Spherical particles of halophilic archaea correlate with exposure to low water activity – implications for microbial survival in fluid inclusions of ancient halite

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ABSTRACT

Viable extremely halophilic archaea (haloarchaea) have been isolated from million-year-old salt deposits around the world; however, an explanation of their supposed longevity remains a fundamental challenge. Recently small roundish particles in fluid inclusions of 22 000- to 34 000-year-old halite were identified as haloarchaea capable of proliferation (Schubert BA, Lowenstein TK, Timofeeff MN, Parker MA, 2010, Environmental Microbiology, 12, 440–454). Searching for a method to produce such particles in the laboratory, we exposed rod-shaped cells of Halobacterium species to reduced external water activity (aw). Gradual formation of spheres of about 0.4 μm diameter occurred in 4 M NaCl buffer of aw/C20.75, but exposure to buffered 4 M LiCl (aw/C20.73) split cells into spheres within seconds, with concomitant release of several proteins. From one rod, three or four spheres emerged, which re-grew to normal rods in nutrient media. Biochemical properties of rods and spheres were similar, except for a markedly reduced ATP content (about 50-fold) and an increased lag phase of spheres, as is known from dormant bacteria. The presence of viable particles of similar sizes in ancient fluid inclusions suggested that spheres might represent dormant states of haloarchaea. The easy production of spheres by lowering aw should facilitate their investigation and could help to understand the mechanisms for microbial survival over geological times.

INTRODUCTION

During the past years, renewed interest in the presence of viable prokaryotes in ancient salt deposits has surfaced, which was partly stimulated by the detection of halite on Mars (Treiman et al., 2000; Squyres et al., 2006) and elsewhere in the universe (Zolensky et al., 1999). The first cultivations of halophilic micro-organisms from Permian salt deposits (about 250 million years old) were reported in the 1960s (Reiser & Tasch, 1960; Dombrowski, 1963).
on the possibility of microbial contamination during isolation and processing of samples, as well as uncertainty about their real age (McGenity et al., 2000; Park et al., 2009; Schubert et al., 2010). Retrieval of DNA from ancient sediments (Radax et al., 2001; Fish et al., 2002), repeated isolations from the same site (Stan-Lotter et al., 1999), and recent reports of successful haloarchaeal cultivations from well-dated bore cores (Schubert et al., 2010; Gramain et al., 2011) supported the presence of biological material in evaporites of great geological age. Survival of cells over millennia of years in dry sediments or sedimentary rocks on Earth would have important implications for the search for life on other planets, where sediment-like structures exist (Squyres & Knoll, 2005).

Plausible mechanisms for the long-term survival of extremely halophilic archaea in salt sediments have yet to be clarified. Genome sequences suggested that they do not produce spores (Onyenweke et al., 2004), which are resting states of some bacteria and eukaryotes for survival of unfavorable conditions. The occurrence of other types of haloarchaeal resting states, such as cysts, has been discussed (Grant et al., 1998), but could not yet be demonstrated unequivocally. Fluid inclusions in halite were considered early as possible habitats for microorganisms (Norton & Grant, 1988). Indeed, a strain of Halobacterium salinarum was isolated from a single fluid inclusion in a 97 000-year-old halite crystal from Death Valley (Mormile et al., 2003). Recently, the presence of small spherical particles with <1 μm diameter in fluid inclusions of modern and ancient halite was reported (Schubert et al., 2009). The occurrence of roundish cells in laboratory-grown halite had also been described previously (Norton & Grant, 1988). Importantly, the successful propagation of spherical particles, which had been observed microscopically in fluid inclusions of 22 000- to 34 000-year-old halite, was demonstrated (Schubert et al., 2010); the resulting cultures were identified as three different genera of haloarchaea. Improved visualization by staining cells with fluorescent dyes had earlier revealed the preferential accumulation of haloarchaea (Fendrihan & Stan-Lotter, 2004; Fendrihan et al., 2006) and bacteria (Adamski et al., 2006) within fluid inclusions of laboratory-grown halite and not in the host crystal matrix. After prolonged entrapment, rod-shaped species of Halobacterium were apparently converted to roundish particles (Fendrihan & Stan-Lotter, 2004). In the absence of spore formation, it is tempting to assume that the spheres in ancient fluid inclusions represent a form of haloarchaeal resting state. Most haloarchaea possess an S-layer (surface layer), which forms the outer envelope of the cell and consists of glycoproteins, held together by non-covalent interactions (see Sára & Sleytr, 2000; for a review). They do not contain the covalently linked rigid peptidoglycan, which constitutes the cell wall of many bacteria. The haloarchaeal S-layer can be removed by chelating agents, leaving large fragile spheroplasts (Cline & Doolittle, 1992). Nothing is known yet about the mode of formation and properties of small haloarchaeal spheres, although some early reports described spheres of various sizes and stabilities when exploring the effects of different salts on halophilic archaea (Abram & Gibbons, 1961; Kushner & Bayley, 1963; Mohr & Larsen, 1963). Here we show that sphere formation is apparently a response to low external water activity (a_w or aH_2O) of several haloarchaeal species. We also describe a surprisingly simple and rapid procedure for the preparation of viable spheres, which should be useful for further molecular and biophysical studies to elucidate the long-term survival of microorganisms in sediments.

