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Claudia Gruber · Andrea Legat · Marion Pfaffenhuemer
Christian Radax · Gerhard Weidler
Hans-Jürgen Busse · Helga Stan-Lotter

Halobacterium noricense sp. nov., an archaeal isolate from a bore core of an alpine Permian salt deposit, classification of *Halobacterium* sp. NRC-1 as a strain of *H. salinarum* and emended description of *H. salinarum*

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Abstract Two rod-shaped haloarchaeal strains, A1 and A2, were isolated from a bore core from a salt mine in Austria. The deposition of the salt is thought to have occurred during the Permian period (225–280 million years ago). The 16S rDNA sequences of the strains were 97.1% similar to that of the type species of the genus *Halobacterium*, which was also determined in this work. Polar lipids consisted of C_{20}–C_{20} derivatives of phosphatidylglycerol, methylated phosphatidylglycerol phosphate, phosphatidylglycerol sulfate, triglycosyl diether and sulfated tetraglycosyl diether. Optimal salinity for growth was 15–17.5% NaCl; Mg^{++} was tolerated up to a concentration of 1 M. The DNA–DNA reassociation value of strain $A1^T$ was 25% with *H. salinarum* DSM 3754^T and 41% with *Halobacterium* sp. NRC-1, respectively. Based on these results and other properties, e.g. whole cell protein patterns, menaquinone content and restriction patterns of DNA, strains A1 and A2 are members of a single species, for which we propose the name *H. noricense*. The type strain is A1 (DSM 15987^T, ATCC BAA-852^T, NCIMB 13967^T). Since we present evidence that *Halobacterium* sp. NRC-1 is a member of *H. salinarum*, an emended description of *H. salinarum* is provided.

Keywords *Halobacterium noricense* · *Halobacterium salinarum* · *Halobacterium* sp. NRC-1 · Haloarchaea · Salt mine · Bore core · Longevity

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C. Gruber · A. Legat · M. Pfaffenhuemer · C. Radax
G. Weidler · H. Stan-Lotter (✉)
Fachbereich für Molekulare Biologie,
Abteilung Mikrobiologie, Hellbrunnerstrasse 34,
5020 Salzburg, Austria
E-mail: helga.stan-lotter@sbg.ac.at
Tel.: +43-662-80445756
Fax: +43-662-8044144

H.-J. Busse
Institut für Bakteriologie, Mykologie und Hygiene,
Veterinärplatz 1, 1210 Vienna, Austria

H.-J. Busse
Institut für Mikrobiologie und Genetik, Dr. Bohrgasse 9,
1030 Vienna, Austria

Introduction

From ancient salt deposits of Permian and Triassic age, viable halophilic Archaea (Haloarchaea) have been isolated in recent years (see McGenity et al. 2000, for a review). The first such strain to be described as a novel species was *Halococcus salifodinae*, which was isolated from pieces of rock salt, following blasting operations in the salt mine near Bad Ischl, Austria (Denner et al. 1994), and also from ancient salt deposits in Germany and England (Stan-Lotter et al. 1999). Another coccoid strain, *H. dombrowskii*, was isolated from similar alpine rock salt samples (Stan-Lotter et al. 2002). In addition, analysis of dissolved alpine rock salt with molecular methods (amplification and sequencing of 16S rRNA genes) provided evidence for the occurrence of numerous haloarchaea, which have not yet been cultured (Radax et al. 2001). In this work, a haloarchaeal species is described, which was isolated from a deep drilling bore core of alpine rock salt of Permian age. The strains, designated A1 and A2, are rod-shaped cells and show some similarity to members of the genus *Halobacterium*. However, the strains differ from the known representatives of the genus *Halobacterium*, including *Halobacterium* sp. NRC-1, the only haloarchaeon so far whose whole genome sequence is known (Ng et al. 2000), but which has not yet been included in a taxonomic description. During comparisons with strains $A1^T$ and A2, phenotypic information about *Halobacterium* sp.

NRC-1 and strains of *H. salinarum*, which has not been reported previously, was obtained and is included here. The data support the classification of *Halobacterium* sp. NRC-1 as a strain of the species *H. salinarum*.

Materials and methods

Isolation of haloarchaea from salt bore cores

Samples of bore cores of 47 mm diameter and approximate length of 100–120 mm were obtained from the salt mine in Altaussee, Austria, during deep drilling operations performed by the mining company (Salinen Austria). The drilling was carried out in a horizontal tunnel approximately 350 m below the surface; the bore cores stemmed from a depth of an additional 120 m below the tunnel floor. The bore cores were immediately processed. They consisted of 92–95% water-soluble material, which was taken as the approximate halite content. The geological setting of the salt mine has been described previously, as have the general methods for sterile dissolving of salt pieces and initial steps for culturing of isolates (Radax et al. 2001; Stan-Lotter et al. 1993).

