Survival and death of the haloarchaeon *Natronorubrum* sp. strain HG-1 in simulated early Earth and martian environments


Abstract

Halophilic archaea are of interest to astrobiology due to their survival capabilities in desiccated and high salt environments. The detection of extraterrestrial salts in meteorites and the possibility of former 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, since it grows on acetate and is stimulated by tetrathionate (Sorokin et al. 2005). We investigated the effects of high and low temperature as well as desiccation on *Natronorubrum* strain HG-1. Growth curves following exposure to extreme conditions showed that the viability of the strain in liquid medium was not affected by a 24 h storage at 4 or −20 °C. Lag phases of cultures were increased when samples were desiccated and/or stored at low temperatures. Samples stored at 70 °C did not show any growth at all. We exposed *Natronorubrum* strain HG-1 to ultraviolet (UV) radiation, similar to the levels currently prevalent on Mars. Samples irradiated with UV radiation for only 30 min showed a survival rate of <10^{-4} %, while samples irradiated for 24 h did not show any growth. Samples desiccated onto soil from the Atacama desert showed no growth after rehydration. From the results it can be concluded that *Natronorubrum* strain HG-1 cannot survive for more than a few minutes when exposed to UV radiation equivalent to that at the martian equator during local noon. Even when protected from UV radiation, viability may be impaired by a combination of desiccation and low temperature. In addition, the lethal effect of exposure to 70 °C indicates that this strain could not have existed during the Precambrian era when ocean temperatures were thought to be high.

6.1 Introduction

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

A possible connection between the origin of life on Earth and the ability of microorganisms to grow at high salt concentrations has been described by Dundas (1998), who suggested that microvesicles may form easily under the isohaline conditions of hypersaline brines. Litchfield (1998) concluded that 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. Mars is
thought to have had liquid water present at its surface for a geologically long period (Squyres et al. 2004). The progressive desiccation of the surface in a later phase led to an increase in the salt content of the remaining bodies of water. Since on Earth microbial life is thriving in similar environments, mechanisms for tolerance of high salt concentrations could be envisaged to have evolved in potential martian organisms.

The effects of simulated martian environmental stresses have been tested on a few microorganisms including cyanobacterium Chroococcidiopsis sp. 029 (Cockell et al. 2005), several Bacillus species (Schuerger et al. 2006), Escherichia coli and Deinococcus radiodurans (Diaz & Schulze-Makuch 2006), and 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. Rettberg et al. (2004) studied the survival of Bacillus subtilis spores mixed with thin layers of Mars soil simulant aboard the Biopan facility. They found that thin layers of clay, rock, or meteorite could only successfully protect spores against solar radiation when in direct contact.

The halophilic archaean Natronorubrum sp. strain HG-1 was isolated and described by Sorokin et al. (2005). It displays typical haloarchaeal features, including pink to red colour due to carotenoids and bacterioruberin. These pigments were suggested to have a protective effect against damage by UV radiation in the related Halobacterium salinarum (Shahmohammadi et al. 1998). In contrast to most other haloarchaea, Natronorubrum sp. strain HG-1 uses simple compounds (acetate) as a carbon source and is able to reduce tetrathionate. These features make this organism well-suited to study the responses to environmental stresses with defined growth media.

In this paper, we report the results of survival experiments, where Natronorubrum strain HG-1 was exposed to similar levels of UV radiation as found on the surface of Mars. In addition, we tested the survival of Natronorubrum strain HG-1 stored at high and low temperatures. The cells were subjected to these conditions either in suspension or after desiccation, i.e. while embedded in salt crystals. We also tested the survival of desiccated cultures of Natronorubrum strain HG-1 mixed with soil from the Atacama desert, as a Mars soil analogue. The results of these experiments are discussed and the chance for survival of Natronorubrum strain HG-1 on Mars is evaluated.

6.2 Experimental

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 7 days at 37 °C the cultures reached maximum density. The growth medium included 240 g L⁻¹ NaCl, 2 g L⁻¹ K₂HPO₄, and 0.5 g L⁻¹ (NH₄)₂SO₄. The pH was adjusted to
7.3 before sterilisation by heating for 20 minutes at 120 °C. After sterilisation, the medium was supplemented with 2 mM MgCl$_2$, 10 mM sodium acetate, 0.05 g L$^{-1}$ yeast extract, and 1 mL L$^{-1}$ of trace elements solution (Pfennig & Lippert 1966).

