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Aqueous extracts of a Mars analogue regolith that mimics the Phoenix landing site do not inhibit spore germination or growth of model spacecraft contaminants *Bacillus subtilis* 168 and *Bacillus pumilus* SAFR-032

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ABSTRACT

Because Mars is a primary target for life detection and habitability assessment missions, its exploration is also by necessity a Planetary Protection issue. The recent finding of significant levels of perchlorate (ClO_4^-) in regolith sampled from the Phoenix landing site raises the question of its potential biotoxicity to putative indigenous martian life, microbial forward contaminants from Earth, or future human visitors. To address this issue, an analogue regolith was constructed based on regolith chemistry data from the Phoenix landing site. A Mars Aqueous Regolith Extract (MARE) was prepared from the Phoenix analogue regolith and analyzed by ion chromatography. The MARE contained (mg/L) the cations Na^+ (1411 ± 181), Mg^{2+} (1051 ± 160), Ca^{2+} (832 ± 125), and K^+ (261 ± 29), and the anions SO_4^{2-} (5911 ± 993), ClO_4^- (5316 ± 1767), Cl^- (171 ± 25) and F^- (2.0 ± 0.4). Nitrogen-containing species NO_3^- (773 ± 113) and NO_2^- (6.9 ± 2.3) were also present as a result of regolith preparation procedures, but their relevance to Mars is at present unknown. The MARE was tested for potential toxic effects on two model spacecraft contaminants, the spore-forming bacteria *Bacillus subtilis* strain 168 and *Bacillus pumilus* strain SAFR-032. In *B. subtilis*, spore germination and initial vegetative growth (up to ~5 h) was not inhibited in a rich complex medium prepared with the MARE, but growth after 5 h was significantly suppressed in medium prepared using the MARE. Both *B. subtilis* and *B. pumilus* exhibited significantly higher rates of spore germination and growth in the MARE vs. DW with no additions (likely due to endogenous spore nutrients), but germination and growth was further stimulated by addition of glucose and a combination of buffered inorganic salts (K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, and $MgSO_4$). The data indicate that the aqueous environment in the regolith from the Phoenix landing site containing high levels of perchlorate does not pose a significant barrier to growth of putative forward contaminants such as *B. subtilis* and *B. pumilus* under Earth laboratory conditions.

1. Introduction

The search for conditions of habitability on Mars was given a boost in 2008 when the Phoenix lander performed a series of regolith and water chemistry experiments at its Vastitas Borealis landing site in the martian north polar region (68.22°N 125.75°W). Water ice, potential nutrients, and compounds capable of supporting oxidation–reduction reactions (i.e., redox couples) were discovered at the site (Boynton et al., 2009; Hecht et al., 2009; Sutter et al., 2012). However, high levels of perchlorate ion (ClO_4^-; up to 1.5 wt.%) were also discovered in the Phoenix regolith samples (Hecht et al., 2009). It was speculated that the localized distribution of perchlorate salts in the Phoenix regolith trench could have resulted from its interaction with liquid water films during downward percolation (Cull et al., 2010). Because liquid water is a prerequisite for life, an important issue both for Mars life detection and for Planetary Protection is whether wetted regolith around the Phoenix landing site would present a beneficial or a toxic environment for microorganisms.

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Perchlorate ion (ClO_4^-) is widely found as a soil and groundwater contaminant on Earth, mostly from the manufacture and use of solid rocket fuels, fireworks, flares, automotive airbags, etc. Perchlorate can also be formed naturally as a result of direct photochemical or ozone-mediated oxidation of chloride (Cl^-) in the atmosphere (Miller et al., 2006; Catling et al., 2010; Kounaves et al., 2010) or by photooxidation of Cl^- by oxide minerals in aqueous solution (Schuttlefield et al., 2011). In humans, perchlorate inhibits iodine uptake by the thyroid gland, and from the 1950s to 1960s was used medically at concentrations of 70–300 ppm in treatment of hyperthyroidism; since that time, however, it has been replaced by other drugs (National Research Council, 2005). Perchlorate in the ppb range has been identified as a contaminant in food and drinking water, but the evidence that it is harmful to humans at ppb levels is currently the subject of debate (National Research Council, 2005).

In contrast to the situation in humans, perchlorate is much more benign to microbes. At least 40 distinct species of bacteria have been identified that can reduce perchlorate sequentially via chlorate and chlorite to chloride, and such bacteria have been considered as good candidates for bioremediation of perchlorate-contaminated sites (Wallace et al., 1996; Coates et al., 1999; Coates and Achenbach, 2004). It has even been suggested that microbes in martian regolith environments might actually take advantage of perchlorate and reduced iron as a redox couple from which energy could be derived (Stoker et al., 2010). Thus it is possible that areas such as the Phoenix landing site could contain three important prerequisites for life: liquid water, nutrients, and an energy source.

