Very similar strains of *Halococcus salifodinae* are found in geographically separated Permo-Triassic salt deposits

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The authors have previously isolated a novel extremely halophilic archaeon, *Halococcus salifodinae* BIp, from Austrian rock salt deposited about 250 million years ago. In this study they compared strain BIp with two other halococci isolated independently from geographically distant salt deposits of similar age, and with four recent isolates (N1 and H2) from the same site as strain BIp. Strain BG2/2 was from a salt mine in Germany and strain Br3 from a halite deposit in England; both resembled *Hc. salifodinae* BIp in cellular and colonial morphology. Strains BIp, BG2/2 and Br3 had identical 16S rRNA sequences, very similar whole-cell protein patterns, which were different from those of other halococci, similar G+C contents and identical sequences in a 108-base insertion in their 5S rRNA gene. Other similarities included composition and relative abundances of polar lipids, antibiotic susceptibility, enzymic activities and Fourier-transform infrared spectra. Strains N1 and H2 showed similar morphology, whole-cell protein patterns and biochemical characteristics as strains BIp, Br3 and BG2/2. Their partial 16S rRNA sequences (682 and 641 bases, respectively) were indistinguishable from those of strains BIp, Br3 and BG2/2. Therefore strains N1 and H2 can be considered as reisolates of *Hc. salifodinae* which were obtained 8 years after the first samples were taken from that mine. The results presented suggest that viable halophilic archaea, which belong to the same species, occur in widely separated evaporite locations of similar geological age, and support the notion that these halophilic isolates from subterranean salt deposits may be the remnants of populations which inhabited ancient hypersaline seas.

**Keywords:** archaea, *Halococcus*, subterranean microbiology, salt deposits, prokaryotic longevity

INTRODUCTION

The possibility of microbial survival for very long periods of time has been discussed over several decades (for recent reviews see Kennedy et al., 1994; Grant et al., 1998), and gained renewed interest in light of the reports on ancient amplifiable DNA and the viability of bacterial spores found in amber-embedded insects 25–40 million years old (e.g. DeSalle et al., 1992; Cano & Borucki, 1995). We reported the isolation of viable extremely halophilic archaea from Permian and Triassic salt sediments in England and Austria (Norton et al., 1993; Stan-Lotter et al., 1993); one of the isolates, which showed pink pigmentation, was identified as a novel species belonging to the *Halobacteraeaceae* (*Halococcus salifodinae* BIp; Denner et al., 1994). The microorganisms which were recovered from the salt deposits may represent relics of populations which lived in the original brines, as we and others have suggested (Norton et al., 1993; Denner et al., 1994; Grant et al., 1998).

**Abbreviations:** FT-IR, Fourier-transform infrared; PG, glycerol diether of phosphatidylglycerol; PGP-Me, glycerol diether of phosphatidylglycerol methylphosphate; PGS, glycerol diether of phosphatidylglycerol sulphate; SDGD-1, sulphated mannosylglucosylglycerol diether; C20 :C20, 2,3-di-O-phytanyl-sn-glycerol diether; C20 :C20, 2-O-sesterterpanyl-3-O-phytanyl-sn-glycerol diether.

The GenBank accession numbers for the nucleotide sequence data reported in this paper are Z28387 (*Hc. salifodinae* BIp DSM 8989), AJ238897 (strain Br3), AJ131458 (strain BG2/2), AJ245422 and AJ245423 (strain N1), AJ245424 and AJ245425 (strain H2). Accession numbers for other 16S rRNA sequences used for comparisons in this study have been reported previously (McGenity et al., 1998).

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Similar claims had been made earlier by Dombrowski (1963) and Reiser & Tasch (1960); the notion of revival of micro-organisms from dormancy after several millenia predictably elicited scepticism. It is sometimes argued that the isolates result from laboratory contamination. We decided that a detailed comparison of micro-organisms from rock salt from different geographical locations should provide information on the presence of similar or perhaps identical strains. One pink coccoid isolate, designated as strain Br3, from a British halite formation (Norton et al., 1993) had a similar whole-cell protein pattern and an identical partial 16S rRNA sequence (212 bases) to \textit{Hc. salifodinae} Blp (Denner et al., 1994). These results suggested a close relationship between the two micro-organisms, which were isolated from geographically distant ancient salt sediments of Triassic to Permian age. Another pink coccoid isolate, designated as strain H4, was obtained from deep core salt sediment drillings in the salt mine in Berchtesgaden, Germany (50 km from the salt mine near Bad Ischl). In this study we present evidence for a very close relationship between the three strains by phenotypic and molecular analyses, which justifies their designation as members of a single species; in addition, we describe further isolates from the Bad Ischl salt mine, which were similar enough to \textit{Hc. salifodinae} Blp to consider them strains of the same species, obtained 8 years after the initial rock salt samples were taken.

