Identification and Characterization of Early Mission Phase Microorganisms Residing on the Mars Science Laboratory and Assessment of Their Potential to Survive Mars-like Conditions

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Abstract

Planetary protection is governed by the Outer Space Treaty and includes the practice of protecting planetary bodies from contamination by Earth life. Although studies are constantly expanding our knowledge about life in extreme environments, it is still unclear what the probability is for terrestrial organisms to survive and grow on Mars. Having this knowledge is paramount to addressing whether microorganisms transported from Earth could negatively impact future space exploration. The objectives of this study were to identify cultivable microorganisms collected from the surface of the Mars Science Laboratory, to distinguish which of the cultivable microorganisms can utilize energy sources potentially available on Mars, and to determine the survival of the cultivable microorganisms upon exposure to physiological stresses present on the martian surface. Approximately 66% (237) of the 358 microorganisms identified are related to members of the *Bacillus* genus, although surprisingly, 22% of all isolates belong to non-spore-forming genera. A small number could grow by reduction of potential growth substrates found on Mars, such as perchlorate and sulfate, and many were resistant to desiccation and ultraviolet radiation (UVC). While most isolates either grew in media containing $\geq 10\%$ NaCl or at 4°C, many grew when multiple physiological stresses were applied. The study yields details about the microorganisms that inhabit the surfaces of spacecraft after microbial reduction measures, information that will help gauge whether microorganisms from Earth pose a forward contamination risk that could impact future planetary protection policy. Key Words: Planetary protection—Spore—Bioburden—MSL—Curiosity—Contamination—Mars. *Astrobiology* 17, 253–265.

1. Introduction

THE SEARCH FOR extraterrestrial life is bolstered by our long-standing quest to determine if we are alone in the Universe. Mars and Europa are two likely candidates to target in the search for extraterrestrial life, since both have carbon, potential energy sources, and water in some form (Malin and Edgett, 2000, 2003; McKay, 2004; Leshin, 2013; Ming *et al.*, 2014; Roth *et al.*, 2014). The focus to search for life on Mars is supported by recent findings that ancient environments on Mars could have supported microbial life (Grotzinger *et al.*, 2014). Despite the fact that present-day Mars is quite cold and dry, these conditions are thought to be analogous to

conditions on early Earth when single-celled life was gaining a foothold (Rasmussen *et al.*, 2009; Carr, 2012). Current explorations of Mars confirm that all the basic necessities for microbial life are present, but it remains unclear whether microorganisms that are metabolically capable of living on Mars can actually survive in the martian environment. The present-day martian surface presents a very inhospitable habitat for life because of the intense radiation, highly oxidizing conditions, concentrated evaporative salts, and extremely low water activity (Davila *et al.*, 2010). Since a diversity of microorganisms thrive in the most inhospitable habitats on Earth, it is not unreasonable to think that microorganisms could live on present-day Mars (Rummel *et al.*,

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2014). Determining whether microbes can survive exposure to conditions similar to those on the martian surface is vital to understanding whether microorganisms transported on spacecraft launched from Earth have the potential to contaminate Mars.

One of the primary issues hindering assessment of the likelihood of the forward contamination of Mars is that there is not enough experimental information accumulated about the survival of Earth microorganisms in the martian environment. Over the last decade, a number of studies have reported on the microorganisms that are detectable in spacecraft assembly facilities during prelaunch preparations despite the precautions that were taken to reduce the microbial load (La Duc *et al.*, 2004, 2007b; Kempf *et al.*, 2005; Benardini *et al.*, 2014). Previous studies testing survival of Earth microorganisms in simulated martian conditions have focused on spore-forming microorganisms, particularly bacteria of the *Bacillus* genus, since spores are hardy forms of terrestrial life that can survive Mars-like conditions (Horneck, 1993; Nicholson *et al.*, 2000; Riesenman and Nicholson, 2000; Schuerger *et al.*, 2003; Link *et al.*, 2004; Newcombe *et al.*, 2005; Tauscher *et al.*, 2006; Zenoff *et al.*, 2006; Fajardo-Cavazos *et al.*, 2010). Several studies have reported on the survival of spore-forming *Bacillus* species isolated from clean room facilities after exposure of microbes to specific Mars-like environmental conditions (Setlow, 2001, 2006; Nicholson *et al.*, 2002), but recent culture-independent analyses have shown that spore-formers are not the only types of microorganisms present in assembly and clean room facilities (Venkateswaran, 2001; La Duc *et al.*, 2007a, 2009, 2012; Moissl *et al.*, 2007, 2008; Vaishampayan *et al.*, 2010, 2013). Some studies have focused on testing survival of extremophilic microbes that have the metabolic capabilities to inhabit Mars (La Duc *et al.*, 2007a; Morozova *et al.*, 2007; Gómez *et al.*, 2010; Bauermeister *et al.*, 2014), while a lesser number of studies have addressed survival of non-extremophilic, non-spore-forming microbes exposed to Mars-like environmental conditions (La Duc *et al.*, 2007b; Osman *et al.*, 2008; Berry *et al.*, 2010). Few studies have investigated the isolates collected from spacecraft during mission preparations regarding their potential to contaminate Mars.

Protection of Mars is governed by international planetary protection policy, since microorganisms transported on the surface of spacecraft to Mars could hinder the search for past or present life on Mars. This policy restricts the number of microorganisms on a spacecraft's exposed surface areas, mated surface areas, and total encapsulated volume to a bio-burden level of less than or equal to 5×10^5 spores (Science Mission Directorate, 2011, NPR 8020.12D). During the preparation stages of a spacecraft such as the Mars Science Laboratory (MSL), a microbial sampling campaign is undertaken to verify the microbial bio-burden requirement throughout the mission buildup and testing phases. The microorganisms are collected during the campaign using the NASA Standard Assay (Benardini *et al.*, 2014). Isolates are heat shocked, plated on tryptic soy agar (TSA) at 32°C, enumerated, isolated, and preserved for future study. Although this does not take into account all microbial isolates present on the spacecraft, and leads to the enrichment of mostly spore-forming organisms, the preserved isolates do allow for the study of a selection of organisms residing on the spacecraft.