Growth was monitored by measuring the optical density (OD) at 600 nm on a double-beam UV/Vis spectrometer (Cary 3 Bio, Varian). The OD of the cells was correlated to absolute numbers by cell counting with an optical microscope. We found that an OD of 0.033 corresponded to 1.5 x 10$^8$ cells mL$^{-1}$ or 5 x 10$^9$ cells mL$^{-1}$ OD$^{-1}$. The OD was assumed to increase in proportion with the number of cells in suspension up to an OD of ∼0.3. The maximum cell density of a culture in stationary phase reached an OD of ∼0.56.

**Atacama desert soil**

Soil obtained from the Atacama desert in Chile (Flat Top Hill, S25°29’50.9” W69°50’22.5”) was used as a Mars regolith analogue. This soil was previously used as a Mars soil analogue, to determine the stability of intrinsic amino acids under simulated Mars condition (Peeters et al. 2007). To test for the presence of halophilic microorganisms in the Atacama soil that could interfere with our experiments, we added 2 g of soil to 5 mL of medium and incubated this suspension at 37 °C. No growth was observed after several weeks.

**Sample preparation**

The primary culture from which all samples were derived was grown to stationary phase (OD = 0.51 or 2.6×10$^9$ cells mL$^{-1}$). 3 mL of this primary culture was added to 42 mL fresh medium, yielding 45 mL of stock culture with OD = 0.034. This stock culture was divided into 18 sterile plastic Petri dishes (Greiner Bio-One, 3.5 cm diameter, with spacer in lid) and 10 glass bottles, 1 mL per Petri dish or bottle, yielding 28 samples of 1.5×10$^8$ cells. The samples were then divided into two groups: 10 wet samples and 18 dry samples. The wet samples, in the bottles, were used without further treatment and directly subjected to their test environment, see section 6.2. The dry samples, in the Petri dishes, were placed without lids in a 40 °C oven. After 4 h, the samples were dry as was apparent from the formation of salt crystals and the absence of liquid. During the drying period, the weight of the samples was frequently measured and the samples were considered dry when the weight did not show any further decrease. All samples, both wet and dry were then placed in the environment to be tested.

Additional samples for the soil experiment were prepared by transferring 1 mL of stationary cells (OD = 0.51) to 6 empty Petri dishes and 4 to Petri dishes each containing 1.0 g Atacama soil. The Petri dishes with samples were placed in a 37 °C oven for 24 h with open lids to dry the samples. Two samples without soil were wrapped in Parafilm to prevent evaporation of the medium and were used as liquid controls.

**Conditions of exposure**

Each condition (except exposure to the deuterium lamp, see below) was tested with three samples, using exposure times of 24 h. Wet sam-
Table 6.1
Overview of the environments to which samples of *Natronorubrum* strain HG-1 were exposed. Growth curves were measured for the top half of the table, while the bottom half was measured with Most Probable Number (MPN) method.

<table>
<thead>
<tr>
<th>sample</th>
<th>state</th>
<th>environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>liquid</td>
<td>—</td>
</tr>
<tr>
<td>wet blank</td>
<td>liquid</td>
<td>4 °C, dark, 24 h</td>
</tr>
<tr>
<td>wet hot</td>
<td>liquid</td>
<td>70 °C, dark, 24 h</td>
</tr>
<tr>
<td>wet cold</td>
<td>liquid</td>
<td>−20 °C, dark, 24 h</td>
</tr>
<tr>
<td>dry cold</td>
<td>dry</td>
<td>−20 °C, dark, 24 h</td>
</tr>
<tr>
<td>dry solar simulator</td>
<td>dry</td>
<td>18 °C, solar simulator, 24 h</td>
</tr>
<tr>
<td>dry deuterium</td>
<td>dry</td>
<td>18 °C, deuterium lamp, 24 h</td>
</tr>
<tr>
<td>dry blank batch 1</td>
<td>dry</td>
<td>18 °C, ambient light, 24 h</td>
</tr>
<tr>
<td>dry blank batch 2</td>
<td>dry</td>
<td>18 °C, ambient light, 48 h</td>
</tr>
<tr>
<td>control</td>
<td>liquid</td>
<td>—</td>
</tr>
<tr>
<td>dry</td>
<td>dry</td>
<td>—</td>
</tr>
<tr>
<td>dry with soil</td>
<td>dry</td>
<td>Atacama soil</td>
</tr>
<tr>
<td>dry irradiated</td>
<td>dry</td>
<td>solar simulator, 30 min</td>
</tr>
<tr>
<td>soil irradiated</td>
<td>dry</td>
<td>Atacama soil, solar simulator, 30 min</td>
</tr>
</tbody>
</table>