Haloarchaeal strains and culture conditions

The following haloarchaeal strains were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ): *H. salinarum* DSM 668, *H. salinarum* DSM 670, *H. salinarum* DSM 3754^T, *Halobaculum gomorrense* DSM 9297^T and *Halorubrum saccharovorum* DSM 1137^T. *Halobacterium* sp. NRC-1 ATCC-700922 was purchased from LGC Teddington (UK). Initial streaking of dissolved salt bore cores was done on solidified DSM 823 medium, which contained (per liter): 125 g NaCl, 160 g $MgCl_2{\cdot}6H_2O$, 5 g K_2SO_4, 0.1 g $CaCl_2{\cdot}2H_2O$, 1 g yeast extract, 1 g casamino acids and 2 g starch. For solid media, 20 g l^{-1} agar were added. *Halobacterium* sp. NRC-1 was grown in ATCC 2185 medium, which contained (per liter): 250 g NaCl, 20 g $MgSO_4{\cdot}7H_2O$, 3 g trisodium citrate $\cdot$ $2H_2O$, 2 g KCl, 5 g tryptone, 3 g yeast extract, and 0.1 ml of a filter-sterilized trace metal solution (per 200 ml: 1.32 g $ZnSO_4{\cdot}7H_2O$, 0.34 g $MnSO_4{\cdot}H_2O$, 0.78 g $Fe(NO_4)(SO_4)_2{\cdot}6H_2O$, 0.14 g $CuSO_4{\cdot}5H_2O$) was added per liter. The pH of the growth media was 7.0, unless indicated otherwise; incubation of cultures was at 37°C, or as indicated below. Growth in liquid culture was monitored at 600 nm with a spectrophotometer.

Experiments for the description of a new halobacterial taxon

All tests for phenotypic properties were carried out as specified in the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren et al. 1997), with some additions and alterations as outlined below.

Physiological and biochemical tests

The range of salt concentrations which permitted growth was determined as described by Stan-Lotter et al. (2002), except that DSM 823 medium was substituted for M2 medium. The requirement for Mg^{++} was tested with agar plates containing final concentrations of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 M $MgCl_2$ in the presence of 17.5% NaCl. Cultures of strains on agar plates were incubated for 14–20 days. The temperature range for growth was tested by incubating cultures on agar plates at ambient temperature (21–23°C) and at 28, 37, 45 and 50°C. The pH range for growth was determined on solidified DSM 823 medium, which was made with 50 mmol of the following buffer substances and adjusted to the desired pH value (pH values as measured after autoclaving are in brackets): no buffer (pH 4.8–5.2), MES (pH 5.86), MOPS (pH 6.80), Tris (pH 7.70) and Tris (pH 8.83). Plates containing the indicated media were inoculated in duplicates with 100 µl of a washed culture and incubated at 37°C for 3 weeks. Standard tests (cytochrome oxidase, catalase, nitrate reduction, starch hydrolysis) were performed as described by Smibert and Krieg (1994), using appropriate haloarchaeal control strains. The Analytical Profile Index system (API Zym, API 20NE; bioMerieux) was used for the analysis of additional enzyme activities (Humble et al. 1977) and for assimilation tests, as described by Stan-Lotter et al. (2002). All API tests were done at least three times. Utilization of carbohydrates was tested in a semi-defined medium, modified from DSM 823, but containing (per liter) 175 g NaCl, 160 g $MgCl_2{\cdot}6H_2O$, 5 g K_2SO_4, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.1 g yeast extract, 0.1% SL-6 trace elements solution (Malik 1983) and 1% of the respective carbohydrates. Test tubes were inoculated with cultures, which had been adapted to growth without starch, and incubation was done at 37°C with shaking for 4–8 weeks. Growth was assessed by monitoring the optical density of the cultures. 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; appropriate positive controls (*Halobaculum gomorrense* DSM 9297^T; see Oren et al. 1995) and negative controls (no carbohydrates; no yeast extract) were included. Anaerobic growth on L-arginine was tested as described by Oren and Litchfield (1999), except that incubation times were extended for up to 3 weeks; anaerobic growth on nitrate was tested in a similar manner with medium containing 0.5% $NaNO_3$. Anaerobic growth in the presence of dimethylsulfoxide (DMSO) was determined as described by Oren and Trüper (1990), except that initial incubation was for 33 days at 37°C, followed by 2 months at ambient temperature in the dark. The determination of antibiotic susceptibility was tested as described by Stan-Lotter et al.

(2002); the antibiotics tested are listed in the Results and in the description of strain A1^T. At least three determinations per antibiotic were carried out.

Analysis of menaquinones and polar lipids

Menaquinones were extracted from 1 g of cells (wet weight) and purified as described by Stan-Lotter et al. (2002). Identification and comparisons were done with menaquinones from strains A1^T, A2, *Halobacterium salinarum* DSM 3754^T, *Halobacterium* sp. NRC-1 ATCC-700922 and *Halorubum saccharovorum* DSM 1137^T. The methanol phase from the extraction procedure contained the polar lipids, which were further purified and separated by two-dimensional thin-layer chromatography as described by Stan-Lotter et al. (2002). Total lipids were visualized by spraying the chromatograms with phosphomolybdic acid and subsequent heating to 120°C. Phospholipids were detected with molybdenum blue, amino groups with ninhydrin, carbohydrates with α-naphthol and carbohydrates and sulfate groups with Bial's reagent. The equivalence of spots was determined by co-chromatography of extracts of known haloarchaea in two dimensions and by comparison with published data (e.g. Holt et al. 1994).

