Halococcus dombrowskii sp. nov., an archaeal isolate from a Permian alpine salt deposit

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Several extremely halophilic coccoid archaeal strains were isolated from pieces of dry rock salt that were obtained three days after blasting operations in an Austrian salt mine. The deposition of the salt is thought to have occurred during the Permian period (225–280 million years ago). On the basis of their polar-lipid composition, 16S rRNA gene sequences, cell shape and growth characteristics, the isolates were assigned to the genus Halococcus. The DNA–DNA reassociation values of one isolate, strain H4T, were 35 and 38% with Halococcus salifodinae and Halococcus saccharolyticus, respectively, and 65–8–67% with Halococcus morrhuae. The polar lipids of strain H4T were C20–C25 derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate. Whole-cell protein patterns, menaquinone content, enzyme composition, arrangements of cells, usage of carbon and energy sources, and antibiotic susceptibility were sufficiently different between strain H4T and H. morrhuae to warrant designation of strain H4T as a new species within the genus Halococcus. It is proposed that the isolate be named Halococcus dombrowskii, and the type strain is H4T (= DSM 14522T = NCIMB 13803T = ATCC BAA-364T).

Keywords: archaea, Halococcus dombrowskii sp. nov., salt mine, subsurface, longevity

INTRODUCTION

In recent years, viable halophilic archaea have been isolated from various ancient salt deposits of the Permian and Triassic ages (see review by McGenity et al., 2000). The first such strain to be described and validated as a novel species was Halococcus salifodinae, which was isolated from rock salt from an Austrian salt mine (Denner et al., 1994). Further work, which included the culturing of strains as well as the analysis of uncultured samples (Norton et al., 1993; Grant et al., 1998; Stan-Lotter et al., 1999; Radax et al., 2001), revealed vast biodiversity within the viable haloarchaeal community in rock salt, but this biodiversity has yet to be fully comprehended. In view of the potential longevity of these haloarchaeal organisms (Grant et al., 1998), it has been suggested that they may be interesting candidates to be considered when targeting the search for extraterrestrial forms of life, particularly since halite has been detected in outer space (Gooding, 1992; McCord et al., 1998; Zolensky et al., 1999; Whitby et al., 2000). To be able to identify potential signatures of micro-organisms in extraterrestrial materials, it is necessary to have as extensive a record of terrestrial forms of extremophilic life as possible. This record should also include prokaryotes isolated from subsurface materials, such as evaporites.

Here we describe a coccoid haloarchaeal species that was isolated from Permian rock salt and which is distinct from the known representatives of the genus Halococcus.

METHODS

Isolation of haloarchaeae from rock salt. Samples of freshly blasted rock salt taken from a depth of about 650 m below the Earth’s surface were obtained from a salt mine near Bad Ischl in Austria, three days after blasting operations for the creation of new tunnels had been done. The geological setting of the salt mine has been described previously, as

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The GenBank/EMBL accession number for the 16S rDNA sequence of Halococcus dombrowskii is AJ420376.
have the methods for sterile dissolution of the salt pieces and the initial steps for the culturing of isolates (Radax et al., 2001; Stan-Lotter et al., 1993).

Culture conditions and bacterial strains. Initial streaking of dissolved rock salt was done onto solidified M2 medium (Tomlinson & Hochstein, 1976) that had been prepared with commercially available NaCl. In later experiments, dissolved rock salt was occasionally used as an additive to all growth media at a final concentration of 10%, since it enhanced cell yields considerably (M. Pfaffenhuemer, unpublished data). The pH of the growth medium was 7.4, unless indicated otherwise. Growth of strains in liquid culture was monitored spectrophotometrically at 600 nm.

The following haloarchaeal strains were obtained from the DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1B, D-38124 Braunschweig, Germany): Halobacterium salinarum DSM 668; Halococcus morrhuae DSM 13077, DSM 1308 and DSM 1309; Halococcus saccharolyticus DSM 5530T; Halococcus salifodinae BIP DSM 8989T and BG2/2 DSM 13045; Natronococcus occultus DSM 3396T. Halococcus morrhuae NRC 16008 (= NCIMB 746) was obtained from the NCIMB (National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK). Halorubrum saccharovorum M6T (ATCC 29225T), Halobacterium salinarum (R1), Haloferax denitrificans (ATCC 35960T) and Haloarcula vallismortis (ATCC 29715T) were obtained from L. I. Hochstein, formerly of the NASA Ames Research Center, USA.

