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Survival and death of the haloarchaeon *Natronorubrum* strain HG-1 in a simulated martian environment

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Abstract

Halophilic archaea are of interest to astrobiology due to their survival capabilities in desiccated and high salt environments. The detection of remnants of salty pools on Mars stimulated investigations into the response of haloarchaea to martian conditions. *Natronorubrum* sp. strain HG-1 is an extremely halophilic archaeon with unusual metabolic pathways, growing on acetate and stimulated by tetrathionate. We exposed *Natronorubrum* strain HG-1 to ultraviolet (UV) radiation, similar to levels currently prevalent on Mars. In addition, the effects of low temperature (4, −20, and −80 °C), desiccation, and exposure to a Mars soil analogue from the Atacama desert on the viability of *Natronorubrum* strain HG-1 cultures were investigated. The results show that *Natronorubrum* strain HG-1 cannot survive for more than several hours when exposed to UV radiation equivalent to that at the martian equator. Even when protected from UV radiation, viability is impaired by a combination of desiccation and low temperature. Desiccating *Natronorubrum* strain HG-1 cells when mixed with a Mars soil analogue impaired growth of the culture to below the detection limit. Overall, we conclude that *Natronorubrum* strain HG-1 cannot survive the environment currently present on Mars. Since other halophilic microorganisms were reported to survive simulated martian conditions, our results imply that survival capabilities are not necessarily shared between phylogenetically related species.

Keywords: Halophiles; *Natronorubrum*; Mars simulation; Mars soil analogue; Survival

1. Introduction

Halophiles are a class of extremophilic organisms that not only tolerate, but also thrive in environments with high salt concentrations, up to full saturation. These salt tolerant organisms evolved early in the Earth's history (Oren, 2002). Geological formations millions of years old still contain viable halophilic archaea and bacteria (e.g. the Permian–Triassic era, 290–206 million years ago (Norton and Grant, 1988; Stan-Lotter et al., 1999; Vreeland et al., 2000; McGenity et al., 2000)). Mancinelli et al. (1998) have shown that predried osmophilic microbes *Synechococcus Nägeli* and *Haloarcula*-G survived a two week exposure to the space environment while in Earth orbit aboard the Biopan facility.

The Shergotty, Nakhla, and Chassigny (SNC) meteorites, all of which originated on Mars, contain traces of halite (Gooding, 1992). The Nakhla rock may even have been in contact with seawater-like brine on Mars (Sawyer

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et al., 2000). Furthermore, halite and sulfate evaporite minerals have been detected on Mars by the Mars Exploration Rovers (MER) and Mars Express (e.g. Klingelhöfer et al., 2004; Langevin et al., 2005; Squyres et al., 2006). Even if temperatures on early Mars were well below 273 K (Gaidos and Marion, 2003), liquid water may still have been present due to the availability of dissolved minerals which lowered the cryoscopic constant (Fairén et al., 2009). The ability of halophilic archaea to survive within low water-activity environments, such as evaporites, and their requirement for elevated salt concentrations make them model organisms for life on Mars (Litchfield, 1998). The effects of simulated martian environmental stresses and martian soil analogues have been tested so far on a few microorganisms from the bacterial domain, including cyanobacterium *Chroococcidiopsis* sp. 029 (Cockell et al., 2005), several *Bacillus* species (Rettberg et al., 2004; Schuerger et al., 2006), *Escherichia coli* and *Deinococcus radiodurans* (Baumstark-Khan and Facius, 2002; Diaz and Schulze-Makuch, 2006). Much less data are available for prokaryotes from the archaeal domain, such as *Halobacterium salinarum* NRC-1 and *Haloarcula*-G (Mancinelli et al., 2004). In those experiments the response to high salt concentration, low temperature, desiccation, and UV irradiation was studied.

