Survival of the halophilic archaeon *Halovarius luteus* after desiccation, simulated Martian UV radiation and vacuum in comparison to *Bacillus atrophaeus*

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Abstract

The detection and identification of life on planetary objects other than Earth is one of the most important questions in current science. Extraterrestrial environments impact the biochemistry of organisms with high levels of radiation, vacuum, temperature extremes and a lack of water and nutrients. A wide variety of terrestrial microorganisms, counted amongst the most ancient inhabitants of Earth, can cope with high levels of salinity, extreme temperatures, desiccation and 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. The haloarchaea's response to these conditions was compared with the response of the bacteria *Bacillus atrophaeus*. *Bacillus atrophaeus* was selected as a point of comparison for its well-described resistance to extreme conditions and its capability to produce strong spore structures consisting of coat, cortex, outer membrane, germ cell wall, inner membrane and core (Zandomeni *et al.*, 2005b). Thin films of different thickness were produced to investigate viability without the protective influence of cell multi-layers. *Hvr. luteus* and *B. atrophaeus* were placed in brine and phosphate buffered saline (PBS) media, respectively. The solutions were allowed to evaporate and cells were encapsulated, consequently. Samples were exposed to desiccation and vacuum conditions, and their post-exposure viability was studied by the Most Probable Number (MPN) method. The proteome was analyzed by electrophoresis. Results showed that the changes in viability of the spore-forming bacteria *B. atrophaeus* were only minor whereas the *Hvr. luteus* demonstrated a range of viability under different conditions. At the peak radiation flux of 10^5 J/m^2 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.

Key words: *Halovarius luteus*, *Bacillus atrophaeus*, desiccation, Mars simulation chamber, simulated Martian UV radiation, vacuum.

1. Introduction

Various missions with an astrobiology component have been sent to Mars, and two upcoming flagship missions, ExoMars (Parnell *et al.*, 2007) and Mars2020 (Mustard *et al.*, 2013), will soon 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 their survival capabilities are important to understand as the most stable signatures may still be preserved and detected in future missions.

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). There are multiple methods for studying the survival of microorganisms in simulated extraterrestrial environments, both in Earth orbit (Cottin *et al.*, 2017) and in simulated conditions on Earth (Martins *et al.*, 2017). 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 mostly responsible for the lethality of microorganisms exposed to it due to the high absorption of this wavelength range by DNA (Henning *et al.*, 1995, Mancinelli, 2015).

To date, biologically relevant objects studied include not only microorganisms and spores, but also isolated biomolecules such as DNA, amino acids and liposomes (de La Torre et al., 2010, Nicholson et al., 2000b, Stan-Lotter and Fendrihan, 2015). Although UV radiation with a wavelength below 290 nm can reach the surface of the Earth, with most of the solar UV-radiation absorbed in the atmosphere, the entire solar UV radiation spectrum has to be

considered in most studies of the extraterrestrial environment due to the lack of a protective atmosphere in these environments. The stress factor of solar UV radiation was very important during the Archean era as the Earth lacked an ozone layer and the surface was exposed to the complete spectrum of solar radiation (Monk *et al.*, 1994). Thus, it is hypothesized that microorganisms that evolved on the Archean surface under these conditions may retain cellular mechanisms which evolved to cope with elevated levels of full-spectrum UV radiation (Wynn-Williams *et al.*, 2001).

Most DNA lesions formed during short-term exposure to space conditions are due to UV-radiation. However, ionizing radiation is probably more lethal as it produces active oxygen species that are considered an important toxic compound (Horneck, 1993, Horneck *et al.*, 2010). The majority of DNA breaks caused by UV and ionizing radiation are due to molecular damage to nucleotides, their organic bases and the breaking of single or double DNA strands (Cadet and Wagner, 2013).

Vacuum conditions and desiccation in space are the other key detrimental factors affecting microbial survival. If there are no internal or external matrices protecting the cells, vacuum and desiccation will cause severe lesions (Dose and Gill, 1995). Nucleic acids, proteins and carbohydrates would undergo amino-carbonyl reactions (Maillard reactions) that result in cross-linking and, ultimately, polymerization of the biomolecules containing DNA (Horneck *et al.*, 1995, Beckman and Ames, 1998, Mancinelli, 2015). Structural variations would 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 accompanied with long duration desiccation could impose lethal effects on microorganisms, especially non-spore forming

strains. Desiccation tolerance is known for 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 can endure the extreme conditions of desiccation, starvation and radiation exposure for millions of years (Stan-Lotter and Fendrihan, 2015). 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, and display their tolerance against desiccation (Fendrihan *et al.*, 2009, Huu *et al.*, 1999, McGenity *et al.*, 2000, NORTON and GRANT, 1988, Vreeland *et al.*, 2000). It is likely that many of these coping strategies 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 include *Halobacterium salinarium* NRC-1 under UV radiation, vacuum and desiccation (Fendrihan *et al.*, 2009, Kottemann *et al.*, 2005, Leuko *et al.*, 2015), *Haloccoccus morrhuae*, *Halococcus hamelinensis*, *Natronorubrum* sp. strain HG-1, *Hcc. Dombrowskii* under UV radiation (Vreeland *et al.*, 2000, Leuko *et al.*, 2015, Peeters *et al.*, 2010), *Natrialba magadii*, *Haloferax volcanii* under vacuum (Abrevaya *et al.*, 2011a), and *Halorubrum chaoviator* under UV radiation and vacuum (Mancinelli, 2015).

Developing a mechanism for repairing DNA lesions is essential for life in hypersaline environments (Kottemann *et al.*, 2005). Microorganisms that live in salt deposit-evaporated crusts demonstrate high resistant against desiccation, vacuum conditions and UV radiation (Rothschild and Lister, 2003). Thus, the main role of DNA repair systems in these microorganisms is to provide resistance to some extreme environmental conditions such as drought and UV-radiation (Kottemann *et al.*, 2005). 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 of the halophilic archaea. Genetic analysis of *Halobacterium* sp. indicates that its genes are active in all DNA damage repair pathways such as photo-reactivation, base excision repair, nucleotide excision repair, mismatch repair homologous recombination, and translation synthesis that can be found either in eukaryotes or bacteria (Chuang *et al.*, 1997). While most microorganisms use organic compounds to protect against osmotic pressure, halophilic archaea use K^+ to fulfil the same function (Mancinelli, 2015). 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). Oxidative damage caused by ionizing radiation is mitigated via detoxification mechanisms such as superoxide dismutase enzyme activity (Sun *et al.*, 1998). Almost all of these microorganisms possess catalyst enzymes that accelerate the lyzing reaction to water and oxygen.

Desiccated osmophilic microorganisms *Synechococcus* Nägeli and *Haloarcula*-G survived after 2 weeks exposure to space conditions in Earth orbit (Mancinelli *et al.*, 1998). Varying amounts of salt have been found in each of the Shergotty, Nakhla, and Chassigny (SNC) Mars meteorites (Gooding, 1992). It even seems that the Nakhla meteorite may have been in contact with sea water/brine on Mars (Sawyer *et al.*, 2000). Additionally, the Mars Exploration Rovers (MER) and the Mars Express orbiter detected salt and evaporated mineral surfaces on 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 (Fairén *et al.*, 2009).