The goals of this study were to (1) identify microorganisms isolated and preserved from the surfaces of MSL and (2) investigate the potential of the microorganisms to survive specific physiological stresses and utilize energy sources available on Mars. The resulting information will be a step toward recognizing the potential threat of contamination of Mars by microorganisms transported on spacecraft.

2. Materials and Methods

2.1. Sample collection

Samples were collected and processed by the Jet Propulsion Laboratory (JPL) MSL Planetary Protection Implementation Team (Benardini *et al.*, 2014). Cotton swabs and polyester wipes were used to collect samples ($\sim 25 \text{ cm}^2$ and 1 m^2 , respectively) from exterior surfaces of MSL. Swabs were then placed into water and gently sonicated to liberate microorganisms from the swab. Wipes were then submersed in PP rinse solution (NASA, 2010) and gently sonicated to liberate microorganisms from the wipe. Both swab and wipe extracts were heat shocked at 80°C for 15 min in a water bath and a subsample plated onto TSA. Plates were incubated for 3 days at 32°C. Colonies from TSA plates were re-streaked for purity onto the same media, and pure isolates were stored at -80°C in tryptic soy broth (TSB) amended with 50% glycerol. The glycerol stocks (350) were shipped to the University of Idaho for further studies.

2.2. Identification of isolates

A sample from the glycerol stocks was streaked onto TSA and incubated for 24–72 h, until colonies were present, at 30°C. Resulting colonies were re-streaked to ensure purity before proceeding with identification of isolates. Once pure, a colony was inoculated into TSB and grown overnight in a shaking incubator at 30°C at 250 rpm. Cells from grown cultures were collected by centrifugation at 4000g for 7 min. Chromosomal DNA was extracted from cells with a Wizard SV 96 Genomic DNA Purification System (Promega, Madison, WI) or an Ultra Clean Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA). This method was only successful with cultures that did not form floating biofilms. For cultures where biofilm formation was an issue for DNA extraction using kits, the following method was used. Cell pellets were suspended in 250 μL of sterile dH_2O . Cells were lysed by using three rounds of a freeze-thaw method (15 min at -80°C followed by 15 min at 80°C). Silica beads (1.0 mm) were added to the cells, and the suspension was mixed by vortexing for 30 s. Cell debris was pelleted by centrifugation at 2576g for 3 min, and 10 μL of the resulting cell lysate was used for amplification of 16S rRNA using PCR.

For each isolate, the 16S rRNA gene was amplified with universal bacterial primers 8F and 1525R (Reysenbach *et al.*, 1994; Suzuki and Giovannoni, 1996), and PCR was performed as previously described (Smith *et al.*, 2009). Briefly, reactions were 50 μL , and template DNA was either cell lysate (10 μL) or chromosomal DNA (1 μL) to a final DNA concentration of approximately 200 ng. PCR reagents were added to each tube in the following volumes: 25 μL of Dream Green Taq 2X Master Mix (Fermentas-Thermo Scientific, Glen Burnie, MD), 2.5 μL of 12.5 μmol primer

8F, and 2.5 μL of 12.5 μmol primer 1525R; water was added to bring the final volume up to 50 μL after the addition of cell lysate or chromosomal DNA. PCR conditions were as follows: an initial denaturation step at 94°C for 5 min was followed by 32 cycles of 95°C for 1 min, 51.4°C for 1.5 min, and 72°C for 1.5 min. Completion of the 32 cycles was followed by a final elongation step at 72°C for 5 min. PCR-amplified fragments were purified with Exonuclease I, 10 U, and Antarctic Phosphatase, 2 U, per 5 μL of PCR product. The reaction was heated at 37°C for 15 min followed by heat inactivation of the enzymes at 80°C for 15 min.

Polymerase chain reaction products were sequenced with the primer 27F (Lane, 1991) and a commercial sequencing service (Macrogen, Rockville, MD). The partial 16S rRNA gene sequences were analyzed by the University of Idaho rRNA analysis pipeline, HiSTA, as previously described (De Gelder, 2005). Briefly, sequences of 500 bp or longer were analyzed with BLAST (Altschul *et al.*, 1997) to search for similarity among eubacterial type strains in the Ribosomal Database Project (RDP) (Cole *et al.*, 2003). The RDP sequence for the closest relative of each input sequence was determined based on the highest sequence similarity. From results of the initial identification, isolates with potentially unique 16S rRNA sequences, or one representative of isolates with identical 16S rRNA sequences, were further sequenced to obtain a more complete 16S rRNA gene sequence with primers 27F, 518F, and 1492R (Muyzer *et al.*, 1993; Frank *et al.*, 2008). Contiguous sequences were assembled with Vector NTI Advance 11 (Invitrogen, Carlsbad, CA), and assembled sequences were reanalyzed as described above. Sequences are deposited in the NCBI GenBank under accession numbers KX453857–KX454155.

2.3. Anaerobic growth studies

Pure isolates were inoculated into 1000 μL of TSB in 96 well plates. Plates were incubated at 250 rpm at 30°C for 24 h. Cells were pelleted by centrifugation at 5000g for 12 min, and the supernatant was discarded. Cell pellets were suspended in 260 μL of phosphate-buffered saline (PBS) and inoculated into modified ATCC #2106 medium, without acetate or sodium chlorate, in an anaerobic chamber (Table 1). The media was boiled, autoclaved, and then placed in an anaerobic

chamber for a minimum of 24 h prior to use. Sterile electron acceptor and carbon source concentrates were added to the medium to reach the desired concentrations prior to use. All tests were performed in triplicate. Growth with perchlorate, sulfate, or arsenate was determined by measuring turbidity at 600 nm at 0, 7, 14, 21, and 28 days. Growth of cultures with selenite or selenate was determined visually by the formation of a red precipitate (insoluble Se). Growth of cultures by the reduction of insoluble Fe(III) was determined visually by a color change of the solid-phase iron from brown to black.