The halophilic archaeon *Natronorubrum* sp. strain HG-1 was cultivated from hypersaline lakes in the Kulunda steppe, Altai, Russia by Sorokin et al. (2005). *Natronorubrum* strain HG-1 is characterized as an extremely halophilic, neutrophilic, heterotrophic archaeon, with a rod-shaped morphology (see Fig. 1). It displays typical haloarchaeal features, including pink to red colour due to the carotenoid bacterioruberin. Shahmohammadi et al. (1998) showed that wild-type *H. salinarum* was more resistant against UV light (254 nm), hydrogen peroxide, and γ-radiation than a bacterioruberin-deficient (colourless) mutant. The protecting effect of bacterioruberin was strongest for UV light.

In contrast to most other haloarchaea, *Natronorubrum* strain HG-1 uses simple compounds (acetate) as a carbon source and is able to oxidize thiosulfate into tetrathionate. On Mars, sulfates have been found ubiquitously by the MER and Mars Express (e.g. Klingelhöfer et al., 2004; Langevin et al., 2005). While it is difficult to imagine thiosulfate being formed from sulfate in the highly oxidizing environment of present day Mars, the formation of thiosulfates may have occurred in the past when Mars had a more reducing environment and active volcanism.

We report on the results of exposure experiments of *Natronorubrum* strain HG-1 cells to different aspects of the martian environment. We have exposed this microorganism to UV radiation, similar to the levels of radiation found on the surface of Mars. In addition, we tested the survival of *Natronorubrum* strain HG-1 stored at low temperatures (4, −20, and −80 °C), after desiccation, and when mixed with a Mars soil analogue from the Atacama desert.

2. Experimental

2.1. Strain and culture conditions

Natronorubrum sp. strain HG-1 was isolated and described by Sorokin et al. (2005). *Natronorubrum* strain HG-1 cultures were grown by inoculating 10 mL fresh medium in 60 mL glass bottles (4.3 cm diameter, with not fully tightened screw caps) with 0.2 mL of a previous culture. After incubating for 4 days at 37 °C the cultures reached a maximum density of 3×10^9 cells mL^{-1}. The growth medium included 240 g L^{-1} NaCl, 2 g L^{-1} K_2HPO_4, and 0.5 g L^{-1} $(NH_4)_2SO_4$. The pH was adjusted to 7.3 before sterilisation (20 min at 120 °C). After sterilisation the medium was supplemented with 2 mM $MgCl_2$, 20 mM sodium acetate, 0.05 g L^{-1} yeast extract, and 1 mL L^{-1} of trace elements solution (Pfennig and Lippert, 1966).

2.2. Mars simulations

Samples of *Natronorubrum* strain HG-1 were prepared by placing 1 mL of an active exponential growth phase culture into a sterile plastic Petri-dish (Greiner Bio-One, 3.5 cm diameter, with spacer in lid). A portion of the samples was wrapped in parafilm, the rest of the samples

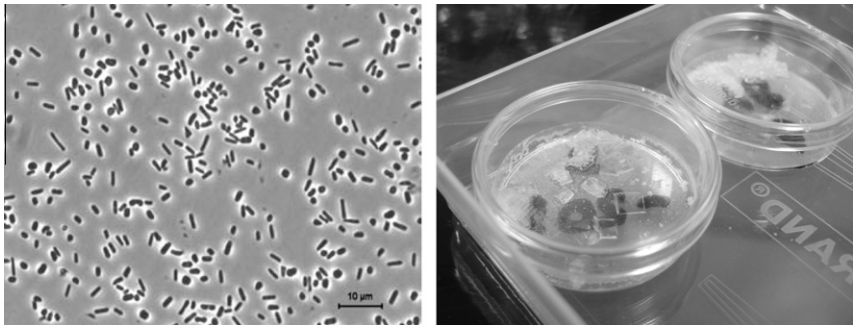

Fig. 1. Phase contrast microphotographs of a pure culture of *Natronorubrum* strain HG-1 (left) and a picture of *Natronorubrum* strain HG-1 in desiccated state (right), showing the halite crystals.