The capability of haloarchaea to survive in low water-activity, such as evaporating environments, and their requirement for living in high salt concentrations make them suitable models to study life on Mars (Litchfield, 1998, DasSarma *et al.*, 2019).

In the present work, we investigated the survival of two different microorganisms, the halophilic archaea *Hvr. luteus* strain DA50^T and the spore-forming bacteria *B. atrophaeus* for the first time in simulated space and Mars-surface conditions. Samples were desiccated in petri dishes. They were exposed to simulated Mars UV-radiation, vacuum and desiccated conditions and their survivability was studied by a semi quantitative method Most Probable Number (MPN) (Sutton, 2010) while the total protein of the halophiles was analyzed by electrophoresis (SDS-PAGE). The result of this research illustrates the potential capability of halophilic archaea under simulated space and Mars conditions. It is well known that haloarchaea are excellent for astrobiological studies not only for their capability of living in the environment with salinity near saturation like 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 temperatures (Abrevaya *et al.*, 2011b). Many studies have been conducted into the effects of UV radiation on microorganisms (Abrevaya *et al.*, 2011a, Kottemann *et al.*, 2005, Mancinelli *et al.*, 2004, Marion *et al.*, 2003, Sella *et al.*, 2015, Zandomeni *et al.*, 2005a). Besides, the effect of simulated Mars stresses on a variety of microorganisms, such as *Chroococcidiopsis* sp. 029 (Cockell *et al.*, 2005), bacillus strains (Rettberg *et al.*, 2004), *Escherichia coli* and *Deinococcus radiodurans* (Baumstark-Khan and Facius, 2002, Diaz and Schulze-Makuch, 2006) have been studied.

2. Materials and Methods

2.1 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. The novel halophilic strain was chosen due to some specific characteristics of its natural habitat discussed in the following and lack of studies in simulated space environments. Urmia Salt 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). This hypersaline environment provides a habitat for a plethora forms of lifeforms including various species of bacteria, archaea, algae, micro fungi and plants (Asem *et al.*, 2014). Over the last four decades, Urmia Salt Lake has been in a critical condition due to overexploitation of water for agricultural purposes, the reduction in precipitation, increased evaporation, and the construction of dams to supply potable water to the 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 instead be used to study biological response under simulated extraterrestrial conditions.

To compare the survival of this novel halophile strain under extreme conditions a bacterium *Bacillus atrophaeus* (formerly *Bacillus subtilis* var. *niger*) was used (Sella *et al.*, 2015). The bacterium was selected 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 (Zandomeni *et al.*, 2005). The strain was obtained from Spordex Biological Indicator Strips (Bacillus atrophaeus nrrl#4418, Steris, USA) and were activated in modified soy bean casein digest broth. After activation in liquid culture medium, the strain was cultivated in Trypticase Soy Broth. The late log phase samples were used for analysis. *Bacillus atrophaeus* was grown at 35º 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 $MgCl_2$. The optimum temperature and pH for growth was 45º 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, MGM 23% (Dyall-Smith, 2009), contains a 23% salt mixture which consists of [g l^{-1}]: 184 NaCl, 26.83 $MgSO_4 \cdot 7H_2O$, 23 $MgCl_2 \cdot 6H_2O$, 7.76 KCl, 1 $CaCl_2 \cdot 2H_2O$, 0.61 NaBr, 0.15 $NaHCO_3$. The culture medium is 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 µL of trace element SL-10 solution (10 mg/L HCl (25%; 7.7 M), 1.5 g $FeCl_2 \cdot 4H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2 \cdot 4H_2O$, 6 mg H_3BO_3, 190 mg $CoCl_2 \cdot 6H_2O$, 2 mg $CuCl_2 \cdot 2H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, 36 mg $Na_2MoO_4 \cdot 2H_2O$, 990 mL distilled water) was added to the medium. The medium was sterilized by autoclaving at 121º C. The liquid cultures were prepared at 38º 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 hrs with UV-VIS spectroscopy (Spectrophotometer UV-visible, Hach Langer Dr 5000, USA) at OD_{600}.

2.2 Preparing and growth of microorganisms

Films were produced at three different thicknesses from cultures in the late log phase. 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 (density A = 5 mL x cell number/mL, density B = 10 mL x cell number/mL, density C = 20 mL x cell number/mL). Cells were harvested by centrifugation at 4,000 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 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 in 5.6 cm diameter petri dishes. The samples were shaken inside the laminar hood for at least 5 hrs at RT and 20 rpm and allowed to dry completely.

2.3 Space Conditions

2.3.1 UV-radiation

Desiccated samples were exposed to UV radiation under both ambient and N_2 atmosphere (a pipe between UV source and sample was continuously vented by N_2), using a broadband, high pressure xenon short arc polychromic lamp without reflector (wavelength of 200 – 400 nm, ScienceTech, model 100150XUV, XBO 150 W/4, XBO, Japan) for various time periods (0 , ~1.8 s, 18 s, 182 s, 1825 s). UV transmission is higher under the N_2 atmosphere as less UV radiation is absorbed. The irradiance of the broadband lamp was calculated to be 54.78 W/m^2 (lamp power 150 W; distance between UV source and sample 47 cm) whereas the lamp intensity at the monochromatic wavelength of 254 nm was 2.90 W/m^2 under ambient and 3.15 W/m^2 under N_2 atmosphere (an increase of about 8%). The irradiance at monochromic wavelength 254 nm was measured in all tests before and after exposure using a UV sensor (VLX-3W, UVC 254 nm, Vilber, Germany). In comparison, Mars receives an estimated 34

W/m^2 (Peeters *et al.*, 2010). The UV + air and UV + N_2 test controls were kept in a laminar hood and an N_2 chamber, respectively.

2.3.2 Vacuum Tests

Films (at density B, see Section 2.2) of the cell suspensions were pipetted into 5.6 cm diameter petri dishes and dehydrated following Baqué *et. al.* (2013). To investigate the impact of vacuum conditions the samples 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 hr and 7 days. High-vacuum measurements were conducted at 1.6×10^{-5} mbar for 1 hr, and 7.6×10^{-8} mbar for 7 days. Control samples were kept under ambient pressure and temperature in dark conditions.

2.3.3 Desiccation

Cultures of both strain cells at density A were desiccated in two different ways 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) for desiccation. In the second method, the dishes were placed in the laminar hood 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. 1 D). Control samples were processed in the same way instantly after culture preparation, however, without being inside a laminar hood or desiccator for given time.

2.4 Determination of survivability

The concentration of living cells after the sample treatment was determined using the MPN method (Sutton, 2010, Blodgett, 2006). For growth and reproductive viability, nine test tubes consisting of 9 mL of relevant liquid culture medium (either MGM 23% and Tryptic Soy Broth)

were prepared for each sample. For recovery of the cells, 3 mL of 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 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 38º C for 3 weeks for *Hvr. luteus* strain DA50^{T}, and 35º C for 2 days for *B. atrophaeus* to reach late log phase.