2.4. Aerobic growth studies (temperature, pH, and NaCl)

Cells were grown overnight and suspended in PBS as described for the anaerobic studies. To study growth of isolates in medium containing NaCl, aliquots of cells were inoculated into TSB containing 0.5, 5, 10, or 20% (w/v) NaCl. To determine growth of isolates at alkaline pH, cells were inoculated into buffered TSB media at pH 7, 8, 9, 10, 11, and 12. Buffers (100 mM) used in the pH range of 7–10 were previously described by Nielsen *et al.* (1995), and buffers used at pH 11 and 12 were 1 M Na_2HPO_4 and 1 M KCl, respectively. Cells were grown at 30°C and turbidity monitored at 600 nm at 0, 1, 3, 7, and 14 days. To determine growth of isolates at low temperature, cells were inoculated into TSB, incubated at 4°C, and turbidity monitored at intervals of 0, 1, 3, 7, 14, and 28 days.

2.5. Desiccation studies

Isolates were inoculated into TSB in 96 well plates and incubated at 30°C overnight. Approximately 30 μL of cultures was added to 96-well flat-bottom plates, covered with a gas-permeable film, and placed in a biosafety cabinet overnight to allow evaporation of the medium to occur. The resulting plates were placed into a glass desiccation chamber containing silica gel desiccant for 14 days at room temperature ($\sim 20^\circ\text{C}$) at Rh <10%. After 14 days of desiccation, plates were removed, and cells were rehydrated with 200 μL of TSB. Initial turbidity readings were recorded immediately after rehydration and again at 24, 48, 72, and 96 h to determine culturability of rehydrated cells.

TABLE 1. COMBINATIONS OF ELECTRON DONORS AND ELECTRON ACCEPTORS USED TO TEST GROWTH OF THE MSL ISOLATES UNDER ANAEROBIC CONDITIONS

Media	Electron acceptor	Electron donor	Number of isolates showing growth
1	Sodium perchlorate (10 mM)	Acetate (20 mM)	2
2	Sodium perchlorate (10 mM)	Lactate (20 mM)	5
3	Sodium arsenate (10 mM)	Acetate (10 mM)	0
4	Sodium arsenate (10 mM)	Lactate (20 mM)	10
5	Sodium selenite (5 mM)	Acetate (20 mM)	0
6	Sodium selenite (5 mM)	Lactate (20 mM)	0
7	Sodium selenate (10 mM)	Acetate (10 mM)	0
8	Sodium selenate (10 mM)	Lactate (20 mM)	0
9	Sodium sulfate (50 mM)	Acetate (20 mM)	0
10	Sodium sulfate (50 mM)	Formate (20 mM)	2
11	Ferrihydrite (80 mM)	Acetate (20 mM)	0
12	Ferrihydrite (80 mM)	Lactate (20 mM)	0

2.6. Peroxide tolerance studies

Peroxide tolerance assays were performed by using a modified method of that described by Riesenman and Nicholson (2000). Cells were grown overnight in TSB and then transferred into PBS. Unopened bottles of hydrogen peroxide (30%) were purchased from the University of Idaho ChemStore on the day of use. Hydrogen peroxide was added to cell suspensions to a final concentration of 5%, and suspensions were incubated at room temperature with gentle mixing for 1 h. A 100 μ L aliquot of the suspension was removed to a new 96-well plate, and 900 μ L of bovine catalase (100 μ g/mL) was added to the sample. After incubation for 1 h, treated cells (10 μ L) were inoculated into 240 μ L of TSB, and turbidity was monitored at 0, 24, and 48 h. Catalase activity was confirmed by placing cells on microscope slides, adding 3% and 5% H₂O₂, and observing for the formation of O₂ bubbles (Lim *et al.*, 2004).

2.7. UVC exposure studies

Ultraviolet C exposure studies were performed by using a modified method previously described by Marshall *et al.*

(2003). All strains were grown in TSB and then plated onto TSA for UVC exposure studies. Single colonies of each strain were inoculated into 5 mL TSB and grown for 24 h at 30°C with shaking to a density of 10⁸ to 10⁹ CFU/mL prior to UVC treatment. Cultures were serially diluted in 10-fold steps to 10⁻⁵, and 10 μ L of each dilution from 10⁻⁵ to 10⁰ was spotted onto TSA in square grid plates. The spots were allowed to dry completely prior to UVC exposure, and control plates as well as treated plates were spotted in duplicate.

A UVP multiple-ray lamp (UVP, Upland, CA, P/N 95-0313-01) with a UVC bulb (Sankyo Denki G8T5) was utilized for all UVC treatments, and all treatments were conducted entirely in a dark room in order to minimize the effects of light-dependent repair mechanisms. Prior to treatment, the lamp was turned on and allowed to stabilize for approximately 20 min, after which lamp output was measured with a UVP MS-100 optical radiometer with a 254 nm sensor attached (P/N 97-0100-01 and 97-0099-01, respectively). Exposure time (in seconds) to achieve targeted fluence in J/m² was calculated by dividing target dose by the meter reading (mW/cm²) multiplied by 10 ($T = \text{target fluence} / [\text{meter} * 10]$). Plates were uncovered and exposed to UVC for the calculated exposure times to obtain

FIG. 1. Isolates identified by genus.

the targeted dose, immediately wrapped with aluminum foil, and incubated at 30°C in the dark for 24 h. Resulting colonies were counted, and the survival fraction was calculated by comparing control and treated plates. Each experiment for all doses was conducted on a minimum of three independent occasions.