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remained open to air. All samples were then placed overnight in a stove at 37 °C (relative humidity 10–20%), to dry the open samples. These samples are labelled 'dry', while the parafilm-wrapped samples retained their liquid medium and are labelled 'wet'. Both wet and dry samples were then, in duplo, exposed to the following aspects of the martian environment. Wet and dry samples were stored for 24 h at either −80 °C, −20 °C, 4 °C, or room temperature. Also, dry samples were exposed for 30 min to a high-pressure xenon solar simulator (ScienceTech, model 100150XUV, fitted with an Osram XBO150 lamp, output 200–2500 nm). The samples were UV-irradiated at room temperature and the temperature of the samples did not significantly change during the exposure. The spectrum of the solar simulator is shown in Fig. 2, along with the spectrum of the Sun on the martian surface, as modelled by Patel et al. (2002). Integrating both spectra over the 200–400 nm wavelength range, the solar simulator produces 28 W m^{-2}, scaled to the distance of the sample from the lamp, while Mars receives 34 W m^{-2}. This means that irradiating a sample for 20 h with the solar simulator is roughly equivalent to one sol (a day on Mars, or 24.6 Earth hours). The fluence received by the sample in 30 min is 50 kJ m^{-2}, the total dose is 48 J.

A Mars soil analogue was obtained from the Chilean Atacama desert, Yungay region (Flat Top Hill, S25° 29′ 50.9″ W69° 50′ 22.5″). X-ray diffraction measurement (Peeters et al., 2009) revealed that the sample was rich in quartz (45% quartz, 25% cristobalite). The water-dissolved fraction contained mainly $CaSO_4$. HPLC analysis showed that the Atacama desert soil sample had a low abundance of organics (~500 ppb amino acids), see Peeters et al. (2009) for a more extended description of the soil sample. A 1 mL aliquot of an exponential growth phase culture of *Natronorubrum* strain HG-1 was added to 1 g of soil in the Petri-dish. The samples were then dried overnight in an oven at 37 °C as described above. Two of the dried samples containing the Mars soil analogue were kept in the dark at room temperature, while two others were irradiated at room temperature using the same xenon solar simulator as described above. To test for the presence of organisms in the Mars soil analogue with the ability to grow in high salt medium, 1 g of Atacama soil was added to 10 mL of fresh medium. Additionally, the ability of *Natronorubrum* strain HG-1 to grow in medium in the presence of Atacama soil, was tested by adding 1 g of Atacama soil to 10 mL of fresh medium and inocculating that with 1 mL of active *Natronorubrum* strain HG-1-culture.

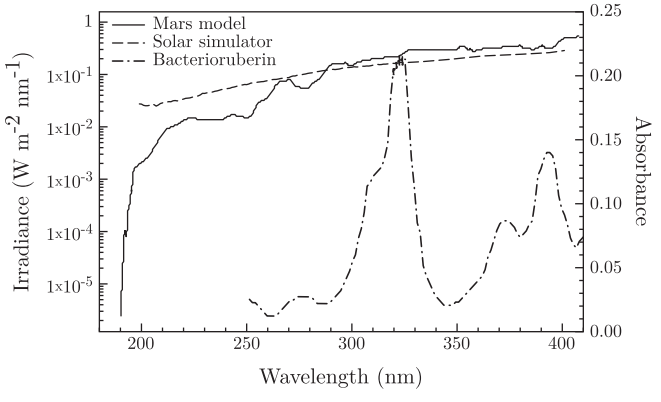

Fig. 2. UV–Vis spectra of the solar simulator lamp (dashed line), the solar spectrum on Mars as modelled by Patel et al. (2002) (solid line), and the absorption spectrum of the C_{50}-carotenoid bacterioruberin (dash-dotted line, right ordinate), extracted from *Halobacterium salinarum* (Kushwaha et al., 1975, species named *Halobacterium cutirubrum* in the reference, later renamed to *Halobacterium salinarum*).

