International Journal of Astrobiology 16 (3): 229–235 (2017)
doi:10.1017/S1473550416000434

Bacterial growth tolerance to concentrations of chlorate and perchlorate salts relevant to Mars

Amer F. Al Soudi[1], Omar Farhat[1], Fei Chen[2], Benton C. Clark[3] and Mark A. Schneegurt[1]

[1]*Department of Biological Sciences, Wichita State University, Wichita, KS, USA e-mail: mark.schneegurt@wichita.edu*
[2]*Jet Propulsion Laboratory, Pasadena, CA, USA*
[3]*Space Science Institute, Boulder, CO, USA*

Abstract: The Phoenix lander at Mars polar cap found appreciable levels of (per)chlorate salts, a mixture of perchlorate and chlorate salts of Ca, Fe, Mg and Na at levels of ~0.6% in regolith. These salts are highly hygroscopic and can form saturated brines through deliquescence, likely producing aqueous solutions with very low freezing points on Mars. To support planetary protection efforts, we have measured bacterial growth tolerance to (per)chlorate salts. Existing bacterial isolates from the Great Salt Plains of Oklahoma (NaCl-rich) and Hot Lake in Washington ($MgSO_4$-rich) were tested in high concentrations of Mg, K and Na salts of chlorate and perchlorate. Strong growth was observed with nearly all of these salinotolerant isolates at 1% (~0.1 M) (per)chlorate salts, similar to concentrations observed in bulk soils on Mars. Growth in perchlorate salts was observed at concentrations of at least 10% (~1.0 M). Greater tolerance was observed for chlorate salts, where growth was observed to 2.75 M (>25%). Tolerance to K salts was greatest, followed by Mg salts and then Na salts. Tolerances varied among isolates, even among those within the same phylogenetic clade. Tolerant bacteria included genera that also are found in spacecraft assembly facilities. Substantial microbial tolerance to (per)chlorate salts is a concern for planetary protection since tolerant microbes contaminating spacecraft would have a greater chance for survival and proliferation, despite the harsh chemical conditions found near the surface of Mars.

Received 5 August 2016, accepted 6 October 2016, first published online 22 November 2016

Key words: chlorate, extremophiles, growth tolerance, Mars, perchlorate, planetary protection.

Introduction

Oxyanions of chlorine have been detected on the surface of Mars (Hecht *et al.* 2009; Kounaves *et al.* 2010; Ming *et al.* 2014; Clark & Kounaves 2015). The Phoenix lander at Mars' polar region measured levels of ~0.4–0.6% in regolith that represent chlorate and perchlorate salts and perhaps other oxychlorines. (Per)Chlorate also has been detected at the Curiosity landing site and in Moon and meteorite samples (Glavin *et al.* 2013; Jackson *et al.* 2015b). Common cations include calcium, iron, magnesium and sodium (Kounaves *et al.* 2014; Ming *et al.* 2014). While their planetary distribution is uneven, chloride and (per)chlorate salts are widespread (Clark & Kounaves 2015). The (per)chlorate salts are highly hygroscopic and therefore have relevance to astrobiology studies of Mars. There is reason to believe that enough humidity exists in the atmosphere of Mars to create deliquescent brines through absorption of water by (per)chlorate salts (Chevrier *et al.* 2009; Martín-Torres *et al.* 2015; Ojha *et al.* 2015). Equally as important is the fact that brines of (per)chlorate salts have very low eutectic temperatures, some remaining liquid even to below –70°C (Möhlmann & Thomsen 2011; Clark & Kounaves 2015).

The intense aridity and extremely low temperatures at the Martian surface create environmental conditions particularly challenging to organisms. It has been suggested that (per)chlorate salts may be important sources of liquid water in the Martian near surface (Zorzano *et al.* 2009; Davila *et al.* 2010; Nuding *et al.* 2014). Spectral evidence is consistent with the idea that recurring slope lineae result from brines of Mg perchlorate, Mg chlorate and Na perchlorate (Ojha *et al.* 2015). Strong oxidants, such as oxychlorines, are typically incompatible with living systems. However, due to mechanistic kinetic barriers, (per)chlorate salts are quite stable in aqueous environments and are not easily reduced (Urbansky 1998; Gu *et al.* 2003).

Perchlorate salts on the Earth tend to result from industrial processes that produce explosives, herbicides, lubricants, paints and paper (Motzer 2001; Urbansky 2002). These salts can contaminate drinking water and be toxic to humans, through competition with iodine in the thyroid (Hooth *et al.* 2001; Kalkhoff *et al.* 2010; EPA 2011). Natural perchlorate salts have been detected in the very arid regions of the Atacama Desert and Antarctica (Kounaves *et al.* 2010; Jackson *et al.* 2015a). This includes Chilean nitrate deposits, which have levels of perchlorate approaching 0.6% (Ericksen 1981). Trace levels of perchlorate salts have been detected in a variety of other aqueous and soil environments (Smith

et al. 2004; Rajagopalan *et al.* 2006, 2009; Rao *et al.* 2007). Given the rarity of these salts worldwide, it might be expected that growth tolerances to high concentrations of (per)chlorate would be rare among microorganisms.

An early study by Durand (1938) found appreciable microbial growth tolerance to Na perchlorate. *Bacterium coli* (*Escherichia coli*) grew in the presence of 2.5% (~0.25 M) Na perchlorate and *Staphylococcus pyogenes aureus* (*S. aureus*), showed slow growth in the presence of 7.5% (~0.75 M), but no growth at 10% (~1.0 M), Na perchlorate. *Sterigmatocystis nigra* (*Aspergillus niger*) exhibited strong mycelial growth at 1% (~0.1 M) Na perchlorate and approximately 25% as much growth at 4% (~0.4 M) Na perchlorate. A recent preliminary report suggests that the haloarchaeon *Haloarcula argentinensis* can grow in the presence of 0.5 M (~5%) perchlorate, even in medium containing 15% (~3.0 M) NaCl (Thombre *et al.* 2015). A study of halotolerant *Haloarcula, Haloferax* and *Halomonas* demonstrated strong growth in the presence of 0.4 M Na perchlorate, with weak growth of *Haloferax* at 0.6 M (Oren *et al.* 2014). Two other recent studies examine another clade of *Archaea*. Methanogenesis was demonstrated to proceed in the presence of 1% perchlorate, but no higher, for strains of *Methanobacterium, Methanosarcina* and *Methanothermobacter* (Kral *et al.* 2016). However, when adapted to higher concentrations of perchlorate salts, these methanogens appeared to metabolize despite the presence of up to 5% (but not 10%) perchlorate. A similar study with *Methanobacterium* and *Methanosarcina* isolates from permafrost observed methanogenesis only at very much lower concentrations of Mg and Na perchlorates, 0.1% (~10 mM) or less (Shcherbakova *et al.* 2015). Another previous study with a facultative anaerobic consortium did not detect growth at 0.4% perchlorate (Bardiya & Bae 2005).