Experiments for the description of the new halobacterial taxon. All tests for determining the phenotypic properties of strain H4T were carried out as specified in the proposed minimal standards for the description of new taxa in the order Halobacterales (Oren et al., 1997), with some additions and exceptions as outlined below.

Physiological and biochemical tests. The range of salt concentrations that permitted growth of strain H4T was determined by spreading 100 µl of a growing culture of the strain onto agar plates containing M2 medium supplemented with NaCl to a final concentration of 5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5 or 30%. Similarly, the requirement of the strain for Mg2+ was tested with M2 agar plates containing MgCl2 at a final concentration of 0, 5, 10, 20, 100 or 500 mM. Agar plates inoculated with strain H4T were incubated for 14–20 days. The pH range for growth was determined in M2 medium, which was made with 50 mM of the following buffer substances and adjusted to the desired pH values (pH values as measured after autoclaving are in parentheses): MES (pH 5.13); MES (pH 5.83) and MOPS (pH 6.79); Tris (pH 8.09); Tris (pH 9.01); CAPS (pH 9.77). Tubes containing 20 ml of the media were inoculated in duplicate with 100 µl of a washed culture and then incubated at 37 °C without shaking for 4 weeks.

Standard tests (cytochrome oxidase production, catalase production, nitrate reduction and gelatin liquefaction) were performed on strain H4T as described by Smibert & Krieg (1994). The Analytical Profile Index systems API ZYM and API 20NE (both from bioMérieux) were used for analysis of additional enzyme activities (Humble et al., 1977) and for assimilation tests, respectively. Strips were inoculated with a cell suspension in Tris-buffered 4 M NaCl and incubated for up to 24 h (API ZYM) or 3 weeks (API 20NE). All API tests were done at least three times.

The utilization of carbohydrates or amino acids by strain H4T was tested in a semi-defined medium (M2A) which consisted of 50 mM Tris/HCl, 4 M NaCl, 0.02% yeast extract, 1 mM NH4Cl, 27 mM KCl, 100 mM MgCl2, 1.4 mM CaCl2, 0.1% trace elements solution SL-6 (Malik, 1983) and 1% of the respective carbohydrates or 0.01% of the respective amino acids. Incubation was done in test tubes at 37 °C without shaking for 10–12 weeks. The most unambiguous results were obtained by determining the size of cell sediments, following growth of the strain on carbon sources. The diameter of the sedimented inoculum was approximately 3 mm and did not change over the course of the experiments. Diameters of cell pellets were considered positive evidence of archaeal growth when they were > 6 mm; some pellet diameters ranged between 7 and > 10 mm. Acidification was determined with special pH indicator test strips (Merck) for the range pH 6.0–10.0. All carbon utilization experiments were done at least twice.

Determination of the antibiotic susceptibility of strain H4T was tested by spreading cell suspensions onto agar plates and then applying filter-paper discs (6 mm in diameter) on which the antibiotics were dispensed in 20 µg amounts, with the exception of novobiocin, which was used in 10 µg amounts. Zones of inhibition were measured following 2 weeks incubation at 37 °C; sensitivity was deemed strong when the zone of inhibition extended more than 3 mm beyond the antibiotic disc. At least three determinations were carried out for each antibiotic.