3. Results

3.1. Identification

Though ~1100 isolates were collected from the MSL surfaces during the assembly and testing phases, only the 358 isolates collected from the JPL spacecraft assembly facility prior to May 2010 were used in this study. Of the 358 isolates, 339 were identified based on comparative sequence analyses of 16S rRNA genes. All were identified as bacteria, and the majority of the isolates were representatives of known spore-forming genera (78%), with most (85% of spore-formers) belonging to the *Bacillus* genus (Figs. 1 and 2). The most commonly identified *Bacillus* species was *B. pumilus* (16%), with *B. amyloliquefaciens* (11%) and *B. megaterium* (7%) comprising another 18% of the *Bacillus* population (Fig. 3). Overall, there were 26 different species represented within the *Bacillus* genus, with an additional 88 isolates that were not identifiable to the species level, due to partial sequence analysis. The remaining spore-forming isolates belonged to five genera: *Paenibacillus* (5%), *Brevibacillus* (1.5%), *Sporosarcina* (2%), *Oceanobacillus* (0.3%), and *Gracilibacillus* (0.3%). A number of microorganisms (22%) that were not related to spore-forming genera were identified within the pool of isolates. The most common non-spore-forming isolates comprising 10% of the entire microbial population belonged to the *Staphylococcus* genus, with seven different species represented (*S. aureus*, *S. capitis*, *S. epidermidis*, *S. hominis*, *S. lugdunensis*, *S. pasteurii*, and *S. warneri*). Of those identified to the *Staphylococcus* species level, more than half were identified as close relatives of *S. epidermidis* (43%) or *S. warneri* (17%). The non-spore-forming isolates were quite diverse, with over 19 genera represented, and included *Acinetobacter* (11%), *Streptococcus* (11%), *Morax-*

ella (5%), *Leclercia* (5%), *Pseudomonas* (4%), *Enhydrobacter* (2%), *Leuconostoc* (1%), and *Stenotrophomonas* (1%).

Four of the isolates identified represented novel species, based on a 16S rRNA sequence identity of <97% to any known microorganism. Two of the four isolates were most closely related to the *Bacillus subtilis* and *Bacillus niabensis* (85% and 89% identity, respectively). Two isolates were most closely related to the *Brevibacillus borstelensis* and *Brevibacillus invocatus* (87% and 93% identity, respectively), and the remaining isolate was most closely related to *Enhydrobacter* sp. KB3-12 (95% identity).

3.2. Anaerobic growth studies

All 358 isolates were tested for the ability to grow under anaerobic conditions by using a variety of terminal electron acceptors. Table 1 shows the various combinations of electron donors and acceptors that were tested and the number of isolates that grew under each condition. Of the 358 isolates tested, only 19 isolates grew under anaerobic conditions. Of the 19 isolates, 7 grew using perchlorate coupled with acetate or lactate as the electron donor. Five of the seven perchlorate-respiring isolates grew with lactate only, while the other two grew only with acetate. The isolates that could use perchlorate as a terminal electron acceptor were identified as either *Bacillus* (6) or *Gracilibacillus* (1). Two of the 19 isolates grew using sulfate as a terminal electron acceptor, and both isolates were identified as belonging to the *Bacillus* genus. Both isolates grew on sulfate using lactate only, and neither grew in medium containing acetate. Ten isolates grew with lactate and arsenate, but none of these isolates were able to grow in medium containing acetate in place of lactate. No isolates grew in medium containing selenite, selenate, or insoluble iron as the terminal electron acceptor, and no isolates grew using more than one of the terminal electron acceptors tested.

3.3. Aerobic growth studies (temperature, pH, and NaCl)

All isolates were tested separately for their ability to grow aerobically at 4°C and in the presence of high salt. Growth

FIG. 2. Isolates by phylum.

FIG. 3. *Bacillus* isolates by species.

at 4°C was demonstrated by 132 (37%) of the isolates, which were identified as belonging to the *Bacillus*, *Staphylococcus*, *Acinetobacter*, *Stenotrophomonas*, *Leclercia*, *Virgibacillus*, *Williamsia*, *Arthobacter*, *Gracilibacillus*, or *Streptomyces* genera. Approximately 35% of the *Bacillus* species and 14% of the *Staphylococcus* species grew at the low temperature. There was minimal difference in the number of spore-forming isolates versus non-spore-forming isolates that grew at 4°C (Table 2).

The majority of isolates (97% of the total) grew in medium containing 5% NaCl. Growth was apparent for 93% (333) of the isolates in media containing 10% NaCl, while only 22% (77) of all isolates grew in media containing 20% NaCl. Of the two most abundant genera, *Staphylococcus* and *Bacillus*, 63% of isolates identified as *Staphylococcus* could grow with elevated salt (20% NaCl), while only 17% of isolates identified as belonging to the *Bacillus* genus could do the same.

Isolates were tested for growth in media buffered within a pH range of 7–12. As expected, all isolates grew at pH 7, since this is the pH of the medium used in the NASA Standard Assay procedure. Of the 358 isolates, 80% (284) grew at pH 8, and 55% (197) grew at pH 9, while the number of isolates that grew in media buffered higher than pH 9 decreased significantly. Eleven isolates (3%) grew at pH 10, and only one isolate grew in media buffered to pH 11. None of the isolates grew in media adjusted to pH 12. The isolates that grew at > pH 10 were all spore-formers, with one isolate identified as belonging to the *Paenibacillus* genus and the remaining 11 isolates belonging to the *Bacillus* genus. Two isolates

identified as *Bacillus clausii* grew in medium at pH 10, but only one of the isolates grew in medium at pH 11.

