International Journal of Astrobiology

cambridge.org/ija

Bacterial growth in saturated and eutectic solutions of magnesium sulphate and potassium chlorate with relevance to Mars and the ocean worlds

Jonathan M. Wilks[1], Fei Chen[2], Benton C. Clark[3] and Mark A. Schneegurt[1]

[1]Department of Biological Sciences, Wichita State University, Wichita, KS, USA; [2]Planetary Protection Group, Jet Propulsion Laboratory, Pasadena CA, USA; and [3]Space Science Institute, Boulder CO, USA

Research Article

Cite this article: Wilks JM, Chen F, Clark BC, Schneegurt MA (2019). Bacterial growth in saturated and eutectic solutions of magnesium sulphate and potassium chlorate with relevance to Mars and the ocean worlds. *International Journal of Astrobiology* **18**, 502–509. https://doi.org/10.1017/S1473550418000502

Received: 27 September 2018
Revised: 30 November 2018
Accepted: 11 December 2018
First published online: 28 January 2019

Key words:
Astrobiology; deliquescence; epsotolerance; extremophiles; habitability; halotolerance; Mars; planetary protection; psychrotolerant; salinotolerance

Author for correspondence:
Mark A. Schneegurt, E-mail: mark.schneegurt@wichita.edu

Abstract

Liquid water on Mars might be created by deliquescence of hygroscopic salts or by permafrost melts, both potentially forming saturated brines. Freezing point depression allows these heavy brines to remain liquid in the near-surface environment for extended periods, perhaps as eutectic solutions, at the lowest temperatures and highest salt concentrations where ices and precipitates do not form. Perchlorate and chlorate salts and iron sulphate form brines with low eutectic temperatures and may persist under Mars near-surface conditions, but are chemically harsh at high concentrations and were expected to be incompatible with life, while brines of common sulphate salts on Mars may be more suitable for microbial growth. Microbial growth in saturated brines also may be relevant beyond Mars, to the oceans of Ceres, Enceladus, Europa and Pluto. We have previously shown strong growth of salinotolerant bacteria in media containing 2M $MgSO_4$ heptahydrate (~50% w/v) at 25°C. Here we extend those observations to bacterial isolates from Basque Lake, BC and Hot Lake, WA, that grow well in saturated $MgSO_4$ medium (67%) at 25°C and in 50% $MgSO_4$ medium at 4°C (56% would be saturated). Psychrotolerant, salinotolerant microbes isolated from Basque Lake soils included *Halomonas* and *Marinococcus*, which were identified by 16S rRNA gene sequencing and characterized phenetically. Eutectic liquid medium constituted by 43% $MgSO_4$ at −4°C supported copious growth of these psychrotolerant *Halomonas* isolates, among others. Bacterial isolates also grew well at the eutectic for K chlorate (3% at −3°C). Survival and growth in eutectic solutions increases the possibility that microbes contaminating spacecraft pose a contamination risk to Mars. The cold brines of sulphate and (per) chlorate salts that may form at times on Mars through deliquescence or permafrost melt have now been demonstrated to be suitable microbial habitats, should appropriate nutrients be available and dormant cells become vegetative.

Introduction

For water to remain liquid at the frigid temperatures near the Martian surface, it must contain high concentrations of solutes. Salts, sugars, and ammonia can depress the freezing point of water dramatically (Table 1). For instance, sodium and magnesium chlorates depress the freezing point of water to −23 and −69°C, respectively (Hanley *et al.*, 2012). Oxyanions of chlorine have been detected in Mars soils, reaching 0.6% near the Phoenix lander and likely exist as separate hygroscopic phases (Hecht *et al.*, 2009; Kounaves *et al.*, 2010; Ming *et al.*, 2014; Clark and Kounaves, 2016). Ammonia at high concentrations can depress the freezing point of water to −100°C and may be relevant to the oceans of Ceres and Pluto (Nimmo and Pappalardo, 2016). Even NaCl can lower the freezing point of water to −21°C, while $MgSO_4$ reduces it to a modest −4°C (Toner *et al.*, 2014). Extremely salty and cold waters created by permafrost melt or deliquescence may be the only wet habitats for microbes near Mars' surface (Chevrier and Altheide, 2008; Davila *et al.*, 2010; Hanley *et al.*, 2012; Rummel *et al.*, 2014; Clark and Kounaves, 2016; Rivera-Valentin *et al.*, 2018).

The eutectic point for a solution is the combination of the highest salt concentration and the lowest temperature at which the solution remains free of ice and free of precipitate. Adding more salt leads to precipitation, while lowering the temperature leads to ice formation. Liquid water on Mars may be found at times as saturated or eutectic solutions from permafrost melt or deliquescence. For instance, it has been hypothesized that saturated ferrous sulphate brines might be the longest-lived liquids near the surface (Chevrier and Altheide, 2008). The eutectic concentrations of relevant brines are typically high, above 20 wt% salt (Table 1). The eutectic conditions for (per)chlorate salts are typically at concentrations of ~40 wt% or more. Eutectic brines could form on Mars by deliquescence when these hygroscopic salts absorb atmospheric moisture (humidity). Sufficient moisture is present at the Curiosity landing site to support this

CAMBRIDGE UNIVERSITY PRESS

Table 1. Chemical compositions of saturated salt solutions at their eutectic temperatures

Salt	Eutectic conditions	
	Concentration (wt %)	Temperature (°C)
$MgSO_4$	17	−4
$MgCl_2$	22	−33
NaCl	27	−21
Mg chlorate	47	−69
K chlorate	3	−3
Na chlorate	39	−23
Mg perchlorate	44	−67
Na perchlorate	52	−37

process with certain perchlorate salts (Chevrier *et al.*, 2009; Hu *et al.*, 2016). Modelling suggests that permafrost melt would create saturated salt solutions in pore spaces given the high salt concentrations in Mars regolith (Crisler *et al.*, 2012). The current work demonstrates bacterial growth in extremely high salt concentrations, including the eutectic conditions for $MgSO_4$ and K chlorate, the first demonstration of microbial growth in a brine that has a reasonable likelihood of being present on Mars at certain times.