2.5 Protein assay

A simple and fast method was developed for the extraction of proteins from haloarchaea strain *Hvr. luteus* DA50^{T}. 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 4,000 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 ºC to pellet cell membranes and unbroken cells. The supernatant was transferred to a fresh tube and stored at -20° C for later analysis. One-dimensional SDS-polyacrylamide gel electrophoresis was carried out to separate protein molecules of different shapes and sizes of bacterial cell lysate. 30 µL sample buffer (4x) and 90 µL of protein were added to gel electrophoresis to be separated gradually in 45 min. Protein bands were visualized

by Coomassie Blue R staining after 4 hrs at RT by soft shaking. Distaining was performed by 10% acetic acid in 24 hrs by shaking at RT.

Following this, 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 – 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. TFA (containing trifluoroacetic acid 0.1%, acetonitrile 30%, and milli-Q water) solution of 50 µL 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.

3. Results

3.1 Determination of survival after UV irradiation

Whereas no loss of viability of *B. atrophaeus* occurred following an UV irradiation exposure of up to 10^5 J/m^2 the viability of haloarchaea was clearly reduced (Fig. 2). At the lowest dose (10^2 J/m^2) the results of MPN tests demonstrated that the thinnest layer (A= 5 mL) of haloarchaea showed less growth even in the control samples in comparison with other groups (10 and 20 mL), most likely due to a too low primarily thickness in combination either with air

or N_2. Cell multi-layers aiding in survivability is well characterized (Mancinelli, 2015). Conversely, 10 ml and 20 mL concentration samples showed no loss of viability in air, with the 10 mL samples decreasing in viability 23% under UV exposure with N_2.

By increasing the dose of UV radiation, the samples with least thickness showed the largest decrease in viability, especially in combination with N_2: 10^3 J/m^2 and 10^4 J/m^2 in 5 mL samples showed 59% and 32% viability in combination with air, and 39% and 30% in combination with N_2. For 10 ml and 20 mL, a slight decline continued following exposure of 10^3 and 10^4 J/m^2 so that thickness B (10 mL) showed 76% and 52% viability in combination with air and 61% and 40% viability under N_2. Thickness C demonstrated 82% viability for both doses in air and 65% and 52% with N_2. With 10^5 J/m^2 irradiation, thickness A showed 12% viability in combination with air and N_2 while thickness B showed 16% and 15% viability in air and N_2. Thickness C demonstrated the highest viability: 40% and 18% in air and N_2 after exposure to 10^5 J/m^2. In all other combinations of culture density and radiation it is clear that radiation transmitted through a N_2 atmosphere has a higher impact compared to ambient air, which contains, on average, 21% oxygen. This is an expected result as the intensity arriving at the sample is higher under N_2 than under air, as there is less absorption. No reduction of viability was detected for *B. atrophaeus* under either atmospheric test.

Based on the UV radiation measurements using the UV sensor (254 nm), the substitution of air with N_2 caused an increase of ~ 8% in intensity (measured at the sample location). Nonetheless, the 8% increase of one wavelength (254 nm) is only part of the irradiance spectrum of the broadband lamp (200 – 400 nm). The intensity increase influences the viability percent significantly at all thicknesses, with the highest effect observed at the smallest thickness, A. As can be seen in Fig. 2, the viability of *Hvr. luteus* strain DA50^T declined after the replacement of the air with N_2 at all doses. For *B. atrophaeus*, no decrease in viability, even after exposure up to 10^5 J/m^2, was observed in either the air or N_2 experiments.

3.2 Effect of vacuum exposure

The survival of *Hvr. luteus* strain DA50^T and *B. atrophaeus* to low- and high-vacuum conditions were evaluated. Under these conditions, the MGM 23% medium formed large halite crystals in which the cells were trapped. A 10 mL thickness film was prepared for these exposure experiments. For both strains there was no significant difference in survival after 1 hr in low and high vacuum condition exposures, and for *B. atrophaeus* no significant effect was observed even after 7 days exposure. The reduction of survival of *Hvr. luteus* to 61% after 7 days is statistically different from the controls in the 7-day groups (Fig. 3).

3.3 Determination of survival after desiccation

As seen in Fig. 4, both cell strains did not show a significant viability change after 1 week desiccation in the laminar flow hood. 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 hood and a desiccator. For *B. atrophaeus* the viability was 55% in a desiccator and 85% in a laminar flow hood after 2 weeks. For *Hvr. luteus* the final viabilities were 15.1% and 59.2%, respectively. Percentages were acquired based on MPN analysis.

3.4 Proteome analysis

After cells of *Hvr. luteus* DA50^T were exposed to UV radiation, low- and high-vacuum conditions their whole cell proteins were separated on SDS polyacrylamide gels (Fig. 5). One-dimensional protein patterns of cells under UV and vacuum conditions displayed some significant differences (indicated by red arrows), with respect to intensity and location of bands to the patterns of control samples. By converting the quality data to quantity and column with ImageJ software (version 1.51m9; ImageJ curves in supplementary parts S. 1 and S. 2),

significant differences in weights 42 and 15 kDa for UV-air, and 70 and 15 kDa for UV-N_2 and 38, 25 and 15 kDa in high vacuum conditions can be observed (Figs. 6 and 7).

3.5 HPLC and MALDI-TOF

The chromatograms of UV-radiation and vacuum exposed samples are presented in Figs. 8 and 9. Fractions were separated in minutes and subsequently analyzed by MALDI-TOF. The MALDI-TOF protein spectrums of one of the clearer qualities of vacuum samples, minute 13, is shown in Figs. 10 – 12. Several changes in protein content between the controls and vacuum exposed samples in the 13-minute fractions can be observed from these figures. For instance, in the proteins below 3 kDa in the control sample, some proteins appearing at masses 2267, 2629, 2854 Da. In samples exposed to low vacuum condition (Fig. 11), the weights are 2288, 2690 and 2854 Da, so similar to the control sample. Though, in high vacuum condition exposed sample (Fig. 12), protein in weight 2959 Da and some others are deviating some more from the control. In the proteins above 3 kDa, the control sample shows peaks at 3108, 3414, 3593, 3729, 4086, 4683, and 5062 Da and some other proteins around them. For above 3 kDa in low vacuum condition sample, weights 3350, 3531, 3721, 4102 Da are appeared. In high vacuum exposed samples, peaks at 3360, 3723 and 4100 Da can be observed.

4. Discussion

In this study, we explored the resistance of halite-embedded cells of *Hvr. luteus* strain DA50^T and PBS-embedded of resistant bacteria *B. atrophaeus* after exposure to UV radiation (200 – 400 nm), low and high vacuum conditions, and desiccation. *Hvr. luteus* stain DA50^T was

exposed to various UV fluxes in both air and N_2 atmospheres, and it remained vital even after more than half an hour exposure to simulated Mars UV radiation.