3.4. Desiccation studies

Isolates showing positive anaerobic or aerobic growth study results, novel isolates (<97% identity based on 16S rRNA gene sequences), or representatives from each clade, were chosen for desiccation studies. A total of 189 isolates were tested for their ability to survive a 2-week desiccation period. Isolates chosen for experiments consisted of representatives from 14 different genera, with 144 isolates representing spore-forming genera and 39 isolates from non-spore-forming genera. The identities of six of the isolates tested have not been determined. Of the isolates subjected to desiccation, 142 (75%) survived the 2-week desiccation period, while 15 isolates did not (Table 3). Desiccation survival on an additional 32 isolates could not be determined due to varying results. Of the spore-forming genera, 80% of the *Bacillus* isolates and 75% of the *Paenibacillus* isolates tested survived desiccation. One *Gracilibacillus* isolate was also tested and survived desiccation. Results obtained from non-spore-forming isolates were similar to those of spore-forming isolates. Of the non-spore-forming isolates, 25 (66%) survived desiccation, while 21% did not (Tables 2 and 4).

3.5. Peroxide tolerance studies

All isolates were tested for their tolerance to hydrogen peroxide by measuring survival after exposure to 5% H₂O₂

TABLE 2. ISOLATES SHOWING ANAEROBIC GROWTH USING VARIOUS SUBSTRATES AND GROWTH RESULTS UNDER ANAEROBIC CONDITIONS

Isolate	Identification	Growth conditions	pH	NaCl	4°C	5% H ₂ O ₂	Desiccation	UVC (J/m ²)
2.1	<i>Bacillus subtilis</i>	Ac + ClO ₄ ⁻	8	10	-	+	NT	NT
8.1	<i>Bacillus niacini</i>	Ac + ClO ₄ ⁻	8	10	-	-	NT	NT
276.1.2	<i>Bacillus</i> sp.	Lac + ClO ₄ ⁻	7	10	-	-	NT	NT
279.1.1	<i>Bacillus subtilis</i>	Lac + ClO ₄ ⁻	7	10	-	-	NT	NT
279.1.2B	<i>Gracilbacillus dipsosauri</i>	Lac + ClO ₄ ⁻	9	10	+	-	+	NT
285.1.2	<i>Bacillus niacini</i>	Lac + ClO ₄ ⁻	7	5	-	-	NT	NT
286.1	<i>Bacillus subtilis</i>	Lac + ClO ₄ ⁻	9	5	-	-	NT	NT
222.1.1	<i>Bacillus subtilis</i>	For + SO ₄ ²⁻	9	10	+	-	+	NT
236.1.1	<i>Bacillus aerophilus</i>	For + SO ₄ ²⁻	10	10	-	+	+	500
243.1	<i>Bacillus licheniformis</i>	Lac + As ⁺⁵	7	10	-	-	+	NT
249.1	<i>Bacillus licheniformis</i>	Lac + As ⁺⁵	7	10	-	-	-	NT
272.1.1	<i>Sporosarcina</i> sp. SF238	Lac + As ⁺⁵	9	10	+	-	NT	NT
272.1.2	<i>Sporosarcina</i> sp. SS-2009-PA3	Lac + As ⁺⁵	9	10	+	-	NT	NT
273.1.1	<i>Bacillus subtilis</i>	Lac + As ⁺⁵	9	10	+	-	NT	NT
273.1.2	<i>Bacillus megaterium</i>	Lac + As ⁺⁵	7	10	-	-	NT	NT
273.1.3	<i>Bacillus megaterium</i>	Lac + As ⁺⁵	9	10	-	+	NT	2000
277.1.1	<i>Bacillus thuringiensis</i>	Lac + As ⁺⁵	9	10	-	-	NT	NT
277.1.2.1	<i>Bacillus</i> sp. SC104	Lac + As ⁺⁵	9	10	-	-	NT	NT
284.1.2	<i>Bacillus niacini</i>	Lac + As ⁺⁵	9	10	-	-	NT	NT

Also shown are results from H₂O₂, desiccation, and UVC experiments. Ac = Acetate, Lac = lactate, For = formate, NT = not tested.

for 1 h. Approximately 19% (70) of isolates grew in TSB after exposure to 5% H₂O₂, some of which are included in Table 4. Both spore-formers and non-spore-formers representing eight different genera were tolerant to hydrogen peroxide, with 84% of the tolerant strains being spore-formers and 71% of those being *Bacillus* species. Other isolates tolerant to hydrogen peroxide included representatives from the following genera: *Staphylococcus*, *Acinetobacter*,

Brevibacillus, *Streptococcus*, *Paenibacillus*, *Moraxella*, and *Sporosarcina*.

3.6. Isolates surviving exposure to multiple independent stresses

Table 4 shows that a small percentage (11%) of the isolates, 41 of the total, grew after exposure to more than one

TABLE 3. NUMBER OF ISOLATES FROM DIFFERENT GENERA THAT GREW AFTER BEING SUBJECTED TO DESICCATION FOR A 2-WEEK PERIOD

Genus	Number of isolates tested	Number of isolates with undetermined survivability	Number of isolates with positive growth	% of isolates showing growth
<i>Bacillus</i>	134	19	107	98%
<i>Pseudomonas</i>	3	1	1	33%
<i>Staphylococcus</i>	13	2	9	69%
<i>Streptomyces</i>	2	0	2	100%
<i>Acinetobacter</i>	3	0	3	100%
<i>Leclercia</i>	4	0	4	100%
<i>Paenibacillus</i>	8	1	6	75%
<i>Streptococcus</i>	1	0	1	100%
<i>Arthrobacter</i>	1	0	1	100%
<i>Enhydrobacter</i>	2	0	2	100%
<i>Moraxella</i>	2	0	2	100%
<i>Gracilbacillus</i>	1	0	1	100%
<i>Sphingopyxis</i>	2	0	0	0%
<i>Brevibacillus</i>	1	1	0	0%
<i>Williamsia</i>	1	1	0	0%
<i>Oceanobacillus</i>	1	1	0	0%
<i>Sporosarcina</i>	1	1	0	0%
<i>Stenotrophomonas</i>	1	1	0	0%
<i>Neisseria</i>	1	1	0	0%
<i>Brevibacterium</i>	1	1	0	0%
Unknown	6	2	3	50%

Total isolates tested = 189.