Samples were stored in the dark at −20 °C, 70 °C, or 4 °C. Desiccated samples were stored at −20 °C (wrapped in Parafilm to prevent the attraction of moisture) or at room temperature. The desiccated samples were irradiated at room temperature with either a short-arc xenon solar simulator (ScienceTech, SF150) or a deuterium discharge lamp (Heraeus-Noblelight, DX 202). The solar simulator was used without the optional AM0 filter, to use the full output of the lamp down to 200 nm. The intensity of the light was measured for both lamps in the UV and visible regions, using a Solartech UV-C sensor (model 8.0) and an Extech light meter (EA30). The Solartech sensor measures irradiance over a known sensitivity profile over the wavelength range 240–280 nm with a peak at 257 nm. The Extech sensor is a broadband optical sensor and was used to ensure that the solar simulator’s output was stable for the duration of the experiment. Relative intensity spectra of both the solar simulator and the deuterium lamps were provided by the respective manufacturers of the lamps. The output of the Solartech UV sensor integrated over the 240–
280 nm wavelength range, was used to scale the spectra of the lamps. The resulting spectra are shown in figure 6.1, along with a the surface illumination spectrum of an equatorial region on Mars for a dust-free atmosphere at noon-time, as modelled by Patel et al. (2002). With the control box of the lamp set to 8.0 A constant current, the solar simulator produced an output of 30 W m$^{-2}$ in the wavelength range 200–400 nm. Both lamps had an output of 3.4 W m$^{-2}$.

The solar simulator produced a beam spot with a diameter of 11.3 cm at a distance of 31.0 cm. This was large enough to irradiate 3 Petri dishes simultaneously. The deuterium lamp produced a spot with a diameter of 4 cm at a distance of 8.5 cm. Since the deuterium lamp could be used for irradiation of only 1 sample at a time, two identical lamps were used simultaneously. The irradiation experiments were repeated after 24 h; the two experiments are referred to as batch 1 and 2. The samples of batch 2 were prepared at the same time as batch 1 and were stored at room temperature during the intervening 24 h period. A set of control samples was also included in the two batches.

**Growth curves**

After the 24 h storage period, the samples were allowed to equilibrate to room temperature for 1 h. The desiccated samples were rehydrated by adding 1 mL sterile demineralized water and 4 mL medium to the Petri dishes. Then, both the rehydrated samples and the stored cell suspension samples were transferred to sterile 60 mL bottles. The Petri dishes were then washed three times with 5 mL medium. The medium used for washing was added to the rest in the bottles, resulting in a final volume of 20 mL of culture. These cultures were incubated in a water bath at 37 °C, while shaking at 40 rpm. A control culture was included by adding 1 mL of the original primary culture from which the samples were derived, to 19 mL of fresh medium. The start of the incubation was noted as t = 0. The growth of the samples and the control was followed in time by taking 1 mL aliquots every few hours and measuring the OD.

**Analysis of the data**

The OD values were converted into cell density, using a conversion factor of $5 \times 10^9$ cells mL$^{-1}$ OD$^{-1}$ obtained from optical cell counting (see above). The resulting data points were fitted with a model based on the Gompertz equation, which describes bacterial growth using three parameters: the maximum growth rate ($\mu_{max}$), the lag (L), and an asymptotic value (A) for the maximum cell density in the stationary phase (Zwietering et al. 1990).

$$\ln \left( \frac{N(t)}{N_0} \right) = A \exp \left[ \frac{\mu_{max} e}{A} (L - t) + 1 \right]$$ (6.1)

In equation 6.1, N(t) is the cell density at time t, $N_0$ is the cell density at time t = 0, and $e$ is the base of the natural log. Since $N_0$ is unknown, and is in fact the number we are looking for, equation 6.1 was expressed using $A = \ln(N_{max}/N_0)$, with $N_{max}$ the maximum cell density, see equation 6.2. $N_0$ was allowed to vary as a fitting parameter besides $N_{max}, \mu_{max}$, and L. $N_0$
Figure 6.1
UV spectrum of the solar simulator (dashed line), the deuterium lamp (dotted line), and the lighting spectrum of Mars (solid line) in the range 190–400 nm. The Mars spectrum is the predicted equatorial noon-time irradiation of the martian surface, as modelled by Patel et al. (2002) for a dust-free atmosphere. The solar simulator was used without any filters, giving the full output of the lamp down to 200 nm.

is expected to lie between 0, when none of the cells survive, and \(7.5 \times 10^6\) cells mL\(^{-1}\), when all cells survive. This is below the detection limit of \(\sim 1\times10^7\) cells mL\(^{-1}\). Extrapolating \(N_o\) to a small number below the detection limit causes \(N_o\) to have a large uncertainty interval. Because \(N_o\) can not easily be determined from the data, the results will be discussed based on differences in lag (L) and maximum specific growth rate (\(\mu_{\text{max}}\)).