Analysis of menaquinones and polar lipids. All steps were done under a nitrogen atmosphere. Menaquinones were extracted from about 200 mg of cells (wet weight) with methanol/hexane (2:1, v/v) as described by Tindall (1990), except that pellets of growing cells were used instead of lyophilized cells. The hexane phase contained the menaquinones, which were purified by TLC on silica-gel plates using hexane/diethyl ether (85:15, v/v) as the developing solvent, and then separated by HPLC (Denner et al., 1994). Identifications and comparisons were done with the menaquinones from strain H4T, Halococcus salifodinae BIP DSM 8989T and Halococcus morrhuae DSM 13077. The methanol phase contained the polar lipids, which were further purified by extraction with chloroform/methanol/0.3% NaCl (1:2:0.05, v/v) and heating of the mixture to 60 °C for 15 min. Following removal of the cell debris by centrifugation, the extraction with chloroform was repeated. The dried polar lipids were resuspended in chloroform/methanol (2:1, v/v) and separated by two-dimensional TLC (Ross et al., 1985). Polar-lipid extracts were spotted onto the corner of a thin-layer 10 x 10 cm silica-gel plate (60 F254; Merck). These were first developed in chloroform/methanol/water (65:25:4, v/v) and then in chloroform/methanol/glacial acetic acid/water (80:12:15.4, v/v). Total lipids were visualized by spraying the chromatograms with phosphomolybdic acid and then heating them to 160 °C. Phospholipids were detected with molybdenum blue, amino groups were detected with ninhydrin, carbohydrates were detected with anisaldehyde and carbohydrates and sulfate groups were detected with Bial’s reagent (ornil ferric chloride spray reagent). The equivalence of spots was determined by comparison of chromatograms of extracts of known haloarchaea in two dimensions and by comparison with published data.

Analysis of whole-cell-protein patterns. SDS-PAGE of whole-cell proteins was performed as described previously (Stan-Lotter et al., 1989, 1993). Briefly, approximately 50 mg wet weight cells ml−1 were lysed by boiling them in SDS sample buffer (Laemmli, 1970) for 10 min. The lysate was then centrifuged at 10000 g for 15 min, to remove any precipitates. The gel system of Laemmli (1970) was used to separate the proteins. Proteins were visualized by staining

1808 International Journal of Systematic and Evolutionary Microbiology 52

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with Coomassie blue. Marker proteins of 2.5 to 200 kDa in size (Mark 12) were from Novex. Electrophoresis of the whole-cell proteins from strain H4T and from the type strains used in this study was repeated more than four times.

**DNA base composition.** Cells of strain H4T were harvested in the early-stationary phase of growth. The determination of the G + C content of this strain was carried out by the DSM Identification Service. DNA was isolated on hydroxyapatite by the procedure of Cashion et al. (1977), following disruption of cells by passage through a French press, and degraded as described by Mesbah et al. (1989). The resulting deoxyribonucleosides were analysed by HPLC according to Mesbah et al. (1989) and Tamaoka & Komagata (1984). Non-methylated λ DNA was used for calibration (Mesbah et al., 1989).

**DNA–DNA hybridization.** DNA was isolated as described by Cashion et al. (1977). Levels of DNA–DNA hybridization between strain H4T and three haloarchaeal type strains were determined spectrophotometrically by the renaturation method of De Ley et al. (1970), with the modifications by Huss et al. (1983) and Escara & Hutton (1980). Renaturation rates were computed by the program TRANSFER.BAS (Jahnke, 1992). These experiments were carried out by the DSM Identification Service.

**Sequencing of the 16S rRNA genes of the rock-salt isolates and phylogenetic analyses.** For each strain isolated from the rock salt, the 16S rRNA gene was amplified by PCR using primers Archae21F (DeLong, 1992) and 1525R (McGenity et al., 1998). Template DNA was purified from cells grown on M2 agar plates as follows. A loopful of cells was suspended in 500 µl water and an equal volume of buffer-saturated phenol (pH 8.0) was added to the suspension. The DNA from the aqueous phase was then precipitated with ethanol. After enzymic treatment with RNase A (Sigma) and Proteinase K (Sigma), the DNA was extracted with phenol/chloroform/isoamyl alcohol (24:24:1), precipitated, washed and dissolved in water. Amplification reactions were performed in a 50 µl reaction volume in a programmable thermal cycler (Biorad). The reaction mixtures were composed of 50 pmol of each primer, 12.5 mM deoxyribonucleotides, 1.5 mM MgCl2, 1 U Taq DNA polymerase (Promega), appropriately diluted DNA polymerase buffer B (Promega) and 0–1–1 ng template DNA. Thermal cycling consisted of a primary heating step (94 °C for 5 min), followed by 30–35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. The amplified PCR product was purified from ethidium-bromide-stained agarose gels by using a commercially available kit (NucleoTrap; Macherey & Nagel). Sequences were determined by automated dideoxynucleotide methods using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 310 Genetic Analyser (both from Applied Biosystems), according to the manufacturer’s instructions. Primer 1525R and primers described by Radax et al. (2001) were used as sequencing primers.

