Survival of the Halophilic Archaeon *Halovarius luteus* after Desiccation, Simulated Martian UV Radiation and Vacuum in Comparison to *Bacillus atrophaeus*

Niloofer Feshangsaz, et al. [full author details at the end of the article]

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Abstract

Extraterrestrial environments influence the biochemistry of organisms through a variety of factors, including high levels of radiation and vacuum, temperature extremes and a lack of water and nutrients. A wide variety of terrestrial microorganisms, including those counted amongst the most ancient inhabitants of Earth, can cope with high levels of salinity, extreme temperatures, desiccation and high levels of radiation. Key among these are the haloarchaea, considered particularly relevant for astrobiological studies due to their ability to thrive in hypersaline environments. In this study, a novel haloarchaea isolated from Urmia Salt Lake, Iran, *Halovarius luteus* strain DA50^T, was exposed to varying levels of simulated extraterrestrial conditions and compared to that of the bacteria *Bacillus atrophaeus*. *Bacillus atrophaeus* was selected for comparison due to its well-described resistance to extreme conditions and its ability to produce strong spore structures. Thin films were produced to investigate viability without the protective influence of cell multi-layers. Late exponential phase cultures of *Hvr. luteus* and *B. atrophaeus* were placed in brine and phosphate buffered saline media, respectively. The solutions were allowed to evaporate and cells were encapsulated and exposed to radiation, desiccation and vacuum conditions, and their post-exposure viability was studied by the Most Probable Number method. The protein profile using High Performance Liquid Chromatography and Matrix Assisted Laser Desorption/Ionization bench top reflector time-of-flight are explored after vacuum and UV-radiation exposure. Results showed that the change in viability of the spore-forming bacteria *B. atrophaeus* was only minor whereas *Hvr. luteus* demonstrated a range of viability under different conditions. At the peak radiation flux of 10^5 J/m² under nitrogen flow and after two weeks of desiccation, *Hvr. luteus* demonstrated the greatest decrease in viability. This study further expands our understanding of the boundary conditions of astrobiologically relevant organisms in the harsh space environment.

Keywords *Halovarius luteus* · *Bacillus atrophaeus* · Desiccation · Mars simulation chamber · Simulated Martian UV radiation · Vacuum

Introduction

Various missions with an astrobiology component have been sent to Mars, and two upcoming flagship missions, ExoMars (Vago et al. 2017) and Mars2020 (Mustard et al. 2013), will search for indicators of life, past or present, with a greater capacity than ever before.

There are many signatures of life, from biomolecules to viable microbes, and it is important to understand the ability of these biosignatures to survive and persist, as the most stable signatures may still be preserved and have the potential to be detected during future missions.

There are numerous methods to study the survival of microorganisms in simulated extra-terrestrial environments, both in Earth orbit (Cottin et al. 2017) and in simulated conditions on Earth (Martins et al. 2017).

Ultraviolet (UV) radiation is ubiquitous in the Solar System and it is understood that UV radiation causes damage to cells and increases the rate of DNA mutation and eventually leads to death (Horneck 1999). UV radiation is grouped into three bands: UVA (315–400 nm), UVB (280–315 nm), and UVC (200–280 nm). Although UVB and UVC make up only 2% of the entire solar spectrum, these wavelengths are the ones primarily responsible for cell damage due to the high absorption of this wavelength range by DNA (Henning et al. 1995; Mancinelli 2015).

The stress factor of solar UV radiation was also very important during the Archean era as the Earth lacked an ozone layer and thus the surface was exposed to the complete spectrum of solar radiation (Monk et al. 1994). As a result, it is hypothesized that microorganisms which evolved on the Archean surface under these conditions may retain cellular mechanisms which developed to cope with elevated levels of full-spectrum UV radiation (Wynn-Williams et al. 2001).

In addition to whole microorganisms and spores, isolated biomolecules such as DNA, amino acids and liposomes have also been studied (De La Torre et al. 2010; Nicholson et al. 2000; Stan-Lotter and Fendrihan 2015).

Vacuum conditions and desiccation are two other key detrimental factors which affect microbial survival in the space environment. If there are no internal or external matrices protecting the cells, vacuum and desiccation can cause severe lesions (Dose and Gill 1995). Structural variations lead to functional alterations, such as changes in membrane permeability, inhibited or altered enzyme activity and variations of genetic information that can give rise to cell death or mutagenesis (Mancinelli 2015). Humidity reduction by desiccation can likewise impose ultimately lethal effects on microorganisms, especially in the case of non-spore forming strains. A degree of desiccation tolerance is present in a variety of organisms, including bacteria, archaea, yeast, lichens, fungus, plants, insects and crustaceans (Crowe et al. 1992; Csonka and Hanson 1991; Tepfer et al. 2012).