The lag phase (referred to as lag) is the time it takes for a newly inoculated culture to enter the logarithmic growth phase. It is defined as the

\[
N(t) = N_o \exp\left\{ \ln\left( \frac{N_{\text{max}}}{N_o} \right) \exp\left[ -\exp\left( \frac{\mu_{\text{max}}}{\ln(N_{\text{max}}/N_o)}(L - t) + 1 \right) \right] \right\}
\]  
(6.2)
X-intercept of the tangent to the inflection point, while $\mu_{\text{max}}$ is equal to the slope of the curve in the inflection point (see figure 6.2). Lag is a property of the total cell culture and does not directly relate to the lag of the individual cells. The lag and the other fitting parameters $N_{\text{max}}$, $N_0$, and $\mu_{\text{max}}$ are related to the inflection point, the point at which the maximum specific growth rate ($\mu_{\text{max}}$) occurs (figure 6.2). Combining equations 4, 9, and 10 from Zwietering et al. (1990) and replacing A with $\ln\left(\frac{N_{\text{max}}}{N_0}\right)$, lead to the following expression for $t_i$, the time at which the inflection point occurs.

$$t_i = \frac{\ln\left(\frac{N_{\text{max}}}{N_0}\right)}{\mu_{\text{max}}e} + L \quad (6.3)$$

For two curves with the same inflection point, an increase in $\mu_{\text{max}}$ will lead to an increase in L. This change is marked by arrow 1 in figure 6.2. Alternatively, when two curves have a different $\mu_{\text{max}}$, and also a different inflection point, then not all the change in L can be attributed to an alteration of $\mu_{\text{max}}$ and an additional growth retarding mechanism must be in effect, depicted by arrow 2 in figure 6.2. Therefore, both the growth rate and the inflection point can contribute to the lag.

**Most Probable Number analysis**

The liquid samples of the soil experiment were transferred to a bottle containing 5 mL medium. The Petri dish was washed twice with 2 mL medium, which was also added to the bottle, resulting in a total culture volume of 10 mL. The dried samples were rehydrated by adding 2 mL medium and shaking on a flat-bed rotary shaker for 15 min at 110 rpm. The dissolved cell suspension was then transferred to a bottle containing 5 mL medium. The Petri dish was washed once with 2 mL medium and once with 1 mL medium. For the samples mixed with soil the same procedure was followed. A small amount of soil being transferred from the Petri dish to the bottle could not be avoided.

At this stage, all samples were in a $10^{-1}$ dilution. The samples were then diluted further to $10^{-2}$ and $10^{-3}$. From the $10^{-3}$ dilution step the dilution series was continued in steps of 2 for another 10 dilutions (viz. $5 \times 10^{-4}$, $2.5 \times 10^{-4}$, $1.3 \times 10^{-4}$ ... $1.0 \times 10^{-6}$). All dilutions were incubated in a 37 °C water bath without shaking for two weeks and were checked regularly for growth. The cell concentration in the original sample was calculated from the bottle with the highest dilution that still showed cell growth.

### 6.3 Results and discussion

Samples of *Natronorubrum* strain HG-1 were exposed to high (70 °C) and low (−20°C) temperature in suspension in growth medium. Dried cultures of *Natronorubrum* strain HG-1 were exposed to low (−20 °C) temperature, light from a solar simulator or a deuterium lamp. After exposure, the cultures were rehydrated and al-

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1. Each cell, provided that it is still viable, goes into growth phase at a different time. This leads to a distribution of cell lags, but the shape of the distribution is unknown, preventing the determination of $N_0$ from the lag (see also Baranyi 1998, Robinson et al. 2001).
Figure 6.2
A simulated growth curve, to show the parameters that determine the shape of the curve. $N_o$ is the cell density at time $t=0$ and $N_{\text{max}}$ is the maximum cell density. $\mu_{\text{max}}$ is the maximum specific growth rate, which is equal to the slope of the curve in the inflection point. Lag is defined as the X-axis intercept of the tangent to the curve in the inflection point and depends therefore on $\mu_{\text{max}}$. If the growth rate ($\mu_{\text{max}}$) increases, the lag will increase (to lag’’ by rotation around the inflection point, as depicted by arrow 1. However, if the time at which the inflection point occurs ($t_i$) increases, the lag will also increase (to lag”’, arrow 2). Therefore, both the growth rate and the inflection point can contribute to the total lag. The relation between the lag, the inflection point and the growth rate is given by equation 6.3.