Growth of salinotolerant (exhibit growth at high salt concentrations) microbes has been demonstrated in the presence of high concentrations of solutes, including saturated NaCl and $MgSO_4$ solutions (Crisler *et al.*, 2012; Schneegurt, 2012; Fox-Powell and Cockell, 2018). We have examined culture collections captured from these natural environments rich in salts: Great Salt Plains of Oklahoma (GSP; saturated with NaCl) and Hot Lake, WA (HL) and Basque Lake, BC (BLE) (both saturated with $MgSO_4$) (Caton *et al.*, 2004; Crisler *et al.*, 2012, 2018; Kilmer *et al.*, 2014). Halotolerance (exhibits growth at high NaCl concentrations) and epsotolerance (exhibits growth at high $MgSO_4$ concentrations) appear to be widespread in both types of environments, those rich in monovalent ions and those rich in divalent ions. Water activities are low in both environments and ionic strength is particularly high for epsomic lakes rich in sulphates. Low water activity is associated with the inhibition of microbial growth, whether the solute be salts or sugars, due to specific solute effects, which involve interplay of several physicochemical parameters (Grant, 2004; Schneegurt, 2012). Salinotolerant and sucretolerant (exhibit growth at high sugar concentrations) microbes are readily found in common soils and thus are not found only in extreme environments (Echigo *et al.*, 2005; Al Soudi *et al.*, 2017; Fredsgaard *et al.*, 2017). We have recovered remarkably tolerant microbes from spacecraft assembly facilities (SAFs) as well, suggesting that microbes contaminating spacecraft may be able to grow under the extreme chemical conditions expected at certain times near the surface of Mars (Eberl and Schneegurt, 2015; Wilks *et al.*, 2017).

While high salinities appear to be widely tolerated, microbial growth at low temperatures seems to be more restricted (Clarke *et al.*, 2013; Bakermans, 2017). Although microbial metabolism is generally slowed at low temperatures, methanogenesis has been detected in permafrost at temperatures as low as −16.5°C (Rivkina *et al.*, 2000, 2007). Record microbial growth is limited to about −20°C, higher than the freezing points of many eutectic brines relevant to Mars and the ocean worlds. The suggestion is that the high salt concentrations of eutectic brines may be less inhibitory to growth than their low temperatures on frigid celestial bodies. We have reported growth of bacteria at >27% Na chlorate and survival at the eutectic concentration (Al Soudi *et al.*, 2017; Schneegurt *et al.*, 2018). However, growth at the Na chlorate eutectic temperature of −23°C has yet to be demonstrated for salinotolerant bacteria.

The current study examines microbial responses to two eutectic solutions that appear suitable for growth and that are potentially found at times on Mars. We have previously demonstrated strong bacterial growth in the presence of 50% (w/v; 2M) $MgSO_4$, which is higher than the eutectic concentration for $MgSO_4$ of 43% (w/v) (Crisler *et al.*, 2012, 2018; Kilmer *et al.*, 2014). While the eutectic temperature for $MgSO_4$ is −3.9°C, we had previously reported growth of salinotolerant bacteria in 10% NaCl at 4–7°C (Caton *et al.*, 2004; Litzner *et al.*, 2006; Crisler *et al.*, 2012, 2018). The second eutectic solution we have examined is that of K chlorate, which is unusual among the (per)chlorate salts in that its eutectic concentration of ∼3% is low and its eutectic temperature is moderate, near −3°C (Hanley *et al.*, 2012). In the current report, we demonstrate the growth of psychrotolerant isolates from saline soils at each of these eutectics. This is the first demonstration that terrestrial microbes can grow in the densest brines potentially present at times near the surface of Mars or near the oceans of icy worlds.

Materials and methods

Site description and sample collection

Water and lake margin soils (∼100 g from top 4 cm) were collected using sterile tools and containers from Basque Lake, BC at three locations: S1 (50°36′02.5″N, 121°21′32.2′W), S2 (50°35′59.8″N, 121°21′27.6″W) and S3 (50°35′58.9″N, 121°21′27.2″W) (Fig. 1). Aliquots for live culture work were held and transported fresh at ambient temperature. The soils were predominantly grey or black fine clays and silts, often with a strong sulfidic odour, and were saturated with salts.

Organisms and enrichment of microbial isolates

Salinotolerant bacterial isolates were obtained previously from the Great Salt Plains of Oklahoma, an environment rich in NaCl (Caton *et al.*, 2004; Litzner *et al.*, 2006). Two previous studies of epsomic lakes, Hot Lake, WA and Basque Lake, BC, provided bacteria tolerant to 2M (50%) $MgSO_4$ (Kilmer *et al.*, 2014; Crisler *et al.*, 2018). Finally isolates from an existing collection of epsotolerant bacteria from the SAFs at NASA Jet Propulsion Laboratory (JPL) were included in the current study (Eberl and Schneegurt, 2015; Wilks *et al.*, 2017). Additional psychrotolerant, salinotolerant isolates were obtained from Basque Lake for the current study. Enrichment cultures were started with fresh samples of Basque Lake soils in SP medium supplemented with 50% $MgSO_4$. Seven isolates were obtained that grew well at salt saturation for the growth temperature, namely, 67 and 56% (w/v) $MgSO_4$ at 25 and 4°C, respectively, and in some cases, under eutectic conditions with 43% (w/v) $MgSO_4$ at −4°C.

Cultures were maintained on SP medium (Caton *et al.*, 2004), a nutrient-rich medium containing per litre: NaCl, 1 g; KCl, 2.0 g; $MgSO_4{\cdot}7H_2O$, 1.0 g; $CaCl_2{\cdot}2H_2O$, 0.36 g; $NaHCO_3$, 0.06 g; NaBr, 0.23 g; $FeCl_3{\cdot}6H_2O$, 1.0 mg; trace minerals, 0.5 ml; Bacto tryptone, 5.0 g; yeast extract, 10.0 g; glucose, 1.0 g; final pH 7.0. This was

https://doi.org/10.1017/S1473550418000502 Published online by Cambridge University Press