Halophilic archaea are some of the most primitive inhabitants of hypersaline environments in Earth's history (Oren and Mana 2002). Geological structures from the Permian and Triassic (290–206 Ma ago) have been found to contain viable archaea and bacteria, demonstrating their tolerance of desiccation (Fendrihan et al. 2009; Huu et al. 1999; McGenity et al. 2000; Norton and Grant 1988; Vreeland et al. 2000). Stromatolites 3.5 billion years in age may have been one of the first microenvironments to shelter life (Awramik 2006). Ancient halite has been demonstrated to preserve living microorganisms for more than 400 Ma; both stromatolites and halite are habitats for the archaeal family *Halobacteriaceae* (Leuko et al. 2010) which may have high degree of longevity (Grant et al. 1998; Landis 2001). It has also been shown that halophiles can endure the extreme conditions of desiccation, starvation and radiation exposure

for millions of years inside stromatolites and ancient halite (Leuko et al. 2010; Stan-Lotter and Fendrihan 2015; Vreeland et al. 2007).

It is likely that many of the coping strategies of these cells relate to the Precambrian era when the UV-shielding ozone layer was not yet formed (Baqué et al. 2013). Particularly relevant studies of halophilic archaea have been performed which include *Halobacterium salinarum* NRC-1 under UV radiation, vacuum and desiccation (Fendrihan et al. 2009; Kottemann et al. 2005; Leuko et al. 2015), *Halococcus morrhuae*, *Halococcus hamelinensis*, *Natronorubrum* sp. strain HG-1, *Hcc. Dombrowskii* under UV radiation (Leuko et al. 2015; Peeters et al. 2010; Vreeland et al. 2000), *Natrialba magadii*, *Haloferax volcanii* under vacuum (Abrevaya et al. 2011), and *Halorubrum chaoviator* under UV radiation and vacuum (Mancinelli 2015).

Developing a mechanism for repairing DNA lesions is essential for life in hyper-saline environments (Kottemann et al. 2005). Microorganisms that live in salt deposit-evaporated crusts demonstrate high resistance against desiccation, vacuum conditions and UV radiation (Rothschild and Lister 2003). Survival mechanisms for prokaryotic microorganisms under desiccating conditions include spore-formation (Nicholson et al. 2000) and the production of extracellular polysaccharide (Hill et al. 1997). However, these two mechanisms are not present in most halophilic archaea. Genetic analysis of *Halobacterium* sp. indicates that its genes are active in all DNA damage repair pathways that can be found either in eukaryotes or bacteria, pathways such as photo-reactivation, base excision repair, nucleotide excision repair, mismatch repair homologous recombination and translation synthesis (Crowley et al. 2006; Kottemann et al. 2005). While most microorganisms use organic compounds to protect against extreme osmotic pressure, halophilic archaea use K^+ to fulfil the same function for salt-in mechanism and Na^+/H^+ for salt-out mechanism (Mancinelli 2015; Singh et al. 2013). They also use Na^+/H^+ antiporter proteins related to Na efflux to extrude Na out of the cell in exchange for H^+ in order to keep cytoplasm isosmotic with the environment and thus elude intoxication of living cells (Han et al. 2019; Roberts 2005; Yang et al. 2006). For radiation protection, and associated damage due to hydroxyl radical production, halophilic archaea utilize protective mechanisms such as polyploidy, membrane pigments such as C50, bacterioruberin and intracellular KCl (Shahmohammadi et al. 1998; Siefermann-Harms 1987).

Varying amounts of salt have been found in each of the Shergotty, Nakhla, and Chassigny (SNC) Mars meteorites (Gooding 1992), and indeed the Nakhla meteorite may have been in contact with sea water/brine on Mars (Sawyer et al. 2000), while the Mars Exploration Rovers (MER) and the Mars Express orbiter have detected salt and evaporated mineral surfaces on present-day Mars (Bibring et al. 2005). Even if the temperature of early Mars was below 273 K (Gaidos and Marion 2003), the probability of liquid water is high based on the presence of dissolved minerals in the system (Fairén et al. 2009). The capability of haloarchaea to survive in low water-activity conditions, such as evaporating environments, and their requirement for a high salt concentrations, make them suitable models to study life on Mars (Dassarma et al. 2019; Litchfield 1998).

In the present work, we investigated the survival of two different microorganisms in simulated space and Mars-surface conditions: the halophilic archaeon *Hvr. luteus* strain DA50^T and the spore-forming bacteria *B. atrophaeus*. Samples were desiccated in petri dishes. They were exposed to simulated Mars-relevant levels of UV-radiation, vacuum and desiccation conditions and their survivability was studied by the semi-quantitative Most Probable

Number (MPN) method (Sutton 2010) and shift in protein profiles of the halophiles was analyzed by HPLC and MALDI-TOF. The result of this research illustrates the survival capacity of halophilic archaeon under simulated space and Mars conditions. It is well known that haloarchaea are excellent candidates for astrobiological studies, not only for their ability to survive salinity near saturation like on Mars (Mancinelli et al. 2004) or Jupiter's moon Europa (Marion et al. 2003), but also because of their ability to cope with a variety of extreme conditions such as desiccation, radiation, extreme pH and temperature (Abrevaya et al. 2011).