The resulting growth curves are shown in figure 6.3. A summary of the results, relative to the control experiment is given in table 6.3. The modified Gompertz equation was fitted to the growth curve data. No fitting was applied to the high temperature and the irradiated samples (WH, DX and DD in figure 6.3, (a) and (c)), because those samples did not show any growth for the duration of the experiment. Some curves in figure 6.3 showed a poor fit towards the stationary phase. The Gompertz equation (as indeed many other growth models) does not account for cell death and a decrease in population size that occurs after the maximum cell density has been reached. Some curves showed a drop in OD (traces labelled WB and WC in figure 6.3(a) and (b)) right after the maximum was reached. Coagulation of the cells into clumps might explain the drop in OD, but it does not explain why other samples (control and DC) did not show this effect. The parameters that resulted from the fitting procedure were used to calculate the inflection point, according to equation 6.3. The results are shown in table 6.2.

High temperature
Sorokin et al. (2005) found that Natronorubrum strain HG-1 does not grow above 46 °C. No further tests have been performed to determine viability at higher temperatures. From the data points in 6.3a (growth curve labelled WH) it is clear that Natronorubrum strain HG-1 does not survive a 24 h period at 70 °C, since no growth was measurable following 120 h of incubation. Recent data of oxygen and silicon isotope ratios in cherts indicate that the temperature of the Earth’s oceans ∼3.5 billion years ago was ∼70 °C (Knauth 2005, Robert & Chausse-
Figure 6.3
Growth curves with regression lines. The data points in this figure reflect the cell densities over time. The lines represent the curves resulting from fitting equation 6.2 to the data points. (a) Shows the control sample (crosses), the wet hot samples (WH, open circles), and wet blanks (WB, solid dots). (b) Shows the wet cold samples (WC, solid dots) and dry cold samples (DC, open circles), while (c) shows the dry solar simulator and dry deuterium samples (DX & DD, open circles) and dry blanks (DB, solid dots). The dry blanks designated batch 1 were stored in desiccated state for 24 h, those labelled batch 2 were stored 48 h.
don 2006). Around 2 billion years ago, the ocean temperature started to drop until it reached \( \sim 30 \, ^\circ\text{C} \) approximately 1.2 billion years ago. It remained at that temperature with some fluctuations for around 400 million years. According to the ocean temperature scale of Robert & Chaussidon (2006), \textit{Natronorubrum} strain HG-1 could have existed only later than 1.7 Gyr ago, when the temperature of the Earth’s oceans dropped below 45 °C. Furthermore, \textit{Natronorubrum} cells lyse in water with low ionic strength, e.g. less than 1.5–2 M NaCl (Grant et al. 2001), and are inactivated by such conditions in the laboratory. Unless special structures (spores, cysts, or other morphological cellular adaptations) are formed as considered by Grant et al. (1998), they would not survive in today’s oceans (\( \sim 0.6 \) M NaCl), nor in the early Earth’s oceans where the salinity was 1.5–2 times higher (Knauth 1998). Approximately 2.5 Gyr ago, large scale salt deposition by evaporation and brine sequestration into groundwater pockets led to the lowering of ocean salinity, which continued until \( \sim 1 \) Gyr ago (Knauth 1998). In the evaporites and pockets of brine storage the salt concentration would have been high enough for \textit{Natronorubrum} strain HG-1 to survive, but only after \( \sim 1.7 \) Gyr ago, due to the temperature limitation.

**Low temperature**

Figure 6.3a shows a comparison between the wet blanks (WB) and a control sample. The wet blank samples were stored at 4 °C for 24 h, while the control sample was inoculated with 1 mL of an exponentially growing culture at 37 °C. This resulted in the control having a maximum specific growth rate (\( \mu_{\text{max}} \)) almost double that of the stored wet blank samples. A reason for this higher growth rate could be the enrichment of the medium with growth stimulating factors. If \textit{Natronorubrum} strain HG-1 cells excrete growth-stimulating factors into the surrounding medium, neighbouring cells can be stimulated into cell division. For a cell culture in logarithmic growth phase, the medium would be enriched with these growth-stimulating factors. When the control sample was inoculated, these growth-stimulating factors would have been transferred along with the cells, thereby enriching the fresh medium and enhancing the growth rate compared to cells without enriched medium. However, it is not known how medium enrichment influences the growth rate of \textit{Natronorubrum} strain HG-1. Besides a doubling in growth rate for the control culture compared to the wet blanks, also the lag increased. But since the inflection point (\( t_i \)) for the wet blanks and the control occur at the same time, any difference in lag can be attributed to a change in growth rate (cf. equation 6.3).