Analysis of whole-cell protein patterns

Sodium dodecyl sulfate (SDS) gel electrophoresis of whole-cell proteins was performed as described by Stan-Lotter et al. (1989, 1993, 2002). Briefly, approximately 50 mg ml^{-1} of cells (wet weight) were lysed by boiling in SDS sample buffer (Laemmli 1970) for 10 min and were then centrifuged at 10,000 *g* for 1 min, to remove any precipitates. The gel system of Laemmli (1970) was used to separate the proteins. Visualization of proteins was performed by staining with Coomassie blue. Electrophoresis of the whole cell proteins from strains A1^T, A2 and other haloarchaea used in this study was repeated more than four times.

DNA base composition

Cells of strains A1^T and A2 were harvested in the early stationary phase of growth. The determination of the G + C content was carried out by the DSMZ Identification Service. Details of the method were provided by Stan-Lotter et al. (2002).

DNA–DNA hybridization

DNA was isolated as described by Cashion et al. (1977). Levels of DNA–DNA hybridization between strains A1^T, A2 and the haloarchaeal strains *Halobacterium salinarum* DSM 3754^T and *Halobacterium* sp. NRC-1 were determined spectrophotometrically by standard methods (see Stan-Lotter et al. 2002). These experiments were carried out by the DSMZ Identification Service.

Sequencing of 16S rRNA gene and phylogenetic analysis

The 16S rRNA genes from strains A1^T, A2 and *H. salinarum* DSM 3754^T were amplified by PCR, using primers Archae21F (DeLong 1992) and 1525R (McGenity et al. 1998). Template DNA was purified from cells grown on agar plates, containing DSM 823 medium, as described by Stan-Lotter et al. (2002). Amplification reactions and sequencing were performed as described, using primer 1525R and the primers described by Radax et al. (2001), except that PCR products were purified by precipitation with polyethylene glycol and ethanol (http://gator.biol.sc.edu/CGEL_ABI/PEG_Precip.html). Sequences used for comparison with the 16S rDNA sequence from strains A1^T and A2 were obtained from the European Molecular Biology Laboratory (EMBL) web interface or from the Ribosomal Database Project II (RDP; Maidak et al. 2001) and fitted in a subset of aligned archaeal sequences obtained from the RDP, using BioEdit software (Hall 1999). The alignment was subjected to phylogenetic analysis with distance-matrix (Jukes–Cantor correction; Jukes and Cantor 1969), maximum-likelihood and maximum-parsimony methods, using programs in the PHYLIP package, ver. 3.5.1c (Felsenstein 1993) and Clustal X (Thompson et al. 1997). Confidence in the branching pattern was assessed by analysis of 100 bootstrap replicates.

The GenBank/EMBL accession numbers for the 16S rDNA sequence data are AJ548827 (*H. noricense* strain A1^T) and AJ496185 (*H. salinarum* DSM 3754^T).

Restriction analysis and plasmids

Haloarchaeal cells were embedded in agarose, using plug-forming molds (BioRad) and were treated and prepared for restriction digestion, as described by Hackett (1995). The following restriction enzymes were used: *Bsp*TI (Fermentas), *Dra*I (Promega) and *Pme*I (New England Biolabs). Fragments were separated by pulsed field gel electrophoresis (PFGE; Hackett et al. 1994), using a CHEF-DR III apparatus (BioRad), and visualized by staining with ethidium bromide. Plasmids were detected following separation of both undigested and restrictase-digested haloarchaeal DNA by PFGE. In some cases, minipreparations of plasmids were made (Ng et al. 1995) to corroborate data obtained from PFGE.

Electron microscopy

Cells were harvested following growth in liquid medium (DSM 823 or ATCC 2185) and prepared for scanning

electron microscopy (SEM) by fixing with 4% glutaraldehyde in 0.15 M cacodylate buffer, containing 4.7 M NaCl, 80 mmol $MgSO_4 \cdot 7H_2O$ (for *Halobacterium* sp. NRC-1) or 3.8 M NaCl, 250 mmol $MgCl_2 \cdot 7H_2O$ (for strain $A1^T$), post-fixing with 1% osmium tetroxide, critical point-drying and sputter-coating with approximately 2 nm Pt. A Hitachi S-900 field-emission SEM was used. This work was done by Chris Frethem at the University of Minnesota Characterization Facility.

Other methods

Unstained cells were observed with a Nikon Eclipse E200 microscope, using phase contrast. Gram staining of cells was carried out as described by Dussault (1955). Assays to determine lysis of halobacterial cells in water and isoelectric focusing of whole cell proteins were performed as described by Stan-Lotter et al. (1989) and Denner et al. (1994). Carotenoid pigments were extracted from freeze-dried cells with methanol/acetone (1:1, v/v) and the absorption spectra were determined (Gochnauer et al. 1972) with a Beckman DU-650 spectrophotometer in the range 330–600 nm.