There is a substantial body of literature on microbes that can use perchlorate as a terminal electron acceptor for anaerobic respiration (Wallace *et al.* 1996, 1998; Coates *et al.* 1999; Herman & Frankenberger 1999; Okeke *et al.* 2002; Coates & Achenbach 2004; Shete *et al.* 2008). Typically perchlorate is added to media at concentrations below 1 mM. The biological reduction of perchlorate is performed by perchlorate reductase, producing a chlorite anion, which is toxic to cells if not further reduced to chloride. It has been suggested that microorganisms with nitrate reductase can convert perchlorate into chlorite, but that this is then cytotoxic. Certainly (per)chlorate respiration has clear astrobiology relevance given that (per)chlorate brines may be present on Mars. However, perchlorate respirers would still need to be tolerant to very high concentrations of (per) chlorate salts.

Microbes exhibiting high tolerance to (per)chlorate anion also need to be tolerant of high concentrations of the accompanying cations. We have examined (per)chlorate growth tolerance in bacteria isolated from hypersaline environments, including terrestrial hyperhaline soils rich in NaCl from the Great Salt Plains of Oklahoma and epsomite lake margins and sediments rich in $MgSO_4$ from Hot Lake in Washington. Our study has relevance to potential forward contamination of (per)chlorate brines on Mars by spacecraft carrying soil particles or the salinotolerant bacteria commonly found in spacecraft assembly facilities (SAF).

Table 1. *Salinotolerant bacteria used for this study from Hot Lake* (*Kilmer et al.* 2014) *and the Great Salt Plains* (*Caton et al.* 2004)

Isolate	Identification	GenBank accession
HL11	*Marinococcus halophilus*	KC705247
HL12	*Halomonas venusta*	KC705248
HL14	*Halomonas venusta*	KC705250
HL20	*Planococcus maritimus*	KC705257
HL54	*Marinococcus halophilus*	KC705293
HL55	*Bacillus licheniformis*	KC705294
HL64	*Nesterenkonia halotolerans*	KC705304
HL68	*Bacillus licheniformis*	KC705308
HL76	*Nesterenkonia halotolerans*	KC705317
HL80	*Planococcus salinarum*	KC705322
HL82	*Halomonas venusta*	KC705324
HL91	*Planococcus salinarum*	KC705334
GSP3	*Halomonas venusta*	AY505527
GSP10	*Bacillus megaterium*	AY505510
GSP11	*Terribacillus halophilus*	AY553069
GSP17	*Salibacillus marismortui*	AY505533
GSP21	*Halomonas salina*	AY553072
GSP63	*Bacillus licheniformis*	AY553106

Materials and methods

Organisms

Bacterial isolate collections previously generated from hypersaline environments were used to measure growth tolerances. We previously characterized 93 halotolerant aerobic heterotrophic bacteria isolated from the Great Salt Plains of Oklahoma, a wet terrestrial environment saturated with NaCl (Caton *et al.* 2004). Similar work at Hot Lake, WA, an environment saturated with $MgSO_4$ (epsomite), yielded a collection of 64 bacterial isolates from sediments and lake margin samples (Kilmer *et al.* 2014). A group of 18 isolates from these collections was chosen for further study based on salinotolerance and taxonomy (Table 1).

Media and growth measurements

Bacterial cultures were grown on Salt Plains (SP) medium supplemented with various (per)chlorate salts (Caton *et al.* 2004). Shake tubes (2 ml in 13 × 100 mm tubes) were lightly loop-inoculated in triplicate (below 0.05 OD units) from agar slants and maintained at room temperature on a rotary shaker (150 rpm, 1-in stroke dia). Culture density was determined as A_{600} by spectrophotometry (ThermoFisher Genesys 10S) using a medium blank at the time of inoculation and at 2 days intervals for 12 days after inoculation. Error bars of SD were generally smaller than the symbols used for plotting growth curves.

The water activity of each medium (Table 2) was measured using an AqualLab Series 3 water activity meter (Decagon Devices, Inc., Pullman, WA). The instrument was calibrated with standard NaCl solutions and run at room temperature.

https://doi.org/10.1017/S1473550416000434 Published online by Cambridge University Press

Table 2. *Water activities of (per)chlorate salt solutions*

Solute	a_w
0.1 M $NaClO_3$	0.99
0.5 M $NaClO_3$	0.98
1.0 M $NaClO_3$	0.96
1.5 M $NaClO_3$	0.94
2.0 M $NaClO_3$	0.91
2.75 M $NaClO_3$	0.89
0.1 M $KClO_3$	0.99
0.5 M $KClO_3$	0.98
1.0 M $KClO_3$	0.98
0.1 M $NaClO_4$	0.99
0.5 M $NaClO_4$	0.98
1.0 M $NaClO_4$	0.96
0.1 M $KClO_4$	0.99
0.5 M $KClO_4$	0.98
1.0 M $KClO_4$	0.97
0.05 M $Mg(ClO_4)_2$	0.99
0.25 M $Mg(ClO_4)_2$	0.98
0.5 M $Mg(ClO_4)_2$	0.97

(Per)chlorate measurements

Chlorate and perchlorate concentrations were measured by ion chromatography as previously described (Carlström *et al.* 2015). Briefly, a mobile phase of 36 mM NaOH was used with a Dionex Ion Pac AS 25 column (4 × 250 mm) in a Dionex ICS 500 instrument in recycle mode with a Dionex ASRS 300 (4 mm) and suppressor control at 90 mA. Samples of culture media were clarified by filtration (0.22 μm) before dilution and analysis.