The potential habitability of the Phoenix landing site makes it interesting as a location for life detection experiments, but at the same time makes it a location with enhanced risk for forward contamination. Bacterial spores are common contaminants of spacecraft and are notoriously resistant to spacecraft disinfection treatments. Thus, spores are considered a Planetary Protection concern, as the potential exists for spore-forming bacteria to be viable forward contaminants of martian surface or near-subsurface environments (reviewed in Nicholson et al., 2009; Schuerger, 2004). Most perchlorate-reducing bacteria have been taxonomically identified as belonging to the Gram-negative *Proteobacteria* (Coates et al., 1999), but at least 2 examples of perchlorate-reducing Gram-positive spore-forming bacteria have been identified: *Moorella* (formerly *Clostridium*) *perchloratireducens* (Balk et al., 2008) and *Sporomusa* sp. (Balk et al., 2010).

The spore-forming bacteria *Bacillus subtilis* strain 168 and *Bacillus pumilus* strain SAFR-032 have commonly been used as model surrogate organisms in Planetary Protection research (Schuerger et al., 2006) and in testing of spacecraft disinfection efficacy (Link et al., 2004; Kempf et al., 2005; Nicholson et al., 2009). In a previous communication (Schuerger et al., 2012, in preparation), air-dried *B. subtilis* spores and *Enterococcus faecalis* cells were exposed directly to a Mars regolith simulant, fashioned using data obtained at the Phoenix landing site, under simulated Mars environmental conditions of temperature, atmospheric composition and pressure, and solar UV flux. It was observed that the Phoenix regolith simulant exerted only mild biotoxic effects on spore or cell viability in the dry state (Schuerger et al., 2012, in preparation). However, in order to be a significant Planetary Protection risk a potential forward contaminant would actually have to proliferate in its new location (Nicholson et al., 2009) – i.e., must be able to grow in a liquid water environment containing the soluble ions leached from the surrounding Mars regolith. Therefore, in this communication we report the results of experiments testing the potential biotoxic effects of an aqueous leachate from a Mars analogue regolith modeled after the Phoenix landing site (Boynton et al., 2009; Hecht et al., 2009; Schuerger et al., 2012, in preparation), using the model spacecraft contaminants *B. subtilis* 168 and *B. pumilus* SAFR-032.

2. Materials and methods

2.1. Preparation of a Mars Aqueous Regolith Extract (MARE)

A Mars regolith simulant closely resembling the weakly alkaline/perchlorate regolith characterized from the Phoenix landing site was prepared as described in detail previously (Schuerger et al., 2012, in preparation). The Phoenix analog regolith was composed of anhydrite ($CaSO_4$; 2.0 wt.%), basalt (MSL-1 basalt from Duluth, MN; 63 wt.%), calcium carbonate ($CaCO_3$; 4 wt.%), ferrihydrite [np-Ox; synthesized from ferric nitrate according to Schwertmann and Cornell (1991); 12.0 wt.%], hematite (Fe_2O_3; 0.5 wt.%), kieserite ($MgSO_4{\cdot}H_2O$; 2.0 wt.%), Ti-magnetite [$(Fe^{2+}(Fe^{3+},Ti^{4+})_3O_4$); 2.25 wt.%], magnesite ($MgCO_3$; 2.0 wt.%), olivine [$((Mg,Fe)_2SiO_4$); 9.75 wt.%], pyroxene ($CaAlSi_2O_6$; 8.75 wt.%), and sodium perchlorate ($NaClO_4{\cdot}H_2O$; 1.5 wt.%).

To prepare the Mars Aqueous Regolith Extract (MARE) used in this work, 50 g of Mars regolith simulant (Schuerger et al., 2012, in preparation) were placed in a 250 ml baffled flask and mixed with 100 ml of sterile deionized (18 MΩ) water. The regolith suspension was then vigorously mixed at 150 rpm for 2 h on a rotary shaker. To completely remove regolith particles >0.22 μm in diameter from the extract, the entire regolith/water slurry was filtered through Whatman No. 4 paper (Whatman, Inc., Clifton, NJ, USA), then through a 0.45 μm polyethersulfone membrane filter (Whatman), and finally through a 0.22 μm polyethersulfone membrane filter (Whatman) into a sterile container. The MARE liquids were stored frozen at −20 °C until used.