Results and discussion

Isolation of strains from bore cores

A piece of bore core of about 300 g was obtained from deep drilling operations in the salt mine at Altaussee, Austria, transported to the laboratory on the same day and surface-sterilized. Portions of the dissolved rock salt were spread on agar plates and incubated at 37°C for up to 3 months. Colonies with a light red pigmentation were detected; two of these were purified by re-streaking and designated as strains A1 and A2.

Cell and colonial morphology

Strains $A1^T$ and A2 grew as single small rods. Occasionally, pleomorphic forms (irregular rods, coccoid forms) were observed in liquid cultures, a property which is characteristic for most members of the genus *Halobacterium* (Grant 2001). Individual cells of strain $A1^T$ are shown in Fig. 1 (right panels). Their size range was 1.2–1.8 μm; coccoid forms were about 1.0 μm. In comparison, cells of *Halobacterium* sp. NRC-1 (left panel) were on average 2.8–3.5 μm in length.

Cells of strain $A1^T$ grown in liquid medium or on agar were motile. Cells stained uniformly Gram-negative, whether from 5- or 14-day-old cultures; endospores were not produced. Colonies on solidified complex medium at pH 7 were circular, convex, with entire margins, about 0.4 mm in diameter (following 18 days of incubation at 37°C), of light red pigmentation and had a shiny surface.

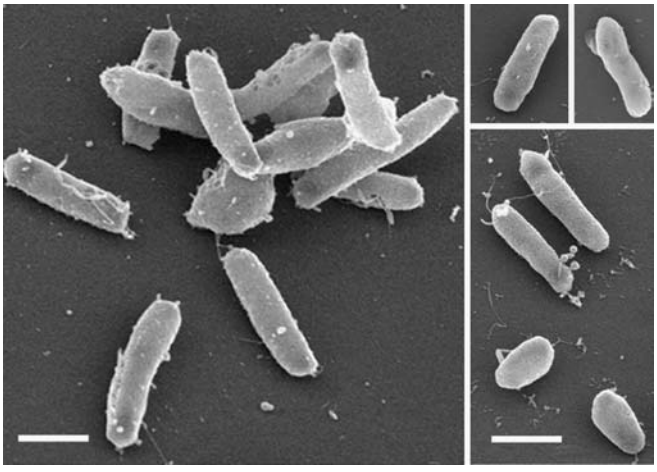

Fig. 1 Scanning electron microscopy of *Halobacterium* sp. NRC-1 (*left panel*) and strain $A1^T$ (*right panels*). *Bars* 1 μm

Physiological and biochemical characteristics

Strains $A1^T$ and A2 grew aerobically with doubling times of approximately 15 h at 37°C in liquid DSM 823 medium with shaking. Optimum growth occurred at NaCl concentrations of 3 M and Mg^{++} concentrations in the range of 0.6 M and 0.9 M. No growth was observed below 2.1 M NaCl and below 100 mmol Mg^{++}. Growth of strains $A1^T$ and A2 was supported by DSM 823 medium at pH 5.2, 5.8, 6.8, or 7.7. No growth occurred at pH above 8.8 or below 5.1. Compared with *Halobacterium* species, which have a range of growth between pH 6.8 and pH 9.5, strain $A1^T$ grew well at lower pH values. The temperature range for growth was between 28°C and 50°C, with an optimum at 45°C. Growth in test tubes with broth created a sediment. Like the cells of other non-coccoid haloarchaea, cells of strain $A1^T$ and A2 lysed within 1–2 h when suspended in distilled water (Grant and Larsen 1989; Grant 2001), with a concomitant increase in the viscosity of the suspension, which indicated the release of DNA.

No gas vesicles were present in strains $A1^T$ and A2 under the growth conditions used in this study. This was in contrast to *H. salinarum* strains, where the presence of gas vesicles was readily apparent by the floating of cultures. Further phenotypic characteristics of strains $A1^T$ and A2 are shown in Table 1, in comparison with *H. salinarum* DSM 3754^T and *Halobacterium* sp. NRC-1. Growth on carbon sources was tested in semi-defined media containing 0.01% yeast extract in standing tubes. No growth was observed on D(+) glucose, D(+) galactose, D(+) maltose, D(+) sucrose, or D(+) xylose; slow growth was observed with glycerol or starch.

Growth of strains $A1^T$ and A2 was strongly inhibited by the antibiotics anisomycin and novobiocin. No inhibition of growth occurred with ampicillin, bacitracin, chloramphenicol, erythromycin, streptomycin, tetracycline, gentamycin, kanamycin, vancomycin or nalidixic acid.