**METHODS**

**Strains and culture conditions.** Several independently isolated strains were investigated in this study: (a) \textit{Halococcus salifodinae} Blp (DSM 8989\textsuperscript{T} = ATCC 51437\textsuperscript{T} = JCM 9578\textsuperscript{T}), isolated in 1989 from surface-sterilized rock salt of the Bad Ischl salt mine, Austria; its isolation and initial characterization were described previously (Stan-Lotter et al., 1993; Denner et al., 1994); (b) strain Br3, isolated by W. D. Grant from solution-mined brine at Lostock, Cheshire, UK (Norton et al., 1993); (c) strain BG2/2, isolated by one of us (K.O.S.) in 1988 from a deep drilling core taken in the salt mine near Berchtesgaden, Germany; (d) recent isolates from the Bad Ischl salt mine, which were similar enough to \textit{Hc. salifodinae} Blp to consider them strains of the same species, obtained 8 years after the initial rock salt samples were taken.

**16S rRNA sequences and analysis.** Nucleotide sequences of the 16S rRNA genes of \textit{Hc. salifodinae} Blp DSM 8989\textsuperscript{T}, and strains BG2/2, N1 and H2, were determined by the Identification Service of the DSMZ; the 16S rRNA gene of strain Br3 was sequenced as described previously (McGenity & Grant, 1995). Extraction of the genomic DNA, amplification of the 16S rDNA by PCR and purification of the products were performed as described by Rainey et al. (1996). The purified 16S rDNA was sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) as indicated by the manufacturer. The DNA fragments of the sequencing reactions were separated and analysed using an Applied Biosystems 373 DNA Sequencer. The resulting sequences were read into the Alignment Editor (Maidak et al., 1996), aligned manually and compared with 16S rRNA gene sequences of representative archaean. Sequences used for comparisons were obtained from the EMBL database or the database of the RDP (Maidak et al., 1996). The phylogenetic tree was constructed using the neighbour-joining method of Saitou & Nei (1987) in the PHYLIP program (Felsenstein, 1993), following transformation of sequence distances according to Jukes & Cantor (1969). Confidence of the branching pattern was assessed by 100 bootstrap analyses, using the PHYLIP package.

**DNA base composition.** Cells were harvested in the late-exponential phase of growth and G+C content was determined by the Identification Service of the DSMZ. Cells were broken by passage through a French press (Aminco) and DNA was isolated and purified according to the procedures of Cashion et al. (1977) and Viswanathan et al. (1989). Analysis was tested as described by Smibert & Krieg (1994). Additional characterization with respect to the presence of enzymes was carried out with API ZYM (bioMerieux) strips. These were used according to the instructions of the manufacturer, except that cells were suspended in mineral solution I (Tomlinson & Hochstein, 1976), which contained 200 g NaCl l\textsuperscript{-1}.

**Electron microscopy.** Cells were harvested after 1 week of incubation in M2 medium and prepared for scanning or transmission electron microscopy as described previously (Denner et al., 1994).

**Polar lipid analysis.** Strains were grown on halophile medium agar, until colonies were nearly confluent (\textsim 14 d). Colonies were removed from the surface of three agar plates by spreading with 2 ml 20\% (w/v) NaCl, and transferring the cell suspension to a centrifuge tube. Cells were harvested by centrifugation and freeze-dried. Approximately 0.5 g freeze-dried cells were stirred with 5 ml chloroform/methanol (1:1, v/v) at 50 °C for 16 h. Extracted lipids were separated from cells by filtration through a 0.2 µm pore-size PTFE filter, and dried under a stream of oxygen-free nitrogen. The dried polar lipids were resuspended in ~0.3 ml chloroform/methanol (1:1, v/v). For two-dimensional thin-layer chromatography the method of Collins et al. (1980) was used. Polar lipid extracts were spotted onto the corner of a thin-layer 10×10 cm silica gel plate (60 F254, Merck), and were developed first in chloroform/methanol/water (65:25:4, by vol.), and then in chloroform/methanol/glacial acetic acid/water (80:12:15:4, by vol.). For one-dimensional development the latter solvent mixture was used. Lipids were visualized by dipping dry TLC plates in 0.1\% (w/v) ceric sulphate in 1 M sulphuric acid, and charring at 150 °C for 5 min. Glycolipids appeared as purple spots for up to 1 h after charring, while other lipids appeared brown. The equivalence of spots was determined by co-chromatography in two dimensions and by determining R\textsubscript{f} values based on one-dimensional chromatography.