Sequences used for comparison with the 16S rDNA sequence from strain H4T were obtained from the European Molecular Biology Laboratory (EMBL) web interface or from the Ribosomal Database Project II (RDP) (release 8; Maidak et al., 2000) and fitted in a subset of aligned archaean sequences obtained from the RDP, using the software BioEdit (Hall, 1999). The alignment was subjected to phylogenetic analyses by distance-matrix (Jukes–Cantor correction; Jukes & Cantor, 1969), maximum-likelihood and maximum-parsimony methods using programs of the PHYLIP package (version 3.5.1c; Felsenstein, 1993) and CLUSTAL X (Thompson et al., 1997). The confidence of the branching pattern was assessed by analysis of 100 bootstrap replicates.

**Other methods.** Unstained haloarchaeal cells were observed with a Zeiss Axioskop microscope using phase-contrast. Gram-staining of cells was carried out as described by Dussault (1955). Assays to determine the lysis of haloarchaeal cells in water and the isoelectric focusing of whole-cell proteins were performed as described previously (Stan-Lotter et al., 1989; Denner et al., 1994). Carotenoid pigments were extracted from freeze-dried cells with methanol/acetone (1:1, v/v) and their absorption spectra were determined (Gochnauer et al., 1972) with a Beckman DU-650 spectrophotometer in the range 330–600 nm.

**RESULTS AND DISCUSSION**

**Isolation and selection of strains from rock salt**

Following incubation of the dissolved-rock-salt samples on M2 medium at 37 °C for 5–6 weeks, light-red or pink colonies were obtained. Smears taken from the pure cultures of these colonies were examined by phase-contrast microscopy. Two different arrangements of coccoid cells could be recognized among the strains. One consisted mainly of cells growing in *Sarcina*-like packets, which were often in very large clusters; several representative strains with this arrangement of cells (e.g. strains H1 and N2) were identified recently as belonging to the species *Halococcus salifodinae* (Stan-Lotter et al., 1999). The other arrangement consisted of cells that appeared as single cells, diplococci or small clusters of only a few cells. For further differentiation of the strains isolated from rock salt, the whole-cell protein patterns of the strains were examined (see below). In addition, partial sequences (about 400 bp long) for the 16S rRNA gene of the strains (i.e. a total of eight strains) were determined (see Methods). On the basis of this preliminary characterization, several of the strains isolated from the rock salt were found to be similar to *Halococcus salifodinae*. On the basis of the sequence data, some strains were more similar to *Halococcus morrhuae* than to other halococcal species, but they appeared as single cells, diplococci or small clusters upon microscopic examination rather than in *Sarcina*-like packets. These strains are the subject of the present study.

**Cell and colony morphology**

One strain isolated from the rock salt, strain H4T, was a coccoid organism of about 0.8–1.2 µm in diameter. When grown in M2 medium or on M2 agar, cells of strain H4T were non-motile and appeared as single cells or diplococci. This was a different arrangement than that seen with cells of *Halococcus morrhuae* DSM 1307T, which exhibited predominantly classical *Sarcina*-like packets when grown in M2 medium or on M2 agar. Cells of strain H4T stained uniformly Gram-negative, whether they were taken from 5-day-old or 14-day-old cultures; endospores were not produced by the strain. Colonies of strain H4T on solidified complex
medium (pH 7-4) were circular, convex, had entire margins, were about 1–2 mm in diameter following 14 days of incubation at 37 °C, light red in colour and had a shiny surface. In contrast, colonies of *Halococcus morrhuae* DSM 1307<sup>T</sup> grown under the same conditions as strain H4<sup>T</sup> were irregular and dark red in colour.