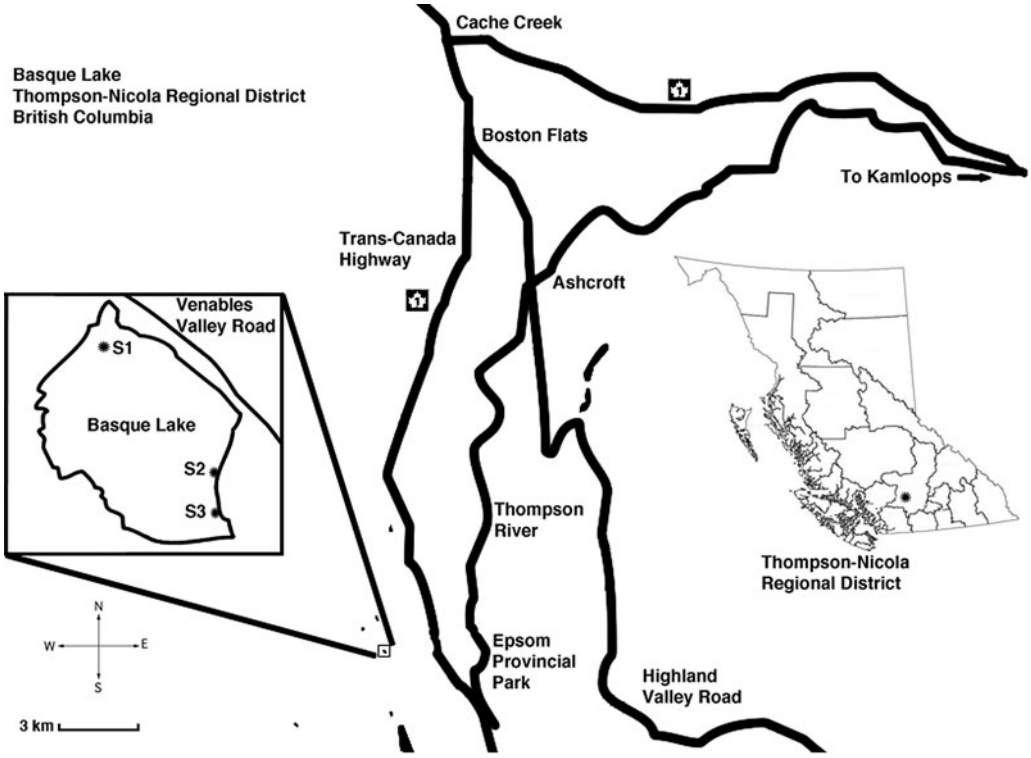

Fig. 1. Contextual map of Basque Lake showing sampling sites.

prepared with various concentrations of $MgSO_4$, K chlorate or NaCl. Throughout this work, $MgSO_4$ is used to represent epsomite, $MgSO_4 \bullet 7\ H_2O$.

Measurement of growth in saturated and eutectic media

Bacterial growth was tested in SP medium (Caton *et al.*, 2004), supplemented with high concentrations of $MgSO_4$, K chlorate or NaCl. Note that SP medium supplemented with salts at the eutectic concentration formed brines that were near-eutectic for a single salt, but by definition were not true eutectic solutions since nutritive media components were added to support microbial growth. Cultures were maintained in saturated $MgSO_4$ media at 4°C before growth experiments under eutectic conditions. The temperatures of the incubators were monitored with traceable wire thermometers. There were no temperature anomalies during the course of these experiments. Culture flasks and tubes were partially buried in sand to damp the effects of temperature changes due to thermostat cycling (~2°C range) or opening the incubator for sampling. Cultures were kept in a moist box when incubations were above 0°C. Growth was measured by turbidity as optical density at 600 nm using a Thermo Scientific (Thermo Fisher Scientific, Waltham, MA, USA) Genesys 10S UV-Vis spectrophotometer. An OD of 0.1 was chosen as the threshold for positive growth. Growth also was measured by standard plate count, with serial dilution and spread plating, to enumerate colony-forming units. Plating was typically on SP medium supplemented with 10% NaCl, since agar with high concentrations of $MgSO_4$ did not gel. While culture turbidity is the product of live cells, dead cells, and any materials that scatter or absorb light, the standard plate count reflects only live cells capable of forming a colony on agar. Not all replicate subcultures were successful under the most extreme conditions.

Characterization of bacterial isolates

Characterization of the bacterial isolates followed protocols described previously (Caton *et al.*, 2004; Litzner *et al.*, 2006). SP medium composition was modified with different concentrations of NaCl or $MgSO_4$ to measure salinity tolerances in liquid shake-tubes. A threshold of 0.1 OD units was chosen for positive growth. All isolates were Gram-stained using the Protocol Gram-staining kit (Thermo Fisher Diagnostics, Waltham, MA, USA) following the manufacturer's instructions. The addition of 3% hydrogen peroxide solution to confluent plates or smears of culture on slides was used to detect catalase. Oxidase testing was performed using the BBL DrySlide system (BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Amylase was determined after a 5-day incubation on Starch agar (BD Difco, Franklin Lakes, NJ, USA), followed by flooding of plates with 25% stabilized Gram's iodine solution. Nitrate reduction tests were done as previously described (Caton *et al.*, 2004). Production of acid and gas from carbohydrates was tested using 0.5% (w/v) glucose, lactose or sucrose in culture tubes containing inverted Durham tubes, using a 10% NaCl solution supplemented with Bacto tryptone ($10\ g\ l^{-1}$) and phenol red ($0.018\ g\ l^{-1}$; pH 7.3).

Crude DNA extracts from each isolate were prepared using a freeze–thaw technique as described in Caton *et al.* (2004). Genomic DNA in the supernatant was the target of PCR amplification of nearly complete 16S rRNA gene fragments using universal bacterial primers (EUBPA: 5′–AGAGTTTGATCCTGGCT CAG–3′ and EUBPH: 5′–AAGGAGGTGATCCAGCCGCA–3′) (Edwards *et al.*, 1989). PCR was performed in a thermal cycler (Eppendorf Mastercycler; Hamburg, Germany) as 25-µl reactions containing 0.2 µM of each primer, 1 U of ExTaq DNA polymerase and associated master mix (Takara, Kusatsu, Japan), and 5 µl of

https://doi.org/10.1017/S1473550418000502 Published online by Cambridge University Press

cell extract. DNA was denatured at 95°C for 2 min, followed by 40 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min, with a final 5 min extension at 72°C. PCR amplicons were single-pass sequenced by a commercial vendor (Eurofins Genomics, Louisville, KY, USA) using the EUBPA primer. Initial sequences were trimmed to remove remaining vector regions. All sequences appear in GenBank with accession numbers MH286503 to MH286509 and MH503928 to MH503933, for BLE and JPL sequences, respectively. Sequences were aligned using SILVA and maximum likelihood analysis was used to build trees in MEGA v7.0 (Kumar *et al.*, 2016). Known contextual 16S rRNA gene sequences from type organisms were identified in GenBank using BLAST.

Results

Isolation and characterization of psychrotolerant bacterial isolates

Basque Lake, Hot Lake and Spotted Lake have been recognized as Mars analogues due to their high divalent ion concentrations and low water activities (Kilmer *et al.*, 2014; Pontefract *et al.*, 2017; Crisler *et al.*, 2018). Our current bacterial collections include more than 150 isolates from epsomic lakes, but these organisms were not selected for psychrotolerance. Seven new isolates were obtained from enrichments at 4°C in SP medium supplemented with 50% $MgSO_4$. Designated as BLE isolates, the bacteria were identified by phylogenetic analysis of 16S rRNA gene sequences (Fig. 2). Six BLE isolates were Gram-negative *Halomonas* and BLE4 was a Gram-positive *Marinococcus*. The isolates were closely related to those obtained earlier in this laboratory from HL and the GSP.