**Phenotypic characterization.** Biochemical assays and antibiotic susceptibility tests were described by Denner et al. (1994). Hydrolysis of casein, starch, Tween 20 and Tween 80...
Fig. 1. Scanning electron micrographs of strains BG2/2 (A), Br3 (B) and *Hc. salifodinae* Blp DSM 8989\(^T\) (C), grown in liquid culture (M2 medium); and electron micrographs of ultrathin sections of *Hc. morrhuae* DSM 1307\(^T\) (a), *Hc. salifodinae* Blp DSM 8989\(^T\) (b) and strain BG2/2 (c).
Detection and sequencing of an insertion in halococcal SS rRNA genes. Primers were designed to flank the region of the SS rRNA gene in which an insertion of 108 bases has been reported in *Hc. morrhuae* ATCC 17082T (Luehrs en *et al.*, 1981; see Fig. 4). An alignment of 19 halobacterial SS rRNA sequences demonstrated that there was a highly conserved region towards the 5′ end (primer 5SF), but none at the 3′ end of the gene, and so the reverse primer was designed from the cysteine tRNA gene just downstream of the SS rRNA gene (primer cysR). The sequences of the PCR primers were: 5SF, 5′-CGTACCCAT(T/C)CGGAA-C-3′, and cysR, 5′-CTGCC-ACCTTGGGC(A/G)CA-3′. DNA was extracted by the method of Pitcher *et al.* (1989), using guanidinium thiocyanate. The PCR included BioXact polymerase (2 units) in buffer (Bioline), with the indicated final concentrations of MgCl₂ (2.5 mM); dNTPs (200 µM), BSA (400 ng µl⁻¹), and DNA (~10 ng). Cycle conditions were: 2 min denaturation at 94 °C, followed by 30 cycles of denaturation (15 s at 94 °C), annealing (20 s at 55 °C) and extension (1 min at 68 °C). Reactions were carried out in thin-walled tubes in a Progene thermal cycler (Techne). PCR products (4–7 µl) were separated by electrophoresis at 105 V for 60 min in a 2% (w/v) agarose gel in TAE buffer, and visualized after staining with ethidium bromide using a UV transilluminator (Sambrook, 1989). The PCR product was cleaned using a Qiagen column, and sequenced with a primer (5′-AGTACTGGAGTGTGCGA-3′) labelled at the 5′ end with Cy5 (Pharmacia). For the cycle sequencing reaction and electrophoresis on an ALF-Express (Pharmacia) automated sequencer the procedures described by the manufacturer were followed.

Fourier-transform infrared (FT-IR) spectroscopy. Bacterial strains were streaked on agar plates containing M2 medium and incubated for 1–2 weeks. Bacterial films were prepared by suspending one loopful (1 mm diameter) in 80 µl distilled water and applying an aliquot of 35 µl to a preformed sample area on a zinc selenite optical plate. Following drying of samples under reduced pressure, spectra were recorded between wave numbers 4000 cm⁻¹ to 500 cm⁻¹ in an FT-IR spectrometer type IFS 28/B (Bruker) as described by Helm *et al.* (1991a, b). Data analysis was carried out using the OPUS 3.0 software for bacterial identification from the same manufacturer. At least six independent preparations of each investigated strain were measured and a mean spectrum was calculated.

Other methods. SDS-PAGE of whole-cell proteins was performed following lysis of archaea as described previously (Stan-Lotter, 1989, 1993). At least six gels were run with each of the samples. Protein was determined by the Lowry method, with bovine serum albumin as standard.