2.3. Viability

After exposure of the *Natronorubrum* strain HG-1 cells to different aspects of the martian environment, the concentration of living cells was determined using the Most Probable Number (MPN) method. Three milliliters of medium was added to each sample and stirred for homogenization. After dissolution of the samples, the medium was transferred to a 50 mL culture flask (4.3 cm diameter). For the dry samples, 1 mL H_2O was added to replace the liquid removed in the drying process. *Natronorubrum* strain HG-1 cells are obligate halophiles, requiring a minimum of 2 M NaCl, therefore medium was added before water to prevent cell lysis due to osmotic shock. Additional medium was added to a total volume of 10 mL. From these solutions (a 10^{-1} dilution from the original 1 mL sample), a dilution series was made. The dilution series consisted of six 10× dilution steps ($10^{-1}\cdots10^{-6}$), followed by ten 2× dilution steps ($5.0\times10^{-7}\cdots9.8\times10^{-10}$). The dilution series was allowed to grow for 20 days at 37 °C, after which the culture flasks were checked for growth. For each sample, two separate dilution series were made.

3. Results and discussion

We have exposed cultures of *Natronorubrum* strain HG-1 to a wide temperature range, in wet and dry conditions, and investigated its UV stability in air and embedded in a martian soil analogue. Table 1 shows the experimental conditions to which samples of *Natronorubrum* strain HG-1 cells were exposed and the survival rates under these conditions. After recovery, the initial viable cell concentrations were determined from a dilution series (MPN method). The samples that were kept wet and at room temperature served as a control. The control samples contained 5×10^8 cells mL^{-1}. The samples that were dried and kept at room temperature showed a cell concentration of 7×10^8 cells mL^{-1} after reconstitution in fresh medium, showing an approximately 150% survival rate. Similar results were found for the samples (both dry and wet) stored for 24 h at 4 °C and −20 °C. At −80 °C, survival of the *Natronorubrum* strain HG-1 cells dropped to 25% for the wet samples and to 1% for the dried samples.

Mancinelli et al. (2004) observed that *H. salinarum* NRC-1 and *Haloarcula*-G (which was recently reclassified as *Halorubrum chaoviator* (Mancinelli et al., 2009)) could

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Table 1
Experimental conditions and final cell concentrations, determined by dilution assay. The values are an average of two separate experiments. Survival is measured as fraction of the control. RT = room temperature.

Experimental conditions	Cell concentration (mL^{-1})	Survival (%)
Wet, RT (control)	5×10^8	≡100[a]
Dry, RT	7×10^8	~150
Wet, 4 °C	5×10^8	~100
Dry, 4 °C	1×10^9	~200
Wet, −20 °C	1×10^9	~200
Dry, −20 °C	5×10^8	~100
Wet, −80 °C	1×10^8	~25
Dry, −80 °C	8×10^6	~2
Dry, RT, UV[b]	1×10^1	$\sim 10^{-6}$
Dry, RT, soil[c]	$<10^1$	$<10^{-6}$
Dry, RT, soil, UV	$<10^1$	$<10^{-6}$

[a] By definition.
[b] Thirty minutes under a xenon arc lamp, $\lambda > 200$ nm, 28 W m^{-2} integrated over 200–400 nm range. Mars receives 34 W m^{-2} at noon-time at the equator, see Fig. 2.
[c] Mars soil analogue obtained from the Flat Top Hill site in the Yungay region of the Atacama desert in Chile, see Peeters et al. (2009).