Table 1 Comparison of phenotypic characteristics of *Halobacterium* species and strains A1^T and A2. Tests were performed at salinities of at least 15% NaCl and 0.57 M $MgCl_2$ (for details, see Materials and methods). All strains were Gram negative, motile and showed lysis in distilled water. They were able to grow anaerobically in the presence of Arg or nitrate, but were unable to liquefy gelatine and hydrolyze starch. Catalase was present in all strains. None of the strains showed β-glucosidase, β-galactosidase, urease, esterase lipase (C8) or leucine arylamidase acitivity. + Positive reaction or growth, – no reaction or growth

Property	*H. salinarum* DSM 3754^T	*Halobacterium* sp. NRC-1	Strain A1^T	Strain A2
pH range for growth	5.5–8.0[a]	5.2–8.0	5.2–7.0	5.2–7.0
Temperature range for growth (°C)	22–50[b]	22–50	28–50	28–50
Oxidase	+[b]	+	–	–
Anaerobic growth in the presence of DMSO	+[b]	+[c]	–	–
Enzyme assays (API-ZYM)				
Alkaline phosphatase	+	+	–	–
Esterase (C4)	–	–	+	+
Cystine arylamidase	–	–	+	+
Valine arylamidase	–	–	+	+
Acidic phosphatase	+	+	–	–
Naphthol-AS-BI phosphohydrolase	+	+	–	–

[a]Data taken from Grant (2001); all other data were determined in this work
[b]Data in agreement with Grant (2001)
[c]Data in agreement with Oren and Trüper (1990)

Menaquinones, polar lipids and carotenoids

The predominant types of menaquinones in *Halobacterium* species were MK-8 (85% of total quinones) and dihydromenaquinone MK-8[H_2] (12–13%), in accordance with Collins et al. (1981). Traces of MK-6, MK-7 and MK-7[H_2] were also present. Strains A1^T and A2 possessed all of these menaquinones, although the amounts of MK-8 and MK-8[H_2] were nearly equal (50% of MK-8, 49% of MK-8[H_2] in strain A2; 32% and 68%, respectively, in strain A1^T), while MK-7[H_2] was present at 1% each and MK-6, MK-7, and MK-9 were present in trace amounts (< 0.5%). Extracts of *Halobacterium* sp. NRC-1 and *H. salinarum* DSM 3754^T consisted almost exclusively of MK-8 (89% and 81%, respectively), with the remainder being made up of MK-8[H_2] (9% and 15%, respectively), MK-7[H_2] (1% and 2%, respectively) and traces of MK-7 and MK-6.

Two-dimensional thin layer chromatography of lipids of strains A1^T and A2 revealed C_{20}–C_{20} archaeal core lipids, as detected by the absence of double spots. Phosphatidylglycerol (PG), phosphatidylglycerol methylphosphate (Me-PGP), phosphatidylglycerol sulfate (PGS), triglycosyl diether (TGD) and sulfated tetraglycosyl diether (S-TeGD) were present, but sulfated triglycosyl diether (S-TGD) was not present. The overall polar lipid pattern was most similar to that of members of the genus *Halobacterium* (Ross et al. 1985; Holt et al. 1994; Wainø et al. 2000), except for the absence of S-TGD. The polar lipids of *Halobacterium* sp. NRC-1 and *H. salinarum* DSM 3754^T both consisted of C_{20}–C_{20} derivatives of PG, Me-PGP, PGS, S-TGD-1, S-TeGD and TGD-1.

Strain A1^T produced characteristic carotenoid pigments, as determined with methanol/acetone extracts. Absorption peaks at wavelengths of 370, 388, 497 and 526 nm and a shoulder at 466–476 nm were observed with extracts from strains A1^T, *Halobacterium* sp. NRC-1 and *H. salinarum* DSM 3754^T; these peaks corresponded to C_{40} and C_{50} (bacterioruberin) pigments (Gochnauer et al. 1972; Oren 1983).

G + C content

The DNA base composition of strains A1^T and A2 was 54.5 mol% and 54.3 mol% G + C, respectively. The range of the G + C content of *Halobacterium* species is 57–60 mol% for the minor and 67.1–71.2 mol% for the major DNA component, respectively (Grant 2001).

DNA–DNA hybridization

The measurement of DNA–DNA hybridization showed the following reassociation values: 41% between strain A1^T and *Halobacterium* sp. NRC-1, 27% between strain A2 and *Halobacterium* sp. NRC-1 and 25% between strain A1^T and *H. salinarum* DSM 3754^T.

Whole-cell protein patterns

SDS-polyacrylamide gel electrophoresis (PAGE) of whole-cell proteins is a rapid method for distinguishing prokaryotic species and has a similar level of discrimination to DNA–DNA hybridization, when cells grown under identical conditions are analyzed (Jackman 1987). The protein pattern of strain A1^T following SDS-PAGE did not resemble the profile of any of the haloarchaeal strains analyzed in this study (Fig. 2). Strain A2 possessed a protein pattern identical to that of strain A1^T (data not shown). A high degree of similarity of protein profiles of different strains from the same species can be expected (Stan-Lotter et al. 1999); this was evident from