2.2. Analyses of the MARE

Electrical conductivity and pH of the MARE working solutions were measured using a portable Orion 3 Star conductivity meter (model 1214000, Thermo Scientific, Inc., Beverly, MA, USA) and a portable Oakton pH meter (model PD-300, Oakton Instruments, Vernon Hills, IL, USA), respectively. Ion chromatography (IC) analysis of the MARE was performed on a dual Dionex ICS-2100 system (Dionex, Sunnyvale, CA, USA), configured to simultaneously analyze anions and cations. The system was equipped with a conductivity cell (DS6), vacuum degasser, column heater, eluant generator, and self-regenerating suppressor (Dionex ASRA 300 4 mm and CSRS 300 4 mm). IC was performed using a modification of EPA Method 300.1 (Hautman and Munch, 1997). Separation was achieved on a Dionex IonPac AS18 and IonPac CS12A column (4 × 250 mm) in isocratic mode using 32 mM potassium hydroxide and 20 mM methanesulfonic acid, both with a flow rate of 1 mL/min and a column temperature of 35 °C. Samples (25 μL injection loop) were introduced to the column by an autosampler (Dionex AS-DV) using 5-mL sample vials. Certified anion and cation standards (Inorganic Ventures, Inc., Christiansburg, VA, USA) were used for calibration. The instrument was further calibrated to detect and quantify perchlorate ion using sodium perchlorate (Sigma–Aldrich). Under the IC conditions used, perchlorate eluted as a broad peak centered at 70.9 min.

2.3. Preparation of growth media

Luria–Bertani (LB) medium (Miller, 1972) was used for growth and maintenance of bacterial cultures. For production of spores, Schaeffer Sporulation Medium (SSM) (Schaeffer et al., 1965) was used. As a minimal defined medium, Spizizen Minimal Medium (SMM) (Spizizen, 1958) to which tryptophan (50 μg/ml final concentration) was used. Spizizen salts (10× stock solution) consisted of (g/100 mL): K_2HPO_4 (14.0), KH_2PO_4 (6.0), $(NH_4)_2SO_4$ (2.0), sodium citrate·$2H_2O$ (1.0), and $MgSO_4{\cdot}7H_2O$ (0.2), pH 7.0. Spizizen

salts were used at 0.1× working concentration, because use of higher concentrations in the MARE resulted in formation of an insoluble precipitate consisting presumably of $MgPO_4$ and $CaPO_4$. Media prepared with DW were sterilized in an autoclave before use, however all liquid solutions prepared in which the MARE replaced DW were filter-sterilized (0.45-μm filter) prior to use.

2.4. Bacterial strains tested and preparation of spores

The bacteria used were *B. subtilis* strain 168 (*trpC2*) and *B. pumilus* strain SAFR-032 from the corresponding author's strain collection. Both strains have been described in detail elsewhere (Venkateswaran et al., 2001; Zeigler et al., 2008). Spores were obtained by cultivation of cells under vigorous aeration in liquid SSM and purified by lysozyme treatment and buffer washing as described previously (Nicholson and Setlow, 1990). Spore preparations consisted of single spores with no detectable clumps, and were free (>99%) of growing vegetative cells, germinated spores and cell debris, as seen in the phase-contrast microscope.

2.5. Germination and growth of spores in the MARE

All liquid cultures were incubated with aeration. *B. subtilis* and *B. pumilus* were cultivated at 37 °C and 30 °C respectively. Growth was monitored by optical density (OD) measurements either at 660 nm in a spectrophotometer or at 620 nm in a microtiter plate reader. Viable cell counts were determined by performing serial tenfold dilutions of cells in phosphate-buffered saline (PBS; 10 mM potassium phosphate, 150 mM NaCl, pH 7.2), plating aliquots of dilutions on LB plates and counting the resulting colonies after overnight incubation at the appropriate temperature (Nicholson and Setlow, 1990).

2.6. Statistical analyses

Unless otherwise indicated, data points represent the averages and standard deviations of triplicate determinations. Basic statistical parameters and Analyses of Variance (ANOVA) were computed using commercial statistical software (Kaleidagraph version 3.6.2, Synergy Software, Reading, PA, USA). Differences with P values <0.05 were considered statistically significant.

3. Results

3.1. Composition of the MARE

From the published elemental analysis of regolith from the Phoenix landing site (Boynton et al., 2009; Hecht et al., 2009), a high-fidelity Phoenix landing site Mars regolith simulant was constructed (Schuerger et al., 2012, in preparation) and was used to prepare the MARE. The pH and electrical conductivity of the MARE were on average 6.1 and 11.7 mS cm^{-1}, respectively, (n = 5 replicates). The lower pH of the MARE extract compared to the Phoenix solution chemistry was attributed to reaction time (i.e., kinetics) of the dissolution of the carbonates in the MARE and the dilution factor differences between the MARE regolith extraction (2:1 solution:regolith) vs. the Phoenix Wet Chemistry Laboratory extraction (approx. 25:1). Longer reaction times (i.e., stirring of the MARE regolith and solution) and a 25:1 dilution (similar to the Phoenix experiment) resulted in a pH of over 8 (data not shown). The electrical conductivity of the MARE solution is considerable higher than the Phoenix solutions; however, this is attributed to the dilution factors between the two extractions. A 25:1 solution:regolith extraction of the MARE regolith resulted in an EC of 1685 μS cm^{-1}, which is well within the error of the Phoenix measurement (1900 μS cm^{-1} ±50%) (Hecht et al., 2009). Cations detected in the MARE consisted of sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) (Fig. 1A). Anions detected included fluoride (F^-), chloride (Cl^-), nitrate (NO_3^-), nitrite (NO_2^-), sulfate (SO_4^{2-}), and perchlorate (ClO_4^-). The concentration of perchlorate ion in the leachate was determined to be 5316 mg/L (ppm), indicating that ~87% of the perchlorate had been leached from the Mars regolith simulant into the MARE.