Results

Growth tolerance to perchlorate salts

Growth tolerances to high concentrations of (per)chlorate salts were determined for 18 salinotolerant bacterial isolates. Tests were performed at several concentrations of Mg, K and Na perchlorate salts and with K and Na chlorate salts. Representative growth curves are presented for HL12, a *Halomonas venusta* isolate from Hot Lake, which was particularly tolerant to (per) chlorate exposure (Figs. 1–3).

Magnesium perchlorate is a likely Mars salt and is a major contributor to the ∼0.6% (∼0.06 M) level of (per)chlorate salts observed by Phoenix (Hecht *et al.* 2009). Growth of HL12 in the presence of 0.05 M (∼1%) Mg perchlorate was rapid and robust (Fig. 1). Growth in the presence of 0.25 M (∼5%) Mg perchlorate was slower and the cultures did not become as dense as those cultures grown with 0.05 M Mg perchlorate. The small amount of growth that HL12 may have exhibited in medium supplemented with 0.5 M Mg perchlorate was near the threshold that was chosen for faint growth (0.1 OD unit). All of the salinotolerant isolates grew robustly in the presence of 0.05 M Mg perchlorate, except HL 54 and 82 (Table 3). Half of the isolates exhibited positive growth in the presence 0.25 M Mg perchlorate. While some growth may have occurred at levels as high as 0.5 M (∼10%) Mg perchlorate for certain isolates, it was limited and inconsistent.

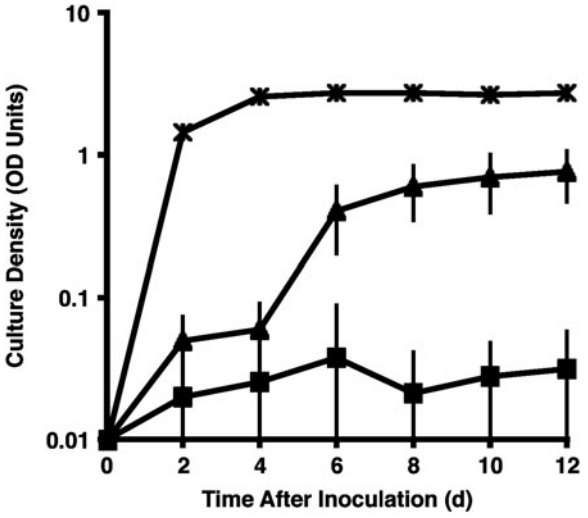

Fig. 1. Growth of HL12 in SP medium supplemented with Mg perchlorate. Bacterial growth in shake-tube cultures was measured by turbidity and is presented in OD units. SD of some triplicate cultures were smaller than the point markers. Stars, 0.05 M Mg perchlorate; triangles, 0.25 M Mg perchlorate; squares, 0.5 M Mg perchlorate.

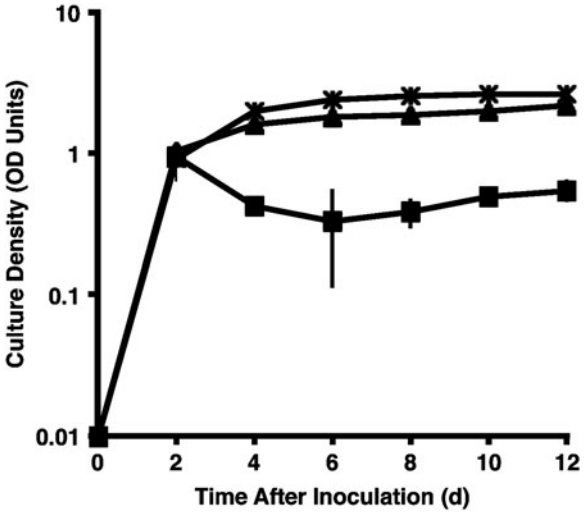

Fig. 2. Growth of HL12 in SP medium supplemented with Na perchlorate. Bacterial growth in shake-tube cultures was measured by turbidity and is presented in OD units. SD of some triplicate cultures were smaller than the point markers. Stars, 0.1 M Na perchlorate; triangles, 0.5 M Na perchlorate; squares, 1.0 M Na perchlorate.

Growth of HL12 was robust in the presence of 0.1 and 0.5 M Na perchlorate (Fig. 2). At 1.0 M Na perchlorate, growth was slower and the maximum density reached was <30% of that attained in the presence 0.5 M Na perchlorate. Only HL12 showed substantial growth at 1.0 M Na perchlorate. HL11, 12 and 55 grew robustly with 0.5 M Na perchlorate. Overall, 12 of 18 isolates showed growth in 0.5 M Na perchlorate. All showed robust growth at 0.1 M Na perchlorate, except for HL82.

HL12 showed robust growth in the presence of K perchlorate at ≤1.0 M (Fig. 3). The growth tolerance of HL12 to the

https://doi.org/10.1017/S1473550416000434 Published online by Cambridge University Press

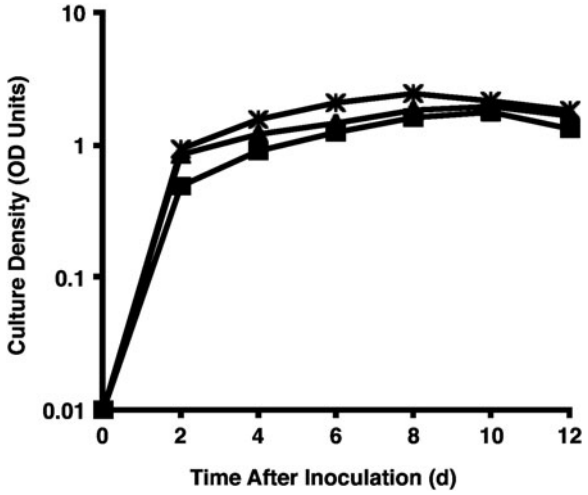

Fig. 3. Growth of HL12 in SP medium supplemented with K perchlorate. Bacterial growth in shake-tube cultures was measured by turbidity and is presented in OD units. SD of triplicate cultures were smaller than the point markers. Stars, 0.1 M K perchlorate; triangles, 0.5 M K perchlorate; squares, 1.0 M K perchlorate.