The same inflection point was also found for the wet cold (WC) samples that were stored at \( -20 \) °C. Besides the inflection point, all other fitting parameters of the wet cold samples were comparable to the wet blanks, which shows that lowering the storage temperature from 4 to \( -20 \) °C for 24 h has a negligible effect. The wet cold samples were not frozen at \( -20 \) °C, due to the high salt content. Cell damage under cold conditions is usually attributed to the formation of ice crystals in the cell, which damage the cell’s membranes. While the high salt concentration pre-
Table 6.2
Parameters resulting from fitting equation 6.2 to the data shown in figure 6.3. The error limits are the propagated standard errors of the fits. Also shown is the time at which the inflection point occurs, \( t_i \), calculated from the other fitting parameters using equation 6.3.

<table>
<thead>
<tr>
<th>experiment</th>
<th>( N_{\text{max}} ) (( \times 10^9 ) cells mL(^{-1} ))</th>
<th>( N_0 ) (( \times 10^8 ) cells mL(^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>2.74 ± 0.01</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>wet blanks</td>
<td>2.60 ± 0.08</td>
<td>1.0 ± 1.2</td>
</tr>
<tr>
<td>wet cold</td>
<td>2.61 ± 0.08</td>
<td>0.93 ± 1.3</td>
</tr>
<tr>
<td>dry cold</td>
<td>2.83 ± 0.04</td>
<td>0.95 ± 0.30</td>
</tr>
<tr>
<td>dry blanks</td>
<td>2.64 ± 0.07</td>
<td>0.71 ± 0.70</td>
</tr>
<tr>
<td>dry blanks batch 1</td>
<td>2.66 ± 0.09</td>
<td>0.68 ± 1.1</td>
</tr>
<tr>
<td>dry blanks batch 2</td>
<td>2.62 ± 0.09</td>
<td>0.77 ± 0.47</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>experiment</th>
<th>( \mu_{\text{max}} ) (cells mL(^{-1} ) h(^{-1} ))</th>
<th>( L ) (h)</th>
<th>( t_i ) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.16 ± 0.01</td>
<td>29 ± 1.2</td>
<td>37 ± 2.2</td>
</tr>
<tr>
<td>wet blanks</td>
<td>0.085 ± 0.03</td>
<td>21 ± 9.7</td>
<td>35 ± 21</td>
</tr>
<tr>
<td>wet cold</td>
<td>0.089 ± 0.03</td>
<td>20 ± 10</td>
<td>34 ± 23</td>
</tr>
<tr>
<td>dry cold</td>
<td>0.13 ± 0.01</td>
<td>53 ± 2.1</td>
<td>63 ± 4.1</td>
</tr>
<tr>
<td>dry blanks</td>
<td>0.11 ± 0.03</td>
<td>34 ± 6.6</td>
<td>46 ± 13</td>
</tr>
<tr>
<td>dry blanks batch 1</td>
<td>0.11 ± 0.04</td>
<td>31 ± 10</td>
<td>43 ± 23</td>
</tr>
<tr>
<td>dry blanks batch 2</td>
<td>0.11 ± 0.01</td>
<td>39 ± 4.4</td>
<td>51 ± 8.0</td>
</tr>
</tbody>
</table>

vents the liquid from freezing at −20 °C, it is not known if *Natronorubrum* strain HG-1 employs additional mechanisms to prevent the formation of ice crystals at lower temperatures. These results suggest that *Natronorubrum* strain HG-1 could have survived cold periods that might have existed in the Earth’s history, e.g. 0.7–0.5 Gyr ago (Evans et al. 1997), but only if the salt concentration of the surrounding environment was high enough to keep the water liquid.