After 3 weeks incubation, the minimum percentage of viable halophile cells (as compared to controls) for both air and N_2 experiments was 12 % (Fig. 2). During UV in combination with air exposure experiment, the presence of oxygen has an attenuating effect on the effective radiation, although the oxygen may be source of oxygen radicals, which can directly damage the cells. If the latter effect is indeed present and dominant, such an effect was not reflected in our results. These data suggest that a lower initial cell thickness (density A) may have contributed the lower survival rate of the *Hvr. luteus* for UV radiation. The lethal effects of UV flux surged when the chamber was flushed by N_2. Another factor that may contribute to the resistance of halophilic archaea to high levels of ionizing 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). Therefore, the intracellular salts might have a protective role against radiation damage by sweeping reactive oxygen species, as shown in work with *Hbt. Salinatrum* NRC-1 (Abrevaya *et al.*, 2011, Oren, 2014). 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 $OH^{\cdot}$ radicals produced by UV radiation from residual water molecules inside the halite crystals and/or intercellular fluid.

Leuko and colleagues (Leuko *et al.*, 2015) assayed the viability of three haloarchaea, *Halobacterium salinarum* NRC-1, *Halococcus morrhuae* and *Halococcus hamelinesis* after exposure to simulated solar radiation. The first two demonstrated good resistances against radiation, whilst *Halococcus hamelinesis* showed less viability. *Halobacterium salinarum* is also quite resistant to ionizing radiation probably due to the fact that repairing DNA lesions from ionizing radiation occurs as fast as repairing under desiccation (Kottemann *et al.*, 2005).

This is possibly related to their variety of chromosome sets (Soppa, 2013). Fendrihan and colleagues exposed halite-entrapped *Halococcus dombrowskii* to simulated Mars UV radiation and it survived at doses even up to 10^7 J/m^2. The survival rate of *Hcc. dombrowskii* when embedded inside halite was two times more than in liquid medium with the same dose (Fendrihan *et al.*, 2009). 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 is able to withstand the UV radiation exposure only for a couple of hours. It cannot withstand the combination of radiation with desiccation and low temperatures. A related species, *H. chaoviator*, can tolerate UV radiation dose of up to 10^7 J/m^2, showing that survival capabilities are not necessarily shared between phylogenetically related species (Mancinelli et al., 1998). In this regard, MacCready and colleagues (McCready *et al.*, 2005) tested 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. *D. radiodurans* is not considered a spore-forming bacterium, however it is extremely resisting desiccation and radiation. In a 10-day experiment with the cells embedded in a Mars soil analogue under low pressure they showed 30% viability, while *E. coli* did not survive (Diaz and Schulze-Makuch, 2006). Shahmohammadi and colleagues (Shahmohammadi *et al.*, 1998) suggested the importance of high concentration of intracellular KCl for reducing DNA strand breaks induced by ionizing radiation is probably due to scavenging of free radicals by KCl similar to bacterioruberin. In fact, the reaction between chloride anions with $^{\bullet}$OH radicals will lead to a chain reaction for other anion chlorides. However, $^{\bullet}$OH is much more reactive than these anions (Kottemann et al., 2005). The data from our study demonstrate that *B. atrophaeus* is more desiccation and vacuum resistant than *Hvr. luteus* strain DA50^T. The ability to cope with these conditions for halophiles is most likely related to its salt tolerance. The Halobacteriaceae family responds to increases in osmolarity by means of K^+ as their osmoticum (Mancinelli, 2015,

Kushner, 1968). Osmotic pressure increases during dehydration similar to a cell in hypersaline environment (Mancinelli, 2015). On the other hand, for survival under desiccation, adaptations of many prokaryotes include sporulation (Nicholson et al., 2000a) and also production of extracellular polysaccharides (Potts, 1994), neither of which is utilized by the well-studied halophile, *Halobacterium sp.* (Kottemann *et al.*, 2005). Instead, the results of genome analysis of *Halobacterium sp.* demonstrated that this strain contains putative genes for variety of major DNA repair pathways such as mismatch repair, nucleotide excision repair, homologous recombination, translesion synthesis, base excision and photoreactivation repair (Ng *et al.*, 2000). These results suggest a major role for DNA repair in *Halobacterium sp.* resistances to desiccation and other extreme conditions (Kottemann *et al.*, 2005).

One-hour exposure to either low or high vacuum had no effect on either cell type, however a 7-day exposure to vacuum did decrease halophile viability. In these tests, the samples were desiccated and washed. So here the viability is related to tolerating all factors. In the 1-week desiccation tests, no changes were observed in *B. atrophaeus* using either a laminar flow hood or a desiccator. *Hvr. luteus* also showed no decrease in percentage viable cells in the laminar flow hood. However, to ~ 21% the viability dropped using a desiccator. After 2 weeks, the negative effect of desiccation especially with the lack of air humidity on *Hvr. luteus* was very significant: ~ 15% of viability inside the desiccator and ~ 59% inside the laminar flow hood (Fig. 4). In another study, Kottemann *et al. (*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. These results suggest that halite crystals that formed during the experiment offered a level of cellular protection. 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 hrs. While the latter remained viable at 75%, all *H. halobium* cells were overserved to be dead (Kottemann *et al.*, 2005, Koike *et al.*, 1992). Abrevaya and colleagues (Abrevaya *et al.*, 2011), compared two haloarchaea, *Natrialba magadii* and *Haloferax volcanii,* with *Deinococcus radiodurans* under high vacuum conditions (10^{-7} mbar). The results demonstrated that *N. magadii* was more resistant compared to *D. radiodurans*. High resistance in *N. magadii* may have been due to the more effective mechanisms of halophiles against desiccation. Actually, they were evolved in environments with a variety of stresses such as hyper-salinity and heat. On the other hand, the viability of *H. volcanii* was hundred times less than *N. magadii* at the same conditions. The point is the salt concentration: *N. magadii* and *H. volcanii*, 3.5 – 4 M and 1.7 – 2.5 M NaCl, respectively.

In our HPLC chromatograms the detection of different proteins between exposed and control samples was difficult due to similarities and interfering peaks. Nonetheless, in all cases the MALDI-TOF analysis demonstrated different mass spectra in low molecular weight regions (between 2–10 kDa) after exposing of the cells to extreme environmental conditions. These observations may indicate that in such extreme conditions the cells are able to change intercellular metabolism, novel metabolic pathways, and protein expressions to survive the harsh environmental conditions. Additionally, interpretations of MALDI-TOF spectra analysis of peptide-protein complexes in the high molecular weight regions are occasionally difficult. In future studies, these complex mixtures could be explored using trypsin digestion of the high mass molecular proteins to small fragments or peptides for the MS-MS analysis and compare these with the library of known MALDI-TOF spectral fingerprints and masses predicted from haloarchaea proteomic databases.

The current MALDI-TOF analysis suggests changes in peptide profiles of the cell lysate fractions by the amino acid degradation or modification, induced during the exposure to the space environment. Changes might have affected single peptides or the polypeptides profile in

the cell involved in stress responses during such extreme conditions in order to adapt their physiology and cellular structures. Also, the archaeal cell envelope is 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 the harsh conditions and it could be investigated in more detail in future experiments.