Table 3. *Maximum culture density (OD units) observed for salinotolerant bacterial isolates grown in the presence of perchlorate salts*

	Solute concentration (M)								
	Na perchlorate			Mg perchlorate			K perchlorate		
Isolate	0.1	0.5	1.0	0.05	0.25	0.5	0.1	0.5	1.0
HL11	2.52	2.09	0.04	2.62	0.14	0.05	1.55	1.26	0.38
HL12	2.63	2.21	0.60	2.69	1.09	0.05	1.81	1.48	1.22
HL14	2.65	0.07	0.04	1.92	0.01	0.04	1.26	1.09	1.08
HL20	1.90	0.01	0.09	2.42	0.21	0.08	0.84	0.06	0.01
HL54	2.02	0.22	0.08	0.69	0.11	0.06	1.78	1.12	1.31
HL55	2.33	0.98	0.09	1.84	0.62	0.05	1.24	1.15	1.10
HL64	2.48	0.03	0.10	2.56	0.30	0.06	1.88	1.46	1.17
HL68	2.56	0.45	0.09	2.25	0.57	0.05	0.98	0.62	0.02
HL76	2.54	0.02	0.10	2.64	0.04	0.15	1.19	0.34	0.02
HL80	2.66	0.01	0.09	1.74	0.02	0.01	2.13	1.23	0.72
HL82	0.05	0.04	0.10	0.01	0.02	0.03	1.12	1.05	1.08
HL91	1.30	0.50	0.09	1.94	0.64	0.11	1.54	1.14	1.01
GSP3	1.35	0.37	0.09	2.47	0.08	0.11	2.12	1.32	0.09
GSP10	1.63	0.15	0.09	2.18	0.03	0.03	1.63	0.15	0.07
GSP11	1.29	0.42	0.09	1.76	0.03	0.32	2.15	1.12	1.09
GSP17	1.44	0.35	0.09	2.14	0.10	0.30	1.02	0.98	0.43
GSP21	1.71	0.16	0.09	2.19	0.05	0.07	1.71	1.32	1.02
GSP63	1.54	0.80	0.09	1.96	0.04	0.07	1.04	0.50	0.01

K salt was substantially greater at 1.0 M than was growth tolerance to the corresponding Mg or Na salts. The salinotolerant isolates overall performed better in the presence of K perchlorate than with Mg or Na perchlorate (Table 3). More than half of the isolates grew well in the presence of 1.0 M K perchlorate, while growth in Mg and Na salts at 1 M perchlorate was barely detectable. All of the isolates grew well at 0.1 M K perchlorate and only HL20 and GSP10 did not grow well at 0.5 M K perchlorate. The solubilities of calcium and iron perchlorate salts in media were too low (<0.1 M) for meaningful growth tolerance experiments.

Table 4. *Maximum culture density (OD units) observed for salinotolerant bacterial isolates grown in the presence of chlorate salts*

	Solute concentration (M)								
	Na chlorate						K chlorate		
Isolate	0.1	0.5	1.0	1.5	2.0	2.75	0.1	0.5	1.0
HL11	2.34	2.48	2.18	2.29	1.65	1.62	2.54	2.32	2.38
HL12	1.95	2.30	2.16	2.03	1.81	1.81	2.46	2.08	1.03
HL14	1.39	1.57	0.76	0.03	0.24	0.15	2.02	1.98	1.08
HL20	1.87	1.67	1.63	0.06	0.06	0.06	0.98	0.87	0.89
HL54	0.28	0.74	0.38	0.11	0.10	0.07	2.00	1.54	1.21
HL55	1.42	1.40	1.18	0.49	0.05	0.04	1.87	1.73	1.54
HL64	2.25	2.55	0.62	0.58	0.07	0.06	2.43	2.31	2.21
HL68	1.02	1.59	1.10	0.46	0.05	0.07	1.27	1.06	0.99
HL76	2.22	2.41	0.76	1.13	0.09	0.08	2.99	2.06	2.01
HL80	1.80	1.81	0.04	0.04	0.11	0.11	0.79	0.52	0.43
HL82	0.01	0.06	0.06	0.02	0.06	0.06	0.18	0.14	0.08
HL91	0.82	1.72	1.01	0.73	0.05	0.03	1.07	0.98	0.87
GSP3	1.38	2.27	1.58	0.81	0.14	0.14	1.21	0.93	0.97
GSP10	2.05	1.97	1.51	1.12	0.05	0.04	1.03	0.93	0.97
GSP11	1.38	2.24	1.69	1.61	0.21	0.21	1.75	1.62	1.09
GSP17	1.38	1.99	0.97	1.49	0.30	0.22	1.65	1.43	1.23
GSP21	1.33	1.94	1.54	1.46	0.39	0.39	1.87	1.84	1.66
GSP63	1.37	2.11	0.77	1.30	0.18	0.18	2.08	2.01	1.98

Growth tolerance to chlorate salts

Growth tolerances to chlorate salts were greater among the isolates than were growth tolerances to perchlorate salts (*cf.* Tables 3 and 4). All of the isolates grew robustly in the presence of 0.1 M Na chlorate, except HL 54 and 82 (Table 4). This trend continued up to 1.0 M Na chlorate, and in medium with 1.5 M Na chlorate, 13 of 18 isolates grew well. At 2.75 M (~25%) Na chlorate, HL 11 and 12 showed strong growth, while several other isolates exhibited weak growth. Note that none of the isolates grew strongly in 1 M perchlorate salts. Growth curves for HL12 at different concentrations of Na chlorate show that, for the highest concentrations tested, lag phases were longer, growth rates were slower, and maximum culture densities were lower (Fig. 4). However, growth was still substantial even at 2.75 M Na chlorate, the highest concentration at which media could be prepared without visible precipitate. In the presence of 1.0 M K chlorate, growth was strong for all isolates, except HL 80 and 82 (Table 4), with this being the highest concentration that could be added to culture medium without visible precipitate. HL12 grew robustly in the presence of 0.1 or 0.5 M K chlorate, but growth was slowed, although still strong, at 1.0 M K chlorate (Fig. 5). Tolerance to K chlorate was not consistently greater than to Na chlorate, in contrast to the corresponding perchlorate salts. We were unable to obtain Ca or Mg chlorate commercially to complete the iterative matrix of salts. The solubility of iron chlorate in media was too low (<0.1 M) for meaningful growth tolerance experiments.