Materials and Methods

Microorganisms and Culture Media

An extremely halophilic archaeon *Halovarius luteus* genus novel, species novel strain DA50^T, belonging to *Halobacteriaceae* family, was isolated from a brine sample collected in 2011 from Urmia Salt Lake (37° 32' N, 45° 43' E) located in north west Iran. This lake was one of the largest supersaturated hypersaline lakes in the world (Alipour 2006; Alipour and Mosavi Onlaghi 2018). Geological formations surrounding the Urmia Salt Lake consist of Jurassic limestone and Eocene volcanic rocks (Alipour 2006) and this hypersaline environment provides a habitat for a plethora of life-forms including various species of bacteria, archaea, algae, micro fungi and plants (Asem et al. 2014). Over the last four decades, Urmia Salt Lake has dramatically reduced in extent due to overexploitation of water for agricultural purposes, reduction in precipitation, increased evaporation, and the construction of dams to supply potable water to urban districts (Amiri et al. 2016). The lake salinity has increased from 166 g/l in 1995 to over 412 g/l in 2015, and its resident halophilic microorganisms, more of which are identified every year, are plentiful (Amiri et al. 2016; Mehrshad et al. 2015). It was therefore decided that, instead of using a model haloarchaeon, a novel strain which evolved in an increasingly saturated brine should be used to study biological response under simulated extraterrestrial conditions.

To compare the survival of this halophile strain under extreme conditions a bacterium *Bacillus atrophaeus* (formerly *Bacillus subtilis* var. *niger*) was used (Sella et al. 2015). The bacterium was selected since it is a well-established control organism for comparisons in extreme environments for its high resistance to extreme conditions and the capability to produce strong spore structures consisting of coat, cortex, outer membrane, germ cell wall, inner membrane and core (Carrera et al. 2007; Halfmann et al. 2007; Irie et al. 2014; Sella et al. 2015). The strain was obtained from Spordex Biological Indicator Strips (*Bacillus atrophaeus* nrrl#4418, Steris, USA) and spores were aseptically transferred to modified soy bean casein digest broth. *B. atrophaeus* is a facultative anaerobic, gram-positive and rod that makes endospores (Sella et al. 2009). After activation in liquid culture medium, the strain was cultivated in Trypticase Soy Broth. The late log phase samples were used for analysis. *B. atrophaeus* was grown at 35 °C in a shaking incubator (Stuart-Orbit incubator S150) at 150 rpm. For *Hvr. luteus* optimum growth was maintained in 4 M NaCl and 0.3 M MgCl₂. The optimum temperature and pH for growth was 45 °C and 7.0, respectively (Mehrshad et al. 2015). This strain was obtained from Iranian Biological Research Center (IBRC) as an active culture. The culture medium, modified growth medium (MGM) 23% (w/v) (Dyall-Smith 2008), contains a 23% salt mixture which consists of [g l⁻¹]: 184 NaCl, 26.83 MgSO₄·7H₂O, 23 MgCl₂·6H₂O, 7.76 KCl, 1 CaCl₂·2H₂O, 0.61 NaBr, 0.15 NaHCO₃. The culture medium

was supplemented with 3 g peptone casein, 1 g peptone proteose, 1 g nutrient broth, 2 g yeast extract, and 18 g agar for solid medium. To increase the growth rate, 0.5 mL vitamin B12, 1 mL B-complex and 400 μ L of trace element SL-10 solution (10 mg/L HCl (25%; 7.7 M), 1.5 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 70 mg ZnCl_2 , 100 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 6 mg H_3BO_3 , 190 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 24 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 36 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 990 mL distilled water) was added to the medium. The medium was sterilized by autoclaving at 121 °C. The liquid cultures were prepared at 38 °C (due to the water temperature at the time of sampling) in a shaking incubator (Stuart-Orbit incubator S150) at 200 rpm. Growth rate was monitored turbidometrically for more than 300 h with a spectrophotometer (Hach Langer Dr. 5000, USA) at OD_{600} .