**Physiological and biochemical characteristics of strain H4<sup>T</sup>**

Strain H4<sup>T</sup> grew aerobically with doubling times of 25–28 h at 37 °C when grown in liquid M2 medium (4 M NaCl, pH 7-4) with shaking. Optimum growth of strain H4<sup>T</sup> occurred when it was grown in the presence of NaCl concentrations ranging from 20 to 30%; it did not grow in medium containing < 15% NaCl. Strain H4<sup>T</sup> required at least 2-5 mM Mg<sup>2+</sup> to be present in growth medium that included 4 M NaCl; optimum growth of the strain occurred when Mg<sup>2+</sup> was present in the medium at a concentration of 20–60 mM. Slight but noticeable differences in the Mg<sup>2+</sup> requirements of *Halococcus morrhuae* DSM 1307<sup>T</sup> (optimum growth in presence of 2-5 and 5 mM Mg<sup>2+</sup>) and *Halococcus saccharolyticus* DSM 5350<sup>T</sup> (optimum growth in presence of 60 mM Mg<sup>2+</sup>) were observed, when compared to the Mg<sup>2+</sup> requirements of strain H4<sup>T</sup>. The growth of strain H4<sup>T</sup> was supported by M2 media of pH 5-2, 5-8, 6-8 or 8-0. No growth of this strain occurred in the media at pH values above 9-0 or below 5-1. Upon their examination under phase-contrast microscopy, cells of strain H4<sup>T</sup> grown at lower pH values (i.e. pH 5-8 and 6-8) appeared larger and with a somewhat irregular shape when compared with strain H4<sup>T</sup> cells grown at pH 7-8 (not shown). Compared to *Halococcus salifodinae* Blp<sup>T</sup>, which grew between pH 6-8 and 9-5, strain H4<sup>T</sup> grew well at lower pH values. *Halococcus morrhuae* and *Halococcus saccharolyticus* have both been reported to grow at pH values as low as 5-5 or 6-0, respectively (Larsen, 1989; Montero et al., 1989). Similar to cells of other halococci, cells of strain H4<sup>T</sup> did not lyse within 1–2 h when suspended in distilled water (Grant & Larsen, 1989). Strain H4<sup>T</sup> grew between 28 and 50 °C, with its optimum temperature for growth between 37 and 40 °C. Its growth in test tubes with broth occurred as sediment.

Further phenotypic characteristics of strain H4<sup>T</sup> are shown in Table 1, along with some characteristics of *Halococcus morrhuae* DSM 1307<sup>T</sup>. The growth of strain H4<sup>T</sup> on various carbon sources was tested in semi-defined media containing 0-02% yeast extract and, due to the known long generation times of members of the genus *Halococcus* (Grant, 2001), incubation was carried out for up to 12 weeks. Acidification of the media was, in general, weak or not detectable. *Halococcus salifodinae* Blp<sup>T</sup> has a similar complement of enzymes as strain H4<sup>T</sup>, except that lipase (C14), leucine arylamidase and cystine arylamidase have not been detected in this strain (Stan-Lotter et al., 1999).

**Table 1. Comparison of phenotypic characteristics of *Halococcus morrhuae* DSM 1307<sup>T</sup> and strain H4<sup>T</sup>**

Tests were performed at salinities of at least 20% NaCl (see Methods). Enzyme assays were done with the API ZYM system. Both strains were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8) and leucine arylamidase activity; no activity was detected for arginine dihydrolase, urease, β-glucosidase or β-galactosidase in either strain. Both strains liquefied gelatin. Neither strain was able to utilize L(+)-arabinose, D(+)-raffinose, D(+)-rhamnose, D,L-Phe nor L-Trp, but they were both able to utilize D(+)-galactose. +, Positive reaction or growth; –, no reaction or growth.