(Per)chlorate retention by cultures

While it appears that these salinotolerant bacterial isolates can grow in the presence of high concentrations of (per)chlorate salts, it was possible that the microbes detoxify the anions,

https://doi.org/10.1017/S1473550416000434 Published online by Cambridge University Press

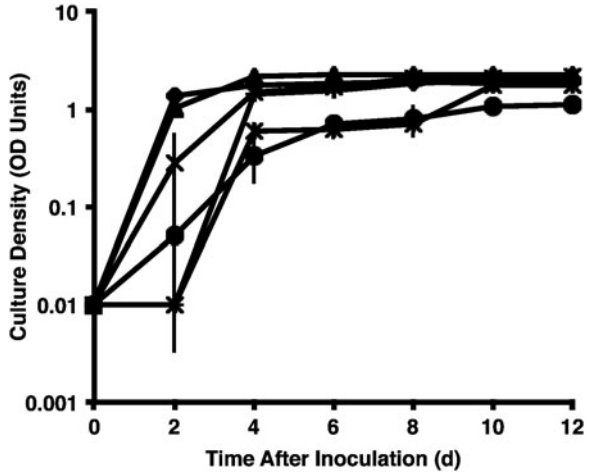

Fig. 4. Growth of HL12 in SP medium supplemented with Na chlorate. Bacterial growth in shake-tube cultures was measured by turbidity and is presented in OD units. SD of some triplicate cultures were smaller than the point markers. Diamonds, 0.1 M Na chlorate; triangles, 0.5 M Na chlorate; X, 1.0 M Na chlorate; +, 1.5 M Na chlorate; stars, 2.0 M Na chlorate; circles, 2.5 M Na chlorate.

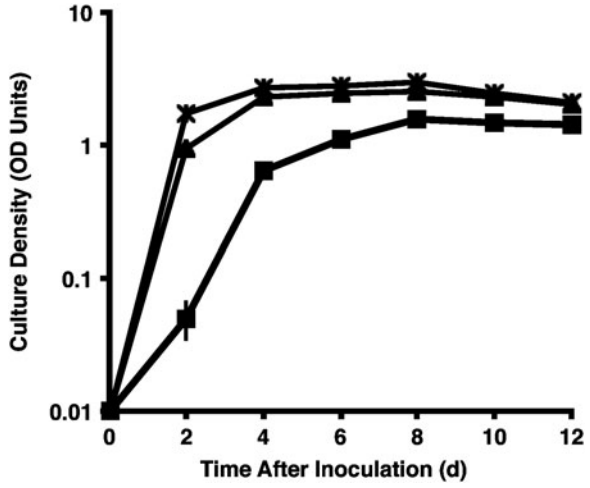

Fig. 5. Growth of HL12 in SP medium supplemented with K chlorate. Bacterial growth in shake-tube cultures was measured by turbidity and is presented in OD units. SD of triplicate cultures were smaller than the point markers. Stars, 0.1 M K chlorate; triangles, 0.5 M K chlorate; squares, 1.0 M K chlorate.

removing them before substantially growing. Chlorate and perchlorate levels were measured by ion-exchange chromatography before and after microbial cultivation. Overall there were only small differences between the (per)chlorate concentrations of media before and after cultivation. For example, in medium containing 0.25 M Mg perchlorate, there was less than a 2% difference between the initial concentration of perchlorate and the concentration of perchlorate after the growth of HL12 in batch culture. Similarly, for medium containing 0.5 M Na chlorate, there was less than a 2% change in chlorate concentration after batch culture of HL12. Tests with other cations, isolates and concentrations gave the same general outcome. In addition, no appreciable chlorate was present after bacterial cultivation in any medium initially containing perchlorate. Perchlorate respiration is an anaerobic process and it does not appear that (per)chlorate was metabolized in our aerobic cultures. (Per)Chlorate added to the medium remained at high concentrations throughout the development of the microbial culture.

Discussion

The ability to grow in the presence of 0.1 M (~1%) perchlorate appears to be widespread among bacteria from hypersaline environments. Nearly all of the salinotolerant bacterial isolates examined grew, not only in the presence 0.1 M perchlorate salts, but also at 0.5 M (~5%), and some grew at 1.0 M (~10%). Only Durand (1938) has demonstrated bacterial growth at such high perchlorate concentrations. Furthermore, we have demonstrated bacterial growth at concentrations of chlorate up to 2.75 M (~25%), where previous tolerance studies have not included chlorate salts.

Soils near the Phoenix lander appear to contain ~0.4–0.6% (per)chlorate salts. If this was evenly distributed in the soil and then incorporated into liquid brine, the bacteria in our study could easily tolerate that level of (per)chlorate. However, it is likely that (per)chlorate salts exist as distinct phases within the soil. These hygroscopic salts may form heavy brines through deliquescence. Hence, the effective concentrations of (per)chlorate salts in solution would be much higher than the 0.6% (per)chlorate observed in bulk soil. Strong arguments can be made that (per)chlorate brines are some of the most likely sources of liquid water on Mars (Zorzano *et al.* 2009; Davila *et al.* 2010). In our study, the highest perchlorate concentration that allowed bacterial growth was 10%. This is far lower than the eutectic concentrations of perchlorate salts. For instance, Na perchlorate has its eutectic point at 52 wt% at –37°C, while the eutectic point of Mg perchlorate is 44 wt% at –67°C (Chevrier *et al.* 2009). It is possible that certain bacteria, perhaps those adapted to an environment high in perchlorate, are capable of growth at higher concentrations of perchlorate than observed in our study. Survival of viable cells likely occurs at higher (per)chlorate concentrations than growth. The great degree of chlorate tolerance we observed, with growth above 25% chlorate, is closer to what would be needed to grow in a eutectic solution of Na chlorate (39 wt% at –23°C) (Hanley *et al.* 2012). Note that the eutectic point for K chlorate is 3 wt% at –3°C and well within the growth tolerances observed for K chlorate among our isolates.

