1992, 1995). Some members of the genera Halobacterium and Haloarcula have been reported to partially produce C\(_{10}\)-carotenoids and ketocarotenoids such as \( \beta \)-carotene, lycopene, 3-hydroxy echinonone and trans-astaxanthin as the minor carotenoids (Kelly et al., 1970; Kushwaha, 1974; Ronnekleiv & Liaaen-Jensen, 1992, 1995).

Downloaded from www.microbiologyresearch.org by
IP:  54.70.40.11
On: Fri, 26 Oct 2018 17:05:50
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 (Rodriguez-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 microorganisms from this saltern. Of the strains isolated, strain TM°, 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 μg (g dry cells)−1] (Asker & Ohta, 1999); however, among the other 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° 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 succinate, 4; CaCl2, 3; MgSO4, 7H2O, 20; KCl, 2; NaCl, 200; FeCl3, 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° 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 ml−1): FeCl3, 4H2O, 2.3; CaCl2, 7H2O, 7; MnSO4, H2O, 0.3; ZnSO4, 0.44; CuSO4·5H2O, 0.05 mM. 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° 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 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. pellitubum IAM 10476° 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 1−1 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): glucose, 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 μl exponential-phase cultures (3–4 d) on the standard growth agar medium plate and applying antibiotic discs (Becton Dickinson microbiology system: chloramphenicol, 30 μg; erythromycin, 15 μg; neomycin, 30 μg; josamycin, 30 μg; rifampicin, 5 μg; novobiocin, 30 μg; bacitracin, 10 μg and tetracycline, 30 μg). 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
Images were scanned and electronically enhanced using the
Micrographs were recorded at magnifications of 3500.
using a JEOL JSM-5800LV electron microscope at 20 kV.
The specimen was examined
adhesive conducting aluminium tape and gold-coated using
Critical Point Dryer. The specimens were placed on self-
slides. These specimens were subsequently dehydrated
2%(w
NaCl, and covered with a cover-slip. For examination with
Micrographs were prepared from exponential-phase
Phase-contrast microscopy and scanning electron micro-
180 and 200
µ
culture were mixed on a microscope slide with an equal
drops of the
cells grown in the standard growth medium. Drops of the
volume of melted 2%(w
NaCl was added 10
µ
l
°
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 F
254 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)
ether/diethyl ether (85:15, v/v) as the developing solvent.
The lipids were visualized by spraying with 10%(w/v)
ethanol with chloroform/methanol/25% 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 (Torre-
blanca 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

---

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

<table>
<thead>
<tr>
<th>Primer name*</th>
<th>Sequence (5'→3')</th>
<th>Position†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1† ‡</td>
<td>attccggttatccctgcgg</td>
<td>1–20</td>
<td>Kamekura &amp; Seno (1992)</td>
</tr>
<tr>
<td>F2‡</td>
<td>ttccgaagtggaggcagaa</td>
<td>688–707</td>
<td>Kamekura &amp; Seno (1992)</td>
</tr>
<tr>
<td>TMF3</td>
<td>cgcagcgcgcgaacctx</td>
<td>329–348</td>
<td>This study</td>
</tr>
<tr>
<td>TMF4</td>
<td>gcagaagcgacgcagcgc</td>
<td>1039–1055</td>
<td>This study</td>
</tr>
<tr>
<td>TMF5</td>
<td>gacagagggcatgcgccc</td>
<td>983–1001</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Reverse</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1† ‡</td>
<td>cccggttatatacgcgtt</td>
<td>720–739</td>
<td>Kamekura &amp; Seno (1992)</td>
</tr>
<tr>
<td>R2‡</td>
<td>agggattggtcaacgcgcacg</td>
<td>1453–1472</td>
<td>Kamekura &amp; Seno (1992)</td>
</tr>
<tr>
<td>TMR3</td>
<td>gtattacccgcggtgcgtgc</td>
<td>457–474</td>
<td>This study</td>
</tr>
<tr>
<td>TMR4</td>
<td>gacaggggctgggtggtc</td>
<td>1340–1357</td>
<td>This study</td>
</tr>
</tbody>
</table>

* 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
16SrDNA.