B. atrophaeus demonstrated a greater resistance to extreme space conditions such as desiccation, UV-radiation and vacuum compared to *Hvr. luteus*. Their increased resistance may be due to the thick spore structure that causes *B. atrophaeus* to be used as an industrial and astrobiological reference strain (Sella *et al.*, 2015).

5. Conclusion

This study is the first work reporting the survival of a novel extremely halophilic archaea 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/m^2), low (1.7×10^{-1} mbar) and high vacuum conditions (1 hr at 1.6×10^{-5} mbar and 7 days at 7.6×10^{-8} mbar), and desiccation, and their survival was evaluated. The total protein content evaluated for *Hvr. luteus*. *B. atrophaeus* showed high resistance against given conditions. *Hvr. luteus* strain DA50^T also showed a good resistance withstanding 30 min of Mars simulated UV radiation within air or N_2 atmosphere, high vacuum conditions, and up to 2 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. Finally, it should be mentioned that the present study suggests that this extremely halophilic archaeon may be used as a suitable candidate for ongoing astrobiological research.

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Author Disclosure Statement

No competing financial interests exist.

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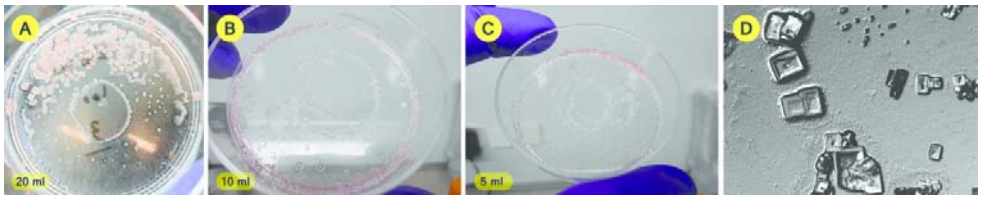

Fig. 1. *Hvr. luteus* strain DA50^T encased in salt crystals during desiccation. Desiccated plates with preliminary volume of (A) 20 mL, (B) 10 mL and (C) 5 mL. (D) Light microscope image of desiccated plate of *Hvr. luteus* strain DA50^T (40X) showing details of the mineral formed

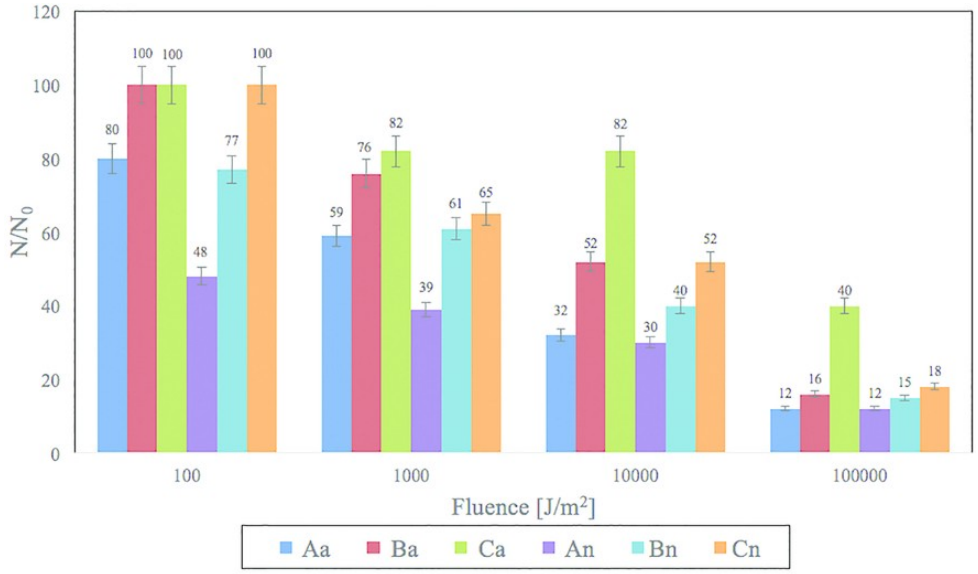

Fig. 2. *Hvr. luteus* strain DA50^T survival after exposure up to 10^5 J/m^2 of UV-radiation in a regular air environment and UV-radiation in combination with 100% N_2. Aa, Ba and Ca are UV-air exposed samples and An, Bn and Cn are UV-N_2 exposed samples (A, B and C are density). The error bars are the 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 survivors after radiation specified by the MPN technic and N_0 is the number of surviving cells in controls.

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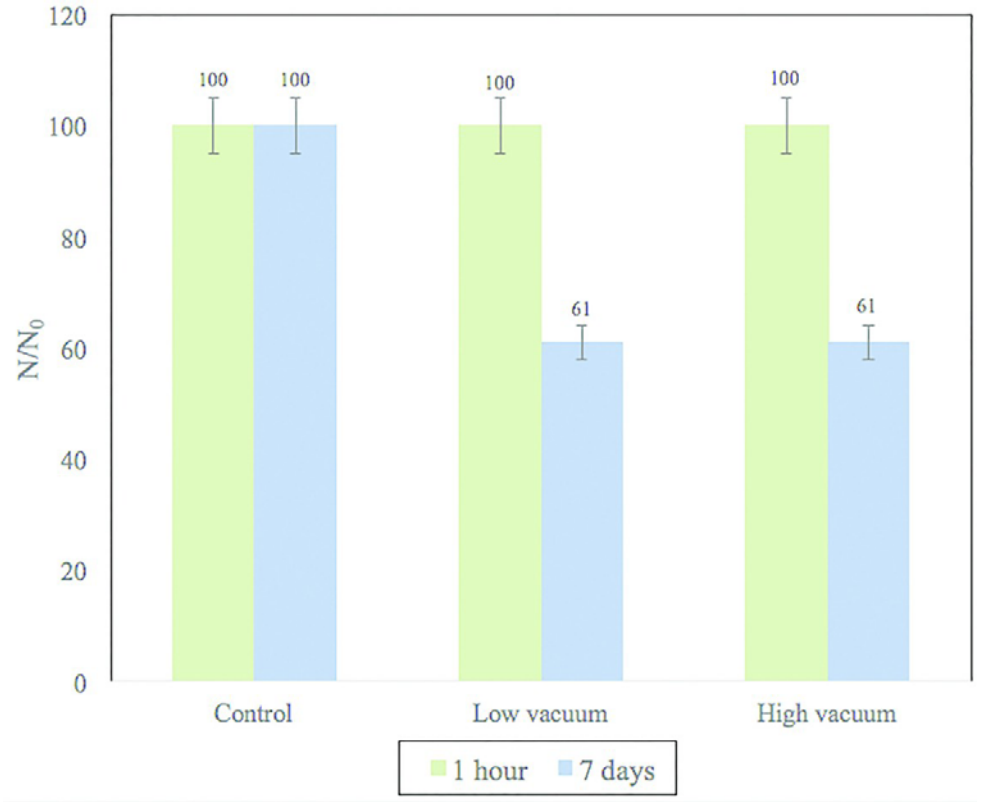

Fig. 3. The percentage of survival of *Hvr. luteus* strain DA50^T exposed to no (control), low (1.7×10^{-1} mbar) and high vacuum (1 hr at 1.6×10^{-5} mbar and 7 days at 7.6×10^{-8} mbar) conditions for one hour or 7 days. The standard error bar of 95% confidence intervals for three independent experiments (n=3) is indicated. The survival percentages N/N_0 are given above each bars, where N is the number of survivors specified by the MPN technic and N_0 is the number of surviving cells in controls.