Tolerance to chlorate salts was greater than tolerance to perchlorate salts by a wide margin. Limited growth in perchlorate salts was not solely due to cation effects, since all of the isolates can grow at concentrations of NaCl and $MgSO_4$ above 1.0 M. At 2.75 M Na chlorate, however, Na concentrations are reaching the Na tolerance limits of even these salinotolerant microbes. Of the isolates in our study, only HL 11, 20 and 54 have been shown to grow above 20% NaCl (Kilmer *et al.* 2014). Inhibition of the growth of HL91 above 1.5 M (~15%) Na chlorate for example may have been due to cation effects, since this organism tolerates 10% (1.7 M) NaCl, but not 20% (3.4 M)

https://doi.org/10.1017/S1473550416000434 Published online by Cambridge University Press

NaCl. While growth of these isolates occurs in ≥50% (~2 M) $MgSO_4$, growth in Mg perchlorate was inhibited at much lower Mg concentrations (Crisler *et al.* 2012). Growth in relatively high concentrations of Mg chlorate may be possible, however, that salt could not be obtained commercially. Strong growth was observed with K perchlorate addition, best seen for isolates that were less tolerant overall. For instance, HL 54 and 82 were particularly sensitive to (per)chlorate salts, with HL82 unable to grow at even 0.1 M Na perchlorate. However, these isolates grew strongly in 1.0 M K perchlorate. Isolates most tolerant to perchlorate seemed to mirror those most tolerant to chlorate, suggesting a common mechanism for growth inhibition.

The observation of high (per)chlorate tolerance in salinotolerant bacteria demonstrates that terrestrial microbes are capable of growing at concentrations of (per)chlorate salts found in Mars soils. However, one of the most likely sources of bacteria that contaminate spacecraft is common oligosaline soil from around the SAF (Foster & Winans 1975; Puleo *et al.* 1977). Common soils appear to harbour bacteria capable of growing at relatively high concentrations of salts and this is a concern for forward planetary protection (Echigo *et al.* 2005; Chen *et al.* 2010). Initial microbial abundance measurements by most probable number analysis of oligosaline turf soils found that 1.7×10^5 and 7.1×10^3 cells g^{-1} soil were tolerant to 10 and 20% NaCl, respectively (Kilmer *et al.* 2010; Porazka *et al.* 2011). Similarly, preliminary measurements of the abundance of bacteria tolerant to (per)chlorate salts indicate that it is not rare to find microbes that are tolerant to concentrations of (per)chlorate salts relevant to Mars (Crisler *et al.* 2013a, b; Al Soudi *et al.* 2016). While our salinotolerant isolates are from rare extreme environments, some of the bacterial species isolated from SAFs match those from hypersaline environments (Caton *et al.* 2004; Moissl *et al.* 2008; Stieglmeier *et al.* 2009). The cleanrooms that act as SAFs are dry environments (≤40% relative humidity) and it has been shown that salinotolerant microbes are present on surfaces and perhaps enriched by selection (Venkateswaran *et al.* 2001, 2003a, b; La Duc *et al.* 2003; Link *et al.* 2003; Kempf *et al.* 2005). It will be interesting to directly test microbial isolates from SAFs and neighbouring soils for (per)chlorate tolerance.

Acknowledgements

The authors are grateful for the contributions of Zonaira Ahmad, Todd Caton, James Crisler, Timothy Eberl, Joshua Fleming, Sascha Khan, Brian Kilmer, Tony Mai, Hieu Nguyen, Anastasiya Nosova and Noah Schneegurt. We thank Fadi Aramouni for determining water activities and Anna Engelbrektson and John Coates for measuring (per)chlorate concentrations. Preliminary accounts of this work have been presented previously and abstracted (Mai *et al.* 2012; Crisler *et al.* 2013a, b; Al Soudi *et al.* 2016). This work was supported by awards from NASA ROSES Planetary Protection Research (09-PPR09-0004 and 14-PPR14-2-0002) and Kansas INBRE IDeA NIGMS NIH (P20 GM103418).

References

Al Soudi, A., Farhat, O., Chen, F., Clark, B.C. & Schneegurt, M.A. (2016). Bacterial growth tolerance to chlorate and perchlorate salts relevant to Mars. In *The 116th Annual Meeting of the American Society for Microbiology.* Abstract No. FR-027.

Bardiya, N. & Bae, J.-H. (2005). Bioremediation potential of a perchlorate-enriched sewage sludge consortium. *Chemosphere* **58**, 83–90.

Carlström, C.I., Loutey, D.E., Wang, O., Engelbrektson, A., Clark, I., Lucas, L.N., Somasekhar, P.Y. & Coates, J.D. (2015). Phenotypic and genotypic description of *Sedimenticola selenatireducens* strain CUZ, a marine (per)chlorate-respiring Gammaproteobacterium, and its close relative the chlorate-respiring *Sedimenticola* strain NSS. *Appl. Environ. Microbiol*. **81**, 2717–2726.

Caton, T.M., Witte, L.R., Ngyuen, H.D., Buchheim, J.A., Buchheim, M.A. & Schneegurt, M.A. (2004). Halotolerant aerobic heterotrophic bacteria from the Great Salt Plains of Oklahoma. *Microb. Ecol*. **48**, 449–462.

Chen, Q., Liu, Z., Peng, Q., Huang, K., He, J., Zhang, L., Li, W. & Chen, Y. (2010). Diversity of halophilic and halotolerant bacteria isolated from non-saline soil collected from Xiaoxi National Natural Reserve, Hunan Province. *Acta Microbiol. Sin*. **50**, 1452–1459. [Chinese].

Chevrier, V.F., Hanley, J. & Altheide, T.S. (2009). Stability of perchlorate hydrates and their liquid solutions at the Phoenix landing site, Mars. *Geophys. Res. Lett*. **36**, L10202.

Clark, B.C. & Kounaves, S.P. (2015). Evidence for the distribution of perchlorates on Mars. *Int. J. Astrobiol*. doi: http://dx.doi.org/10.1017/S1473550415000385.

Coates, J.D. & Achenbach, L.A. (2004). Microbial perchlorate reduction: rocket-fueled metabolism. *Nat. Rev*. **2**, 569–580.

Coates, J.D., Michaelidou, U., Bruce, R.A., O'Connor, S.M., Crespi, J.N. & Achenbach, L.A. (1999). Ubiquity and diversity of dissimilatory (per) chlorate-reducing bacteria. *Appl. Environ. Microbiol*. **65**, 5234–5241.

Crisler, J.D., Newville, T.M., Chen, F., Clark, B.C. & Schneegurt, M.A. (2012). Bacterial growth at the high concentrations of magnesium sulfate found in Martian soils. *Astrobiology* **12**, 98–106.