---

http://ijs.sgmjournals.org
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 McGinley & 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 \textit{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\textsuperscript{T} was determined using the EMBL database and \textsc{fasta} (Pearson & Lipman, 1988). Similarity values were calculated from the alignment of the strain TM\textsuperscript{T} sequence with sequences from the following organisms: \textit{Haloferax volcanii} ATCC 29605\textsuperscript{T} (accession no. K00421\textsuperscript{T}), \textit{Haloferax mediterranei} ATCC 33500\textsuperscript{T} (D11107), \textit{Haloferax denitrificans} ATCC 35960\textsuperscript{T} (D14128) and \textit{Haloferax gibbonisi} ATCC 33959\textsuperscript{T} (D13378), using \textsc{clustal w} (version 1.8) multiple sequence alignments (Thompson \textit{et al}., 1994). Alignment gap base positions were not taken into consideration for the calculation. Each 1467-base sequence of the evolutionary distance matrix was used to determine the similarity (Kimura, 1980). The programs used for this analysis can all be found in the \textsc{phylip} program (version 3.5.1) (Felsenstein, 1993).

Preparation of labelled DNA, and DNA–DNA hybridization. The DNA of strain TM\textsuperscript{T} was nick-translated with [\textalpha-\textsuperscript{32}P]-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 $3 \times 10^8$ d.p.m. (µg 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 × 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 & Tijtgat, 1970). After hybridization, the filters were washed in 2 × 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.

RESULTS

Morphological, biochemical, cultural and physiological characteristics of strain TM\textsuperscript{T}

Colonies of strain TM\textsuperscript{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<sup>T</sup> from the validly described species within the genus *Haloferax*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas vacuoles (production)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Anaerobic growth in the presence of nitrate</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Tween 80 hydrolysis</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gas produced from nitrate</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;S produced from cysteine</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt; requirements for growth (g l&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>10–40</td>
<td>49–9.9</td>
<td>ND</td>
<td>49–9.9</td>
<td>2.5–4.9</td>
</tr>
<tr>
<td>NaCl range for growth (g l&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>100–320</td>
<td>–</td>
<td>88–263</td>
<td>76–270</td>
<td>100–320</td>
</tr>
<tr>
<td>Optimum NaCl for growth (g l&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>250</td>
<td>146</td>
<td>110–180</td>
<td>160</td>
<td>200–250</td>
</tr>
<tr>
<td>Cell stability (NaCl, g l&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>100</td>
<td>30</td>
<td>88</td>
<td>30</td>
<td>30–40</td>
</tr>
<tr>
<td>Acid produced from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Lactose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Lactose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Resistant to antibiotics (µg ml&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Josamycin (180)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rifampicin (100)</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Carotenoid composition†</td>
<td>1, 3, 4, 5</td>
<td>2, 5</td>
<td>5</td>
<td>3, 5</td>
<td>5</td>
</tr>
</tbody>
</table>

*Only sparse growth occurs at 233 g l<sup>−1</sup> (Mullakhanbhai & Larsen, 1975).

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

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<sup>−1</sup> with water, the cells changed in shape from pleomorphic forms to spheres, and the spheres then underwent lysis below 100 g NaCl l<sup>−1</sup>. 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. H<sub>2</sub>S 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<sup>−1</sup> and at 123–2 g MgSO<sub>4</sub> l<sup>−1</sup>. At the same concentration of NaCl, the strain tolerated elevated concentrations of MgSO<sub>4</sub> up to 394·4 g l<sup>−1</sup>, but the lag phase was extended for several days. However, with the reduction in NaCl concentration to 125 g l<sup>−1</sup> in the same medium, strain TM<sup>T</sup> could tolerate up to 493 g MgSO<sub>4</sub> l<sup>−1</sup>. 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<sup>−1</sup>) and thiamine (8 µg ml<sup>−1</sup>) 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-xylene, ribose, sucrose, N-acetyl glucosamine and glycerol) was observed. Lactose, galactose, mannose, citrate and starch were not utilized. Strain TM<sup>T</sup> was susceptible to 30 µg novobiocin ml<sup>−1</sup> and 30 µg baci-
tracín ml⁻¹, but was resistant to 10–20 μg bacitracin ml⁻¹, 30 μg chloramphenicol, 15 μg erthromycin, 30 μg neomycin, 180 μg josamycin, 100 μg rifampicin and 30 μg tetracycline. Furthermore, the susceptibility to antibiotics in comparison with that of four reference strains was tested: *N. pellirubrum* JCM 10476ᵀ, *Haloferax mediterranei* and *Halorubrum saccharovorum* JCM 8865ᵀ. Two small plasmids with of 3 and 2 kbp were identified in cells of strain TMᵀ after DNA extraction and agarose gel electrophoresis. The G+C content of strain TMᵀ was 59.5 ± 0.3 mol %, as determined by HPLC (mean of three independent determinations). The G+C content is within the range of 59.5 ± 0.3 mol % for *Haloferax* species, strain TMᵀ could be differentiated by a distinct 66–51 kDa band of polypeptide. A qualitative analysis of the relatedness of the protein patterns indicated that strain TMᵀ was closely related to species of *Haloferax*.