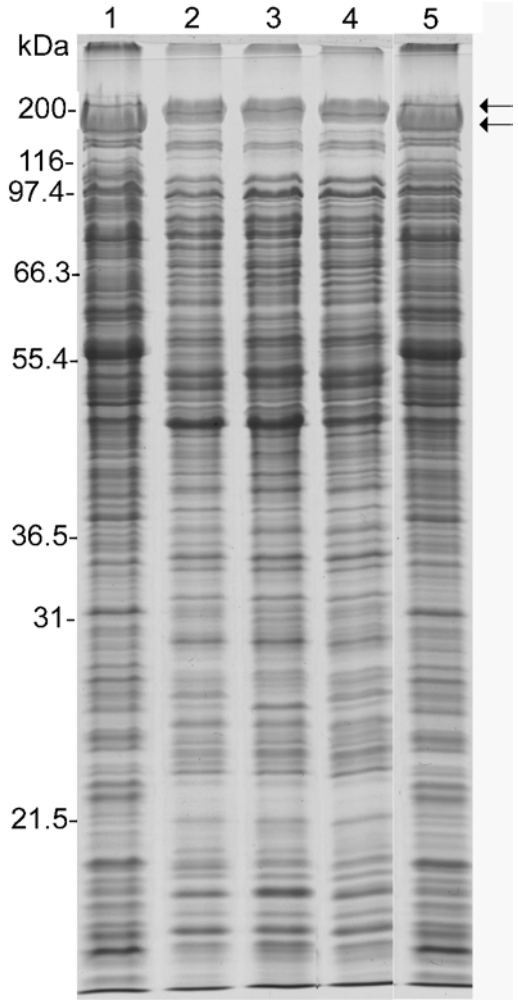

Fig. 2 Whole-cell proteins from haloarchaea and strain A1^T, following separation by SDS-PAGE. Approximately 20 μg of protein were applied per lane. Proteins were stained with Coomassie blue. Molecular mass markers are indicated on the *left*. *Arrows* indicate S-layer protein subunits (see text). *Lanes 1, 5* strain A1^T, *lane 2* *H. salinarum* DSM 3754^T, *lane 3* *Halobacterium* sp. NRC-1, *lane 4* *H. salinarum* DSM 670

the very similar protein patterns of strains *H. salinarum* DSM 3754^T and *Halobacterium* sp. NRC-1 (Fig. 2, lanes 2, 3). Numerous differences to the protein profile of strain A1^T were detectable: one prominent example was the S-layer protein, which runs in this gel system as a broad band of about 200 kDa (Lechner and Sumper 1987; Fig. 2, upper arrow). Strain A1^T apparently also possessed a S-layer protein, but of an apparently smaller size (approximately 160 kDa; Fig. 2, lower arrow). *Halobacterium* sp. NRC-1 and *H. salinarum* DSM 670 are most likely identical strains (Grant 2001); the very similar whole-cell protein patterns of the two strains (Fig. 2, lanes 3, 4) was in agreement with this notion. Haloarchaea are known to contain acidic bulk proteins (Reistad 1970), whose isoelectric points range between pH 3.6 and 5.0 (Stan-Lotter et al. 1989). Isoelectric focusing of whole-cell proteins from strains A1^T and A2

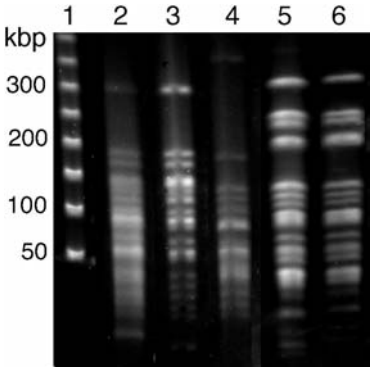

Fig. 3 Restriction patterns of haloarchaeal DNA, following digestion with *Bsp*TI and separation by PFGE. *Lane 1* size standards, *lane 2* *Halobacterium* sp. NRC-1, *lane 3* *H. salinarum* DSM 670, *lane 4* *H. salinarum* DSM 3754^T, *lane 5* strain A1^T, *lane 6* strain A2

revealed bulk proteins with isoelectric points between pH 4.2 and 5.2 (data not shown).

DNA analysis and plasmids

DNA from strains A1^T, A2 and species of *Halobacterium* was digested with restriction enzymes and fragments were separated by PFGE. Strains A1^T and A2 showed very similar fragment patterns (Fig. 3, lanes 5, 6). Differences were apparent between the fragment patterns of *H. salinarum* strains DSM 670 and DSM 3754^T and *Halobacterium* sp. NRC-1 (Fig. 3, lanes 2–4), which among themselves showed high similarity. Comparable results were obtained whether enzymes *Bsp*TI (Fig. 3), *Pme*I or *Dra*I were used for digestion (data not shown). The data suggested that strains A1^T and A2 had less fragments in common with *H. salinarum* strains and *Halobacterium* sp. NRC-1. Restriction maps of the latter group have been compared previously (Hackett et al. 1994) and were found to contain conserved chromosome regions of at least 70%, which is expected to be reflected in the restriction patterns.