Preparing and Growth of Microorganisms

Microbial films were produced at three different thicknesses (A, B and C) from cultures in the late log phase (see Fig. 1A, B, C). OD_{600} of 0.8–1.0 for *Hvr. luteus* strain DA50^T and 1.0–1.2 for *B. atrophaeus*, which corresponded to cell densities of 3×10^8 and 2.5×10^7 per mL, respectively. The approximate cell number in each thickness are $A = 1.5 \times 10^9 / 5 \text{ mL}$, $B = 3 \times 10^9 / 10 \text{ mL}$, $C = 6 \times 10^9 / 20 \text{ mL}$ for halophiles. With a petri dish surface area of 24.6 cm^2 and *Hvr. luteus* cell size of $\sim 1 \mu\text{m}$ (Mehrshad et al. 2015), the average cell coverage for thickness A, B and C was approximately 0.6, 1.2 and 2.4 cells/ μm^2 , respectively. Cells were harvested by centrifugation at 4000 g for 10 min at room temperature (RT). The pellets of *Hvr. luteus* strain DA50^T and *B. atrophaeus* were resuspended three times in the buffers (3 M NaCl, 100 mM Tris, pH = 7.5 and phosphate buffered saline (PBS) 1X, pH = 7.4, for halophile and *bacillus* respectively) to remove the nutrients around the cells. *Hvr. luteus* strain DA50^T cells are obligate halophiles, requiring a minimum of 2 M NaCl. After the final wash, the pellets were suspended in the given solutions and poured into 5.6 cm diameter petri dishes. The samples were shaken inside a laminar flow bench for at least 5 h at RT and 20 rpm and allowed to dry completely.

Simulated Space Conditions

UV-Radiation

Desiccated samples (thicknesses A, B and C) were exposed to UV radiation under both ambient and N_2 atmosphere (a cylinder between UV source and sample was continuously vented by N_2), using a broadband, high pressure xenon short arc polychromatic lamp without reflector (wavelength of 200–400 nm, ScienceTech, model 100150XUV, XBO 150 W/4,

Fig. 1 *Hvr. luteus* strain DA50^T encased in salt crystals during preparation for further experiments. Desiccated plates, diameter 5.6 cm with preliminary volume of (A) 5 mL, (B) 10 mL and (C) 20 mL. (D) Light microscope image of a plate of *Hvr. luteus* strain DA50^T (40X) showing details of the mineral formed after desiccation

XBO, Japan) for various time periods (0, ~1.8, 18, 182 and 1825 s). The irradiance of the broadband lamp was calculated to be 54.78 W/m² (lamp power 150 W; distance between UV source and sample at 47 cm) whereas the lamp intensity was measured at the monochromatic wavelength of 254 nm (VLX-3 W, UVC 254 nm, Vilber, Germany) was 2.90 W/m² under ambient and 3.15 W/m² under N₂ atmosphere, an increase of about 8%. In comparison, Mars receives an estimated 34 W/m² (Peeters et al. 2010). The UV-air and UV-N₂ control samples were kept in a laminar flow bench and an N₂ chamber, respectively.

Vacuum Tests

To investigate the impact of vacuum conditions, cells at thickness B (see Section 2.2) were installed in a vacuum chamber which was evacuated by a scroll pump in combination with a turbomolecular pump. Low-vacuum tests were conducted at 1.7×10^{-1} mbar (only scroll pump operating) for 1 h and 7 days. High-vacuum tests were conducted at 1.6×10^{-5} mbar for 1 h, and 7.6×10^{-8} mbar for 7 days. Control samples were kept under ambient pressure and temperature in dark conditions.

Desiccation

Cultures of both strains at thickness C (see Section 2.2) were desiccated using two different methods inside petri dishes. In the first method, the samples were placed in a desiccator along with a 230 g blue silica gel cartridge (Bel-art SP Scienceware Desiccant, USA). In the second method, the dishes were placed in the laminar flow bench with closed lids and exposed to ambient atmosphere. For both methods the materials were desiccated for 2 weeks. During the tests, salt crystals were formed (see Fig. 1D). Control samples were processed in the same way, but were not placed inside a laminar flow bench nor a desiccator.

Determination of Survivability

The concentration of living cells after the sample treatment was determined using the MPN method (Blodgett 2006; Sutton 2010). For growth and reproductive viability, nine test tubes consisting of 9 mL of the relevant liquid culture medium (either MGM 23% or Tryptic Soy Broth) were prepared for each sample. For recovery of the cells, 3 mL of the given buffer (Tris-NaCl and PBS for halophile and *bacillus*, respectively) were added to each dry sample and stirred for homogenization. After dissolution of the samples at RT, 1 mL of the solution (buffer mixed with recovered cells) was transferred to each prepared test tube (final total volume of 10 mL). From these solutions (a 1:10 dilution from the original 1 mL sample), a dilution series of three tubes were made: 1:10, 1:100, and 1:1000. These dilution series were incubated at 38 °C for 3 weeks for *Hvr. luteus* strain DA50^T, and 35 °C for 2 days for *B. atrophaeus* to reach late log phase.