**MATERIAL AND METHODS**

**Haloarchaeal strains and culture conditions**

Strains Halobacterium salinarum DSM 670, Hbt. salinarum DSM 3754^T, Hbt. noricense DSM 15987^T, and Haloferax mediterranei DSM 1411^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Strain Hbt. salinarum NRC-1 (ATCC-700922) was purchased from LGC Teddington (Teddington, UK). Halobacterium strains DSM 670, DSM 3754^T, and DSM 15987^T were grown in DSM 823 medium (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium823.pdf). Hfx. mediterranei DSM 1411^T was grown in DSM 954 medium (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium954.pdf). Hbt. salinarum NRC-1 was grown in ATCC 2185 medium (http://www.lgcstandards-atcc.org/Attachments/3339.pdf). The pH of the growth media was 7.0. For solid media, 20 g L^-1 agar was added. Incubation of cultures was at 37 °C. Growth in liquid culture was monitored at 600 nm with a spectrophotometer (Novaspec II, Pharmacia, Vienna, Austria).

**Embedding of haloarchaea in salt crystals**

When the cultures reached an optical density (OD) of 0.9–1.3 [equal to approximately 10^8 to 5 × 10^9 colony-forming units (CFU) per mL], cells were harvested by centrifugation at 7000 g for 20 min at 20 °C (Sorvall RC6, rotor SLA 3000). The pellets were washed by resuspending them at a ratio of 500 mg (wet weight) per 10 mL of TN buffer (100 mM Tris–HCl, 4 mM NaCl, pH 7.4, adjusted with HCl) and centrifuged as before. Five milliliters each of the suspension was placed into 94-mm diameter petri dishes and stored in an incubation chamber at 37 °C, unless noted otherwise, for up to 70 days. The weight of the petri dishes was determined gravimetrically; after about 5–6 days of incubation, it did not decrease any further. For small-scale preparations, 50 μL of a haloarchaeal cell suspension, which
was stained with the LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, Lofer, Austria) as described previously (Leuko et al., 2004), was dried onto a glass slide for 2–3 days at ambient temperature in the dark.

**Preparation of haloarchaeal spheres**

Following embedding in halite for several days, salt crystals were dissolved by the addition of modified TN buffer that contained 3 M NaCl (instead of 4 M). If required, concentration of samples was performed by centrifugation at 10 000 g in a 4K-15 centrifuge (Sigma, Vienna, Austria) at 20 °C for 15–20 min. In later experiments, spheres were prepared by suspending haloarchaeal pellets in TL buffer, which consisted of 100 mM Tris prepared by suspending haloarchaeal pellets in TL buffer, g

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**Concentration of samples was performed by centrifugation at 10 000 g in a 4K-15 centrifuge (Sigma, Vienna, Austria) at 20 °C for 15–20 min.**

In later experiments, spheres were prepared by suspending haloarchaeal pellets in TL buffer, which consisted of 100 mM Tris–HCl, 4 M LiCl, pH 7.4 (adjusted with HCl) at a ratio of about 50 mg of cells (wet weight) per ml of buffer. Suspensions were left for several hours or overnight at ambient temperature. The resulting spheres were centrifuged as noted above and washed twice with TN buffer to remove LiCl.

**Electron and light microscopy**

Cells or spheres were prepared for scanning electron microscopy (SEM) by fixing with 4% glutaraldehyde in 0.15 M cacodylate buffer, containing 4.7 M NaCl, 80 mM MgSO4 × 7H2O, 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 carried out by Chris Frethem at the University of Minnesota Characterization Facility. Dimensions of cells and spheres were determined from electron micrographs. Five measurements were made for cells (rods) and 21 measurements for spheres.

Fluorescence microscopy of cells stained with the LIVE/DEAD® BacLight™ bacterial viability kit, referred to as LIVE/DEAD kit, was carried out as described previously (Leuko et al., 2004). For some experiments, a laser confocal microscope (Zeiss KLM 510, Vienna, Austria), equipped with an argon laser for excitation at 488 nm, was used. Photographs were taken with single-track two-channel examination, which allows mixing of images. The instrument was operated by the dedicated software Zeiss LSM 5, version 3.2.

Unstained cells were observed with the Leica (Vienna, Austria) DM 5000B microscope or an Eclipse E200 microscope (Nikon, Vienna, Austria), using phase contrast. Photography of light microscopic images was performed with a Powershot G10 camera (Canon, Vienna, Austria). For quantitative determinations, aliquots of cells and spheres were examined in a Thoma microscopic counting chamber (Lactan, Graz, Austria). Cultures with 3 × 10^8 CFU were used. Ratios of spheres to cells were determined with cultures from mid-log, late-log, and late stationary phase. At least 10 determinations were done for each growth phase.