The presence of plasmids was examined using PFGE; in strains A1^T and A2 at least two plasmids of approximately 200 kbp and 400 kbp were detected, which were similar in size to the known plasmids of *Halobacterium* sp. NRC-1 (191 kbp, 365 kbp; data not shown).

Phylogenetic position

The sequences (1,473 bases) of the 16S rRNA gene of strains A1^T and A2 were determined. In addition, the 16S rDNA sequence of *H. salinarum* DSM 3754^T (ATCC 33171, NRC 34002) was determined in this work, since there has not yet been a phylogenetic analysis of the type strain of *H. salinarum* (Grant 2001). Comparison of the sequence with members of the family Halobacteriaceae placed strains A1^T and A2 within the genus *Halobacterium* (Fig. 4). The greatest similarities,

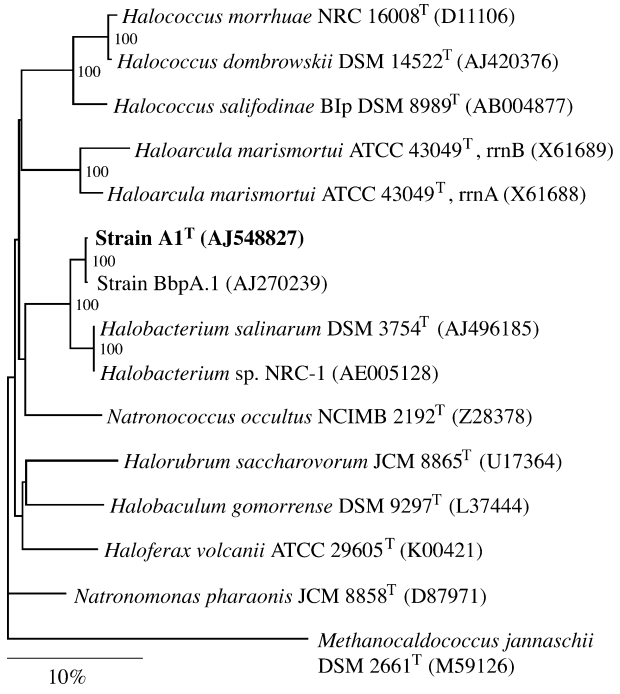

Fig. 4 Distance-matrix neighbor-joining tree, showing the phylogenetic relationships of the haloarchaeal rock salt isolate strain A1^T to several genera of the Halobacteriaceae. The tree is based on an alignment of 16S rRNA gene sequences. Sequence accession numbers are given in *brackets*. Bootstrap values higher than 70 out of 100 subreplicates are indicated at the respective bifurcations. The tree was constructed using the neighbor-joining method of Saitou and Nei (1987). The *bar* represents the scale of estimated evolutionary distance (ten substitutions at any nucleotide position per 100 nucleotide positions) from the point of divergence. *Methanocaldococcus jannaschii* was used as an outgroup

as deduced from this analysis, were with the 16S rDNA sequences of *Halobacterium* sp. NRC-1, *H. salinarum* DSM 668 and the type strain of the genus, *H. salinarum* DSM 3754^T (97.1%). An even closer relationship (99.7% similarity) was evident with cultured haloarchaeal isolates from ancient salt deposits in England (strain BbpA.1 from brine in the Winsford salt mine) and Poland (strain PW5.4 from brine in the Wieliczka salt mine; see McGenity et al. 2000). The three signature 16S rDNA sequences A, B and C, which were proposed for the genus *Halobacterium* (Grant et al. 2001), were found in *H. salinarum* DSM 3754^T and are present in *Halobacterium* sp. NRC-1. In strains A1^T and A2, however, A, B and C contained two (A), two (B) and one (C) base exchange/s, respectively.

Conclusions

On the basis of polar lipid content, 16S rDNA sequences and acidic bulk proteins, strains A1^T and A2 were identified as halophilic archaea. Their growth requirements, overall phospholipid composition, menaquinone content, poor utilization of carbohydrates and antibiotic susceptibility were similar to species of the genus *Halobacterium*. However, strains A1^T and A2 differed from *H. salinarum* with respect to DNA–DNA similarity, G+C content, absence of sulfated triglycosyl diether, whole-cell protein patterns, presence of some enzymes, susceptibility to antibiotics and restriction fragment patterns, (see Table 1, Figs. 2, 3). These data justify the proposal of a new species, for which we suggest the name *H. noricense*. Since the former species *H. salinarium*, *H. halobium* and *H. cutirubrum* were combined, due to their very similar properties, only one recognized species, *H. salinarum*, remains (Ventosa and Oren 1996). *H. noricense* thus represents, at this time, the second species of the genus *Halobacterium*.