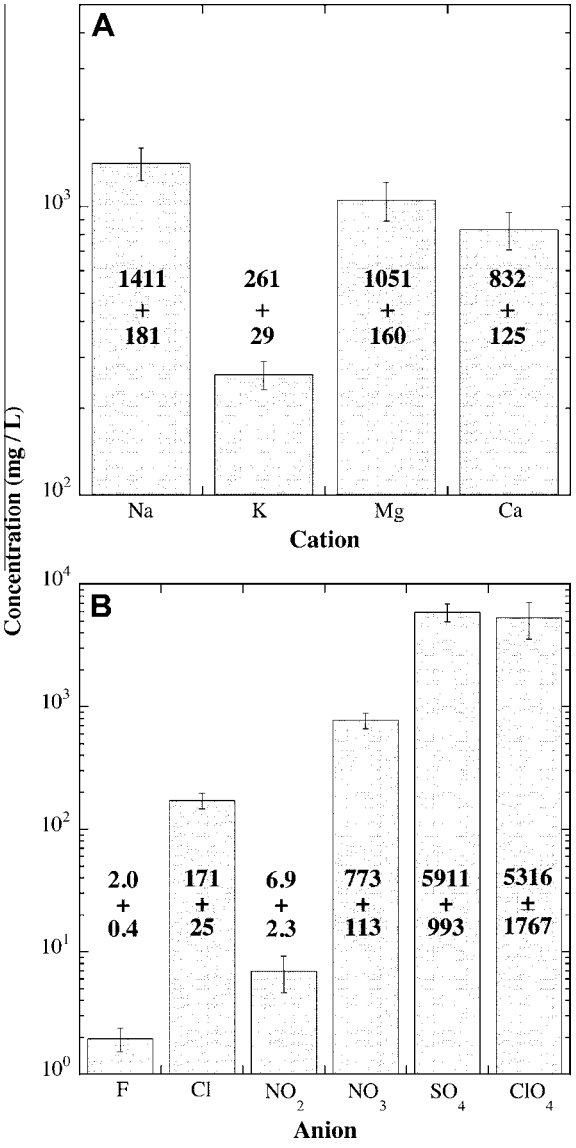

Fig. 1. Concentrations of cations (A) and anions (B) measured in the MARE by IC. Concentrations are averages and standard deviations of triplicate determinations.

3.2. Spore germination and outgrowth in rich complex media

Schuerger et al. (2012, in preparation) demonstrated that a Mars analog regolith prepared from the regolith composition measured at the Phoenix landing site was rather benign to *B. subtilis* strain HA101 spores exposed in the dry state. We were interested in determining if a complex medium, containing all nutrients needed for growth but prepared using the MARE vs. DW, exerted inhibitory or toxic effects on the germination of *B. subtilis* 168 spores or their subsequent vegetative growth. To test this, LB liquid medium was prepared with either DW or MARE. The two broths