TABLE 4. ISOLATES THAT GREW AFTER EXPOSURE TO MULTIPLE INDIVIDUAL STRESSES OF COLD, ALKALINITY, HIGH SALINITY, AND HYDROGEN PEROXIDE

Isolate	Genus	20% NaCl	4°C	5% H ₂ O ₂	Upper pH	Isolate	Genus	20% NaCl	4°C	5% H ₂ O ₂	Upper pH
4.2	<i>Neisseria</i> sp. TM10_4	-	+	-	8	137.1	<i>Bacillus aerius</i>	+	+	+	9
46.1	<i>Sphingopyxis alaskensis</i>	+	+	-	8	149.2	<i>Bacillus</i> sp. MBEA35	+	+	-	9
47.1	<i>Sphingopyxis alaskensis</i>	+	+	-	9	152.1	<i>Virgibacillus</i> sp. PrLB2	+	+	-	9
66.1.1	<i>Bacillus safensis</i>	+	+	-	7	160.1	<i>Bacillus subtilis</i>	+	+	-	8
66.1.2	<i>Staphylococcus</i> sp. EE09C102	+	+	-	9	160.2E	<i>Bacillus amyloliquefaciens</i>	+	+	-	8
66.1.3	<i>Staphylococcus</i> sp. EE09C102	+	+	-	9	164.1.2B	<i>Staphylococcus pastueri</i>	+	+	+	9
68.1	<i>Bacillus safensis</i>	+	+	+	9	166.1	<i>Bacillus</i> sp. T14	+	+	+	9
72.1.1	<i>Staphylococcus epidermidis</i>	+	+	-	7	170.2	<i>Bacillus</i> sp. PSM2	+	+	-	7
73.1	<i>Stenotrophomonas maltophilia</i>	+	+	-	9	173.1	<i>Bacillus</i> sp. BJ-45	+	+	-	7
100	<i>Pseudomonas putida</i>	+	+	-	7	195.1A	<i>Paenibacillus lautus</i>	+	+	+	10
104.1.2	<i>Staphylococcus epidermidis</i>	+	+	+	8	207.1	<i>Bacillus</i> sp. 4014	+	+	-	9
105.1	<i>Bacillus subtilis</i>	+	+	-	7	215.1	<i>Bacillus pumilus</i>	+	+	-	9
105.2	<i>Bacillus thuringiensis</i>	+	+	-	9	218.1	<i>Bacillus</i> sp. TSE34	+	+	-	7
107	<i>Staphylococcus epidermidis</i>	+	+	-	7	225.2.1	<i>Bacillus subtilis</i>	+	+	-	8
112.1	<i>Bacillus</i> sp. NSP483	-	+	-	10	236.1.1	<i>Bacillus aerophilus</i>	+	-	+	10
116.1	<i>Sporosarcina ginsengisoli</i>	+	+	-	9	250.1	<i>Bacillus licheniformis</i>	+	+	-	7
116.2	<i>Bacillus licheniformis</i>	+	+	-	9	251.1	<i>Bacillus</i> sp. PPB8 MB8	+	-	-	10
117.1	<i>Paenibacillus lautus</i>	+	+	-	9	265.1	<i>Bacillus</i> sp. JCM 10530	-	-	+	10
127.1.1B	<i>Bacillus megaterium</i>	+	+	-	9	104594	<i>Streptomyces lanatus</i>	+	+	-	9
130.1	<i>Bacillus</i> sp. JS-2	-	+	-	10	104598	<i>Bacillus stratosphericus</i>	+	+	-	9
133.1	<i>Bacillus subtilis</i>	+	+	-	7						

individually applied stress. For example, *Bacillus* isolate 236.1.1 grew in medium at pH 10, in media with 20% NaCl, and grew after exposure to hydrogen peroxide. Of the 41 isolates found to grow after exposure to multiple individual stresses, 31 belonged to spore-forming genera. Fourteen isolates survived at least two of the extreme conditions, and 20 isolates survived three of the four conditions shown in Table 4. Six isolates were found to survive all four individual stress conditions, with four identified as *Bacillus* and the remaining two representing *Staphylococcus* and *Paenibacillus*.

3.7. Survival to UVC exposure

Thirteen isolates, listed in Table 5, were chosen for UVC exposure survival, based upon their ability to grow after exposure to the physiological stresses used in this study (e.g., desiccation, hydrogen peroxide, high salt). All strains were initially irradiated at 100 J/m². Four strains exhibiting

>10⁴ log survival were singled out for exposure to higher doses. All four isolates survived irradiation at 500 J/m². Three of these survived exposure to 2000 J/m² and exhibited the same level of survival from 500 to 2000 J/m², the highest dose tested. The isolates were identified as being closely related to *Bacillus amyloliquefaciens* (isolate #160.2E), *Bacillus megaterium* (isolate #273.1.3), and an isolate most closely related to *Moraxella osloensis* (isolate #198.1.1B).