**Biochemical and physiological tests**

Lysis of haloarchaea in water was determined by suspending cells or spheres in sterile distilled water and observing cellular integrity, or the loss of it, by light microscopy for 1–2 h. The enzymes cytochrome oxidase and catalase were tested by standard procedures (Smibert & Krieg, 1994). For additional enzyme activities, the Analytical Profile Index system API ZYM (bioMerieux, Vienna, Austria) was used (Humble et al., 1977). All API tests were performed at least three times.

Polar lipids were extracted, separated by thin-layer chromatography, and visualized as described (Stan-Lotter et al., 2002). The equivalence of spots was determined by cochromatography with the extract of Halobacterium salinarum NRC-1, whose polar lipid composition is known (Gruber et al., 2004).

**SDS polyacrylamide gel electrophoresis (SDS–PAGE)**

SDS–PAGE was performed using the gel system by Laemmli (1970). For whole-cell proteins, approximately 50 mg mL⁻¹ of cells or spheres (wet weight) was lysed by boiling in SDS sample buffer (Laemmli, 1970) for 10 min. For examination of released proteins, spheres that had been prepared by treatment with TL buffer were centrifuged at 10 000 g for 20 min. Supernatants were taken off and filtered through 0.22-μm sterile filters to remove any residual spheres. Filtered samples were applied to SDS polyacrylamide gels (criterion gradient gels 10.5–14% acrylamide; Bio-Rad, Vienna, Austria), following mixing with SDS sample buffer (Laemmli, 1970) in a ratio of 1:1, or, if required, were concentrated first about threefold with Centricon devices (Amicon) with a cutoff of 10 000 Da. Proteins were stained with Coomassie blue. Electrophoresis of whole-cell proteins and supernatants from spheres was repeated more than four times.

**Mass spectrometry of proteins**

Sequence analysis of proteins that were released into the supernatant, following sphere preparation by treatment with 4 M LiCl, was performed by LC-MS/MS. Proteins were separated by SDS polyacrylamide gel electrophoresis as described earlier. Coomassie-stained bands 1–7 (see Fig. S1) were excised and in-gel digested using the ProteoExtract All-In-One Trypsin Digestion Kit (Calbiochem, San Diego, CA, USA). Resulting peptides were separated by RP-HPLC (Nanocase Symmetry 300<sup>TM</sup> trap column and Nanocase Atlantis dC18™ separating column; Waters, Milford, MA, USA) directly coupled to an ESI quadrupole TOF mass spectrometer (Q-ToF Ultima Global; Waters/Micromass). For sequence identification, a combined Swiss-Prot/TrEMBL database was used. Experimental
details and instrument parameters used were described previously (Erler et al., 2011).

Other methods

Colony-forming units were determined following plating of aliquots of exposed and resuspended cells or, in the case of liquid cultures, of cell suspensions, on agar plates of 90 mm diameter containing solidified DSM 954 medium or ATCC 2185 medium. Plates were incubated at 37–40 °C for several days. Determination of ATP was performed in a microassay method, using the luciferin–luciferase reaction in 96-well microplates (Kiesslich et al., 2003). Reagents were obtained from Sigma-Aldrich except for luciferin (Invitrogen, Vienna, Austria) and luciferase (Promega, Mannheim, Germany). Luminescence was measured in an Infinite M200 microplate reader (Tecan, Groedig, Austria). Determination of protein was by Lowry’s method (Lowry et al., 1951), with BSA as standard. The number of determinations was at least 5 in both experiments. Water activity (aw) was determined with the portable Pawkit (IUL Instruments GmbH, Königswinter, Germany), using salt standards of known aw, which were supplied by the company (LiCl 0.25 aw, NaCl 0.76 aw), for calibration.

An estimation of sphere size was made in vivo by passing sphere suspensions of *Hbt. salinarum* NRC-1 (ca. 10⁹ CFU mL⁻¹) through filters of pore sizes 0.2, 0.45, 0.8, 1.2, and 5 μm (Minisart; VWR, Vienna, Austria), respectively, and examination of recovered cells in the filtrate by subsequent microscopy, using phase contrast. Suspensions of rod-shaped cells (ca. 10⁸ CFU mL⁻¹) served as controls.

RESULTS

Formation of spheres in fluid inclusions of halite

For slow embedding of haloarchaeal cells in halite, cultures of *Halobacterium* species in Tris-buffered 4 M NaCl (TN buffer) were left to dry at ambient temperature or 37 °C and stored for several days or weeks (see Material and Methods). On a smaller scale, drying was carried out on glass slides with haloarchaeal cells, which had been pre-stained with the LIVE/DEAD® BacLight™ bacterial viability kit, referred to as LIVE/DEAD kit, before embedding. The bright green fluorescence of cells stained with the dye SYTO9 outlined the shapes of the characteristic square or rectangular fluid inclusions of halite (Fig. 1, left). Upon higher magnification, spherical particles of *Halobacterium salinarum* NRC-1, entrapped within a fluid inclusion, became visible (Fig. 1, right). Most cells were fluorescing green, indicating intact membranes and therefore viability. Embedding in halite evidently caused transformation of formerly rod-shaped cells to nearly uniformly spherical particles. Scanning electron microscopy of spheres obtained from *Hbt. salinarum* NRC-1, which were fixed with glutaraldehyde after dissolution of halite crystals (see Methods), showed mainly single globular particles (Fig. 2B,D,F) with a diameter of 0.40 ± 0.02 (SD) μm. The diameter of the rods of *Hbt. salinarum* NRC-1 was about 0.65 μm (Fig. 2A; Kushner & Bayley, 1963). Occasionally spheres were adhering to each other in groups of 2–4 (Fig. 2C,E,G,H); as these arrangements were observed frequently, the assemblies most likely represented intermediate stages in the transition from rods to spheres. Images from fluorescence microscopy following staining of cells with the LIVE/DEAD kit contained frequently groups of three spheres (Fig. 3), corroborating the notion that one cell gave rise to several spheres and that adhesion of spheres persisted for some time.