**Desiccation**

The desiccated blanks (figure 6.3c, DB) showed a slightly increased maximum growth rate (\( \mu_{\text{max}} \)) and lag (\( L \)) compared to the wet (WB and WC) samples. Also, the inflection point (\( t_i \)) occurs later than that of the wet samples, which means that not all change in lag can be attributed to an altered growth rate, but an additional growth retarding mechanism must be in effect, depicted by
Table 6.3
Summary of the results. The results in this table are relative to the control experiment. The relation between the lag, the maximum specific growth rate ($\mu_{\text{max}}$), and the inflection point ($t_i$) is given by equation 6.3. The ‘dry blanks’ sample is the average of batch 1 and 2.

<table>
<thead>
<tr>
<th>experiment</th>
<th>lag (%)</th>
<th>$\mu_{\text{max}}$ (%)</th>
<th>$t_i$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>wet blanks</td>
<td>69 ± 50</td>
<td>51 ± 17</td>
<td>95 ± 58</td>
</tr>
<tr>
<td>wet cold</td>
<td>69 ± 34</td>
<td>55 ± 20</td>
<td>94 ± 63</td>
</tr>
<tr>
<td>dry cold</td>
<td>178 ± 10</td>
<td>77 ± 9</td>
<td>172 ± 15</td>
</tr>
<tr>
<td>dry blanks</td>
<td>117 ± 22</td>
<td>67 ± 16</td>
<td>128 ± 38</td>
</tr>
<tr>
<td>dry blank b. 1</td>
<td>103 ± 35</td>
<td>66 ± 26</td>
<td>119 ± 63</td>
</tr>
<tr>
<td>dry blank b. 2</td>
<td>132 ± 16</td>
<td>69 ± 10</td>
<td>139 ± 24</td>
</tr>
</tbody>
</table>

Table 6.4
Cell concentration and survival of the samples analyzed with the MPN method. The cell concentration is given by the highest dilution that still showed growth. The experiments that included Atacama desert soil did not show any growth, even for the undiluted culture.

<table>
<thead>
<tr>
<th>sample</th>
<th>concentration (cells mL$^{-1}$)</th>
<th>survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>$3 \times 10^5$</td>
<td>100</td>
</tr>
<tr>
<td>dried</td>
<td>$3 \times 10^5$</td>
<td>100</td>
</tr>
<tr>
<td>dried on soil</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dried &amp; UV</td>
<td>$10^2$</td>
<td>$&lt;10^{-4}$</td>
</tr>
<tr>
<td>dried on soil &amp; UV</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Comparing the results for the samples that were in a desiccated state for 24 h (batch 1) with those desiccated for 48 h (batch 2), shows that the lag is increased when the cells remain longer in a desiccated state. These results show that desiccation has a small effect on the cell growth after rehydration, and that the effect is enhanced by prolonged desiccation. The dried samples that were analyzed with the Most Probable Number (MPN) method, have the same viable cell concentration after rehydration as the liquid control sample (table 6.4). This means that from the MPN analysis no change in viability due to desiccation could be detected. In the MPN analysis the first bottle that does not show growth is diluted by a factor 2 compared to the last bottle that displayed growth. This means that the relatively small change in lag between the wet blanks and the desiccated blanks in the growth curves experiment could be missed by the factor 2 step with the MPN analysis.

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. It is likely that a large fraction of the *Natronorubrum* strain HG-1 cells will have been incorporated in inclusions in the salt crystals, similar to *Pseudomonas aeruginosa*. Cells that are incorporated in inclusions are not actually dehydrated, while the cells that remain outside the inclusions are more susceptible to dehydration and might not survive, caus-
ing increased lag in the growth curve. The lag increased further when samples were stored at −20 °C for 24 h (figure 6.3b, DC samples). Those samples have a lag almost double that of the wet samples (WB, WC and control).

Mancinelli et al. (2004) found that *Halobacterium salinarum* NRC-1 and Haloarcula-G could survive repeated freeze-thaw cycles to −20 and −80 °C for 144 days. The cells in those experiments were in the desiccated state. Mancinelli et al. (2004) furthermore found that control samples of *Escherichia coli*, *Pseudomonas fluorescens*, and *Deinococcus radiodurans* could not survive desiccation for 144 days at room temperature, at −20 °C, or at −80 °C. A notable exception was *D. radiodurans*, which survived freeze-thaw cycles to −80 °C in the desiccated state for 144 days. Of the five bacterial strains tested, *H. salinarum* and Haloarcula-G were extreme halophiles which grow at 25 % NaCl. These findings concur with our results that halophilic organisms like *Natronorubrum* strain HG-1 can survive storage at low temperatures in a desiccated state.