survive repeated freeze-thaw cycles both to −20 and −80 °C for 144 days. The cells in those experiments were in a fully desiccated state. Mancinelli et al. (2004) furthermore found that control samples of *E. coli* and *Pseudomonas fluorescens* did not survive desiccation for 144 days at room temperature, at −20 °C, or at −80 °C. The radioresistant *D. radiodurans* did not survive freeze-thaw cycles at room temperature, or at −20 °C, but did survive freeze-thaw cycles to −80 °C in the desiccated state for 144 days. In addition, Pogoda de la Vega et al. (2007) found that desiccated samples of *D. radiodurans* could survive 7 diurnal cycles in temperature between +14 and −63 °C, while in a Mars-like atmosphere. Of the five bacterial species tested, *H. salinarum* and *Haloarcula*-G were obligate halophiles which grow at 4 M NaCl. In our experiments, we found that *Natronorubrum* strain HG-1 cells could survive a freeze-thaw cycle for 24 h to −20 °C. Viability was much lower upon −80 °C storage for both the moist and dry samples, see Table 1. The liquid medium remained liquid at −20 °C due to the high solute concentration, but eventually froze at −80 °C. During freezing large ice crystals can form and damage the cells' membranes. The results of our experiments and comparison to other microorganisms are summarized in Table 2.

Desiccation of *Natronorubrum* strain HG-1 cells for a period of 24 h did not have a measurable effect on the viability of the culture. From this short period of incubation in a desiccated state, it is difficult to extrapolate the survival of *Natronorubrum* strain HG-1 to geologically relevant time scales. Other halophilic archaea, however, have been isolated from salt deposits believed to be millions of years old and presumably survived in inclusions in halite crystals (Stan-Lotter et al., 1999). Adamski et al. (2006) have shown that cells of the bacterium *Pseudomonas aeruginosa* are incorporated in fluid inclusions, which are pockets of saturated brine in the halite crystals, and that these bacteria show motility while incorporated in those inclusions for at least 13 months. Similarly, cells of the extremely halophilic *H. salinarum* NRC-1 were preferentially localized in fluid inclusions of artificial halite (Fendrihan et al., 2006). When cultures of *Natronorubrum* strain HG-1 cells were dehydrated, halite crystals were formed, see Fig. 1. We envisage that *Natronorubrum* strain HG-1 cells may be incorporated in liquid inclusions in salt crystals, in a way similar to *H. salinarum* and *P. aeruginosa* (Adamski et al., 2006). Cells incorporated in inclusions are not completely desiccated, while the cells that remain outside the inclusions are more susceptible to full dehydration and may not survive.

Desiccated *Natronorubrum* strain HG-1 cultures did not survive irradiation with a solar simulator for 30 min. For the UV-exposed samples growth was found only in the bottle with the highest concentration (10^{-1} dilution). The corresponding survival is approximately 10^{-6}%. In general, cell death by exposure to UV radiation is associated with irreparable levels of photochemical DNA damage. This means that the pigments present in *Natronorubrum* strain HG-1 are not protective against considerable doses of UV light in the >200 nm wavelength range. The pigments of *Natronorubrum* strain HG-1, when extracted with a mixture of methanol/acetone (Sorokin et al., 2005) had absorption maxima at 470, 497, and 530 nm, which correspond to well-known features of haloarchaeal bacterioruberins (Oren, 2002). The UV part of the absorption spectrum of C_{50}-carotenoid bacterioruberin (Kushwaha et al., 1975), is shown in Fig. 2, along with the spectra of the solar simulator and the solar illumination spectrum on Mars. The bacterioruberin spectrum has a small absorbance feature around 320 nm with a height of 0.22 absorbance units, which means it absorbs roughly 50% of the incident light. A smaller feature can be seen around 400 nm. There are no absorbance features in the ranges 250–300 nm and 330–370 nm, which means that radiation of these wavelengths can pass through the pigment and cause photochemical damage. Absorption data in the 200–250 nm range were not available for the bacterioruberin pigment, but many other molecules absorb in this region of the light spectrum, such as peptides and carboxylic acids.