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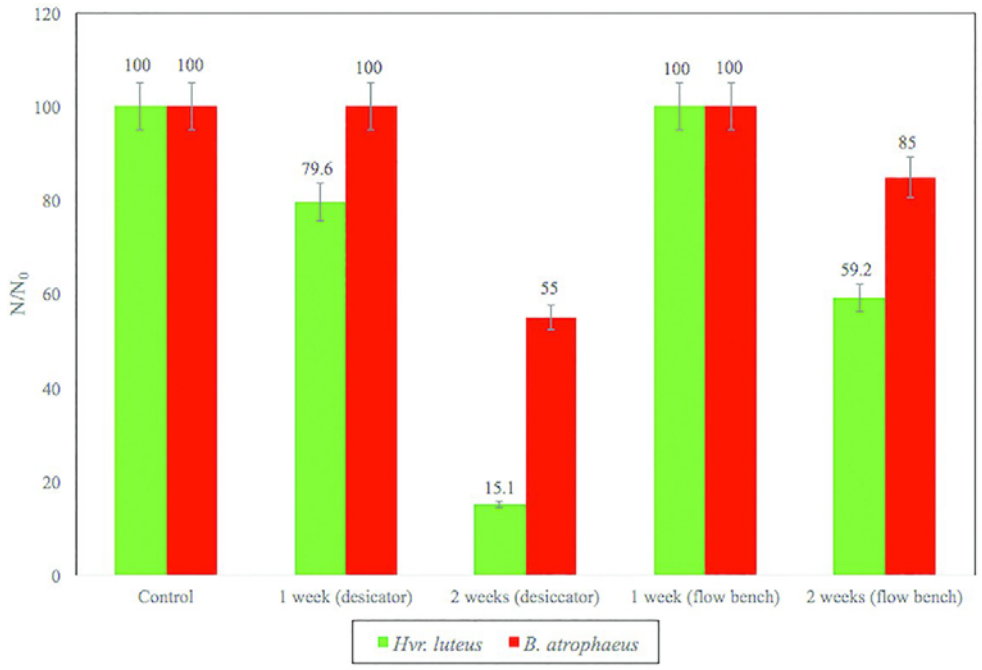

Fig. 4. *Hvr. luteus* strain DA50^T (green) and *B. atrophaeus* (red) exposed to desiccation for 1 and 2 weeks. The standard error bar of 95% confidence intervals for three independent experiments (n=3) is indicated. The survival percentages N/N_0 of both strains are given on top of the bar charts where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

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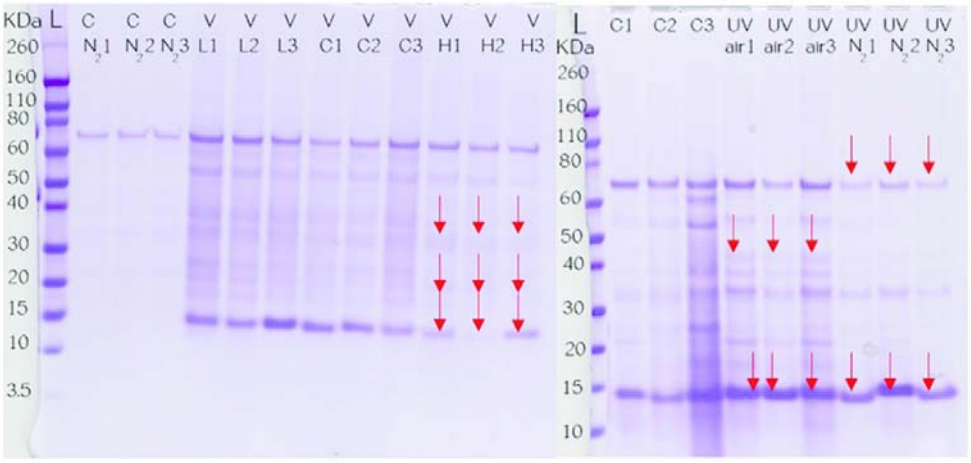

Fig. 5. Whole cell proteins from *Hvr. luteus* strain DA50^T cells under UV-radiation, following separation by SDS polyacrylamide gel electrophoresis. The gel shown on the left demonstrates ladder (first lane, L), 3 lanes for control of UV-N_2 (C-N1-3) which were exposed within a N_2 atmosphere, 3 lanes for low vacuum (V-L1-3), 3 lanes for vacuum control (V-C1-3), and 3 lanes for high vacuum (V-H1-3). The gel on the right shows the ladder (L), controls for UV (C1-3) which were kept in ambient pressure, UV-air (UVair1-3) and UV-N_2 (UV-$N_2$1-3). For each condition, three replicas were made.

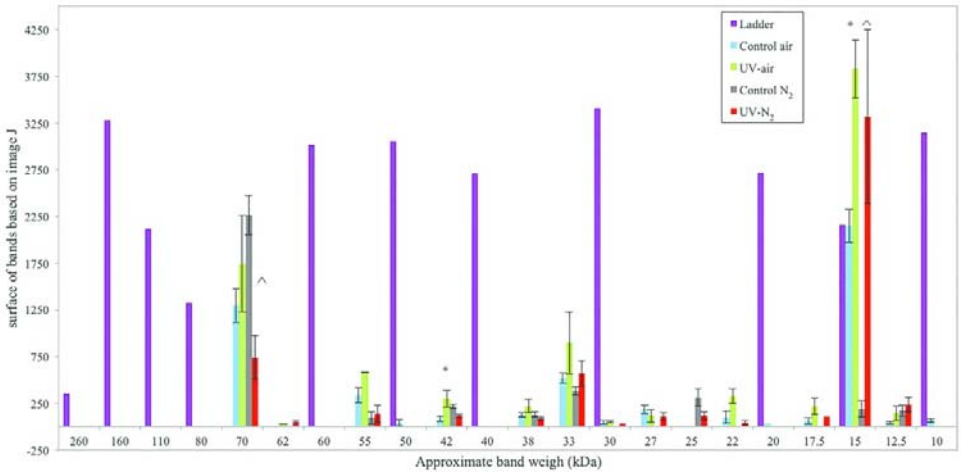

Fig. 6. Bar chart values of UV irradiated samples are shown. The values displayed are derived from the gel electrophoresis measurements (right panel in Fig. 5) discussed in the supplementary information, using the program ImageJ. Some significant differences between the control and UV irradiated samples can be observed. Significance was verified by ANOVA ($p<0.05$) and t-test. Signs are showing differences in UV-air (*) and UV-N_2 (^) measurements compared to the controls.