The water activity (aw) of halophilic growth medium as well as TN buffer was 0.75 at ambient temperature, measured with a capacitance hygrometer (Paw kit; see Material and Methods). Mere storage of haloarchaeal rods in TN buffer did not alter their morphology. Therefore, we concluded that the water activity within fluid inclusions presumably decreased below 0.75 upon prolonged evaporation and that this reduction caused the formation of spherical particles.

Fig. 1 Localization of pre-stained cells of *Halobacterium salinarum* NRC-1 in halite fluid inclusions. Low magnification (left) and higher magnification of an individual fluid inclusion (right). Cells were stained with the LIVE/DEAD® BacLight™ bacterial viability kit prior to embedding in halite; epifluorescence microscopy was performed after entrapment of cells for 3 days.
Rapid conversion of rods to spheres by LiCl

If sphere formation is triggered by lowering of $a_w$, other salt solutions may bring about this effect, too. Replacement of Na$^+$ with other cations (K$^+$, Mg$^{++}$, Ca$^{++}$) or mixtures of such salts resulted in partial conversion of rods into large and small spheres, similar as observed by Abram & Gibbons (1961); in addition, cells or spheres often lost viability. However, suspension of rod-shaped haloarchaea in Tris-buffered 4 M LiCl (TL buffer) of an $a_w$ of 0.73 at 22–23 °C led to nearly immediate appearance of spheres (Fig. 4). The method was a convenient way to produce homogenous preparations of spheres. No influence of growth phase was noted, because cells in mid-log to late-log to stationary phase (with optical densities between 0.8 and 1.3) were always converted to spheres. The viability of haloarchaeal cells, when stored in TL buffer, declined within 2 days to near zero, as determined by CFU. Therefore, spheres made by exposure to 4 M LiCl were re-equilibrated in NaCl-containing buffer shortly after their formation (see Material and Methods).

The ratio of spheres to cells was 2.86 ± 0.97 (SD). This number was determined by using a microscopic counting chamber with cultures from mid-log, late-log, and stationary phase, respectively, which were counted before and after exposure to TL buffer. The ratio corroborated the previous observation that one rod gave rise to three or four spheres (see Figs 2 and 3).

Properties of spheres

A phenotypical characterization of spheres in comparison with rods is shown in Table 1. Besides the obvious differences of their dimensions, rods and spheres differed in ATP content, which was about 50-fold lower in spheres, and a slower sedimentation of spheres. Whole-cell protein SDS gel electrophoresis showed a reduced amount of the

Fig. 2 Scanning electron micrographs of rods (A) and spheres (B–H) of Halobacterium salinarum NRC-1. Spheres had formed in laboratory-grown halite and were obtained after dissolution of salt crystals. Bars, 600 nm (A), 270 nm (B–H).

Fig. 3 Groups of spheres from Halobacterium salinarum NRC-1, following embedding of rod-shaped cells in halite for 30 days, dissolution of salt crystals and staining with the LIVE/DEAD kit.
S-layer protein in spheres (Fig. 5), which is a glycoprotein and forms the outermost coat of \textit{Halobacterium} species (Mescher & Strominger, 1976) and several other haloarchaea. Its apparent molecular mass is about 200 kDa (see Fig. 5). Spheres contained the known polar lipids of \textit{Hbt. salinarum} (Gruber et al., 2004; Table 1); however, the higher centrifugal force needed for sedimentation (Table 1) was probably due to a relative increase of lipids in spheres. The results suggested a loss of proteins, ATP, and probably also other molecules during the conversion from rods to spheres, which decreased the specific weight of spheres. The proteins, which were released upon conversion, were separated by SDS polyacrylamide gel electrophoresis (Fig. S1), and some of the more prominent ones were subsequently identified by mass spectrometry (Table 2).

The S-layer protein of \textit{Halobacterium} made up the bulk of released proteins; other proteins of smaller M_r were also present (Fig. S1; Table 2). Lag phases of growth in liquid and on solid medium were increased for spheres (Table 1); other properties of rods and spheres examined here were very similar.

The size of spheres as determined from SEM micrographs (Fig. 2; Table 1) was obtained after fixing and drying. An estimation of sphere size was also made in \textit{vivo} by passing sphere suspensions of \textit{Hbt. salinarum} NRC-1 through filters of pore sizes in the range between 0.2 and 5 \textmu m (see Material and Methods). A cutoff at 0.45 \textmu m was observed for spheres, corroborating the diameter of spheres as \leq 0.45 \textmu m.