**RESULTS**

**Cellular and colonial morphology**

Cells of the salt mine isolates Br3 and BG2/2 were cocci of about 0.8–1.2 µm in diameter, as was observed previously for *Hc. salifodinae* Blp DSM 8989T (Denner *et al.*, 1994). Strains Br3, BG2/2, N1 and H2 showed very similar behaviour with respect to reduction of nitrate (positive); hydrolysis of casein (negative), TWEEN 80 (positive) and starch (positive); liquefaction of gelatin (negative); presence of catalase and oxidase (both positive), and susceptibility to antibiotics. Optimal growth occurred at NaCl concentrations of 20–25%; no growth was observed at NaCl concentrations below 15%. The following enzyme activities were detected in the salt mine halococci appeared to be partly detachable during the preparation for electron micrography (Fig. 1A, B). Colonies on complex solid medium were circular with irregular margins, 1–2 mm in diameter after 10 d incubation at 37–39 °C, and had pink pigmentation. Several of the new isolates from rock salt (N1 to N7; H1 to H4) exhibited similar colonial appearance, including pigmentation, which is characteristic for *Hc. salifodinae*, and also coccoid morphology and growth in clusters, as observed by light microscopy (not shown).

**Biochemical and physiological characterization**

The initial phenotypic characterization was described previously for *Hc. salifodinae* Blp DSM 8989T (Denner *et al.*, 1994). Strains Br3, BG2/2, N1 and H2 showed very similar behaviour with respect to reduction of nitrate (positive); hydrolysis of casein (negative), TWEEN 80 (positive) and starch (positive); liquefaction of gelatin (negative); presence of catalase and oxidase (both positive), and susceptibility to antibiotics. Optimal growth occurred at NaCl concentrations of 20–25%; no growth was observed at NaCl concentrations below 15%. The following enzyme activities were detected in the saline of distinct layers (Fig. 1a). The envelope of all three of...
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**Fig. 3.** Phylogenetic dendrogram of halobacteria based on 16S rRNA gene sequence data, indicating the position of the salt mine isolates Br3 and BG2/2. The tree was constructed using the neighbour-joining method of Saitou & Nei (1987). *Methanospirillum hungatei* was used as outgroup. The bar represents the scale of estimated evolutionary distance (i.e. a mean of 10 substitutions at any nucleotide position, per 100 nucleotide positions) from the point of divergence of the 16S rRNA gene sequences. Bootstrap percentages are indicated at the nodes, except when values were less than 75%.

all strains, including *Hc. salifodinae* Bp DSM 8989T, using API ZYM: alkaline phosphatase, esterase (C4) and esterase lipase (C8); not present were lipase (C14), leucine arylamidase, cystine arylamidase, trypsin, chymotrypsin, naphthol-AS-biphosphohydrolase, \( \alpha \)-galactosidase, \( \beta \)-galactosidase, \( \beta \)-glucuronidase, \( \alpha \)-glucosidase, \( \alpha \)-mannosidase and \( \alpha \)-fucosidase. Variable reactions were observed for the presence of acidic phosphatase and \( N \)-acetyl-\( \beta \)-glucosamine, and for the hydrolysis of Tween 20. All strains were susceptible to anisomycin, aphidicolin, bacitracin, chloramphenicol and novobiocin, but not to ampicillin, nalidixic acid, tetracycline and streptomycin.

**DNA base composition**

The mol % G + C contents of the strains were as follows (means of three determinations are stated, ± SEM, except for H2, where the mean of four determinations is given): BG2/2, 63.9 ± 0.1; Br3, 61.0 ± 0.1; N1, 62.2 ± 0.5; H2, 62.8 ± 0.4. These values were similar to that of *Hc. saclifodinae* Bp DSM 8989T (62.1 ± 1; Denner et al., 1994) and generally somewhat higher than that of *Hc. saccharolyticus* ATCC 49257T (59.5; Montero et al., 1989).

**Gel electrophoresis of whole-cell proteins**

SDS gel electrophoresis of whole-cell proteins is a rapid method for distinguishing bacterial species and has a similar level of discrimination as DNA–DNA hybridization (Jackman, 1987). Bacterial cells that are grown under carefully standardized conditions produce constant protein patterns, which greatly facilitates the identification of strains (Vauterin et al., 1993). This method has been widely used in the systematics of
numerous bacterial strains (Kersters & De Ley, 1980), including the identification of several novel isolates by our laboratories (Denner et al., 1994; Dang et al., 1996; Nguyen et al., 1999). The whole-cell protein patterns of \textit{H. salifodinae} Bpl DSM 8989\textsuperscript{T}, Br3, BG2/2, N1 and H2, following separation by SDS gel electrophoresis, were very similar (Fig. 2, lanes 2–7); they were distinctly different from the protein patterns of \textit{H. morrhuae} DSM 1307\textsuperscript{T} (lane 1) and \textit{H. saccharolyticus} ATCC 49257\textsuperscript{T} (lane 8) (see also Denner et al., 1994).