H. noricense and several strains, which are probably closely related to this species but which have only a preliminary characterization (Stan-Lotter et al. 2003), were isolated from subterranean salt mines. The 16S rDNA sequence of *H. noricense* was very similar to those of several uncultured haloarchaea from alpine rock salt of the salt mine near Bad Ischl (Radax et al. 2001), e.g. at a length of 918 bp the similarity was 100, 99.8 and 99.7%, respectively, to haloarchaea A175, A153 and A148. A very close relationship (99.7% similarity) was noted for *H. noricense* and cultured, but as yet uncharacterized haloarchaeal isolates, such as strain BbpA.1 from brine in the Winsford salt mine and strain PW5.4 from brine in the Wieliczka salt mine (McGenity et al. 2000). These results suggested a widespread occurrence of similar haloarchaea in salt deposits of similar geological origin, as was concluded previously on the basis of several *Halococcus* isolates (Stan-Lotter et al. 1999). The evidence from data presented in this work and in earlier studies (Norton et al. 1993; Stan-Lotter et al. 1993, 1999, 2000, 2002; McGenity et al. 2000; Radax et al. 2001; Fish et al. 2002) increasingly strengthens the notion that the halophilic salt mine strains are the remnants of populations which originally inhabited paleozoic brines. How they survived is not known, but suggestions for the basis of their longevity have been presented by Grant et al. (1998) and McGenity et al. (2000).

The origin of *Halobacterium* sp. NRC-1 has proven difficult to trace unanimously; according to Grant (2001), *Halobacterium* sp. NRC-1 and *H. salinarum* DSM 670 are probably identical strains. The similarity of whole-cell protein patterns (Fig. 2) and restriction digests (Fig. 3) was consistent with this notion; other properties, which are almost identical to *H. salinarum* DSM 3754^T, are summarized in the emended description below and justify classification of *Halobacterium* sp. NRC-1 as a member of the species *H. salinarum*.

Description of *H. noricense* sp. nov.

noricense (no.ri.cen'se, L. neut. adj.) pertaining to Noricum, the Roman name of the former Austrian province where the salt mines of Altaussee and Bad Ischl are located.

Rods 1.2–2.0 μm in length, growing in liquid media as single cells. Gram-negative, motile, lyse in water, chemoorganotrophic and aerobic, facultatively anaerobic in the presence of L-Arg or nitrate, but not DMSO. Yeast extract and casamino acids support growth. Requires at least 12.5% NaCl for growth, in the presence of 0.6 M $MgCl_2$. Optimum NaCl concentration for growth is 15.0–17.5%, in the presence of 0.7–0.8 M $MgCl_2$. Optimum temperature is 37–45°C, pH range for growth is 5.2–7.0. Small colonies (0.4 mm diameter after 18 days of incubation at 37°C) on complex medium of neutral pH, light red, circular, entire margins. Catalase positive, oxidase negative. Nitrate is not reduced to nitrite. Gelatin is not liquefied. Starch is not hydrolyzed. The following compounds are not utilized for growth in test tubes: *D*(+) glucose, *D*(+) galactose, *D*(+) maltose, *D*(+) sucrose and *D*(+) xylose. Glycerol and starch are utilized slowly, with colonies on agar plates becoming visible after 4–5 weeks. Susceptible to anisomycin and novobiocin. Resistant to ampicillin, bacitracin, chloramphenicol, gentamycin, kanamycin, nalidixic acid, streptomycin, tetracycline and vancomycin. Main polar lipids are C_{20}–C_{20} derivatives of PG, Me-PGP, PGS, TGD and S-TeGD. Menaquinones MK-8 and MK-8[H_2] are the main components and MK-7, MK-7[H_2] and MK-9 are present in traces. Whole-cell proteins are acidic with isoelectric points predominantly between pH 4.2 and 5.2. G+C content is 54.3–54.5 mol%. At least two plasmids of approximate sizes 200 kbp and 400 kbp are present.

The type strain was isolated from a dry bore core of Permian rock salt from the salt mine in Altaussee, Austria, approximately 470 m below surface. The type strain is A1 (=DSM 15987^T, =ATCC BAA-852^T, =NCIMB 13967^T).

Emended description of *H. salinarum* (strains DSM 3754^T, NRC-1)

The previous summarized description (Grant 2001) is emended with the following data, which are similar or identical for the two strains of *H. salinarum*, DSM 3754^T and NRC-1.

Gram negative. Rods 2–4 μm in length, growing in liquid media as single cells, motile, lyse in water, chemoorganotrophic and aerobic, facultatively anaerobic in the presence of L-Arg, nitrate or DMSO. Yeast extract and casamino acids support growth. *D*(+) glucose, *D*(+) galactose, *D*(+) maltose, *D*(+) sucrose and *D*(+) xylose are not utilized for growth in test tubes. Catalase, oxidase, alkaline phosphatase and acidic phosphatase are present. Red pigmentation due to bacterioruberin derivatives.

Menaquinone MK-8 is the main component (>80%), MK-8[H_2] comprises 10–15%, MK-7[H_2] 1–2%. MK-7 and MK-6 are present in traces. Main polar lipids are C_{20}–C_{20} derivatives of PG, Me-PGP, PGS, S-TGD-1, S-TeGD and TGD-1. Susceptible to anisomycin and novobiocin. Resistant to ampicillin, chloramphenicol, gentamycin, kanamycin, nalidixic acid, streptomycin, tetracycline and vancomycin.

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