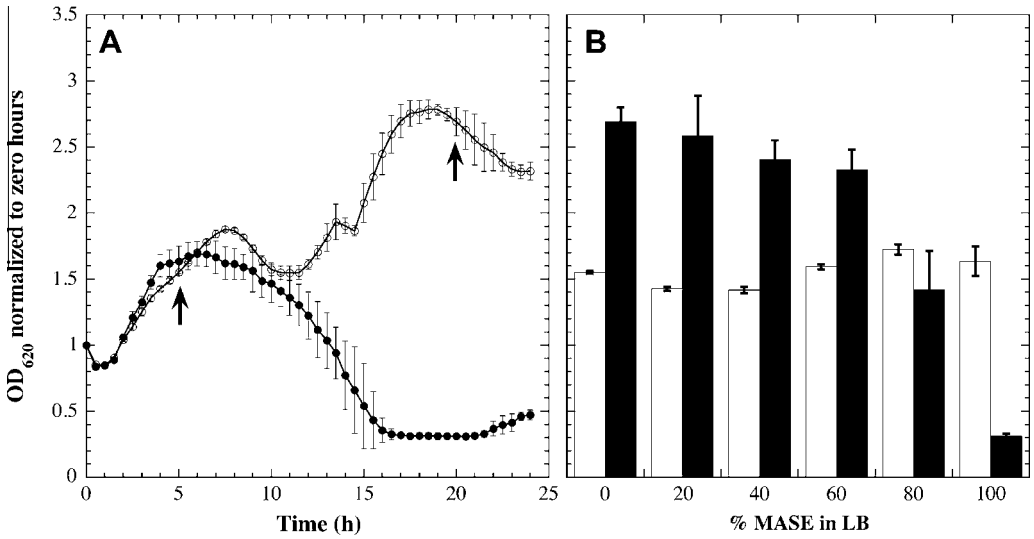

Fig. 2. (A) Spore germination and vegetative growth of *B. subtilis* 168 in LB prepared with DW (open circles) or MARE (filled circles). Vertical arrows at 5 h and 20 h denote times of OD_{620} measurements in panel B. (B) Growth of *B. subtilis* 168 in LB prepared with DW to which MARE had been added at 0, 20, 40, 60, 80, or 100%. Growth was measured at 5 h (open bars) and 20 h (filled bars) post-germination (see panel A). All OD_{620} values were normalized to the OD_{620} of each culture at time zero. Data are represented as averages and standard deviations of triplicate determinations.

were then inoculated with an equal number of spores, and spore germination and growth was measured by OD_{620}. Spore germination, as measured by initial drop in OD_{620}, and subsequent vegetative growth were essentially identical in both cultures up to ~5 h (Fig. 2A). The OD_{620} of *B. subtilis* cells in LB + DW declined slightly between ~5 and 10 h, then a second round of growth occurred until ~20 h, after which OD_{620} declined (Fig. 2A). In sharp contrast, the OD_{620} of the parallel culture incubated in LB + MARE declined dramatically after ~5 h and had not recovered by 24 h (Fig. 2A). It therefore appeared that the MARE did not exert a toxic effect on *B. subtilis* spore germination or vegetative cell growth up to ~5 h, but did exert a toxic effect during the late growth phase (Fig. 2A).

To further explore this phenomenon, a series of LB liquid media was prepared that contained mixtures of DW and MARE such that the final concentration of the MARE was 0%, 20%, 40%, 60%, 80%, or 100%. This series of media were inoculated with an equal number of *B. subtilis* 168 spores and spore germination and growth was again monitored. In this second series of experiments, all cultures germinated normally and grew to essentially the same OD_{620} by ~5 h (Fig. 2B), again indicating that the MARE was benign to germinating spores and vegetatively growing cells. As seen in the previous experiment (Fig. 2A), we again observed a second growth phase in *B. subtilis* cells cultivated in LB + DW, peaking at ~20 h (Fig. 2B). However, in LB cultures containing increasing concentrations of the MARE, this second growth phase was progressively less

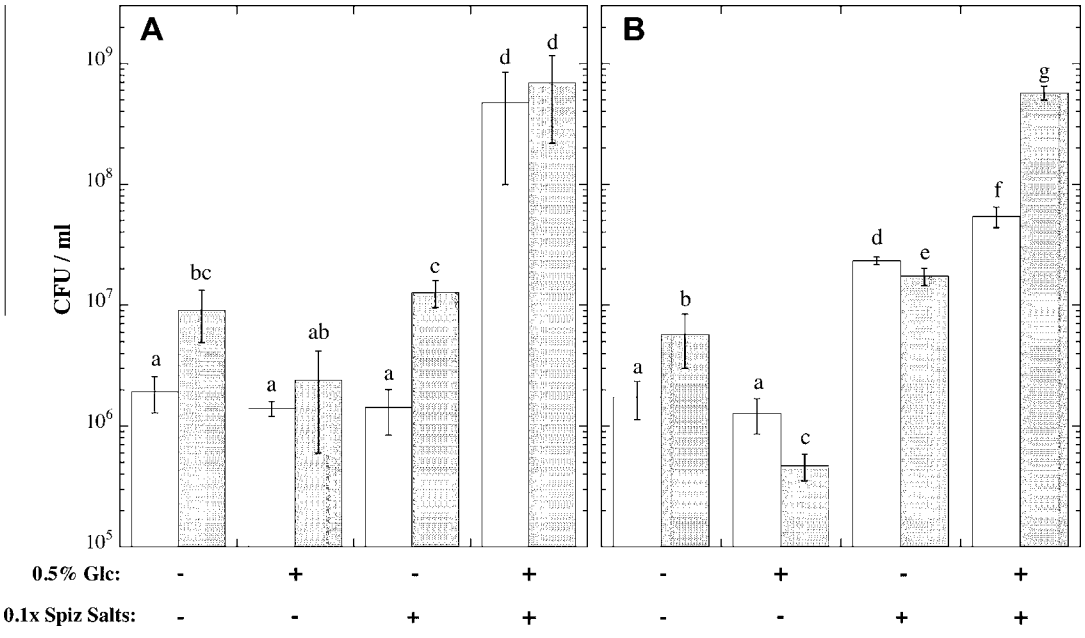

Fig. 3. Spore germination and growth of viable *B. subtilis* 168 (A) and *B. pumilus* SAFR-032 (B) after 72 h incubation in DW (open bars) or the MARE (shaded bars) containing (final concentrations): tryptophan (50 μg/ml in all cultures), glucose (Glc; 0.5%), or Spizizen (Spiz) salts (0.1×). Initial concentration of spores inoculated was 2×10^6/mL. Data are represented as averages and standard deviations of triplicate determinations. Lowercase letters above the bars denote significant differences (Analysis of Variance [ANOVA]; $P < 0.05$). CFU, colony-forming units.

pronounced until it was essentially absent in LB to which MARE had been added at 80% or 100% (Fig. 2B). This clear dose–response implied a toxic effect of the MARE specifically on late-growth-phase cultures.