**Whole-cell protein profiles**

Similarities between strain TMᵀ 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 TMᵀ from members of the genera *Natrinema*, *Halorubrum* and *Haloarcula*. Moreover, among the *Haloferax* species, strain TMᵀ could be differentiated by a distinct 66–51 kDa band of polypeptide. A qualitative analysis of the relatedness of the protein patterns indicated that strain TMᵀ was closely related to species of *Haloferax*.

**Characterization of a plasmid in strain TMᵀ**

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

**DNA base composition and 16S rDNA sequence analysis**

The G+C content of strain TMᵀ was 59.5 ± 0.3 mol %, as determined by HPLC (mean of three independent determinations). The G+C content is within the range of 59.5 ± 0.3 mol % for *Haloferax* species.
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 $^{32}$P-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 phosphatidylglycerosulfate and the presence of SDGD-1 and C$_{29}$C$_{20}$ in the absence of C$_{29}$C$_{25}$ 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; McGinity *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; Rodriguez-Valera *et al*., 1983; Juez *et al*., 1986), but displays a distinctly higher requirement for MgSO$_4$. 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 hydrolyze 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 H$_2$S 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 & Vreeland, 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 *Halofexax* by its different response to the antibiotics listed above. Strain TM\(^4\) differed from *Halofexax volcanii* and other *Halofexax* spp. by its ability to produce bicyclic \(\beta\)-carotene as well as canthaxanthin (Table 1). Strain TM\(^4\) was screened with respect to the production of canthaxanthin – a feature which made it unique within the genus *Halofexax* and among other members of the *Archaeae*. The low DNA–DNA hybridization values between strain TM\(^4\) and *Halofexax volcanii* (49 %) proved that strain TM\(^4\) and *Halofexax volcanii* are not identical and belong to different species. In addition, the DNA–DNA hybridization values between strain TM\(^4\) and the other *Halofexax* spp. indicated that strain TM\(^4\) represents a new species within the genus *Halofexax*. Accordingly, on the basis of chemotaxonomy, phenotypic characteristics and low DNA–DNA hybridization with the type strains of *Halofexax* species, we suggest that strain TM\(^4\) is sufficiently different from the currently recognized species in the genus *Halofexax* to warrant designation as a new species, namely *Halofexax alexandrinus* strain TM\(^4\).

**Description of Halofexax alexandrinus** sp. nov.

*Halofexax 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 (1–1·5 x 1·6–2·0 \(\mu\)m; rod-shaped cells 1·1–1·5 x 3·5–4 \(\mu\)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 \(\beta\)-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 MgSO\(_4\) (40 g l\(^–1\)). Tolerates the elevated concentrations of MgSO\(_4\) 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 \(\beta\)-galactosidase and phosphatase are positive. Lysine decarboxylase and ornithine 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, \(\beta\)-carotene and 3-hydroxy echinenone), including a large amount of canthaxanthin [700 \(\mu\)g (dry cells)\(^–1\)]. Acid hydrolysis of whole cells releases a single diether component identical to 2,3-di-O-phytanoyl-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±0·3 mol\%.

**ACKNOWLEDGEMENTS**

We would also like to thank Professor Ahmed R. El-Mahdy of Alexandria University for useful suggestions and Dr Tarek Awad for his critical reading of the manuscript. This work was financially supported by the Japan Ministry of Education (Monbusho).

**REFERENCES**


Haloferax alexandrinus sp. nov.