<table>
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<tr>
<th>Characteristic</th>
<th>DSM 1307&lt;sup&gt;T&lt;/sup&gt;</th>
<th>H4&lt;sup&gt;T&lt;/sup&gt;</th>
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<tr>
<td>Gram reaction</td>
<td>+/−&lt;sup&gt;∗&lt;/sup&gt;</td>
<td>−</td>
</tr>
<tr>
<td>pH Range for growth</td>
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<td>5–2–8–0</td>
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<td>+&lt;sup&gt;∗&lt;/sup&gt;</td>
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<td>Oxidase</td>
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<td>Nitrate reduction</td>
<td>+&lt;sup&gt;∗&lt;/sup&gt;</td>
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<td>Enzymes assayed for:</td>
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<td>Acid phosphatase</td>
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<td>D(−)-Fructose</td>
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<td>L-Ser</td>
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* Data taken from Grant & Larsen (1989). All other data were determined in this work.

Growth of strain H4<sup>T</sup> was strongly inhibited by the antibiotics bacitracin, novobiocin and rifampicin. Moderate susceptibility of the strain to erythromycin and trimethoprim was observed. No inhibition of strain H4<sup>T</sup> growth was observed when the strain was grown in the presence of ampicillin, anisomycin, aphidicolin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, tetracycline or vancomycin. The susceptibility pattern of strain H4<sup>T</sup> was similar to that described for *Halococcus morrhuae* (Larsen, 1989; Tindall, 1992), except that *Halococcus morrhuae* is moderately susceptible to anisomycin (Larsen, 1989), a result corroborated in this work.

**Gel electrophoresis of whole-cell proteins**

SDS-PAGE of whole-cell proteins is a rapid method for distinguishing bacterial species and has a similar level of discrimination to DNA–DNA hybridization (Jackman, 1987). Strain H4<sup>T</sup> had a unique protein profile following SDS-PAGE that did not resemble the profiles of any of the halococcal representatives analysed in this study (Fig. 1). In addition, the protein pattern of strain H4<sup>T</sup> was also dissimilar to the protein patterns of *Halorubrum saccharovorum*, *Halobac-
terium salinarum (R1), Halobacterium salinarum and Haloferax denitrificans (data not shown). A high degree of similarity between protein profiles of different strains belonging to the same species is usually observed (Stan-Lotter et al., 1999). This phenomenon was observed in this study (Fig. 1), as four different strains of *Halococcus morrhuae* (lanes 2–5) had similar protein patterns and two different strains of *Halococcus salifodinae* (lanes 8 and 9) had similar protein patterns. Haloarchaea are known to possess acidic bulk proteins (Reistad, 1970) whose isoelectric points are between pH 3.6 and 5.0 (Stan-Lotter et al., 1989). Isoelectric focusing of whole-cell proteins from strain H4T and *Halococcus morrhuae* DSM 1307T revealed bulk proteins whose isoelectric points were between pH 4.2 and 5.2, and which differed from each other in their overall pattern (data not shown).

**Menaquinones, polar lipids and carotenoids**

Upon its analysis by HPLC, the quinone extract of strain H4T produced three peaks that had the same retention times as those detected in a quinone extract of *Halococcus morrhuae* DSM 1307T. The predominant types of menaquinones in *Halococcus morrhuae* were MK-8 (15% of total quinones), a menaquinone with eight isoprenoid units in the side chain, and dihydromenaquinone MK-8(H#) (84% of total quinones), in accordance with the results of Collins et al. (1981). Traces of dihydromenaquinone MK-7(H#) were also present (1% of total quinones). Strain H4T possessed all of these menaquinones, although the bulk quinone (94% of total quinones) was MK-8(H#), while MK-8 and MK-7(H#) were present in small amounts (6 and 1% of total quinones, respectively). For comparison, an extract of *Halococcus salifodinae* BlpT was also analysed; this consisted almost exclusively of MK-8(H#) (98% of total quinones). The presence of MK-8(H#) as the dominant menaquinone type in strain H4T indicated that this strain was more closely related to *Halococcus salifodinae* BlpT than to *Halococcus morrhuae* DSM 1307T, as *Halococcus salifodinae* BlpT was also characterized by the presence of MK-8(H#) as its major component, MK-8 (2%) as a minor component and MK-7(H#) as a trace component.