3.3. Spore germination and growth in minimal media

The results from Fig. 2 indicated that spore germination and initial growth of *B. subtilis* in a rich, complete medium (LB) was not adversely affected by high perchlorate levels in the MARE. However, regolith from the Phoenix landing site does not contain complex organic molecules, and certainly not a rich mixture of nutrients as found in LB medium. We were thus interested in asking two related questions: (i) can the MARE on its own support spore germination and growth? (ii) If not, what additional compound(s) is/are needed? To address these questions, spores of *B. subtilis* 168 or *B. pumilus* SAFR-032 were inoculated at an initial concentration of 2×10^6 spores/mL into either DW or MARE with no additions and viable counts of the cultures were determined after 72 h of incubation (Fig. 3). In both species, incubation in DW itself did not promote spore germination or growth, but incubation of spores in the MARE alone supported significantly increased germination and growth of cells than did incubation in DW alone (Fig. 3).

In the case of *B. subtilis* 168, addition of either glucose or Spizizen salts alone did not significantly stimulate growth above that seen in the MARE itself (Fig. 3A). However, addition of both glucose and Spizizen salts to cells stimulated growth to the same high level in both DW and the MARE (Fig. 3A). The response of *B. pumilus* SAFR-032 spores showed some distinct differences compared to *B. subtilis* 168 spores. In DW, SAFR-032 spores did not germinate or grow, and glucose alone did not stimulate germination/growth (Fig. 3B). However, germination and growth in DW was significantly stimulated simply by addition of Spizizen salts alone, and growth was only modestly better in DW containing both additions (Fig. 3B). In the MARE, it appeared that glucose added singly actually lowered germination/growth of *B. pumilus* spores, and that addition of Spizizen salts singly significantly stimulated growth in both DW and MARE (Fig. 3B). Addition of both glucose and Spizizen salts stimulated growth only slightly above that seen by addition of Spizizen salts alone in DW, but the stimulation of growth by both additions in MARE was much more dramatic (Fig. 3B).

4. Discussion

A previous survey of the composition of known martian regoliths from several lander sites and rover traverses indicated that these regoliths might contain a potentially formidable mixture of biotoxic factors such as extremes of pH, heavy metals, or oxidants such as perchlorate (Schuerger et al., 2012, in preparation). To test this hypothesis, Schuerger et al. (2012, in preparation) placed dried *B. subtilis* spores or *E. faecalis* cells in contact with Mars analogue regoliths representing six different martian regolith geochemistries and showed that these regoliths were relatively benign to microbes in the dried state (Schuerger et al., 2012, in preparation). However, in any particular martian regolith, putative indigenous microbes or terrestrial forward contaminants would have to survive and grow in the presence of liquid water containing the soluble components of that regolith. In this communication we first tested the hypothesis that an aqueous Mars Analogue Regolith Extract (MARE), developed from the regolith composition data of the Phoenix landing site (Schuerger et al., 2012, in preparation), would exert biotoxic effects on the spore germination and/or growth of the model spacecraft contaminant, *B. subtilis* strain 168. We found that *B. subtilis* spores germinated and grew well in rich LB liquid medium prepared using the MARE for ~5 h, but after this time OD_{620} declined, in contrast to continued growth in LB prepared with DW (Fig. 2A). Furthermore, a distinct dose–response of late-growth-phase decline in LB prepared with MARE was observed (Fig. 2B), strongly indicating that some presently unidentified component(s) of the MARE inhibited growth, or caused lysis, in *B. subtilis* cultivated longer than 5 h in LB.

Second, we tested the hypothesis that the MARE itself contained sufficient nutrients to support spore germination and growth of model spacecraft contaminants *B. subtilis* or *B. pumilus*. The MARE solution itself, without added glucose or Spizizen salts, was capable of supporting spore germination and growth to a small but statistically significant extent over DW in both *B. subtilis* 168 (~4.7-fold) (Fig. 3A) and *B. pumilus* SAFR-032 (~3.3-fold) (Fig. 3B). It therefore appeared that the MARE contained at least small amounts of elements or compounds capable of triggering spore germination and supporting subsequent growth.