**Table 1** Properties of spheres and rods from \textit{Halobacterium salinarum} NRC-1

| Property                  | Rods                      | Spheres                  |
|---------------------------|---------------------------|--------------------------|
| Shape                     | Cylindrical rods          | Globule                  |
| Dimensions (from SEM)     |                           |                          |
| Length (\mu m)            | 2.5–4                     | –                        |
| Diameter (\mu m)          | 0.65 \pm 0.06 (SD)        | 0.40 \pm 0.02 (SD)       |
| Lysis in distilled water  | Yes                       | Yes                      |
| Main polar lipids         | PGP-Me*                   | PGP-Me*                  |
|                           | PGS*, PG*                 | S-TGD-1*, S-TeGD**       |
| Pigments (carotenoids)    | Dark red                  | Light red                |
| Enzymes assayed for       |                           |                          |
| Alkaline phosphatase      | +                         | +                        |
| Alpha chymotrypsin        | +                         | +                        |
| Acid phosphatase          | +                         | +                        |
| Naphtol AS Bi             | +                         | –                        |
| Phosphohydrolase          | +                         | +                        |
| Oxidase                   | +                         | +                        |
| Catalase                  | +                         | +                        |
| ATP content (ng per mg protein) | 620.0 \pm 85 (SD) | 12.4 \pm 15 (SD) |
| Sedimentation at          |                           |                          |
| 2500–3200 g               | 8000–10 000 g             |
| Lag phase (after dilution | 1–2 days                  | 10–12 days               |
| of 1:50 in fresh medium)  |                           |                          |
| Lag phase (on agar plates)| 4 days                    | \geq 10 days             |

Data for rods are from Gruber et al. (2004), except for ATP, which was determined in this work. For experimental details, see Material and Methods. *PGP-Me, phosphatidyl glycerol phosphate methylester; PGS, phosphatidyl glycerol sulfate; PG, phosphatidyl glycerol; S-TGD-1, sulfated triglycosyl diether; S-TeGD, sulfated tetraglycosyl diether.
Spheres, which were produced in laboratory-grown halite or by LiCl exposure, remained viable for at least 6 and 2 years, respectively, when stored in TN buffer. They preserved their morphology during this time and repeated staining with the LIVE/DEAD kit revealed green fluorescence, indicating viability. Upon streaking on agar plates, spheres produced normal colonies, which confirmed the preservation of propagation.

Several haloarchaeal genera can form spheres

The data reported here were obtained with the closely related strains *Hbt. salinarum* NRC-1 and *Hbt. salinarum* DSM 670. We examined several other haloarchaeal species for the ability of sphere formation under reduced a\(_w\). Under similar conditions as described earlier, cultures of *Hbt. salinarum* DSM 3754\(^T\) and *Hbt. noricense* DSM 15987\(^T\) were exposed to buffered 4 M LiCl and found to be converted to spheres (Fig. 6). The pleomorphic haloarchaeon *Halofex mediterranei* DSM 1411\(^T\) also produced spheres when exposed to 4 M LiCl (data not shown). These results suggested that the capacity for splitting cells into spheres at a\(_w\) below 0.75 is present in several genera of haloarchaea.

### DISCUSSION

**Formation and viability of spheres**

Our results suggested that rod-shaped cells of *Halobacte-rium* species gradually convert to small spheres upon a decrease of the external a\(_w\) to \(<0.75\), as occurs in fluid inclusions of halite during evaporation. In primary halite fluid inclusions, values of a\(_w\) \(<0.75\) have indeed been measured (Yang et al., 1995). We showed that also in a liquid medium, outside of halite fluid inclusions, formation of haloarchaeal spheres can be induced, because upon encountering an environment with a\(_w\) \(<0.75\), cells of *Halobacte-rium* split into spheres nearly instantaneously, when they were exposed to 4 M LiCl solutions of a\(_w\) \(\leq 0.73\). Spheres prepared either way – by evaporation of halite or addition

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### Table 2: Identification of *Halobacterium salinarum* DSM 670 proteins that were released during conversion from rods to spheres by exposure to TL buffer (Tris-buffered 4 M LiCl, pH 7.4), see Fig. S1

| Band | UniProtKB accession number | Number of peptides identified | Sequence coverage (%) | PLGS score | M, (kDa) theor./expt. | Function/name |
|------|---------------------------|------------------------------|-----------------------|------------|-----------------------|---------------|
| 1    | CSG_HALSA                 | 15                           | 31.2                  | 13.2       | 89.76\*/200           | Cell surface glyco-protein (S-layer) |
| 2    | CSG_HALSA                 | 18                           | 31.7                  | 13.2       | 86.76\*/185           | Cell surface glyco-protein (S-layer) |
| 3    | Q9HMW9_HALSA              | 18                           | 39.5                  | 14.8       | 72.28/78              | Dipeptide ABC transporter, dipeptide binding |
| 4    | Q9HM13_HALSA              | 8                            | 20.1                  | 14.0       | 61.88/65              | Dipeptide ABC transporter, ATP binding |
| 5    | Q9HPO4_HALSA              | 8                            | 24.0                  | 14.2       | 49.44/61              | Putative uncharacterized protein |
| 6    | Q9HMUB_HALSA              | 7                            | 24.0                  | 14.2       | 31.85/42              | Copper transport ATP binding protein |
| 7    | DPS_HALSA                 | 5                            | 57.0                  | 13.2       | 20.10/28              | DNA protection during starvation protein/Ferritin |

Theoretical M, were calculated using the Exasy server (http://web.expasy.org/cgi-bin/convert.pl?pl_tool). *Sugar residues were not included.