16S rRNA sequences and phylogeny

The almost complete 16S rDNA sequences of \textit{H. salifodinae} Bpl DSM 8989\textsuperscript{T}, strain Br3 and strain BG2/2 were determined; they comprised 1302, 1465 and 1422 bases, respectively, and were found to be identical. A phylogenetic tree was constructed by alignment of the sequences with those of several representative halophilic archaea and the methanogen \textit{Methanospirillum hungatei} as an outgroup (Fig. 3). The groupings in the tree largely confirmed earlier analyses (Lodwick et al., 1994; Denner et al., 1994; McGenity & Grant, 1995), which suggested that halococci formed a distinct lineage within the halobacteria and shared a common ancestor. The present analysis clearly associated \textit{H. salifodinae} Bpl DSM 8989\textsuperscript{T}, and strains Br3 and BG2/2, with the halococci group. The 16S rRNA gene sequences of these three salt mine isolates were more similar to \textit{H. saccharolyticus} (99% sequence similarity) than they were to \textit{H. morrhuae} (94.2–94.9% sequence similarity). Partial 16S rRNA sequences were determined from strains N1 and H2; bases 6–291 of N1 (Br3 numbering), 6–250 of H2 (Br3 numbering) and 920–1315 of both N1 and H2 were identical to the respective sequences of \textit{H. salifodinae} Bpl DSM 8989\textsuperscript{T}, Br3 and BG2/2.

Insertion sequence in the 5S rRNA gene

A PCR product of the same size was produced by \textit{H. saccharolyticus} ATCC 49257\textsuperscript{T}, \textit{H. salifodinae} Bpl, and strains Br3 and BG2/2, when a region including the 5S rRNA gene was amplified. This indicated that all have an insertion in their 5S rRNA gene similar in size (108 bases) to that reported for \textit{H. morrhuae} ATCC 17082\textsuperscript{T} (Luehrsren et al., 1981). The expected, and obtained, size of the PCR product for \textit{H. morrhuae} ATCC 17082\textsuperscript{T} was 327 nt (Leffers et al., 1981), while that for \textit{H. volcanii} ATCC 29605\textsuperscript{T} was 291 nt (Daniels et al., 1985; accession number X02128; data not shown). Ninety-six bases from the 108-base insert were sequenced, and it was notable that the sequences of Hc. \textit{mor rhuae} and \textit{H. salifodinae} BIp DSM 8989\textsuperscript{T}, strains Br3 and BG2/2 (labelled as ‘others’), which was identical in all four strains. Differences between the sequence of the insert from \textit{H. morrhuae} and the other halococci are indicated by boxes.

Fig. 4. 5S RNA gene and 3’-flanking region of \textit{H. morrhuae} ATCC 17082\textsuperscript{T} (Hc.mor) (adapted from Leffers et al., 1987; accession number X72588). Indicated are the positions of the primers (underlined) used to amplify the region containing the 108-base insertion (bases 30–46 correspond to primer 5SF; bases 341–356 correspond to primer cysR), the position of the sequencing primer (bases 76–92) and the partial sequence of the insert from \textit{H. saccharolyticus} ATCC 49257\textsuperscript{T}, \textit{H. salifodinae} Bpl DSM 8989\textsuperscript{T}, strains Br3 and BG2/2 (labelled as ‘others’), which was almost identical for all four strains. Differences between the sequence of the insert from \textit{H. morrhuae} and the other halococci are indicated by boxes.
Halococci from salt deposits

(a) Strain BG2/2 (b) Hc. salifodinae DSM 8989T (c) Hc. saccharolyticus ATCC 49257

PG, glycerol diether of phosphatidylglycerol; PGP-Me, glycerol diether of phosphatidylglycerol methylphosphate; SDGD-1, sulphated mannosylglucosylglycerol diether.

Fig. 5. Polar lipid patterns of strain BG2/2 (a), Hc. salifodinae DSM 8989T (b) and Hc. saccharolyticus ATCC 49257T (c), following separation by two dimensional thin-layer chromatography. PG, glycerol diether of phosphatidylglycerol; PGP-Me, glycerol diether of phosphatidylglycerol methylphosphate; SDGD-1, sulphated mannosylglucosylglycerol diether.