Crisler, J.D., Mai, T.T., Ahmad, Z., Chen, F., Clark, B.C. & Schneegurt, M. A. (2013a). Bacterial growth at high concentrations of deliquescent salts potentially relevant to Mars. In *The 145th Annual Meeting of the Kansas Academy of Science. Trans. KS Acad. Sci*. **116**, 71.

Crisler, J.D., Mai, T.T., Ahmad, Z., Chen, F., Clark, B.C. & Schneegurt, M. A. (2013b). Bacterial growth in deliquescent lithium and perchlorate salts potentially relevant to Mars. In *The 113th General Meeting of the American Society for Microbiology.* Abstract No. 1053.

Davila, A.F. *et al.* (2010). Hygroscopic salts and the potential for life on Mars. *Astrobiology* **10**, 617–628.

Durand, M.J. (1938). Recherches sur l'élimination des perchlorates, sur leur répartition dans les organes et sur leur toxicité. *Bull. Soc. Chim. Biol*. **20**, 423–433. [French].

Echigo, A., Hino, M., Fukushima, T., Mizuki, T., Kamekura, M. & 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 Syst*. **1**, 8. doi: 10.1186/1746-1448-1-8.

EPA (2011). Drinking water: regulatory determination on perchlorate. *Fed. Regist*. **76**, 7762–7767.

Ericksen, G.E. (1981). *Geology and Origin of the Chilean Nitrate Deposits*. Geological Survey Professional Paper 1188, U.S. Government Printing Office, Washington.

Foster, T.L. & Winans, L. (1975). Psychrophilic microorganisms from areas associated with the Viking spacecraft. *Appl. Microbiol*. **30**, 546–550.

Glavin, D.P. *et al.* (2013). Evidence for perchlorates and the origin of chlorinated hydrocarbons detected by SAM at the Rocknest aeolian deposit in Gale crater. *J. Geophys. Res. Planets* **118**, 1955–1973.

Gu, B., Dong, W., Brown, G.M. & Cole, D.R. (2003). Complete degradation of perchlorate in ferric chloride and hydrochloric acid under controlled temperature and pressure. *Environ. Sci. Technol*. **37**, 2291–2295.

Hanley, J., Chevrier, V.F., Berget, D.J. & Adams, R.D. (2012). Chlorate salts and solutions on Mars. *Geophys. Res. Lett*. **39**, L08201.

Hecht, M.H. *et al*. (2009). Detection of perchlorate and the soluble chemistry of Martian soil at the Phoenix lander site. *Science* **325**, 64–67.

Herman, D.C. & Frankenberger, W.T., Jr. (1999). Bacterial reduction of perchlorate and nitrate in water. *J. Environ. Qual*. **28**, 1018–1024.

Hooth, M.J., DeAngelo, A.B., George, M.H., Gaillard, E.T., Travlos, G.S., Boorman, G.A. & Wolf, D.C. (2001). Subchronic sodium chlorate exposure in drinking water results in a concentration-dependent increase in rat thyroid follicular cell hyperplasia. *Toxicol. Pathol*. **29**, 250–259.

Jackson, W.A. *et al*. (2015a). Global patterns and environmental controls of perchlorate and nitrate co-occurrence in arid and semi-arid environments. *Geochim. Cosmochim. Acta* **164**, 502–522.

Jackson, W.A., Davila, A.F., Sears, D.W., Coates, J.D., McKay, C.P., Brundrett, M., Estrada, N. & Böhlke, J.K. (2015b). Widespread occurrence of (per)chlorate in the Solar System. *Earth Planet. Sci. Lett*. **430**, 470–476.

Kalkhoff, S.J., Stetson, S.J., Lund, K.D., Wanty, B.B. & Linder, G.L. (2010). Perchlorate data for streams and groundwater in selected areas of the United States, 2004. U.S. Geological Survey Data Ser. 495, 43 pp.

Kempf, M.J., Chen, F., Kern, R. & Venkateswaran, K. (2005). Recurrent isolation of hydrogen peroxide-resistant spores of *Bacillus pumulis* from a spacecraft assembly facility. *Astrobiology* **5**, 391–405.

Kilmer, B.R., Chambers, C.A., Akbar, R., Bhakta, S., Beck, A., Brimmerman, J., Lundin, H., DeVries, C., Kasten, L., Pringle-Johnson, B., Ruder, J.S. & Schneegurt, M.A. (2010). Isolation and characterization of halotolerant bacteria from inland oligohaline soils. In *The 110th General Meeting of the American Society for Microbiology.* Abstract No. 298.

Kilmer, B.R., Eberl, T.C., Cunderla, B., Chen, F., Clark, B.C. & Schneegurt, M.A. (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. *Int. J. Astrobiol*. **13**, 69–80.

Kounaves, S.P. *et al*. (2010). Wet chemistry experiments on the 2007 Phoenix Mars Scout Lander mission: data analysis and results. *J. Geophys. Res*. **115**, E00E10.

Kounaves, S.P., Carrier, B.L., O'Neil, G.D., Stroble, S.T. & Claire, M.W. (2014). Evidence of Martian perchlorate, chlorate, and nitrate in Mars meteorite EETA79001: implications for oxidants and organics. *Icarus* **229**, 206–213.

Kral, T.A., Goodhart, T.H., Harpool, J.D., Hearnsberger, C.E., McCracken, G.L. & McSpadden, S.W. (2016). Sensitivity and adaptability of methanogens to perchlorates: implications for life on Mars. *Planet Space Sci*. **120**, 87–95.

La Duc, M.T., Nicholson, W., Kern, R. & Venkateswaran, K. (2003). Microbial characterization of the Mars Odyssey spacecraft and its encapsulation facility. *Environ. Microbiol*. **5**, 977–985.

Link, L., Sawyer, J., Venkateswaran, K. & Nicholson, W. (2003). Extreme spore UV resistance of *Bacillus pumulis* isolates obtained from ultraclean spacecraft assembly facility. *Microb. Ecol*. **47**, 159–163.

Mai, T.T., Nosova, A.O. & Schneegurt, M.A. (2012). Bacterial growth in perchlorate salts at concentrations found in soils on Mars. In *144th Annual Meeting of the Kansas Academy of Science. Trans. KS Acad. Sci*. **115**, 64.