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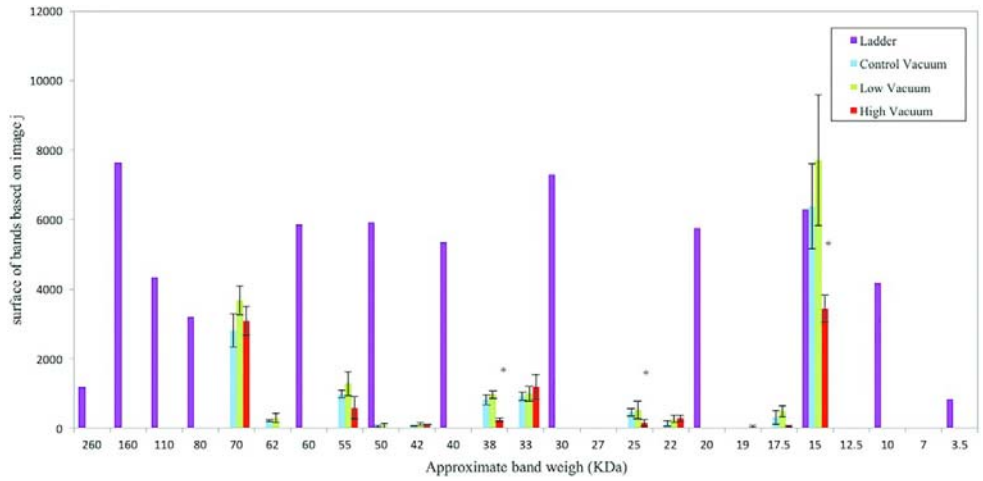

Fig. 7. Bar chart values of vacuum exposed samples are shown. Vacuum exposed samples. The values displayed are derived from the gel electrophoresis measurements shown on Fig. 5, left panel, using ImageJ (information about the calculation can be found in the text). There are some significant differences between the control samples and high vacuum samples. Significance was verified by ANOVA ($p<0.05$) and t-test. Significant differences in comparison to the controls are marked with an *.

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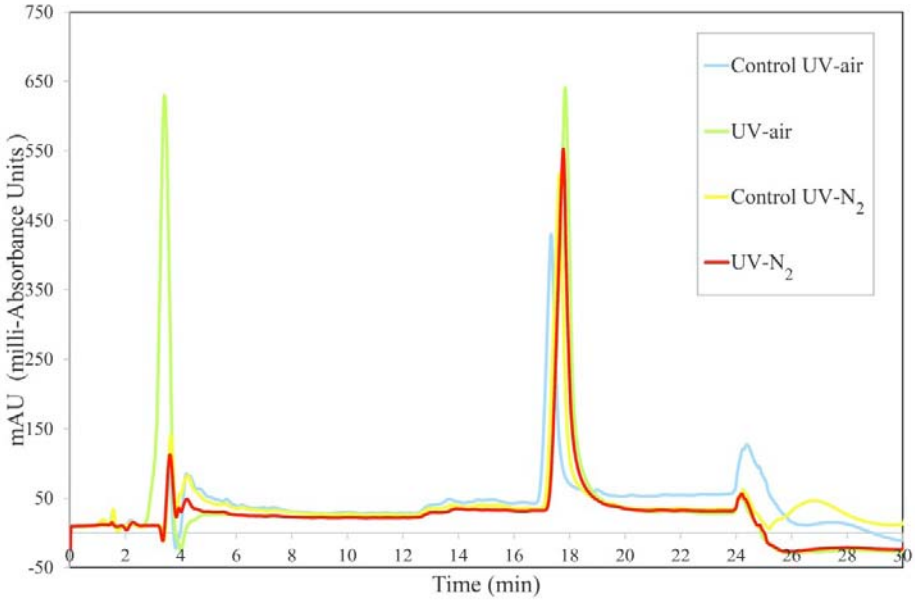

Fig. 8. RP-HPLC chromatograms (milli-Absorbance Units/Time)of proteome analyses of *Hvr. luteus* strain DA50^T after exposure to UV radiation either with air or with N_2.

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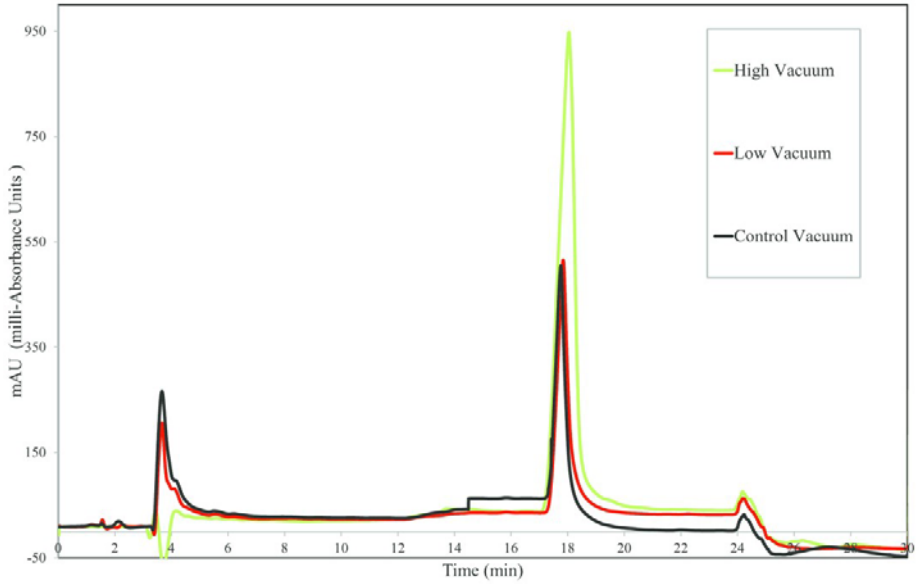

Fig. 9. RP-HPLC chromatograms (milli-Absorbance Units/Time) of proteome analyses of *Hvr. luteus* strain DA50^{T} after exposure to low and high vacuum conditions.