What might be the nature of these elements or compounds? Bacterial cells such as the *Bacillus* species we tested contain ~70% water, and the dried cell fraction consists of [all values are approximate] C (50%); O (20%); N (14%); H (8%); P (3%); K (2%); S (1%); Fe (0.2%); Ca, Mg, and Cl (0.05% each); and a total of 0.3% trace elements such as Mn, Co, Cu, Zn, and Mo (Neidhardt et al., 1990; Madigan et al., 2012). From inspection of the mineral and salt composition of the Mars analogue regolith prepared from the data collected at the Phoenix landing site (Schuerger et al., 2012, in preparation) and the chemical composition of the basalt used as the regolith base (Eick et al., 1996), the Mars regolith simulant used in this study contained all the "macronutrient elements" (C, O, H, P, S, Fe, K, Ca, Mg, and Cl) except N. The macronutrient elements found in the basalt component occur as oxides (FeO, Fe_2O_3, MgO, CaO, K_2O, and P_2O_5) (Eick et al., 1996), and elements in the added salts are in the form of sulfates ($MgSO_4$, $CaSO_4$), carbonates ($MgCO_3$, $CaCO_3$), and sodium perchlorate ($NaClO_4$) (Schuerger et al., 2012, in preparation). Thus it might be expected that the corresponding cation and anion species would be leached into the MARE during its preparation. Indeed, IC analysis of the MARE revealed the cations Na^+, K^+, Ca^{2+}, and Mg^{2+} (Fig. 1A), but not Fe^{2+} or Fe^{3+}. Although high levels of iron were present in the analogue regolith, $Fe^{2+/3+}$ was not detected by IC, likely due to its extremely low solubility in aerobic water at neutral pH (Schwertmann, 1991). In aerobic terrestrial ecosystems microbial growth is often limited by the availability of Fe (Dhungana et al., 2007), and many bacteria including *B. subtilis* (Grossman et al., 1993) and *B. pumilus* (Gioia et al., 2007) produce iron-scavenging compounds called siderophores in order to obtain sufficient iron from these environments. In addition, microbes such as *B. subtilis* growing in terrestrial soils are in close contact with soil particle surfaces (Nicholson and Law, 1999), in locations advantageous for the liberation and chelation of iron.

As expected, IC analysis detected high concentrations of the anion species sulfate (SO_4^{2-}) (Fig. 1B), which is readily utilized by microbes. In addition to the expected perchlorate (ClO_4^-), IC analysis also revealed substantial amounts of Cl^- in the MARE (Fig. 1B). This is likely due to abiotic reduction of ClO_4^- to Cl^- during aqueous extraction of the Mars analog regolith, which has been shown to occur at neutral pH when ClO_4^--containing water is placed in contact with solid-phase iron in batch and column reactors (Moore et al., 2003). In addition, a very minor amount of F^- ion was also detected by IC (Fig. 1B), likely originating from the basalt, which contains ~0.05% F (Seraphim, 1951).

Among the major macronutrients, C, N, and P comprise roughly two-thirds of the microbial cell. Thus it is important to attempt to trace the sources of these elements in Mars analogue regolith and within the MARE itself.

4.1. Carbon

Carbon is the major component of bacterial cells, and considerable theoretical and practical research has centered on trying to (i) elucidate the martian carbon cycle and (ii) identify potential sources of C in the martian environment (reviewed in Grady and Wright, 2006). The presence of organic sources of carbon on Mars has been the subject of much debate since the Viking organic detection experiments. Using the technique of Thermal Volatilization–Gas Chromatography–Mass Spectrometry (TV–GC–MS), it was originally reported that the Viking 1 and 2 landers had failed to detect organic carbon in martian regoliths, although the landers did detect chloromethane (CH_3Cl) and dichloromethane (CH_2Cl_2) which the researchers at the time deemed to be terrestrial contaminant compounds (Biemann et al., 1976, 1977). More recently, spurred by the discovery of perchlorate at the Phoenix landing site (Hecht et al., 2009), Navarro-González et al. (2010) showed that adding magnesium perchlorate to Atacama desert soil containing organic carbon and heating to 500 °C evolved water, CO_2, CH_3Cl and CH_2Cl_2, suggesting that the regolith at the Viking landing sites indeed contained organic carbon. One source of C on Mars is the CO_2-rich martian atmosphere, but neither *B. subtilis* nor *B. pumilus* are capable of CO_2 fixation, and we did not conduct any of our experiments under a CO_2 atmosphere. In the Mars analogue regolith used, C is present as carbonate (CO_3^{2-}) (Schuerger et al., 2012, in preparation). However, at present it is unknown whether *B. subtilis* or *B. pumilus* can utilize carbonate as a C source.