**Atacama soil**

*Natronorubrum* strain HG-1 cultures were mixed with Atacama soil and subsequently dried. After rehydration, the MPN analysis showed no growth at any dilution (table 6.4). When *Natronorubrum* strain HG-1 cells were desiccated without soil, there was no measurable loss of viability. As we have established in the previous section, the cells are likely to survive in brine inclusions in the NaCl crystals (see also Mancinelli et al. 2004). In a hypersaturated brine solution a nucleation point is needed for crystallization to start. In the experiments without soil, the cells acted as nucleation points and crystallization occurred around the cells. The NaCl crystal that formed around the cell, trapped water in an inclusion and prevented the cell from drying out. In contrast, when the cells were mixed with soil, the grains provided far better nucleation points than the cells. It is therefore likely that the cells in this mixture are left without a protective shell and dry out upon evaporation of the medium.

**UV radiation**

Dried samples of *Natronorubrum* strain HG-1 were irradiated for 30 min or 24 h. Figure 6.3c (DX & DD) shows that 24 h irradiation with either the solar simulator or the deuterium lamp completely incapacitated *Natronorubrum* strain HG-1. No growth was apparent after 120 h of incubation. *Natronorubrum* strain HG-1 samples that were irradiated for only 30 min, had a viable cell concentration of $10^2$ cells mL$^{-1}$ as analyzed by the MPN method (table 6.4), which is equivalent to a surviving fraction of $10^{-4}$ %. The *Natronorubrum* strain HG-1 samples that were mixed with Atacama soil, dried and subsequently irradiated, showed no growth at any dilution. This is likely due to the crystallization process that occurs in the soil, described in the previous section.

In general, cell death through interaction with UV radiation is associated with irreparable levels of DNA damage. This means that the pigments present in *Natronorubrum* strain HG-1
are not protective against light in the >200 nm wavelength range (see also figure 6.1). Additionally, OH radicals might be created by the UV photons from water present in the inclusions in which the cells are thought to reside (Adamski et al. 2006). Together with the fact that NaCl crystals are transparent for UV light down to ∼200 nm (Li 1979), the formation of OH radicals that may be damaging to Natronorubrum strain HG-1 in fluid inclusions, is possible.

**Combined effects**

Desiccation of Natronorubrum strain HG-1 had a small effect on the viability (section 6.3), which increased when the desiccation period increased from 24 h to 48 h. From this short period of incubation in desiccated state, it is difficult to extrapolate the survival of Natronorubrum strain HG-1 over geological relevant time scales, but other halophilic archaea have been shown to survive for millions of years in inclusions in halite crystals (Stan-Lotter et al. 1999). Desiccated Natronorubrum strain HG-1 cultures did not survive irradiation with a solar simulator for 24 h (section 6.3). The solar simulator gave an output similar to the solar spectrum on Mars (see figure 6.1). Natronorubrum strain HG-1 would therefore not be able to survive on the surface of Mars for more than a few sols (martian days), unless they are protected from UV radiation, e.g. buried in the martian soil. Even when protected against UV radiation in the martian soil, survival of Natronorubrum strain HG-1 under martian conditions is doubtful, since the viability of desiccated cells was decreased by low (−20 °C) temperature (section 6.3). Although the temperature on the surface of Mars can be above 0 °C near the equator at noontime, the average temperature is around −60 °C. Overall we conclude that Natronorubrum strain HG-1 cannot survive the environment currently present on Mars.

**6.4 Conclusion**

We investigated the survival of the halophilic archaeon Natronorubrum strain HG-1 exposed to high temperature (70 °C), low temperature (4 and −20 °C), desiccation and irradiation with wavelengths >200 nm. Growth of Natronorubrum strain HG-1 was not affected by a 24 h storage at 4 or −20 °C. Desiccation of cells for 24 h or 48 h under ambient conditions had a negligible effect on the growth curves. When desiccated cells were stored at −20 °C for 24 h, there was an increase in the lag of the rehydrated culture. Cell cultures stored at 70 °C or desiccated in the presence of Atacama soil did not show any viability. Desiccated samples irradiated with UV light for 30 min showed a survival of <10⁻⁴ %, while irradiation for 24 h completely sterilized the sample.

From these results we conclude that Natronorubrum strain HG-1 would not be able to survive on the surface of Mars, when unprotected from UV radiation. Even when protected from UV photons, viability would be impaired by a combination of desiccation and low temperature. Natronorubrum strain HG-1 could probably not have survived in the early Earth’s oceans, due to its sensitivity to high temperatures.
Acknowledgements

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