Two-dimensional TLC of lipids of strain H4T revealed the presence of C35C46 and C31C46, archaeal core lipids, as detected by double spots. Phosphatidylglycerol and phosphatidylglycerol methylphosphate were present in strain H4T in greatest abundance, similar to *Halococcus morrhuae* DSM1307T and *Halococcus salifodinae* BlpT; no phosphatidylglycerol sulfate was present. Sulfated mannosylglucosylglycerol diether was detected among the lipids of strain H4T, as well as two unknown glycolipids. The overall phospholipid pattern of strain H4T was characteristic of members of the genus *Halococcus* (Ross et al., 1985; Waino et al., 2000).

Strain H4T produced carotenoid pigments characteristic of members of the haloarchaea, as determined with methanol/acetone extracts. Absorption peaks at wavelengths of 388, 495 and 528 nm and a shoulder at 466–476 nm were observed with the extracts from strain H4T, *Halococcus morrhuae* DSM 1307T and *Halococcus saccharolyticus* DSM 5350T; these peaks corresponded to bacterioruberin (Gochnauer et al., 1972; Oren, 1983). The absorption spectra for extracts of *Halococcus salifodinae* BlpT showed less-well-defined peaks, with shoulders at 490 and 526 nm; this was concomitant with the light-pink pigmentation it produced under the growth conditions used in this study (not shown).

**G+C content**

The G+C content of strain H4T was 61.3 mol%; this was similar to the values determined for *Halococcus morrhuae* (61–66 mol%; Larsen, 1989), *Halococcus saccharolyticus* (59.5 mol%; Montero et al., 1989) and *Halococcus salifodinae* (62 mol%; Denner et al., 1994).

**DNA–DNA hybridization**

The measurement of the levels of DNA–DNA hybridization between strain H4T and representative *Halococcus* spp. showed the following homology values:
35.1% between strain H4T and Halococcus saccharolyticus DSM 5350T; 38.2% between strain H4T and Halococcus salifodinae BG2/2 DSM 9899T; and 65.8 and 67.8% (two determinations) between strain H4T and Halococcus morrhuae DSM 1307T. A DNA homology value of <70% for two strains usually justifies designation of the strains to different species (Wayne et al., 1987); hence, strain H4T represented a new species of the genus Halococcus. The homology values observed for strain H4T and Halococcus morrhuae DSM 1307T indicated that these two species had a closer relationship at the DNA level than strain H4T had with Halococcus salifodinae BG2/2 and Halococcus saccharolyticus DSM 5350T, respectively.

**Phylogenetic position of strain H4T**

The full sequence (1472 bases) of the 16S rRNA gene of strain H4T was determined. Comparison of the 16S rDNA sequence of this strain with the 16S rDNA sequences of members of the family Halobacteriaceae placed strain H4T into the genus Halococcus (Fig. 2). Strain H4T, as deduced from this analysis, was most similar to Halococcus morrhuae. The identity of strain H4T with Halococcus morrhuae ATCC 17082T and Halococcus morrhuae NRC 16008 was 99.3 and 99.5%, respectively. To date, three species of haloneutrophilic [Halococcus morrhuae (Larsen, 1989), Halococcus saccharolyticus (Montero et al., 1989) and Halococcus salifodinae (Denner et al., 1994)] and two species of haloalkaliphilic [Natronococcus occultus and Natronococcus amylolyticus (Tindall, 2001)] archaeal cocci have been recognized. On the basis of the 16S rDNA data presented here and data from previous reports (Ventosa et al., 1999; Stan-Lotter et al., 1999), the genus Halococcus appears to contain at least two lineages – one which contains Halococcus salifodinae and Halococcus saccharolyticus, and one which consists of Halococcus morrhuae and other coccolid strains. Strain H4T forms a branch within the Halococcus morrhuae lineage (Fig. 2). The 16S rDNA sequences of Halococcus salifodinae and Halococcus saccharolyticus are 98.9% identical (Ventosa et al., 1999), but these two halococcal organisms differ in numerous properties, supporting their separation into two distinct species. These two species are also 63.6% related on the basis of DNA–DNA hybridization data (A. Legat, C. Gruber and H. Stan-Lotter, unpublished data). It is therefore reasonable to deduce that with a similar mean value (66.8%) for DNA–DNA hybridization between it and other members of the genus Halococcus, and on the basis of other differentiating characteristics, strain H4T does not belong to the species Halococcus morrhuae.