In the dry state, damage will accumulate to levels that are irreparable later as moisture comes in during the martian night (Möhlmann, 2005). Additionally, OH radicals may be created by the UV photons from water present in the crystal liquid inclusions in which the cells reside. Since NaCl crystals are transparent for UV light down to 200 nm (Li, 1979), it is likely that the formation of OH radicals in fluid inclusions is damaging to *Natronorubrum* strain HG-1. The radiation fluence produced by the solar simulator in 30 min corresponds to the solar fluence received at the martian surface in approximately the same time, see Fig. 2. *Natronorubrum* strain HG-1 is therefore not expected to survive on the surface of Mars for more than a few hours, unless they are protected from UV radiation.

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Table 2
Summary of the findings for *Natronorubrum* strain HG-1, compared to the survival of other microorganisms.

Conditions	*Natronorubrum* HG-1[a]	Others
Dehydration	Survives dehydration	Dried *Halobacterium salinarum* NRC-1 and dried *Halorubrum chaoviator* survive freeze-thaw cycles to −20 and −80 °C for 144 days; dried *D. radiodurans* survives cycles to −80 °C, but not at room temperature or −20 °C[b]; dried *D. radiodurans* also survived diurnal cycles (−63 to +14 °C) under Mars atmosphere[c]
Temperature	Survival lower at −80 °C	
UV radiation	No survival after 30 min	*Chroococcidiopsis* sp. 029 no survival after 30 min[d]; 7 *Bacillus* spp. destroyed in 30–180 min[e, f]; *Bacillus pumilus* reduced but survived UV under Mars-like atmosphere for 5 min–12 h[g]; *Halorubrum chaoviator* survived exposure to space radiation (10^4 kJ m^{-2} UV) for 2 weeks[h]
Atacama soil	No survival in combination with dehydration	*Bacillus pumilus* survived due to shadowing from UV by <60 μm Atacama dust[h]; *Methanosarcina barkeri*, *Methanobacterium formicicum*, and *Methanothermobacter wolfeii* survived dehydration on JSC Mars-1[i]

[a] This experiment.
[b] Mancinelli et al. (2004).
[c] Pogoda de la Vega et al. (2007).
[d] Cockell et al. (2005).
[e] Schuerger et al. (2006).
[f] Rettberg et al. (2004).
[g] Osman et al. (2008).
[h] Mancinelli et al. (1998).
[i] Kendrick and Kral (2006).

In another experiment, *Natronorubrum* strain HG-1 cultures were mixed with Atacama soil, dried, and stored for 24 h at room temperature. After rehydration, no growth was found at any dilution in the dilution series (see Table 1), whereas cells desiccated without soil, showed no measurable loss of viability. In a control experiment, the Atacama soil was mixed with fresh medium and stored at 37 °C. No growth was found after 10 days, showing that no microorganisms with the ability to grow at high salt concentrations were present in the soil. In another control experiment, fresh medium mixed with Atacama soil was inocculated with *Natronorubrum* strain HG-1-culture and allowed to grow for 10 days at 37 °C. After 10 days, the *Natronorubrum* strain HG-1-cells had grown to maximum density, showing that growth of *Natronorubrum* strain HG-1 is not hindered by the presence of Atacama soil. In a hyper-saturated brine solution, solid nucleation particles may be needed for the onset of crystallization. In the absence of soil or dust, cells may act as nucleation objects themselves and crystallization could occur around the cells, resulting in a protective inclusion. In contrast, when the *Natronorubrum* strain HG-1 cells were mixed with soil prior to drying, the soil particles may have provided stronger nucleation points than the *Natronorubrum* strain HG-1 cells. In this case, *Natronorubrum* strain HG-1 cells may not have been incorporated in inclusions and dried out completely upon evaporation of the medium. For samples dried on Mars soil analogue, no growth was found in any of the dilutions; survival was less than 10^{-6}%. In contrast, *Methanosarcina barkeri*, *Methanobacterium formicicum*, and *Methanothermobacter wolfeii* were found to survive dehydration on JSC Mars-1 soil (Kendrick and Kral, 2006), see Table 2. Even while protected against UV radiation in the martian soil, the survival of *Natronorubrum* strain HG-1 under martian subsurface conditions is doubtful, since the viability of desiccated cells was decreased by storage at −80 °C. Although the temperature on the surface of Mars near the equator can be above 0 °C at noon-time, the average temperature is around −60 °C. Summarizing, we conclude that *Natronorubrum* strain HG-1 is unable to survive in the environment on or near the present surface of Mars.