Protein Profile

A simple and fast method was developed for the extraction of proteins from haloarchaea strain *Hvr. luteus* DA50^T (Dyall-Smith 2008; Holland et al. 1999; Wall et al. 1999). In brief, the whole protein from a cell lysate supernatant was extracted after lysis with B-PER Reagent extracts proteins (B-PER® Bacterial Protein Extraction Reagent, Thermo Scientific, USA),

2 mL Tris-NaCl buffer was added to the plates and were shaken for 30 min inside a laminar flow cabinet to avoid sample contamination. Subsequently, cell pellets were collected by centrifugation at 4000 g for 10 min. Pellets were transferred to a 2 mL micro centrifuge tube and centrifuged for a second time at 11,000 g for 10 min, both at RT. At this point, 1 mL of B-PER Reagent extract proteins was added to each sample and vortexed. Cell pellets were sonicated three times for 30 s and cooled on ice between each sonication (Branson Sonifier 250). Samples were then shaken for 30 min and centrifuged at 10,000 g for 20 min at 4 °C to pellet cell membranes and unbroken cells. The supernatant was transferred to a fresh tube and stored at -20 °C for later analysis.

For protein profiling, 700 µL of supernatant was fractionated using Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) (Jasco, Japan) using a C18 column as a qualitative method. Elution was performed with a linear gradient, from 5 to 65% acetonitrile containing 0.1% trifluoroacetic acid (TFA) for 30 min at flow rate of 4 mL/min. The absorbance of the column effluent was monitored at 214 nm (Thermo Finnigan detector, San Jose, CA). The mass identification of the proteins or peptides in each fraction was obtained by Matrix Assisted Laser Desorption/Ionization bench top reflector time-of-flight (MALDI-TOF) mass spectrometer (Micro Flex MALDI-TOF of Bruker Daltonics, Germany). The concentrated fractions were processed with MALDI-TOF. 50 µL of TFA (containing trifluoroacetic acid 0.1%, acetonitrile 30%, and milli-Q water) solution was added to each fraction and the mixture was well vortexed. Next, 4 µL of concentrated protein mixed with TFA was combined with α -Cyano-4-hydroxy-cinnamic acid (α -HCCA) matrix solution (MALDI-MS) in 1:1 ratio and 2 µL of this was spotted on a stainless steel MALDI target and dried at ambient air. After crystallization of the samples, the determination of the protein fragments was obtained in the reflector mode to compare results from different culture conditions.

Results

Determination of Survival after UV Irradiation

Whereas no loss of viability of *B. atrophaeus* occurred following a UV irradiation exposure of up to 10^5 J/m², the viability of haloarchaea was clearly reduced (Fig. 2). At the lowest dose (10^2 J/m²) the results of MPN tests demonstrated that the thinnest film (A, 5 mL), which there is no overlap among cells, of haloarchaeon showed less growth even in the control samples in comparison with other groups (B: 10 and C: 20 mL). Conversely, samples B and C showed no loss of viability in air, with the samples B decreasing in viability 23% under UV exposure with N₂ (Fig. 2). It is worth mentioning that, cells in thickness B has a little bit overlaps ~ 1.2 cell/ μm^2 ; however, thickness C showed an obvious overlap among the cells ~ 2.4 cell/ μm^2 .

By increasing the dose of UV radiation, the samples with least thickness showed the largest decrease in viability, especially in combination with N₂. Samples at 10^3 J/m² and 10^4 J/m² with thickness A showed 59% and 32% viability in combination with air, and 39% and 30% in combination with N₂ (Fig. 2). For thicknesses B and C, a slight decline continued following exposure of 10^3 and 10^4 J/m² so that thickness B showed 76% and 52% viability in combination with air and 61% and 40% viability under N₂. Thickness C demonstrated 82% viability for both doses in air and 65% and 52% with N₂. At 10^5 J/m² irradiation with thickness A there is a 12% viability while thickness B displayed a 16% to 15% viability and thickness C

Fig. 2 *Hvr. luteus* strain DA50^T survival after exposure for 10²–10⁵ J/m² of UV-radiation under a air and under N₂. Aa, Ba and Ca are UV-air exposed samples and An, Bn and Cn are UV-N₂ exposed samples (where A, B and C refer to the sample thickness). The error bars are the 95% confidence intervals for three independent experiments ($n = 3$). The survival percentages N/N_0 are given above each bars, where N is the number of

demonstrated the highest viability: 40% and 18% in air and N₂ at 10⁵ J/m². Note that no reduction of viability was detected for *B. atrophaeus* under either atmospheric test.

Effect of Vacuum Exposure

The survival of *Hvr. luteus* strain DA50^T and *B. atrophaeus* to low- and high-vacuum conditions was evaluated (Fig. 3). Under these conditions, the MGM 23% medium formed large halite crystals in which the cells were trapped. Cultures of strain DA50^T and *B. atrophaeus* at thickness B were prepared for these exposure experiments. For both strains there was no significant difference in survival after 1 h in low and high vacuum condition exposures, and for *B. atrophaeus* no reduction of viability was detected even after 7 days exposure. The reduction of survival of *Hvr. luteus* to 61% after 7 days is statistically lower than the control samples in the 7 days groups.