Biochemical, morphological and physiological characteristics of the BLE isolates are consistent with known related organisms (Table 2). Isolates were all catalase-positive, except *Halomonas* sp. str. BLE2, while only *Halomonas* sp. str. BLE 6 and 7 also were oxidase-positive. Anaerobic nitrate respiration was observed for *Halomonas* sp. str. BLE 5 and 6. Carbohydrate metabolism was limited, as reflected by the absence of amylase activity, as observed previously with microbes from hypersaline environments (Caton *et al.*, 2004; Kilmer *et al.*, 2014; Crisler *et al.*, 2018). It is possible that four of the isolates (*Halomonas* sp. str. BLE 1–3, 5) are slightly salinophilic (requiring high salt for growth), showing no growth in 0.1% NaCl, but growing at 1% NaCl. All of the isolates grew at 20% NaCl and at 50% $MgSO_4$. All of the isolates grew at temperatures from 4 to 37°C. None grew at 50°C and only *Halomonas* sp. str. BLE2 did not grow at −4°C, near the eutectic temperature for $MgSO_4$ and K chlorate.

Screening of salinotolerant bacterial collections

Previous studies of salinotolerant bacteria from the GSP and HL allowed for the selection of organisms with the highest likelihood of growing under extremely salty and sub-zero eutectic conditions (Table 3). A survey of HL bacteria found that only three of 19 salinotolerant isolates (*Halomonas* sp. str. HL 12 and 51 and *Marinococcus* sp. str. HL70) grew well in saturated (67%) $MgSO_4$ medium at 25°C, with four isolates (*Halomonas* sp. str. HL 21, 26, and 62 and *Planococcus* sp. str. HL20) showing slight growth. Representative growth curves by turbidity and plate count are shown for HL12, a particularly tolerant *Halomonas* isolate (Fig. 3). Growth is relatively rapid under these extreme salt conditions, with stationary phase reached ~4 day after inoculation. Several of the BLE isolates were positive for growth in saturated $MgSO_4$ medium at 25°C, including *Halomonas* sp. str. BLE 3 and 7 and *Marinococcus* sp. str. BLE4. Isolates obtained from enrichments of SAF swabs in 50% $MgSO_4$ (*Bacillus* sp. str. JPL10 and *Staphylococcus* sp. str. JPL27) also exhibited growth in saturated $MgSO_4$ medium at 25°C. Doubling times ranged from 9.6 to 46.7 h and maximum culture densities were above 1 OD, similar to those of low-salt cultures. When isolates were grown at 4°C in saturated (56%) $MgSO_4$, doubling times increased substantially, being 300–700 h (12–29 days). Several BLE isolates (*Halomonas* sp. str. BLE 1, 3, 5 and 7) and a HL isolate (*Nesterenkonia* sp. str. HL76) grew under these conditions.

Growth under eutectic conditions

As cultures were transferred into environments that are closer to eutectic conditions for $MgSO_4$, growth was slowed or stopped (Table 4). At −4°C (its eutectic temperature) and 21% $MgSO_4$ (half of its eutectic concentration), several isolates grew, including *Bacillus* sp. str. JPL17; *Halomonas* sp. str. BLE 1, 3, 5 and 6 and HL 12; *Nesterenkonia* sp. str. HL 64 and 76; and *Staphylococcus* sp. str. JPL16.

Growth of *Halomonas* sp. str. BLE 1, 3, 6 and 7 and *Nesterenkonia* sp. str. HL76 was observed under the eutectic conditions for $MgSO_4$. A representative growth curve is presented for *Halomonas* sp. str. BLE7, which grew best, and compared with that of *Marinococcus* sp. str. BLE4, which did not grow at this eutectic (Fig. 4). Note that growth is substantially slowed next to that observed for saturated $MgSO_4$ at 25°C. In saturated $MgSO_4$ at 25°C, stationary phase was reached in about 4 days. However, under eutectic conditions, it took >50 days and perhaps as long as 200 days for *Halomonas* sp. str. BLE7 cultures to reach their maximum cell density. Cells held under these conditions for months appeared somewhat smaller than is typical and formed mucoidy flocs. The characteristic orange coloration of *Nesterenkonia* sp. str. HL76 was maintained even in cultures incubated under eutectic conditions for more than 2 years.

The eutectic state of K chlorate is at approximately the same temperature as the $MgSO_4$ eutectic, but at a much lower salt concentration (3% for K chlorate and 43% for $MgSO_4$). Several isolates showed substantial growth under the eutectic conditions for K chlorate including *Bacillus* sp. str. JPL10, *Halomonas* sp. str. BLE7, *Nesterenkonia* sp. str. HL64 and *Staphylococcus* sp. str. JPL27. Representative data for BLE7 cultured at the K chlorate eutectic point (only one of three replicates grew) demonstrates that growth was slowed, as observed for eutectic $MgSO_4$ (Fig. 5). Stationary phase was reached after 100 days and perhaps not until near 200 days after inoculation. Our previous study reported that growth of salinotolerant isolates in K chlorate up to 1M (10%) at room temperature was much more rapid, reaching stationary phase in less than a week (Al Soudi *et al.*, 2017).

Discussion

Natural brines on Earth are usually dominated by chloride salts. While chlorides are present in Martian soils, often sulphates are found in higher abundance, reaching 30% by weight (Vaniman *et al.*, 2004; Gendrin *et al.*, 2005). Dry conditions near Mars' surface allow the preservation of (per)chlorate salts that are rare on Earth, being found in the driest soils of the Atacama Desert

https://doi.org/10.1017/S1473550418000502 Published online by Cambridge University Press