**Conclusions**

On the basis of its polar-lipid content, antibiotic sensitivity and acidic bulk proteins, coccolid strain H4T was identified as being a halophilic archaean. Strain H4T could be distinguished from Halococcus salifodinae, Halococcus saccharolyticus and natronococci, as described above. Its slow growth, phospholipid content and 16S rRNA gene sequence were similar to characteristics of Halococcus morrhuae. However, strain H4T differed from Halococcus morrhuae with respect to its cellular morphology and arrangement, DNA–DNA similarity, whole-cell protein patterns, menaquinone content, presence of enzymes, pigmentation, susceptibility to some antibiotics, and its usage of carbohydrates and amino acids (see Table 1 and Fig. 1). We believe that these data justify the proposal for assigning H4T to a new species of the genus Halococcus, Halococcus dombrowskii.

Strain H4T and several strains related to it, as well as members of the species Halococcus salifodinae, were isolated from subterranean salt mines in Austria. The salt sediments are thought to have been deposited during the Permian period (Zharkov, 1981). Evidence...
gathered from the data presented in this and earlier studies (Norton et al., 1993; Stan-Lotter et al., 1993, 1999, 2000, 2001; McGenity et al., 2000; Radax et al., 2001) further strengthens the notion that halophilic salt mine strains are the remnants of archaean populations that originally inhabited the Palaeozoic brines. How these organisms survived is not known, but suggestions for the basis of their longevity have been considered by Grant et al. (1998) and McGenity et al. (2000). One of the first scientists to explore subsurface bacteria was H. J. Dombrowski, who isolated bacterial strains from Permian and even pre-Cambrian salt deposits (Dombrowski, 1963, 1966) and, thus, attracted attention to ancient salt sediments as novel and unusual environments for micro-organisms to inhabit.

Description of Halococcus dombrowskii sp. nov.

*Halococcus dombrowskii* (dom.brow.ski.i. L. masc. adj. *dombrowskii* referring to Heinz J. Dombrowski, a pioneer in the isolation of micro-organisms from very ancient materials).

Cocci of 0·8–1·2 μm in diameter, occurring as single cells, diplococci or small clusters of 2–5 cells. Gram-negative, non-motile and aerobic. Small colonies (1–2 mm in diameter after 1 week incubation at 37 °C) when grown on complex medium of neutral pH, with 2 mm in diameter after 1 week incubation at 37 °C; pH range for growth is 5·8–8·0. Optimum NaCl concentration for growth is 20–25%; no growth observed below 15% NaCl. Optimum growth when between 20 and 60 mM Mg2+ is available in the presence of 4 M NaCl. Oxidase- and catalase-positive. Nitrate is reduced to nitrite. Gelatin is liquefied. Table 1 lists compounds that strain H4T can utilize for growth and its active enzymes. Strains H4T is susceptible to bacitracin, novobiocin and rifampicin, slightly susceptible to anisomycin, chloramphenicol and tetracycline, and resistant to ampicillin, aphidicolin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, tetracycline and vancomycin. The G+C content of the DNA is 61·3±1 mol%. Main polar lipids are C20:0 and C20:0 derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate. Sulfated mannosylglucosylglycerol diether is present; phosphatidylglycerol sulfate is absent. Menaquinones MK-7(H6), MK-8(H8), and MK-8 are present. Whole-cell proteins are acidic with isoelectric points predominantly between pH 4·2 and 5·2. The type strain was isolated from dry Permian rock salt from the salt mine in Bad Ischl, Austria, 650 m below the Earth’s surface. The type strain is H4T (= DSM 14522T = NCIMB 13803T = ATCC BAA-364T).

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