Survival after Desiccation

As seen in Fig. 4, both cell strains did not show a viability change after 1 week of desiccation in the laminar flow bench. Further, *B. atrophaeus* did not show a decreased viability when placed in desiccator for 1 week, while *Hvr. luteus* demonstrated a decrease to 79.6% viability. Both organisms showed decreased in viability when exposed to 2 weeks desiccation in a laminar flow bench and a desiccator. For *B. atrophaeus* the viability was 55% in the desiccator and 85% in the laminar flow bench after two weeks. For *Hvr. luteus* the final viabilities were 15.1% and 59.2%, respectively.

HPLC and MALDI-TOF Protein Profiles

The chromatograms of UV-radiation and vacuum-exposed samples are presented in Fig. 5A, B. HPLC was used to separate the mixtures of proteins and peptides based on their affinity to immobilized stationary phase. While the retention time for most peaks are either compatible with the control samples or have only minor shifts, there are some exceptions: peaks at minute 3 in UV-air which show a dramatically high mAU, while at minutes 17 to 19,

Fig. 3 The percentage of survival of *Hvr. luteus* strain DA50^T exposed low (1.7×10^{-1} mbar) and high vacuum 1 h at 1.6×10^{-5} mbar and 7 days at 7.6×10^{-8} mbar in compare with control samples. The standard error bar of 95% confidence intervals for three independent experiments ($n = 3$) is indicated. The survival percentages N/N_0 are given above each bar, where N is the number of survivors specified by the MPN technique and N_0 is the number of surviving cells in control samples

all UV exposed samples show higher peaks than control samples. At minute 25, all exposed samples show weaker peaks than control samples (Fig. 5A).

In the vacuum measurements shown in Fig. 5B, a reverse peak can be observed at 3 min for high vacuum conditions, while for both high and low vacuum conditions an intense signal can be observed in minute 18.

Fractions were separated in minutes and subsequently analyzed by MALDI-TOF. The MALDI-TOF protein spectra of one of the clearer qualities of vacuum exposed samples, minute 13, are shown in Fig. 6. The minute 13 fraction was chosen since this provided clear

Fig. 4 *Hvr. luteus* strain DA50^T (green) and *B. atrophaceus* (red) exposed to desiccation for 1 and 2 weeks. The standard error bar of 95% confidence intervals for three independent experiments ($n = 3$) is indicated. The survival percentages N/N_0 of both strains are given on top of the bar charts where N is the number of survivors specified by the MPN technique and N_0 is the number of surviving cells in control samples

Fig. 5 RP-HPLC chromatograms (milli-Absorbance Units/Time) of proteome profiles of *Hvr. luteus* strain DA50^T after exposure to UV radiation either with air or with N₂ (A) and to low and high vacuum conditions (B). The slice at 13 min is used for additional MALDI-TOF analysis (See Fig. 6)

spectra compared to other time fractions. Several changes in protein profiles between the control sample and vacuum exposed samples in the 13-min fractions can be observed. For instance, in the proteins below 3 kDa in the control sample (Fig. 6 blue line), some proteins appeared at masses 2267, 2629, 2854 Da. In sample exposed to low vacuum condition (Fig. 6 yellow line), the weights are 2288, 2690 and 2854 Da are quite similar to the control sample, while in the high vacuum condition exposed sample (Fig. 6 red line) the protein at weight 2959 Da, as well as some others, are deviating compared to the control sample. In the proteins above 3 kDa, the control sample shows several peaks which are absent in both vacuum samples, with the exception of the peak with the masses of 3531 and 4102 Da in the low vacuum samples. In the high vacuum exposed sample, peaks at 3360, 3723 and 4100 Da can be observed.

Discussion

In this study, we explored the resistance of halite-embedded cells of *Hvr. luteus* strain DA50^T and PBS-embedded cells of resistant bacteria *B. atrophaeus* to UV radiation (200–400 nm), low and high vacuum conditions, and desiccation. *Hvr. luteus* strain DA50^T was exposed to various UV fluxes under both air and a N₂ atmosphere, and it remained viable even after more

Fig. 6 MALDI-TOF spectrum obtained from vacuum control (blue), low vacuum (yellow) and high vacuum (red) samples in minute 13 after purification and fraction by RP-HPLC (See Fig. 5)

than half an hour exposure to simulated Mars UV radiation. In experiments involving UV transmission through air, while the presence of oxygen has an attenuating effect on the effective radiation level, the oxygen may in turn be the source of oxygen radicals, which can directly damage the cells. If the latter effect is indeed present and dominant, such an effect was not observed in our results. Taken into account that NaCl crystals are translucent to wavelengths ≥ 200 nm (Li 1976), damage to *Hvr. luteus* from UV radiation could be due to the formation of $\cdot\text{OH}$ radicals produced by UV radiation from residual water molecules inside the halite crystals and/or intercellular fluid. These data suggest that a lower initial cell thickness (A) may have contributed to the lower survival rate of the *Hvr. luteus* for UV radiation. Cell multi-layers aiding survivability is well characterized (Mancinelli 2015). However, the maximum lethal effect occurred when the single-layer samples exposed to UV fluxes in combination with N_2 . The novel haloarchaeon, *Hvr. luteus* represented more viability when the single-layer arrangement was changed to a multi-layer: 2.4 cells/ $1\mu\text{m}^2$ and more growth under air and nitrogen (Fig. 2). In all other combinations of culture thickness and radiation it is clear that radiation transmitted through a N_2 atmosphere has a higher lethal impact compared to oxygen containing atmosphere.