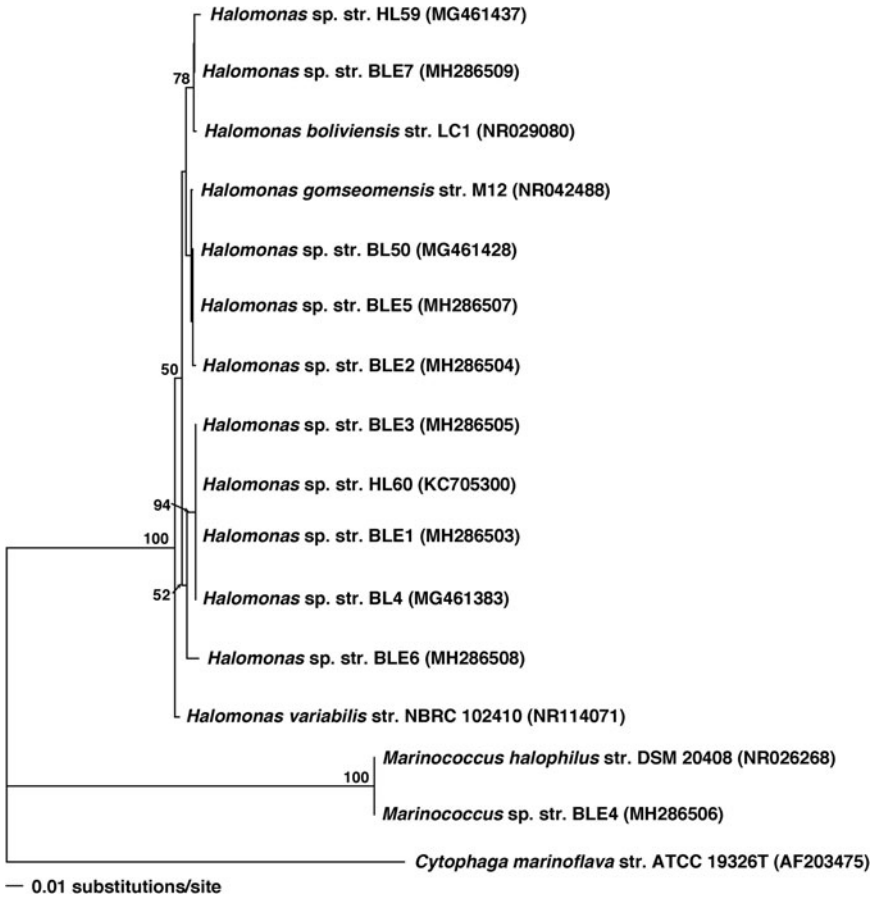

Fig. 2. Phylogenetic tree for psychrotolerant BLE bacterial isolates from Basque Lake based on 16S rRNA gene sequences, with GenBank accession numbers.

Table 2. Phenetic characteristics of psychrotolerant, salinotolerant bacterial isolates from Basque Lake

Trait	BLE1	BLE2	BLE3	BLE4	BLE5	BLE6	BLE7
Gram	–	–	–	+	–	–	–
Catalase	+	–	+	+	+	+	+
Oxidase	–	–	–	–	–	+	+
Nitrate reduction	–	–	–	–	+	+	–
Glucose (acid/gas)	+/+	–/–	+/–	+/–	+/–	+/–	+/–
Sucrose (acid/gas)	–/–	–/–	+/–	+/+	+/+	–/–	+/–
Lactose (acid/gas)	–/–	–/–	–/–	–/–	–/–	+/–	+/+

All isolates were negative for citrate, indole, H_2S, motility and phenylalanine deaminase.

(Kounaves *et al.*, 2010; Jackson *et al.*, 2015). Several of the salts that are most relevant to soils on Mars are hygroscopic enough to form saturated brines through deliquescence. When present, cold liquid brines on Mars, rich in divalent ions and oxidants, may be most limiting for growth when near their eutectic points. Epsomic lakes of southern British Columbia and the Qaidam Basin, China, have divalent ion concentrations similar to potential Martian brines (Hammer, 1978, 1986; Haynes and Hammer, 1978; Kezao and Bowler, 1986; Last and Slezak, 1988; Last and Ginn, 2005). How microbes from analogue sites, such as Hot and Basque Lakes, respond to the chemical conditions on Mars informs our understanding of habitable regions, forward planetary protection and exobiology.

Temperatures at the surface of Mars typically average below −33°C, but can reach over −3°C in summer (Mellon *et al.*, 2004). Maximum daily temperatures do not fall below −68°C. Saturated solutions of some (per)chlorate and sulphate salts would remain liquid under these conditions and could be maintained globally (Chevrier and Altheide, 2008). Heavy brines not only have greatly depressed freezing points; evaporation of water also is slowed, with less potential to boil at low ambient atmospheric pressures. Brines below the surface of Mars would evaporate even more slowly and are not as affected by diurnal temperature changes at the surface. The high concentrations and low eutectic temperatures of most (per)chlorate salts seem non-permissive for microbial growth. Cells might freeze or vitrify

https://doi.org/10.1017/S1473550418000502 Published online by Cambridge University Press

Table 3. Growth of selected Hot Lake bacterial isolates in saturated (67%) $MgSO_4$ at 25°C

	Culture density (OD units)		
Hot Lake isolate	Initial	Final	Score
Marinococcus sp. str. HL3	0.047	0.031	–
Marinococcus sp. str. HL6	0.129	0.063	–
Marinococcus sp. str. HL10	0.015	0.029	–
Marinococcus sp. str. HL11	0.005	0.021	–
Halomonas sp. str. HL12	0.036	1.085	+++
Planococcus sp. str. HL20	0.109	0.276	+
Halomonas sp. str. HL21	0.063	0.171	+
Halomonas sp. str. HL26	0.094	0.157	+
Halomonas sp. str. HL51	0.053	1.234	+++
Marinococcus sp. str. HL54	0.020	0.057	–
Halomonas sp. str. HL62	0.113	0.224	+
Nesterenkonia sp. str. HL64	0.017	0.010	–
Bacillus sp. str. HL68	0.016	0.005	–
Halomonas sp. str. HL69	0.013	0.020	–
Marinococcus sp. str. HL70	0.043	0.794	++
Nesterenkonia sp. str. HL76	0.056	0.021	–
Planococcus sp. str. HL80	0.056	0.063	–
Halomonas sp. str. HL82	0.020	0.000	–
Planococcus sp. str. HL91	0.011	0.024	–

Cultures were grown as shake-tubes in SP medium supplemented with salt. The threshold for positive growth was 0.1 OD and qualitative scores reflect the relative robustness of growth.

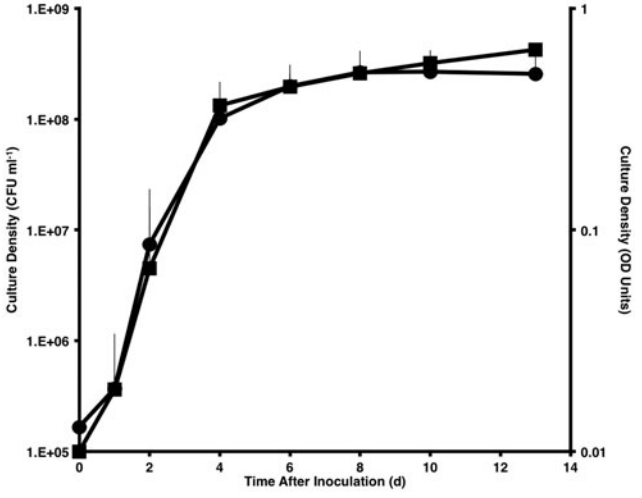

Fig. 3. Growth of *Halomonas* sp. str. HL12 as shake-tubes in SP medium with saturated $MgSO_4$ (67%, w/v) at 25°C. Circles, standard plate count on SP medium supplemented with 10% NaCl; squares, turbidity at 600 nm.

even within liquid brines (Fonseca *et al.*, 2016). The high concentrations of Fe in the sulphate brines that might be the most persistent on Mars (Chevrier and Altheide, 2008) also may prove to be non-permissive for microbial growth, given the high reactivity and hence high toxicity of Fe.