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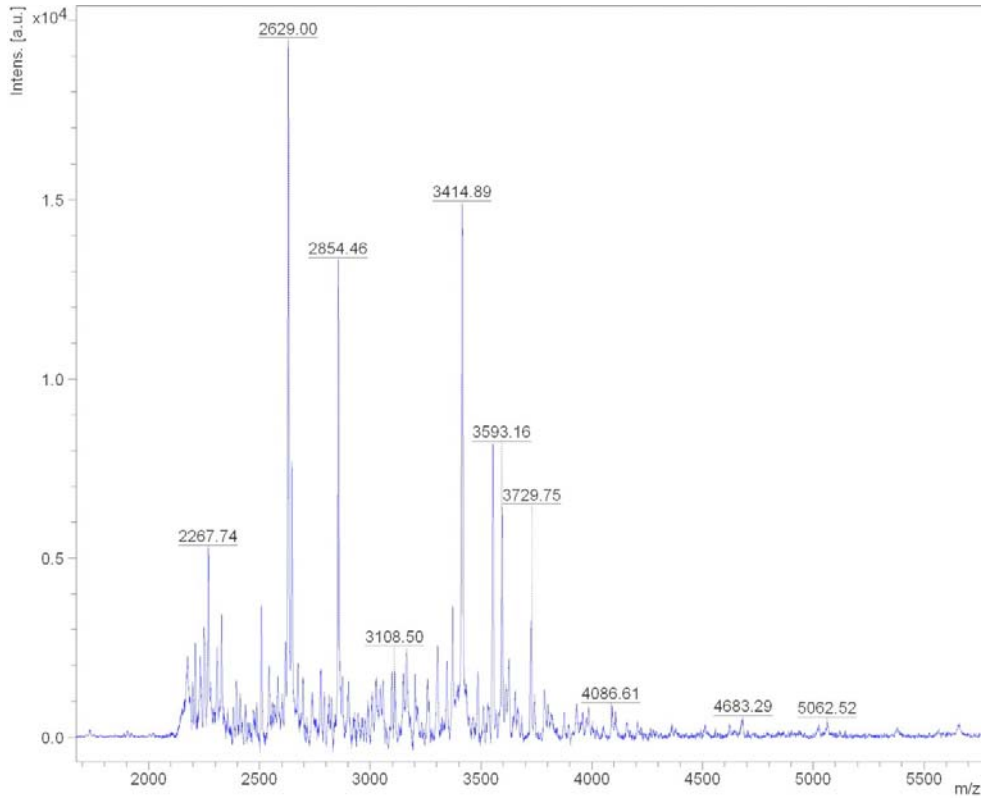

Fig. 10. MALDI-TOF spectrum obtained from vacuum control sample in minute 13 after purification and fraction by RP-HPLC.

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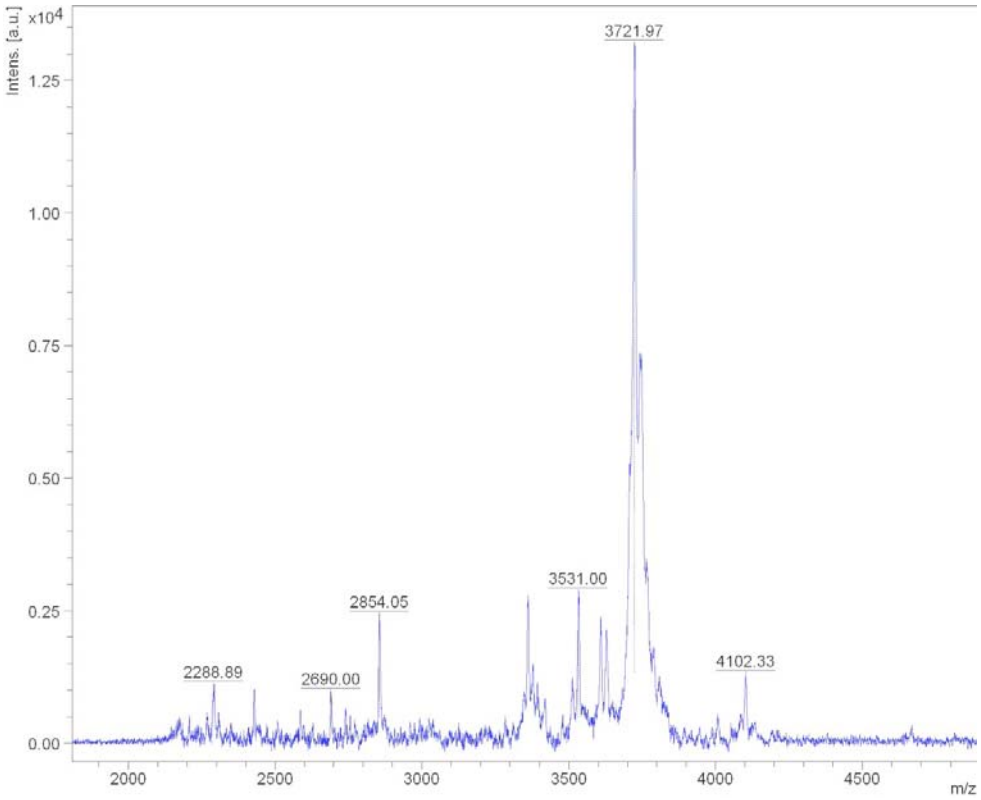

Fig. 11. MALDI-TOF spectrum of low vacuum sample in minute 13 after purification and fraction by RP-HPLC.

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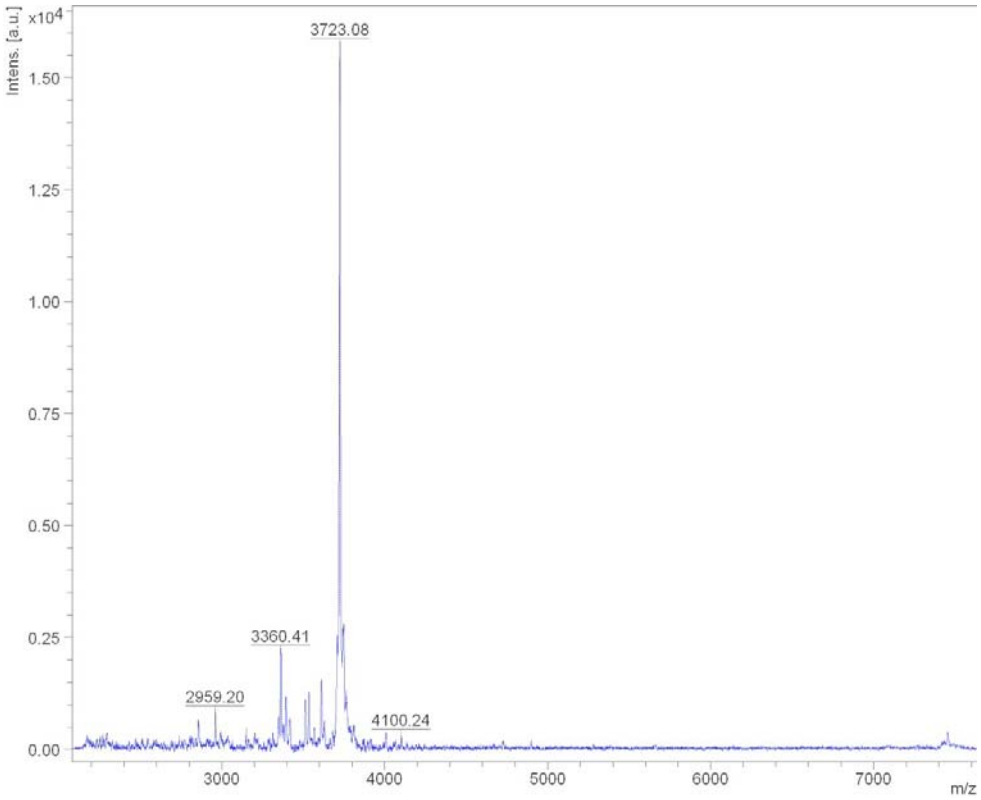

Fig. 12. MALDI-TOF spectrum of high vacuum sample in minute 13 after purification and fraction by RP-HPLC.

138x112mm (600 x 600 DPI)