Fendrihan and colleagues exposed halite-entrapped *Halococcus dombrowskii* to simulated Mars UV radiation and the archaeon survived at doses even up to 10^7 J/m 2 . This survival rate was two times more than in liquid medium with the same dose (Fendrihan et al. 2009). In regard to UV-radiation, MacCready and colleagues (MacCready et al. 2005) tested the resistance of *Halobacterium* NRC-1 and *D. radiodurans* against UVC doses up to 200 J/m 2 . It appeared that both microorganisms are highly resistant. Peeters and colleagues (Peeters et al. 2010) exposed *Natronorubrum* strain HG-1 under desiccation and UV-radiation (200–400 nm, 28 W/m 2) to represent a Mars-like surface. Results showed that the strain was able to withstand

the UV radiation exposure only for a couple of hours. It could not withstand the combination of radiation with desiccation and low temperatures. A related species, *H. chaoviator*, has demonstrated tolerance to UV radiation doses of up to 10^7 J/m², showing that survival capabilities are not necessarily shared between phylogenetically related species (Mancinelli et al. 1998). Zhou and colleagues (Zhou et al. 2007) showed that *Halobacterium* sp. NRC-1, *Haloarcula marismortui* and *Haloquadratum walsbyi* have optimized their proteomic and genomic structures to decrease damage induced by UV irradiation which often exists at high levels in habitats where these organisms thrive. The data from our study demonstrate that *B. atrophaeus* is more UV-radiation, vacuum and desiccation resistant than *Hvr. luteus* strain DA50^T (Figs. 3 and 4). Although, for survival under desiccation, adaptations of many prokaryotes include sporulation (Nicholson et al. 2000) or production of extracellular polysaccharides (Potts 1994), neither of them are utilized by the well-studied halophile, *Halobacterium* sp. (Kottemann et al. 2005). While the positive effect of multi-layering and halite-embedded cells seem irrefutable, it appears that multiple protective mechanisms are influencing haloarchaea survivability.

Another factor that may contribute to the resistance of halophilic archaea to high levels of UV radiation, alongside with the presence of multiple genome copies, is the high concentration of intracellular halide ions including chloride and possibly bromide that play the role of chemical chaperones (Abrevaya et al. 2011; Kish et al. 2009). It has been demonstrated that *H. salinarum* NRC-1 benefits from bromide ions that accumulate with chloride ions in its natural brines (Kish et al. 2009; Sonnenfeld and Perthuisot 1984). It has been proved that the levels of bromide and chloride ions have increased in Urmia Salt-Lake from 1994 to 2009: 2.5 and 6.3 times for chloride and bromide, respectively (Rezvantalab and Amrollahi 2011). With the declining water level in Urmia Salt-Lake continuing for over four decades, the chemical dynamic of the lake could compel some new survival mechanisms to its inhabitants.

The survival of *Hvr. luteus* strain DA50^T at thickness B to low- and high-vacuum and at thickness C to desiccation was evaluated. Under these conditions, the medium formed large salt crystals in which the cells were embedded (Fig. 1). Under vacuum while *B. atrophaeus* showed no loss of viability a moderate decrease in the survival of *Hvr. luteus* was observed (Fig. 3). In a comparable experimental setup, the endurance and stability of the vegetative *Halobacterium* sp. falls short compared to *Bacillus subtilis* while exposed to vacuum (10^{-8} mbar) and low temperatures (-196 °C) for 24 h. While the latter remained viable at 75%, all *H. halobium* cells were overserved to be dead (Koike et al. 1992; Kottemann et al. 2005). In a 10-day experiment with the cells embedded in a Mars soil analogue under low pressure *D. radiodurans* showed 30% viability, while *E. coli* did not survive (Diaz and Schulze-Makuch 2006). Kottemann et al. (2005) demonstrated that *Halobacterium* sp. strain NRC-1 is highly resistant to desiccation and high vacuum (10^{-8} mbar). The viability dropped only gradually, so that after 20 days the viability reduced to only 25% and there was no significant difference between viable cells either in vacuum or in desiccation measurements. These results suggest that halite crystals that formed during the experiment offered a level of cellular protection. Abrevaya and colleagues (Abrevaya et al. 2011), compared two haloarchaea *Natrialba magadii* and *Haloferax volcanii* with *D. radiodurans* under high vacuum conditions (10^{-7} mbar). The results demonstrated that *N. magadii* was more resistant than *D. radiodurans* to such low pressures. High resistance in *N. magadii* may have been due to the more effective mechanisms of halophiles against desiccation. *Hvr. luteus* strain DA50^T indicated a dramatic decline (a decrease of 84.9%) in viability at desiccation experiment since the humidity was highly reduced due to two weeks of desiccation inside desiccator.