Table 4. Growth of selected bacterial isolates from the Great Salt Plains, Hot Lake, Basque Lake and JPL SAFs at −4°C with high salts

	Growth at −4°C	
Isolate	21% $MgSO_4$	3% $KClO_3$
Halomonas sp. str. GSP3	–	–
Bacillus sp. str. GSP10	–	–
Halomonas sp. str. GSP21	–	–
Bacillus sp. str. GSP63	–	–
Marinococcus sp. str. HL11	–	–
Halomonas sp. str. HL12	+	–
Marinococcus sp. str. HL54	–	–
Nesterenkonia sp. str. HL64	+	+
Nesterenkonia sp. str. HL76	+	–
Planococcus sp. str. HL91	–	–
Halomonas sp. str. BLE1	+	–
Halomonas sp. str. BLE2	–	–
Halomonas sp. str. BLE3	+	–
Halomonas sp. str. BLE5	+	–
Halomonas sp. str. BLE6	+	–
Halomonas sp. str. BLE7	+	+
Virgibacillus sp. str. JPL9	–	–
Bacillus sp. str. JPL10	+	+
Bacillus sp. str. JPL14	+	–
Staphylococcus sp. str. JPL16	+	–
Bacillus sp. str. JPL17	+	–
Staphylococcus sp. str. JPL27	+	+

Cultures were grown as shake-tubes in SP medium supplemented with salt. The threshold for positive growth was 0.1 OD. Note that the K chlorate trials were at eutectic conditions.

Copious growth has been demonstrated in saturated (67%) $MgSO_4$ media at 25°C for bacteria isolated from Basque Lake (Crisler *et al.*, 2018). We report here (and elsewhere) growth at very high $MgSO_4$ concentrations for bacteria isolated from the Great Salt Plains, Hot Lake and JPL SAFs (Crisler *et al.*, 2012). The isolates studied in the current report do not appear to be unique and growth at high $MgSO_4$ concentrations is remarkably widespread, heightening planetary protection concerns. For the first time, we demonstrate that bacteria can grow in a brine that may be present at times on Mars. There has been speculation that $MgSO_4$ brines are not suitable for life (Tosca *et al.*, 2008), but we have observed strong microbial growth at high $MgSO_4$ concentrations (Crisler *et al.*, 2012, 2018; Kilmer *et al.*, 2014). We now report that copious bacterial growth is possible in eutectic solutions of $MgSO_4$ that may arise from permafrost melt or deliquescence at certain times. Similar eutectic brines may exist on ocean worlds such as Europa, suggesting that bacteria may be able to grow in these environments too. While K is not a major cation on Mars, it is possible that K chlorate salts could be present and create brines. We demonstrate here for the first time that bacteria can grow in eutectic K chlorate solutions.

https://doi.org/10.1017/S1473550418000502 Published online by Cambridge University Press

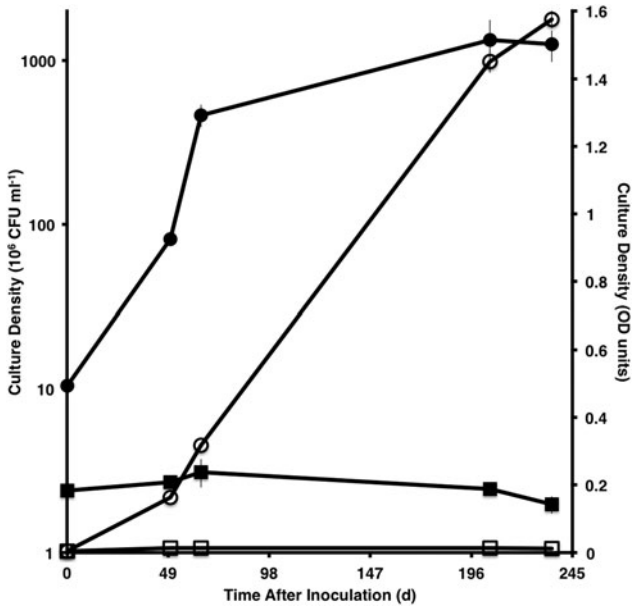

Fig. 4. Bacterial growth at the $MgSO_4$ eutectic in static liquid cultures of SP medium supplemented with 43% (w/v) $MgSO_4$ at −4°C. Closed, standard plate counts on SP medium supplemented with 10% NaCl; open, turbidity at 600 nm; circles, *Halomonas* sp. str. BLE7; squares, *Marinococcus* sp. str. BLE4.

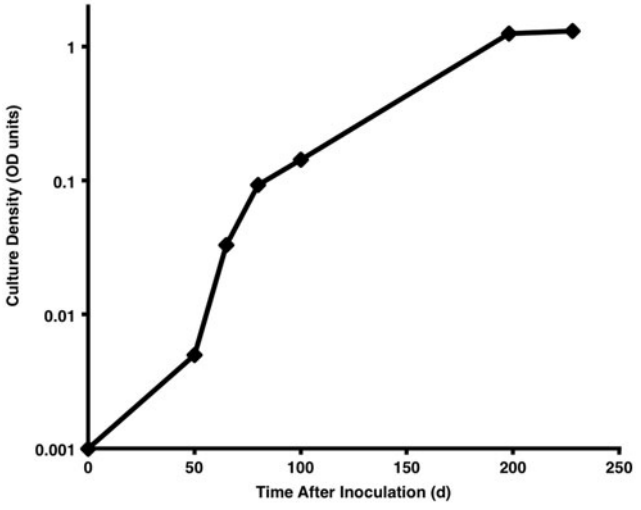

Fig. 5. Bacterial growth of *Halomonas* sp. str. BLE7 at the K chlorate eutectic as a static liquid culture in SP medium supplemented with 3% K chlorate at −4°C.

Suitable conditions for microbial growth may well be intermittent near the surface of Mars. There could be long periods of desiccation punctuated by the formation of heavy brines through deliquescence or permafrost melt when conditions are somewhat warmer than today. Microbes may need to remain cryptobiotic, in a dormant form, for years, perhaps centuries, before revivification. Studies on the survival of microbes under non-permissive growth conditions relevant to Mars would address this possibility, with concomitant measurement of metabolic state and DNA repair. It has been suggested that tolerance to high salt involves many of the same mechanisms leading to tolerance to other extreme environmental conditions and that DNA repair mechanisms are key to survival (Wilson *et al.*, 2004). If microbes existed on Mars during a wetter period of its history, these may well have been deposited with the salts present today and remained viable, reviving when intermittent brines form in their vicinity.