4. Discussion

All Mars-based landers and rovers are subjected to a regiment of microbial reduction protocols to limit the number of microorganisms transported from Earth into space to comply with the Outer Space Treaty. Despite the rigor of the bio-burden reduction procedures employed, <5 × 10⁵ microorganisms as enumerated by the NASA Standard Assay (Science Mission Directorate, 2011, NPR 8020.12D) still persist on spacecraft. The purpose of this study was to identify

TABLE 5. HIGHEST DOSE OF UVC TO WHICH AN ISOLATE WAS EXPOSED AND SURVIVED

Isolate #	Identification	UVC dosage (J/m ²)
2.1	<i>Bacillus subtilis</i>	100
21.1	<i>Bacillus</i> sp. L65	100
42.2	<i>Staphylococcus warneri</i>	100
54.1	<i>Bacillus licheniformis</i>	100
101.2	<i>Acinetobacter</i> sp. KL12010	100
104.1.2	<i>Staphylococcus epidermidis</i>	100
116.1	<i>Sporosarcina ginsengisoli</i>	100
117.1	<i>Paenibacillus lautus</i>	100
160.2E	<i>Bacillus amyloliquefaciens</i>	2000
190.2A	<i>Staphylococcus hominis</i>	100
198.1.1B	<i>Moraxella osloensis</i>	2000
236.1.1	<i>Bacillus aerophilus</i>	500
273.1.3	<i>Bacillus megaterium</i>	2000

microorganisms collected from MSL prior to launch and to characterize those isolates to determine their potential feasibility for survival in transport to and on Mars. The long-term goal is to obtain knowledge of the hardness of isolates residing on spacecraft before deployment to space to assess whether a terrestrial-based microbe could potentially compromise a life-detection mission. The study is unique because it is the only set of comprehensive examinations of the survival of isolates, collected from MSL, exposed to stresses that simulate a Mars-like environment.

Until now, it was largely unknown what types of microorganisms populated the external surfaces of MSL. The assumption has been that the isolates were primarily of spore-forming genera, because samples of the MSL surface collected during various phases of assembly were subject to a brief 80°C heat-shock treatment before plating and enumeration, according to the NASA Standard Assay, which should have selected for non-spore-formers. However, our identification of the MSL isolates surviving the heat-shock treatment indicated that almost one-quarter of the isolates were non-spore-forming microbes. In total, the isolates represented 25 genera of bacteria and at least 65 different species, and four of the isolates appear to be novel. Since the isolates were collected after the NASA Standard Assay was performed, they do not represent the total community of microorganisms on the spacecraft but only those that survived the heat-shock treatment and grow under the selective conditions imposed. Thus, it is expected that there was an even greater diversity of microorganisms on MSL when the total bacterial, archaeal, and fungal community is taken into account (La Duc *et al.*, 2014). Previous studies in which culture-independent techniques were used on samples collected from clean rooms of various NASA facilities have shown a wide variety of microorganisms present, but their activity has not been evaluated, so it is not clear whether all the microbes represented are viable (La Duc *et al.*, 2004, 2009, 2012; Moissl *et al.*, 2008; Vaishampayan *et al.*, 2010). At present, international policy is primarily concerned with the number of spore-forming microorganisms/bacteria on board the spacecraft, since spores have a higher probability of surviving the trip to Mars.

Anaerobic growth studies indicate that a portion of the isolates are able to grow using potentially available energy sources on Mars. Tests performed by the Phoenix lander showed that perchlorate was present on the martian surface at concentrations of 2.1–2.6 mM (Hecht *et al.*, 2009), and continued exploration of Mars has confirmed that perchlorate is widespread (Leshin *et al.*, 2013; Ming *et al.*, 2014; Clark and Kounaves, 2016). Seven of the MSL isolates grew using perchlorate as the sole terminal electron acceptor. Two of the isolates grew using sulfate as a terminal electron acceptor. Sulfate has been shown to be a major constituent of martian soils, and because sulfate systems exhibit high water activity, areas with sulfate salts are potentially more favorable to life (Crisler *et al.*, 2012). Although neither selenium nor arsenic has been detected on Mars, various forms of arsenic and selenium are somewhat common on Earth. It is not unreasonable to expect that they may be present on Mars or other planetary bodies, and current thought is that arsenic may have been important to early life on Earth. It is well known that arsenic and selenium are respired by a number of terrestrial microorganisms to conserve energy (Stolz and Oremland, 1999; Zhu *et al.*, 2014; Nanchaiah and Lens, 2015; Rascovan *et al.*, 2016). Interestingly, this study identified 10 microorganisms that grew in media where arsenic was the only terminal electron acceptor available. Although detailed characterization of arsenic respiration was not a focal point of the current project, future work will investigate arsenic utilization by the isolates, as there is still much to be learned about arsenic metabolism in terrestrial microorganisms.

Tolerance to multiple chemical and physical conditions is critical if life is to survive on Mars. Studies have shown that salt tolerance would be required by microorganisms to survive and grow on Mars due to the high salt concentrations found in martian soils (Crisler *et al.*, 2012). The majority of the isolates collected from MSL were able to grow in media containing elevated NaCl (93% of the isolates grew at ≥10% NaCl). It had previously been thought that the martian soil was likely to be acidic, but results by the Phoenix lander show that the soils were mildly basic with a pH of 7.7 ± 0.5 (Hecht *et al.*, 2009). Most of the isolates from MSL (79%, 284 isolates) were able to grow in media at an elevated pH of 8, and 55% (197) of the isolates could grow in media at a pH ≥ 9, demonstrating that the majority of the isolates tested could survive and thrive in the more alkaline martian soils. Although the average temperature of Mars can range from −10°C to −76°C based on the Mars Pathfinder data, we chose to test all isolates at a baseline of 4°C, with the intent to perform extended experiments at lower temperatures in the future using the isolates that grew at this temperature (Schofield *et al.*, 1997). In this study, 37% (132 isolates) of the isolates grew at 4°C, and future studies will test the resiliency of the 132 isolates to temperatures less than 4°C. Comparing the physical and chemical tolerances exhibited by all of the isolates tested, it is notable that 27% (97) of the isolates were able to thrive under multiple extreme conditions (≥4 of the conditions tested).