Nevertheless, in all desiccation methods *B. atrophaeus* showed a higher viability percentage, *Hvr. luteus* strain DA50^T has shown a good degree of resistance, too. The ability to cope with low active water for halophiles is most likely related to their salt tolerance. The *Halobacteriaceae* family responds to increases in osmolarity by means of K⁺ as their osmoticum (Kushner 1968; Mancinelli 2015). Osmotic pressure increases during dehydration is similar to a cell in a hypersaline environment (Mancinelli 2015).

In regard to protein profiles, the HPLC chromatograms for the UV-exposed samples showed some overlap between groups except for the higher peak at 3 min for UV-air and a slightly higher peak at 25 min for UV-air control sample. There seems to be even more overlap between groups in the vacuum experiment except for the high peak for the samples exposed to high vacuum at 18 min. Initial MALDI-TOF analysis demonstrated different mass spectra in low molecular weight regions after exposing cells to vacuum conditions. This indicates that under such conditions cells are able to change their metabolism, via novel metabolic pathways, or protein expressions to survive the harsh environmental conditions. This demonstrates the need for future studies using a more analytical proteomic approach using, for example, trypsin digestion for high mass molecular proteins to produce small fragments or peptides for MS-MS analysis, comparing these with the library of known MALDI-TOF spectral fingerprints and masses predicted from haloarchaea proteomic databases such as the HAMAP database (Lima et al. 2009). One could also apply Isotope-Coded Protein Labeling (ICPL) for comparing different extreme environments (Tebbe et al. 2009).

The current MALDI-TOF profiles suggest changes of the cell lysate fractions by the amino acid degradation or modification induced during the exposure to the simulated space environment. Changes might have affected single peptides or the polypeptides profile in the cells involved in stress responses during such extreme conditions in order to adapt their physiology and cellular structures. Many archaeal cell envelopes are composed of an atypical cellular membrane constituted by isoprenyl ether glycerol phospholipids surrounded by surface S-layer proteins as the component of the cell wall (Albers and Meyer 2011). This lipid stability of the archaea membrane is able to serve as a shell against harsh conditions and could be investigated in more detail in future work.

Conclusion

This study is the first work reporting the survival of a novel extremely halophilic archaeon compared to a resistant spore-forming bacterium under simulated space conditions. *Hvr. luteus* strain DA50^T and *B. atrophaeus* were exposed to different regimes of simulated solar radiation (200–400 nm, 54.78 W/m²), low ($\sim 10^{-1}$ mbar) and high ($\sim 10^{-5}$ – 10^{-8} mbar) vacuum conditions, and desiccation. *Hvr. luteus* strain DA50^T showed a good resistance, withstanding 30 min of Mars simulated UV radiation under air or N₂ atmosphere, high vacuum conditions, and up to two weeks desiccation. Survival within brine inclusions may be another method by which *Hvr. luteus* strain DA50^T cells can tolerate radiation, high vacuum conditions, and desiccation. General protein profiles show a clear difference between the various extreme conditions used. The present study suggests that this extremely halophilic archaeon may be a suitable candidate for ongoing astrobiological research.

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Compliance with Ethical Standards

Author Disclosure Statement No competing financial interests exist.

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Affiliations

Niloofer Feshangsaz¹ · Farid Semsarha² · Saeed Hesami Tackallou³ · Kamran Nazmi⁴ · Euan P. Monaghan⁵ · Andreas Riedo⁵ · Jack J. W. A. van Loon^{1,6}

✉ Niloofer Feshangsaz
n.feshangsaz@gmail.com

¹ Amsterdam UMC location VUmc, and Academic Centre for Dentistry Amsterdam (ACTA) Department of Oral and Maxillofacial Surgery/Pathology, Amsterdam Movement Sciences, Vrije Universiteit Amsterdam, Gustav Mahlerlaan 3004, 1081 LA Amsterdam, The Netherlands

² Department of Biophysics, Mim Daroo Pharmaceutical Co., Tehran, Iran

³ Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran

⁴ Academic Centre for Dentistry Amsterdam (ACTA) Department of Oral Biochemistry, ACTA-Vrije Universiteit, Amsterdam, The Netherlands

⁵ Laboratory for Astrophysics, Leiden Observatory, Leiden University, Leiden, The Netherlands

⁶ European Space Agency (ESA), Technology Center (ESTEC), TEC-MMG lab, Noordwijk, The Netherlands