Acknowledgements. The authors thank Timothy Eberl for isolating and characterizing JPL SAF microbial isolates and appreciate the technical support of Mark Sutton and Roshni Venagadasamy. We are grateful that Jim Britton (British Columbia Ministry of Energy and Mines) collected and documented Basque Lake samples and that Fadi Aramouni (Kansas State University) performed water activity measurements. Preliminary accounts of this work have been presented previously (Wilks *et al.*, 2017; Schneegurt *et al.*, 2018). This work was supported by awards from National Aeronautics and Space Administration (NASA), Research Opportunities in Space and Earth Science (ROSES), Planetary Protection Research (09-PPR09-0004 and 14-PPR14-2-0002). Additional student support was from Kansas Institutional Development Award (IDeA) Networks of Biomedical Research Excellence (KINBRE) of the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health (NIH) (P20 GM103418).

References

Al Soudi AF, Farhat O, Chen F, Clark BC and Schneegurt MA (2017) Bacterial growth tolerance to concentrations of chlorate and perchlorate salts relevant to Mars. *International Journal of Astrobiology* **16**, 229–235.

Bakermans C (2017) Determining the limits of microbial life at subzero temperatures. In Margesin R (ed.) *Psychrophiles: From Biodiversity to Biotechnology*, 2nd Edn. Basil, Switzerland: Springer, pp. 21–38.

Caton TM, Witte LR, Ngyuen HD, Buchheim JA, Buchheim MA and Schneegurt MA (2004) Halotolerant aerobic heterotrophic bacteria from the Great Salt Plains of Oklahoma. *Microbial Ecology* **48**, 449–462.

Chevrier VF and Altheide TS (2008) Low temperature aqueous ferric sulfate solutions on the surface of Mars. *Geophysical Research Letters* **35**, L22101.

Chevrier VF, Hanley J and Altheide TS (2009) Stability of perchlorate hydrates and their liquid solutions at the Phoenix landing site, Mars. *Geophysical Research Letters* **36**, L10202.

Clark BC and Kounaves SP (2016) Evidence for the distribution of perchlorates on Mars. *International Journal of Astrobiology* **15**, 311–318.

Clarke A, Morris GJ, Fonseca F, Murray BJ, Acton E and Price HC (2013) A low temperature limit for life on Earth. *PLoS ONE* **8**, e66207.

Crisler JD, Newville TM, Chen F, Clark BC and Schneegurt MA (2012) Bacterial growth at the high concentrations of magnesium sulfate found in Martian soils. *Astrobiology* **12**, 98–106.

Crisler JD, Chen F, Clark BC and Schneegurt MA (2018) Numerical taxonomy and phylogenetic analyses of the microbial community of epsomic Basque Lake, BC. *Abstracts and Program, 118th General Meeting of the American Society for Microbiology*, Atlanta, June 2018.

Davila AF, Duport LG, Melchiorri R, Jänchen J, Valea S, de los Rios A, Fairén AG, Möhlmann D, McKay CP, Ascaso C and Wierzchos J (2010) Hygroscopic salts and the potential for life on Mars. *Astrobiology* **10**, 617–628.

Eberl TC and Schneegurt MA (2015) Isolation and characterization of bacterial communities in the clean room environment of NASA's Jet Propulsion Laboratory-spacecraft assembly facility in Pasadena, CA. *Proceedings: 11th Annual Research Symposium on Graduate Research and Scholarly Projects*, Wichita, KS, April 2015.

Echigo A, Hino M, Fukushima T, Mizuki T, Kamekura M and Usami R (2005) Endospores of halophilic bacteria of the family *Bacillaceae* isolated from non-saline Japanese soil may be transported by Kosa event (Asian dust storm). *Saline Systems* **1**, 8.

Edwards U, Rogall H, Blöcker H, Emde M and Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Research* **17**, 7843–7853.

Fonseca F, Meneghei J, Cenard S, Passot S and Morris GJ (2016) Determination of intracellular vitrification temperatures for unicellular micro organisms under conditions relevant for cryopreservation. *PLoS ONE* **11**, e0152939.

https://doi.org/10.1017/S1473550418000502 Published online by Cambridge University Press

Fox-Powell MG and Cockell CS (2018) Building a geochemical view of microbial salt tolerance: halophilic adaptation of *Marinococcus* in a natural magnesium sulfate brine. *Frontiers in Microbiology* **9**, 739.

Fredsgaard C, Moore DB, Chen F, Clark BC and Schneegurt MA (2017) Prevalence of sucretolerant bacteria in common soils and their isolation and characterization. *Antonie van Leeuwenhoek* **110**, 995–1005.

Gendrin A, Mangold N, Bibring JP and Langevin Y (2005) Sulfates in Martian layered terrains: the OMEGA/Mars express view. *Science* **307**, 1587–1591.

Grant WD (2004) Life at low water activity. *Philosophical Transactions of the Royal Society of London. Series B* **359**, 1249–1267.

Hammer UT (1978) The saline lakes of Saskatchewan. III. Chemical characterization. *International Review of Hydrobiology* **63**, 311–335.

Hammer UT (1986) *Saline Lake Ecosystems of the World*. Dordrecht: Junk.

Hanley J, Chevrier VF, Berget DJ and Adams RD (2012) Chlorate salts and solutions on Mars. *Geophysical Research Letters* **39**, L08201.

Haynes RC and Hammer UT (1978) The saline lakes of Saskatchewan. IV. Primary production by phytoplankton in selected saline ecosystems. *International Review of Hydrobiology* **63**, 337–351.

Hecht MH, Kounaves SP, Quinn RC, West SJ, Young SMM, Ming DW, Catling DC, Clark BC, Boynton WV, Hoffman J, DeFlores LP, Gospodinova K, Kapit J and Smith PH (2009) Detection of perchlorate and the soluble chemistry of Martian soil at the Phoenix Lander site. *Science* **325**, 64–67.

Hu R, Bloom AA, Gao P, Miller CE and Yung YL (2016) Hypotheses for near-surface exchange of methane on Mars. *Astrobiology* **16**, 539–550.

Jackson WA, Böhlke JK, Andraski BJ, Fahlquist L, Bexfield L, Eckardt FD, Gates JB, Davila AF, McKay CP, Rao B and Sevanthi R (2015) Global patterns and environmental controls of perchlorate and nitrate co-occurrence in arid and semi-arid environments. *Geochimica et Cosmochimica Acta* **164**, 502–522.