Fig. 6. Dendrogram clustering of FT-IR spectra of halococci. Cluster analysis was performed using the first derivative of the spectra between 500 and 4000 cm⁻¹. Hc. mor., Hc. morrhuae DSM 1307T; Hc. sac., Hc. saccharolyticus ATCC 49257T; Hc. sal. Blp, Hc. salifodinae Blp DSM 8989T; Br 3, strain Br3; BG 2/2, strain BG 2/2.

Distinguishable glycolipids which did not migrate far from the origin. Hc. saccharolyticus ATCC 49257T also had a prominent glycolipid that ran just beyond SDGD-1 in both the x and y directions, labelled as P-1 (Fig. 5).

FT-IR spectroscopy

FT-IR spectra of Hc. morrhuae DSM 1307T, Hc. saccharolyticus ATCC 49257T, Hc. salifodinae Blp DSM 8989T, and strains Br3 and BG2/2 were largely similar from 900 to 4000 cm⁻¹, but showed distinct differences in the region between 600 and 900 cm⁻¹, where characteristic features are often noticed; Helm et al. (1991a) termed this spectral domain the ‘bacterial fingerprint region’. FT-IR spectra were compared by converting absorbance bands to spectral distances (Helm et al., 1991a), where values between 0 and about 10 are to be taken as an indication of identical or indistinguishable spectra, while values of up to 2000 are theoretically possible between unrelated micro-organisms (Helm et al., 1991a). Cluster analysis (Fig. 6), using the first derivative of IR spectra taken between 500 and 4000 cm⁻¹, showed a very close relationship between Hc. salifodinae Blp DSM 8989T, strain Br3 and strain BG2/2 (spectral differences below 18), but lower levels of similarity for Hc. saccharolyticus ATCC 49257T and Hc. morrhuae DSM 1307T (spectral differences of 60 and 120, respectively).

DISCUSSION

Morphological similarities between the salt-deposit halococci Hc. salifodinae Blp DSM 8989T, strain BG2/2 and strain Br3 included the appearance, size and arrangement of the cells (Fig. 1A–C), the appearance of the cell envelope in ultrathin sections (Fig. 1b, c), and colonial features, such as pink pigmentation. On the molecular level, the sequences of the 16S rRNA genes and of an insert in the 5S rRNA gene over a range of 96 bases (Figs 3 and 4) were identical in all investigated strains. Further strong similarities were found between whole-cell protein patterns (Fig. 2), G + C contents, types and abundance of polar lipids (Fig. 5), and FT-IR spectra (Fig. 6). Biochemical and physiological properties such as susceptibility to antibiotics, presence of enzymes, and growth characteristics were nearly identical. The coccoid isolates from salt deposits were distinct from other validly described Halococcus species, such as Hc. morrhuae (DSM 1307T, DSM 1308, DSM 1309) and Hc. saccharolyticus ATCC 49257T with respect to 16S rRNA sequence (see Ventosa et al., 1999), whole-cell protein patterns, polar lipid composition and cellular components (Denner et al., 1994; this work). Taken together, the results unequivocally indicate that the salt-deposit halococci should be assigned to the same species, Hc. salifodinae. Hc. salifodinae is distinct from other
halococi, but, based on 16S rRNA sequences, 5S rRNA insert sequences and FT-IR spectrometry, appears phylogenetically more closely related to *Hc. saccharolyticus* than to *Hc. morrhuae*.

The origins of the halococci isolates were subterranean salt sediments in different parts of northern Europe: walls from a freshly blasted tunnel in the salt mine near Bad Ischl (Austria), a drilling core from a salt deposit in Berchtesgaden (Germany), and solution-mined brine from Lostock, Cheshire (England). Over the past decade it has become increasingly apparent that microorganisms survive in a wide variety of sediments, such as sandstones, chalk and palaeosols, often extending to depths of several hundreds of metres (see Amy & Haldeman, 1997). The geological age of many of these environments dates back thousands or millions of years. However, the origin and transport of microorganisms within these sediments remain uncertain, since numerous ubiquitous microorganisms have been found, which occur also in surface environments. In contrast, halite deposits represent a physically more enclosed environment (see Grant et al., 1998); they are characterized in general by the absence of water (except for fluid inclusions; see below), and species variety is limited. The microorganisms which were previously isolated from surface-sterilized rock salt have been shown to be extremely and obligately halophilic, unable to grow in less than 1·5 M NaCl (Bibo et al., 1983; Norton et al., 1993; Denner et al., 1994; Grant et al., 1998); our strains of *Hc. salifodinae* required at least 2·5 M NaCl for growth.