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Fig. 6 Rods (A1, B1) and spheres (A2, B2) of *Halobacterium salinarum* DSM 3854\(^T\) (A1, A2) and *Halobacterium noricense* DSM 15978\(^T\) (B1, B2), following staining with the LIVE/DEAD kit. Spheres were produced by exposure to Tris-buffered 4 M LiCl.
of LiCl solution – showed identical biochemical properties (Table 1). The morphological shift to spheres was accompanied by release of a portion of the S-layer protein and several other proteins and molecules. The spheres remained stable and viable in NaCl-containing buffer of \( a_w \) of 0.75 under laboratory conditions for at least 6 years. Native cells of haloarchaea survive also under similar conditions, because water samples from the Dead Sea, which were stored for 56 years, contained numerous viable haloarchaeal species (Arah al et al., 2000), and *Halobacterium* species, which were entrapped in halite crystals for 27 months, could be re-grown (Gramain et al., 2011), albeit with extended times for recovery, as was observed here (longer lag phases; see Table 1). Haloarchaeal spheres formed normal rods (similar as shown in Fig. 2A and Figs 4 and 6, upper panels) when nutrients were available and propagation commenced. Obviously, the spheres contained complete genomes and probably multiple copies of them, because up to thirty genome copies can be present in growing cells of *Halobacterium salinarum* (Breuer t et al., 2006). The mode of rapid breaking into spheres at low \( a_w \) suggested that preformed sites may exist in the cells, which allow fast splitting into several viable entities. The exact role of low \( a_w \) in this process is unknown; however, the speed of the conversion upon exposure to lithium ions would be compatible with conformational changes of proteins, perhaps caused by the removal of water shells, which triggers the fission of the cell. In any case, the mechanism constitutes an unknown mode of multiple cell fission, perhaps with similarity to the type of proliferation which has been described for some bacteria and organelles (Angert, 2005), but not yet for archaea. The identification of proteins that were released upon formation of spheres indicated that the bulk consisted of the S-layer protein; in addition, several membrane proteins (Table 2, bands 3, 4 and 6) and one cytoplasmic protein (Table 2, band 7) were also released. The total surface area of three to four spheres of 0.4 \( \mu \text{m} \) diameter each would be much less than the surface area of an average haloarchaeal rod of about 0.65 \( \times \) 2.5 \( \mu \text{m} \) (see Table 1); therefore, the release of a portion of the S-layer protein appears reasonable. A reduced amount of proteins, but retention of other molecules, such as lipids, would be in agreement with the observed lower density of the spheres, compared to rods (Table 1). However, the analysis of released proteins given here should be considered as a preliminary result, which needs to be extended for a more meaningful interpretation, but it could hold the promise of elucidating the mechanism of multiple fission (Angert, 2005).

As mentioned earlier, transformations from rods to spheres of *Hbt. salinarum* had been reported in early studies of haloarchaea, caused by variation of pH, anions, and cations (Abram & Gibbons, 1961; Kushn er & Bayley, 1963; Mohr & Larsen, 1963). In these cases, however, one cell was generally transformed into one large sphere, with a greater diameter than the rod-shaped progenitor and with a propensity to lysis and cell death. Occasionally, occurrence of smaller spheres was noted (Abram & Gibbons, 1961; Mohr & Larsen, 1963), and those derived from exposure to LiCl were deemed quite stable, but the phenomenon was not explored further.

**Dormant haloarchaea?**

Of interest is the ATP content of spheres, which was about 50-fold lower than that of rods (Table 1). Data from dormant or starved bacteria and spores indicated similarly reduced amounts of ATP, for example, the ATP content of dormant spores of several *Bacillus* species was about two orders of magnitude lower than that of actively growing *Bacillus* cells (Setlow & Kornberg, 1970); marine *Vibrio* species, which are deemed dormant when in the oceans, lost ATP during starvation experiments (Oliver & Stringer, 1984); and several strains of *Staphylococcus* showed a decline of ATP when adhering to polymer surfaces and entering a dormant, miniaturized state (Stollenwerk et al., 1998). Prokaryotic dormancy is not easy to define precisely (Kell & Young, 2000), but generally, a low amount of cellular ATP has been associated with a dormant state of various bacteria and spores.