4.2. Nitrogen

Although the Mars analogue regolith was lacking in N, surprisingly IC analysis revealed that the MARE contained a substantial amount of nitrate (NO_3^-) and lesser amount of nitrite (NO_2^-) (Fig. 1B). A likely origin of nitrate and nitrate found in the MARE was from the ferrihydrite added to the Mars analogue regolith (Schuerger et al., 2012, in preparation), which was prepared from ferric nitrate (Schwertmann and Cornell, 1991); some nitrite was likely produced by contact of nitrate with iron, and the subsequent nitrate and nitrite were probably incompletely removed from the ferrihydrite preparation during the final wash phase. Nitrate and nitrite are good N sources for bacterial growth, and likely contributed to the ability of *B. subtilis* and *B. pumilus* to grow in the MARE, but are they relevant to actual regoliths on Mars? At this time, the presence or quantity of biologically relevant N-containing compounds in martian regoliths is unknown, as no instrument capable of measuring them has ever been included on a Mars mission. This issue has been deemed of high scientific importance by members of the Mars research community, and a considerable literature has accumulated speculating that regolith nitrate and nitrite could comprise a major component of a global martian nitrogen cycle sufficient to support a martian subsurface biosphere (for extensive analyses, see Boxe et al., 2012; Mancinelli and Banin, 2003; Manning et al., 2008; Summers et al., 2012). [Note: The Sample Analysis at Mars (SAM) instrument carried on the Mars Science Laboratory (MSL) rover is capable of detecting and quantifying nitrogen, and is scheduled for landing at Mars on 6 August 2012].

4.3. Phosphorus

In terrestrial aquatic ecosystems, microbial growth is often limited by availability of the macronutrient P, usually encountered in the form of phosphate (PO_4^{3-}) (Elser et al., 1995). Phosphorus was not reported in the regolith analyses conducted at the Phoenix landing site, but the basalt component of the Mars analogue regolith does contain P as P_2O_5 (Eick et al., 1996), which reacts readily with liquid water to produce PO_4^{3-} (Van Wazer, 1958). Phosphate was not detected in the MARE by IC; however, P was detected by the Alpha Particle X-ray Spectrometer carried on the MER rovers at both Meridiani Planum (Rieder et al., 2004) and Gusev Crater (Gellert et al., 2006), thus is present on Mars in measurable quantities.

Apart from the components of the MARE, C-, N-, or P-containing compounds could have originated from the spores themselves. Germinating *Bacillus* spp. spores leak significant amounts of peptides, amino acids, dipicolinic acid, and other small C- and N-containing molecules into the surrounding medium (Setlow et al., 2008). It has been shown that *B. subtilis* spores contain substantial amounts of PO_4^{3-} both in their spore coats and as glycerol phosphate teichoic acid in their cell walls (Boylen and Ensign, 1968), and furthermore that *B. subtilis* growing under conditions of PO_4^{3-} limitation can utilize their cell wall teichoic acid as a reserve source of PO_4^{3-} (Grant, 1979).

Recently, Stoker et al. (2010) modeled the habitability of Mars spacecraft landing sites using 19 factors conducive for the development of life that included the availability of water, nutrients, energy sources, and compatible environmental conditions. The Phoenix landing site was given a Habitability Index (*HI*) of 0.47, the highest of the six spacecraft landing sites considered. However the Stoker et al. (2010) habitability modeling did not consider potential biocidal factors that might persist at specific landing sites like the presence of oxidants (Yen et al., 2000) or heavy metals (Newsom and Hagerty, 1999). Perchlorates were considered by Stoker et al. (2010), but only as an electron acceptor for microbial respiration. Beaty et al. (2006) and Schuerger et al. (2012, in preparation) both discuss potential biocidal factors relative to microbial activity in Special Regions on Mars, and suggest that the biocidal factors may dominate some regions of the surface. Results presented here support the conclusion that the habitability of the martian surface at the Phoenix landing site should not be constrained by the presence of perchlorates in the regolith and that limitations to microbial growth may be due to other factors (e.g., lack of adequate reservoirs of organic compounds required for growth, intermittent desiccation of the regolith, or biocidal UV radiation).

Planetary Protection policies aim to minimize the transfer of Earth microbes and biological contaminants on outbound spacecraft destined for exploration targets with a potential for harboring life (for details, see COSPAR, 2011). Spacecraft destined for Mars are known to be contaminated with dormant spores of *Bacillus* spp. that have a high probability of surviving transit through space and deposition on the martian surface in a viable state (reviewed in Horneck et al., 2010; Nicholson et al., 2009; Schuerger, 2004). The results from the present study indicate that the regolith chemistry of the Phoenix landing site would likely prove rather benign to potential terrestrial spacecraft contaminants such as spores of *Bacillus* spp., and indeed may even support their germination and growth. Of course, regolith wet chemistry is only one of several factors limiting growth of terrestrial organisms in the Mars environment; potential forward contaminants would also have to contend with additional harsh environmental factors such as extreme cold, a low-pressure anoxic atmosphere, harsh solar radiation, and a severe lack of organic nutrients, to name but a few. Generalization of the conclusions from this preliminary study will need further support by testing other microorganisms, such as perchlorate-reducers and autotrophs, under environmental simulations more closely approximating Mars conditions. A future goal of our work is to test the ability of terrestrial microbes to survive and grow under progressively more accurate simulations of the Mars environment that incorporate the full complement of potentially biocidal factors.

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