Hansen et al. (2009) found that cultures of indigenous bacteria in a homogenized permafrost, exposed to a simulated martian environment equivalent to 80 sols, were reduced in total number of bacteria in the first 9 mm of soil, while viability was reduced in the first 15 mm of the soil. Biomolecules (polynucleotides and proteins) in the same permafrost were reduced in concentration in the upper 1.5 mm of the soil. They argue that a combination of direct UV photolysis, UV light produced reactive species, and freeze-thaw cycles may have affected the biological and biomolecular content of the permafrost. In our experiments, the *Natronorubrum* strain HG-1 cells were mixed with 1 g Atacama desert soil, which covered the Petri-dish by approximately 1 layer of grains. The soil was not sieved prior to the experiments, yielding a range of grain sizes. The thickness of the soil layer was less than 1 mm, which is well within the range in which Hansen et al. (2009) measured a reduction of both viability and total number of bacteria. Osman et al. (2008) reported that desiccated spores of *Bacillus pumilus* were partially protected against UV radiation through shadowing by a layer of fine (<60 μm) Atacama desert soil dust, see also Table 2. A possible survival strategy for *Natronorubrum* strain HG-1 would be to reside within the soil grains, as a cryptoendolithic organism, although de la Torre et al. (2003) did not detect any representatives of the *Archaea* in cryptoendolithic communities recovered from Antarctic dry desert soil.

Although phylogenetically related to *Natronorubrum* strain HG-1, *H. chaoviator* (formerly *Haloarcula*-G) was

considerably more resistant to UV radiation from space (see Table 2). Viable cells of *H. chaoviator* capable of proliferation were recovered following exposure to about 10^4 kJ m^{-2} integrated over the 200–400 nm wavelength range (Mancinelli et al., 1998). A flux of about 50.4 kJ m^{-2} in the same wavelength range was lethal for *Natronorubrum* strain HG-1. Thus, related microorganisms from the same taxonomic group appear to respond differently to similar high doses of UV radiation. If this is due to different capacities of their DNA repair systems, variations in pyrimidine dimer content, as suggested by Zhou et al. (2007), or other factors, remains to be explored. Future strategies for the search for martian life will most likely include specific molecular probes and/or microarrays, as proposed by various groups (see Parnell et al., 2007). It would not be prudent to design molecular probes towards families of terrestrial extremophilic microorganism, since they may not contain representatives which have a chance to survive under martian conditions. Therefore we feel that more studies like the one presented here are necessary to gain further insight into what type of species are potentially suitable to be present on Mars today, in guidance to direct the design and aim of molecular probes, taking into account that one potential candidate is not illustrative for its entire phylogenetically related group.

4. Conclusion

Natronorubrum strain HG-1 cells have been exposed to different aspects of the martian environment: low temperatures (4, −20, and −80 °C), desiccation, exposure to UV radiation similar to levels found near the equator on the martian surface, and exposure to a Mars soil analogue. We found that storing samples of *Natronorubrum* strain HG-1 cells at 4 or −20 °C (either desiccated or in liquid medium) did not impair viability, while the survival was significantly decreased at −80 °C. When a Mars soil analogue was added, or when the samples were irradiated with UV radiation, cell viability dropped below the detection limit. *Natronorubrum* strain HG-1 cells are furthermore heterotrophic being unable to grow without organic compounds on purely mineral media. Combining these findings, we conclude that *Natronorubrum* strain HG-1 is an unlikely candidate to survive in the present day Mars subsurface environment.

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