The intriguing findings by Schubert et al. (2009, 2010) of spherical particles of about the same size as those described here in fluid inclusions of ancient halite, which can be propagated to haloarchaeal cultures, are probably the best evidence so far for the occurrence of viable long-term survivors in geologically old sediments. Schubert et al. (2010) called the spherical particles miniaturized cocc; however, the haloarchaea that were grown from these particles – members of the genera *Halorubrum*, *Natronomonas* and *Haloterrigena* – were not halophilic cocc, but are known rod-shaped species (http://www.the-icp.org/taxa/halobacterlist.htm). This suggested that the spherical particles in natural fluid inclusions might be in a dormant resting state, which is reversed to growth as rod-shaped or pleomorphic cells, once conditions for proliferation are met. Miniaturization of cells – sometimes called dwarfing (Nyström, 2004) – as consequence of starvation has been reported, besides *Staphylococcus* sp. (see above), for *Arthrobacter crystalllopictes* (Ensing, 1970), *Vibrio* sp. (Amy et al., 1983), and other bacteria (Nyström, 2004).

The data obtained in this work indicated a correlation of the formation of small spheres of halophilic archaea with reduced external water activity, which was brought about by high concentrations of NaCl or LiCl. The water activity of a saturated solution of NaCl at ambient temperature is 0.754 (http://www.emintech.com/1251exps.htm); sphere formation was never observed in such solutions, but rather in the fluid inclusion environment of halite. Lowering the
aw of a saturated NaCl solution could be achieved by the addition of other soluble salts, for example, Mg\textsuperscript{2+} salts; these can lead, however, to denaturation of macromolecules and to cell death (Hallsworth et al., 2007). A buffered solution of 4 M LiCl with aw of 0.73, on the other hand, was conducive for the formation of spheres and did not immediately impair the viability of haloarchaea. A detailed investigation of the mode of sphere formation and their properties should bring about insights into how haloarchaea inside natural fluid inclusions, where low aw is prevalent, may survive. Perhaps spheres can be identified as haloarchaeal resting states, for which no examples are known yet (Grant et al., 1998). Fluid inclusions were also found in billion-year-old meteorites and constitute apparently a very old type of structure in the universe (Zolensky et al., 1999). Thus, such studies may also be useful for the design of experiments aimed at the detection of potential extraterrestrial forms of microbial life in sediments of great geological age.

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REFERENCES

Abram D, Gibbons NE (1961) The effect of chlorides of monovalent cations, urea, detergents, and heat on morphology and the turbidity of suspensions of red halophilic bacteria. Canadian Journal of Microbiology 7, 741–750.

Adamski JC, Roberts JA, Goldstein RH (2006) Entrapment of bacteria in fluid inclusions in laboratory-grown halite. Astrobiology 6, 552–562.

Amy PS, Pauling C, Morita RY (1983) Starvation-survival processes of a marine Vibrio. Applied and Environmental Microbiology 45, 1041–1048.

Angert ER (2005) Alternatives to binary fission in bacteria. Nature Reviews Microbiology 3, 214–224.

Aralsal DR, Gutiérrez MC, Volcani BE, Ventosa A (2000) Taxonomic analysis of extremely halophilic archaea isolated from 56-years-old Dead Sea brine samples. Systematic and Applied Microbiology 23, 376–385.

Breuer T, Allers T, Spohn G, Soppa J (2006) Regulated assembly of the head of the bacteriophage T4. J Virol 80, e92.

Cline SW, Doolittle WF (1992) Transformation of members of the genus Haloarcula with shuttle vectors based on Halobacterium halobium and Haloferax volcanii plasmid replicons. Journal of Bacteriology 174, 1076–1080.

Denner EBM, McGenity TJ, Busse H-J, Wanner G, Grant WD, Stan-Lotter H (1994) Halococcus salifodinae sp. nov., an archaean isolate from an Austrian salt mine. International Journal of Systematic Bacteriology 44, 774–780.

Dombrowski H (1963) Bacteria from Paleozoic salt deposits. Annals of the New York Academy of Sciences 108, 453–460.

Ensign JC (1970) Long-term starvation survival of rod and spherical cells of Arthrobacter crystallopoietes. Journal of Bacteriology 103, 569–577.

Erlar A, Hawranek T, Krückemeier I, Asam C, Egger M, Ferreira F, Briza P (2011) Proteomic profiling of birch (Betula verrucosa) pollen extracts from different origins. Proteomics 11, 1486–1498.

Fendrihan S, Stan-Lotter H (2004) Survival of halobacteria in fluid inclusions as a model of possible biotic survival in martian halite. In Mars and Planetary Science and Technology (eds Teodorescu H, Grieble H). Performantica Press, Iasi, Romania, pp. 9–18.

Fendrihan S, Legat A, Pfaffenhümer M, Gruber C, Weidler G, Gerbl F, Stan-Lotter H (2006) Extremely halophilic archaea and the issue of long-term microbial survival. Reviews in Environmental Science and Biotechnology 5, 203–218.

Fish SA, Shepherd TJ, McGenity TJ, Grant WD (2002) Recovery of 16S ribosomal RNA gene fragments from ancient halite. Nature 417, 432–436. Erratum in: (2002) Nature 420, 202.