Martín-Torres, F.J. *et al*. (2015). Transient liquid water and water activity at Gale crater on Mars. *Nat. Geosci*. **8**, 357–361.

Ming, D.W. *et al*. (2014). Volatile and organic compositions of sedimentary rocks in Yellowknife Bay, Gale Crater, Mars. *Science* **343**. doi: 10.1126/science.1245267.

Möhlmann, D. & Thomsen, K. (2011). Properties of cryobrines on Mars. *Icarus* **212**, 123–130.

Moissl, C., Bruckner, J.C. & Venkateswaran, K. (2008). Archaeal diversity analysis of spacecraft assembly clean rooms. *ISME J*. **2**, 115–119.

Motzer, W.E. (2001). Perchlorate. Problems, detection, and solutions. *Environ. Forensics* **2**, 301–311.

Nuding, D.L. *et al*. (2014). Deliquescence and efflorescence of calcium perchlorate: an investigation of stable aqueous solutions relevant to Mars. *Icarus* **243**, 420–428.

Ojha, L., Wilhelm, M.B., Murchie, S.L., McEwen, A.S., Wray, J.J., Hanley, J., Massé, M. & Chojnacki, M. (2015). Spectral evidence for hydrated salts in recurring slope lineae on Mars. *Nat. Geosci*. **8**, 828–832.

Okeke, B.C., Giblin, T. & Frankenberger, W.T., Jr. (2002). Reduction of perchlorate and nitrate by salt tolerant bacteria. *Environ. Pollut*. **118**, 357–363.

Oren, A., Bardavid, R.E. & Mana, L. (2014). Perchlorate and halophilic prokaryotes: implications for possible halophilic life on Mars. *Extremophiles* **18**, 75–80.

Porazka, T., Kilmer, B.R., Wichita High School Northwest Team, Wichita Northeast Magnet High School Team & Schneegurt, M.A. (2011). Inland oligohaline soils as a habitat for culturable halotolerant bacteria. In *143rd Annual Meeting of the Kansas Academy of Science. Trans. KS Acad. Sci*. **115**, 170.

Puelo, J.R., Fields, N.D., Bergstrom, S.L., Oxborrow, G.S., Stabekis, P.D. & Koukol, R. (1977). Microbiological profiles of the Viking spacecraft. *Appl. Environ. Microbiol*. **33**, 379–384.

Rajagopalan, S., Anderson, T.A., Fahlquist, L., Rainwater, K.A., Ridley, M. & Jackson, W.A. (2006). Widespread presence of naturally occurring perchlorate in high plains of Texas and New Mexico. *Environ. Sci. Technol*. **40**, 3156–3162.

Rajagopalan, S., Anderson, T., Cox, S., Harvey, G., Cheng, Q. & Jackson, W.A. (2009). Perchlorate in wet deposition across North America. *Environ. Sci. Technol*. **43**, 616–622.

Rao, B., Anderson, T.A., Orris, G.J., Rainwater, K.A., Rajagopalan, S., Sandvig, R.M., Scanlon, B.R., Stonestrom, D.A., Walvoord, M.A. & Jackson, W.A. (2007). Widespread natural perchlorate in unsaturated zones in the Southwest United States. *Environ. Sci. Technol*. **41**, 4522–4528.

Shcherbakova, V., Oshurkova, V. & Yoshimura, Y. (2015). The effects of perchlorates on the permafrost methanogens: implication for autotrophic life on Mars. *Microorganisms* **3**, 518–534.

Shete, A., Mukhopadhyaya, P.N., Acharya, A., Aich, B.A., Joshi, S. & Gholem, V.S. (2008). Aerobic reduction of perchlorate by bacteria isolated in Kerala, South India. *J. Appl. Genet*. **49**, 425–431.

Smith, P.N., Yu, L., McMurry, S.T. & Anderson, T.A. (2004). Perchlorate in water, soil, vegetation, and rodents collected from the Las Vegas Wash, Nevada, USA. *Environ. Pollut*. **132**, 121–127.

Stieglmeier, E., Wirth, R., Kminek, G. & Moissl-Eichinger, C. (2009). Cultivation of anaerobic and facultatively anaerobic bacteria from spacecraft-associated clean rooms. *Appl. Environ. Microbiol*. **75**, 3483–3491.

Thombre, R.S., Oke, R.S., Dhar, S. & Shouche, Y. (2015). Survival of haloarchaea in high concentration of perchlorate – Significant requirement for survival on Mars. In *The Astrobiology Science Conf.*, Chicago. Abstract No. 7080.

Urbansky, E.T. (1998). Perchlorate chemistry: implications for analysis and remediation. *Bioremed. J*. **2**, 81–95.

Urbansky, E.T. (2002). Perchlorate as an environmental contaminant. *Environ. Sci. Pollut. Res*. **9**, 187–192.

Venkateswaran, K., Satomi, M., Chung, R., Koukol, R., Basic, C. & White, D. (2001). Molecular microbial diversity of a spacecraft assembly facility. *Syst. Appl. Microbiol*. **24**, 311–320.

Venkateswaran, K., Kempf, M., Chen, F., Satomi, M., Nicholson, W. & Kern, R. (2003a). *Bacillus nealsonii* sp. nov., isolated from a spacecraft assembly facility, whose spores are gamma-radiation resistant. *Int. J. Syst. Evol. Microbiol*. **53**, 165–172.

Venkateswaran, K., Hattori, N., La Duc, M.T. & Kern, R. (2003b). ATP as a biomarker of viable microorganisms in clean-room facilities. *J. Microbiol. Methods* **52**, 367–377.

Wallace, W., Ward, T., Breen, A. & Attaway, H. (1996). Identification of an anaerobic bacterium which reduces perchlorate and chlorate as *Wolinella succinogenes*. *J. Ind. Microbiol*. **16**, 68–72.

Wallace, W., Beshear, S., Williams, D., Hospadar, S. & Owens, M. (1998). Perchlorate reduction by a mixed culture in an up-flow anaerobic fixed bed reactor. *J. Ind. Microbiol. Biotechnol*. **20**, 126–131.

Zorzano, M.-P., Mateo-Martí, E., Prieto-Ballesteros, O., Osuna, S. & Renno, N. (2009). Stability of liquid saline water on present day Mars. *Geophys. Res. Lett*. **36**, L20201.