It has been suggested that obligately halophilic microorganisms isolated from ancient salt deposits are actually the result of laboratory contamination. Our investigation provides the strongest evidence to date that this is not the case. Each isolation was independent; each sample was from a different location; the detailed polyphasic characterization demonstrated that the three salt-deposit strains were almost identical, yet distinct from other halococi. In addition, several very similar strains have been isolated recently from walls of newly blasted tunnels of the Bad Ischl salt mine; two of them, N1 and H2, were characterized in more detail in this study and found to be identical to *Hc. salifodinae*. Besides the halococi described here, we have isolated approximately 30 more extremely halophilic strains from the same pieces of rock salt, all of which possessed red, pink or orange pigmentation (A. Legat & H. Stan-Lotter, unpublished results), similar to the halophilic isolates from the Winsford salt mine in England (Norton et al., 1993).

In order to consider how the strains of *Hc. salifodinae* came to be inside the salt deposits, it is necessary to understand the depositional setting of the evaporites. The age of the Austrian salt sediments is known from under the depositional setting of the evaporites. In order to consider how the strains of *et al*., 1993) isolates from the Winsford salt mine in England (Norton & Grant, 1988) have shown that several halobacteria trapped inside fluid inclusions in laboratory-grown halite remain viable and thus may be capable of dormancy. In ancient evaporites, between 30 and 1000 p.p.m. of brine is present in the form of fluid inclusions (Roedder, 1984), within which bacterial cells could have become enclosed. Halobacteria may be adapted better than most microorganisms to survive long periods of dormancy due to the extremely high

Our working hypothesis is that the halococi from salt deposits represent relict populations from hypersaline Permo-Triassic seas, which became restricted in habitat when the seas evaporated and were buried. A significant question concerns how the halococi could have survived for hundreds of millions of years within salt deposits. Norton & Grant (1988) have shown that several halobacteria trapped inside fluid inclusions in laboratory-grown halite remain viable and thus may be capable of dormancy. In ancient evaporites, between 30 and 1000 p.p.m. of brine is present in the form of fluid inclusions (Roedder, 1984), within which bacterial cells could have become enclosed. Halobacteria may be adapted better than most microorganisms to survive long periods of dormancy due to the extremely high
(approx. 5 M) concentration of KCl inside their cells, which confers considerable stability to DNA (Marguet & Forterre, 1998). Grant et al. (1998) discussed several plausible scenarios for the long-term survival of halo-
bacteria in salt deposits, such as potential energy
sources, or the formation of special structures, such as
cyst-like resting cells. While cysts or spores have not
been detected in H. salifodinae, its cells have some
unalusual features. For example, Fig. 1(b) shows the
presence of a common cell envelope, similar to the
common capsule seen in some halobacteria from soils,
which was suggested to be involved in survival during
unfavourable environmental conditions (Kostrikina et
al., 1991). Therefore, the cell structure of H. salifodinae
may help its possible long-term survival. Furthermore,
studies with Vibrio and other marine bacteria suggested
that non-sporing-forming cells survive long periods of
starvation as well as spores (see Kjelleberg, 1993; Morita,
1997). It is uncertain whether the salt-deposit
halococci have actually been in a state of dormancy for
more than 200 million years, or if extremely slow in situ
reproduction (Kennedy et al., 1994) has occurred. In any
case, the fact that the isolates from different geo-
ographical areas appear so very similar suggests that
perhaps they have not been evolving rapidly. Alterna-
tively, if it is considered that Hc. salifodinae strains
and other halophilic archaea somehow entered the salt
deposits in recent times, and in different geographical
locations, then it would be necessary to propose a
suitable mechanism. To date, Hc. salifodinae has not
been isolated from surface hypersaline environments,
nor from air samples, although further work is required
to specifically search for it, using species-specific DNA
probes.

It is worthy of note that the pink halococci resembling
Hc. salifodinae grow relatively fast, producing colonies
in about 3–4 weeks, but other, red- or mauve-pigmented
halobacteria from the same rock-salt samples grow
much more slowly (in the order of several months) on
agar plates (H. Stan-Lotter & A. Legat, unpublished
results). A characterization of these isolates is under
way.

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