Gramain A, Chong Díaz GC, Demergasso C, Lowenstein TK, McGenity TJ (2011) Archael diversity along a subterranean salt core from the Salar Grande (Chile). Environmental Microbiology 13, 2105–2121.

Grant WD, Gemmell RT, McGenity TJ (1998) Halobacteria – the evidence for longevity. Extremophiles 2, 279–288.

Gruber C, Legat A, Pfaffenhümer M, Radax C, Weidler G, Busse H-J, Stan-Lotter H (2004) Halobacterium noricense sp. nov., an archaean 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. Extremophiles 8, 431–439.

Hallsworth JE, Yakimov MM, Golyshin PN, Gillion JLM, D’Auria G, Alves FDL, La Cono V, Genovese M, McKew BA, Hayes SF, Harris G, Giuliano L, Timmis KN, McGenity TJ (2007) Limits of life in MgCl\textsubscript{2}-containing environments: chaotropicity defines the window. Environmental Microbiology 9, 801–813.

Humble MW, King A, Philips I (1977) API Zym, a simple rapid system for the detection of bacterial enzymes. Journal of Clinical Pathology 30, 275–277.

Kell DB, Young M (2000) Bacterial dormancy and culturability: the role of autocrine growth factors. Current Opinion in Microbiology 3, 238–243.

Kierschlich T, Oberdanner CB, Krammer B, Plaetzer K (2003) Fast and reliable determination of intracellular ATP from cells cultured in 96 wells microplates. Journal of Biochemical and Biophysical Methods 57, 247–251.

Kushner DJ, Bayley ST (1963) The effect of pH on surface structure and morphology of the extreme halophile Halobacterium cutirubrum. Canadian Journal of Microbiology 9, 53–66.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227, 684–685.

Leuko S, Legat A, Fendrihan S, Stan-Lotter H (2004) Evaluation of the LIVE/DEAD BacLight kit for detection of extremophilic archaea and visualization of microorganisms in environmental hypersaline samples. Applied and Environmental Microbiology 70, 6884–6886.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193, 265–275.

McGenity TJ, Gemmell RT, Grant WD, Stan-Lotter H (2000) Origins of halophilic microorganisms in ancient salt deposits. Environmental Microbiology 2, 243–250.

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Squyres SW, Knoll AH, Arvidson RE, Clark BC, Grotzinger JP, Jolliff BL, McLennan SM, Tosca N, Bell JF III, Calvin WM, Farrand WH, Glotch TD, Golombek MP, Herkenhoff KE, Johnson JR, Klingelhöfer G, McSween HY, Yen AS (2006) Two years at Meridiani Planum: results from the opportunity rover. Science 313, 1403–1407.

Stan-Lotter H, McGinity TJ, Legat A, Denner EBM, Glaser K, Stetter KO, Wanner G (1999) Very similar strains of Halococcus salifodinae are found in geographically separated Permo-Triassic salt deposits. Microbiology 145, 3565–3574.

Stan-Lotter H, Pfaffenhuemer M, Legat A, Busse H-J, Radax C, Gruber C (2002) Halococcus dombrowskii sp. nov., an archaeal isolate from a Permian alpine salt deposit. International Journal of Systematic and Evolutionary Microbiology 52, 1807–1814.

Stan-Lotter H, Radax C, McGinity TJ, Legat A, Pfaffenhuemer M, Wieland H, Gruber C, Denner EBM (2004) From intraterrestrials to extraterrestrials - viable haloarchaea in ancient salt deposits. In Halophilic Microorganisms (ed. Ventosa A). Springer Verlag, Berlin, Heidelberg, New York, pp. 89–102.

Stollenwerk M, Fallgren C, Lundberg F, Tegenfeldt JO, Montelius L, Ljungh A (1998) Quantitation of bacterial adhesion to polymer surfaces by bioluminescence. Zentralblatt für Bakteriologie 287, 7–18.

Treiman AH, Gleason JD, Bogard DD (2000) The SNC meteorites are from Mars. Planetary and Space Science 48, 1213–1230.

Vreeland RH, Jones J, Monson A, Rosenzweig WD, Lowenstein TK, Timofeff M, Satterfield C, Cho BC, Park JS, Wallace A, Grant WD (2007) Isolation of live Cretaceous (121–112 million years old) halophilic Archaea from primary salt crystals. Geomicrobiology Journal 24, 275–282.

Yang W, Spencer RJ, Kroupa HR, Lowenstein TK, Casas E (1995) Stable isotopes of lake and fluid inclusion brines, Dabusun Lake, Qaidam Basin, western China: hydrology and palaeoclimatology in arid environments. Palaeogeography, Palaeoclimatology, Palaeoecology 117, 279–290.

Zolensky ME, Bodnar RJ, Gibson EK, Nyquist LE, Reecer Y, Shih CY, Wiesman H (1999) Asteroxial water within fluid inclusion-bearing halite in an H5 chondrite, Monahans (1998). Science 285, 1377–1379.

SUPPORTING INFORMATION

Additional information may be found in the online version of this article:

Fig. S1 SDS PAGE of soluble proteins from Halobacterium salinarum DSM 670 released upon exposure to Tris-buffered 4 M LiCl.

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