Kezao C and Bowler JM (1986) Late Pleistocene evolution of salt lakes in the Qaidam basin, Qinghai province, China. *Palaeogeography, Palaeoclimatology, Palaeoecology* **54**, 87–104.

Kilmer BR, Eberl TC, Cunderla B, Chen F, Clark BC and Schneegurt MA (2014) Molecular and phenetic characterization of the bacterial assemblage of Hot Lake, WA, an environment with high concentrations of magnesium sulphate, and its relevance to Mars. *International Journal of Astrobiology* **13**, 69–80.

Kounaves SP, Hecht MH, Kapit J, Gospodinova K, DeFlores L, Quinn RC, Boynton WV, Clark BC, Catling DC, Hredzak P, Ming DW, Moore Q, Shusterman J, Stroble S, West SJ and Young SMM (2010) Wet chemistry experiments on the 2007 Phoenix Mars Scout Lander mission: data analysis and results. *Journal of Geophysical Research* **115**, E00E10.

Kumar S, Stecher G and Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**, 1870–1874.

Last WM and Ginn FM (2005) Saline systems of the Great Plains of western Canada: an overview of the limnogeology and paleolimnology. *Saline Systems* **1**, 10.

Last WM and Slezak LA (1988) The salt lakes of western Canada: a paleolimnological overview. *Hydrobiologia* **158**, 310–316.

Litzner BR, Caton TM and Schneegurt MA (2006) Carbon substrate utilization, antibiotic sensitivity, and numerical taxonomy of bacterial isolates from the Great Salt Plains of Oklahoma. *Archives of Microbiology* **185**, 286–296.

Mellon MT, Feldman WC and Prettyman TH (2004) The presence and stability of ground ice in the southern hemisphere of Mars. *Icarus* **169**, 324–340.

Ming DW, Archer PD Jr, Glavin DP, Eigenbrode JL, Franz HB, Sutter B, Brunner AE, Stern JC, Freissinet C, McAdam AC, Mahaffy PR, Cabane M, Coll P, Campbell JL, Atreya SK, Niles PB, Bell JF 3rd, Bish DL, Brinckerhoff WB, Buch A, Conrad PG, Des Marais DJ, Ehlmann BL, Fairén AG, Farley K, Flesch GJ, Francois P, Gellert R, Grant JA, Grotzinger JP, Gupta S, Herkenhoff KE, Hurowitz JA, Leshin LA, Lewis KW, McLennan SM, Miller KE, Moersch J, Morris RV, Navarro-González R, Pavlov AA, Perrett GM, Pradler I, Squyres SW, Summons RE, Steele A, Stolper EM, Sumner DY, Szopa C, Teinturier S, Trainer MG, Treiman AH, Vaniman DT, Vasavada AR, Webster CR, Wray JJ, Yingst RA and MSL Science Team (2014) Volatile and organic compositions of sedimentary rocks in Yellowknife Bay, Gale crater, Mars. *Science* **343**, 1245267.

Nimmo F and Pappalardo RT (2016) Ocean worlds in the outer solar system. *Journal of Geophysical Research* **121**, 1378–1399.

Pontefract A, Zhu TF, Walker VK, Hepburn H, Lui C, Zuber MT, Ruvkun G and Carr CE (2017) Microbial diversity in a hypersaline sulfate lake: a terrestrial analog of ancient Mars. *Frontiers in Microbiology* **8**, 1819.

Rivera-Valentin EG, Gough RV, Chevrier VF, Primm KM, Martinez GM and Tolbert M (2018) Constraining the potential liquid water environment at Gale Crater, Mars throughout MSL's traverse. *Lunar and Planetary Science Conference* **2018**, 2752.

Rivkina EM, Friedmann EI, McKay CP and Gilichinsky DA (2000) Metabolic activity of permafrost bacteria below the freezing point. *Applied and Environmental Microbiology* **66**, 3230–3233.

Rivkina E, Shcherbakova V, Laurinavichius K, Petrovskaya L, Krivushin K, Kraev G, Pecheritsina S and Gilichinsky D (2007) Biogeochemistry of methane and methanogenic archaea in permafrost. *FEMS Microbiology Ecology* **61**, 1–15.

Rummel JD, Beaty DW, Jones MA, Bakermans C, Barlow NG, Boston PJ, Chevrier VF, Clark BC, de Vera JP, Gough RV, Hallsworth JE, Head JW, Hipkin VJ, Kieft TL, McEwen AS, Mellon MT, Mikucki JA, Nicholson WL, Omelon CR, Peterson R, Roden EE, Sherwood Lollar B, Tanaka KL, Viola D and Wray JJ (2014) A new analysis of Mars 'special regions': findings of the second MEPAG Special Regions Science Analysis Group (SR-SAG2). *Astrobiology* **14**, 887–968.

Schneegurt MA (2012) Media and conditions for the growth of halophilic and halotolerant bacteria and archaea. In Vreeland RH (ed.) *Advances in Understanding the Biology of Halophilic Microorganisms*. Dordrecht: Springer, pp. 35–58.

Schneegurt MA, Wilks J, Zayed H, Joad MD, Mahdi A, Zbeeb H, Chen F and Clark BC (2018) Bacterial growth and survival under the extreme chemical and physical conditions of Mars and the icy worlds. *Abstracts and Program, 42nd COSPAR Scientific Assembly*, Pasadena, CA, July 2018, F3.1-0016-18, 22238.

Toner JD, Catling DC and Light B (2014) The formation of supercooled brines, viscous liquids, and low-temperature perchlorate glasses in aqueous solutions relevant to Mars. *Icarus* **233**, 36–47.

Tosca NJ, Knoll AH and McLennan SM (2008) Water activity and the challenge for life on early Mars. *Science* **320**, 1204–1207.

Vaniman DT, Bish DL, Chipera SJ, Fialips CI, Carey JW and Feldman WC (2004) Magnesium sulphate salts and the history of water on Mars. *Nature* **431**, 663–665.

Wilks J, Chen F, Clark BC and Schneegurt MA (2017) Bacterial growth at eutectic temperatures and in saturated salt solutions relevant to Mars. *Abstracts and Program, 117th Annual Meeting of the American Society for Microbiology*, New Orleans, LA, June 2017.

Wilson C, Caton TM, Buchheim JA, Buchheim MA, Schneegurt MA and Miller RV (2004) DNA-Repair potential of *Halomonas* spp. from the Salt Plains Microbial Observatory of Oklahoma. *Microbial Ecology* **48**, 541–549.

https://doi.org/10.1017/S1473550418000502 Published online by Cambridge University Press