Although growth of the isolates in Mars-like conditions (*e.g.*, anaerobic substrates, salt, temperature, and pH) provides a means by which to determine the potential for life to proliferate once on Mars, surviving exposure to harsh

environmental stresses is equally important for the organism's survival both in transit to and on Mars. Over 75% (142) of the organisms tested in desiccation studies were able to survive desiccation for 2 weeks. In the future, further studies designed to determine the maximum length of time each of these organisms can survive desiccation will be undertaken, since an organism traveling on spacecraft to Mars would have to endure a 9-month trip without water before even reaching the environment to start growing. Twenty percent of the MSL isolates survived treatment with 5% liquid hydrogen peroxide, indicating that cleaning hardware during spacecraft assembly with a 5% solution of liquid hydrogen peroxide may not be the most effective microbial reduction method. It is noted that liquid hydrogen peroxide was not used during assembly of MSL as a microbial reduction method. Interestingly, it is reported that the surface ice of Europa contains as much as 0.13% hydrogen peroxide generated from the radiolysis of ice (Johnson *et al.*, 2003). Microorganisms traveling to, and surviving on, distant planetary bodies like Europa would need to protect themselves from strong oxidants such as hydrogen peroxide. The ability of some of the isolates to survive oxidizing conditions (*e.g.*, exposure to hydrogen peroxide) and UVC radiation suggests that they may be more likely to survive on a spacecraft destined for Mars or other planetary bodies.

Of the many species belonging to the *Bacillus* genus, the "gold standard" for testing microbial tolerance to UV exposure is *Bacillus pumilus* SAFR-032. *Bacillus pumilus* SAFR-032 was isolated from a spacecraft assembly facility during the time of the assembly of the Viking landers (Puleo *et al.*, 1977). *Bacillus pumilus* SAFR-032 exhibits only a ~ 2 log reduction in viable spores after exposure to 1000 J/m² UVC (Link *et al.*, 2004; Osman *et al.*, 2008). It was unexpected that some of the MSL isolates from this study that were isolated from spacecraft in the spacecraft assembly facility would show resistance to UVC. Not surprising, three of the four isolates exposed to 500 J/m² or higher were *Bacillus* species. The fourth resistant isolate is closely related to *Moraxella osloensis*, a member of a genus well known for having resistance to ionizing radiation and tolerance to UV at doses comparable to those endured by the extremely resistant *Deinococcus radiodurans* (Welch and Maxcy, 1975; Keller *et al.*, 1982). Since isolates obtained from clean rooms must, by default, be resistant to numerous assaults resulting from the various cleaning methods applied throughout the assembly phases, it is not surprising that that some isolates survive multiple extreme environmental conditions including resistance to UVC radiation.

The results of the present study augment our current knowledge of the viable microorganisms present on, or associated with, spacecraft surfaces. The study also provides information regarding some of the physiological traits of those microorganisms that are likely required to grow on Mars, and reports on the ability of some of the isolates to survive exposure to extreme environmental conditions analogous to those expected on Mars. Currently, it is not known how the microorganisms residing on spacecraft that are inside clean room facilities adjust to repeated bioburden reduction applications. Nor is it known how the human-controlled environment may influence the overall evolution of the microbial population within this environment. The

information collected from these types of studies will allow us to assess the current cleaning procedures of spacecraft components during spacecraft assembly and will provide information on the ability of surviving isolates to withstand extreme conditions similar to those present during space travel and on the martian surface. Future work with the isolates can provide us with new genomic and proteomic information that is invaluable for studies exploring the molecular mechanisms that may account for the stress resistances observed in many of the isolates.

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No competing financial interests exist.

References

- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402.
- Bauermeister, A., Rettberg, P., and Flemming, H.C. (2014) Growth of the acidophilic iron-sulfur bacterium *Acidithiobacillus ferrooxidans* under Mars-like geochemical conditions. *Planet Space Sci* 98:205–215.
- Benardini, J.N., III, La Duc, M.T., Beaudet, R.A., and Koukol, R. (2014) Implementing planetary protection measures on the Mars Science Laboratory. *Astrobiology* 14:27–32.
- Berry, B.J., Jenkins, D.G., and Schuerg, A.C. (2010) Effects of simulated Mars conditions on the survival and growth of *Escherichia coli* and *Serratia liquefaciens*. *Appl Environ Microbiol* 76:2377–2386.
- Carr, M.H. (2012) The fluvial history of Mars. *Philos Trans A Math Phys Eng Sci* 370:2193–2215.
- Clark, B.C. and Kounaves, S.P. (2016) Evidence for the distribution of perchlorates on Mars. *International Journal of Astrobiology* 15:311–318.
- Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M., and Tiedje, J.M. (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* 21:442–443.

- Crisler, J.D., Newville, T.M., Chen, F., Clark, B.C., and Schneegurt, M.A. (2012) Bacterial growth at the high concentrations of magnesium sulfate found in martian soils. *Astrobiology* 12:98–106.
- Davila, A.F., Dupont, L.G., Melchiorri, R., Jänicke, J., Valea, S., de Los Rios, A., Fairén, A.G., Möhlmann, D., McKay, C.P., Ascaso, C., and Wierzchos, J. (2010) Hygroscopic salts and the potential for life on Mars. *Astrobiology* 10:617–628.
- De Gelder, L., Vandecasteele, F.P.J., Brown, C.J., Forney, L.J., and Top, E.M. (2005) Plasmid donor affects host range of promiscuous IncP-1₊ plasmid pB10 in an activated-sludge microbial community. *Appl Environ Microbiol* 71:5309–5317.
- Fajardo-Cavazos, P., Schuerger, A.C., and Nicholson, W.L. (2010) Exposure of DNA and *Bacillus subtilis* spores to simulated martian environments: use of quantitative PCR (qPCR) to measure inactivation rates of DNA to function as a template molecule. *Astrobiology* 10:403–411.
- Frank, J.A., Reich, C.I., Sharma, S., Weisbaum, J.S., Wilson, B.A., and Olsen, G.J. (2008) Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl Environ Microbiol* 74:2461–2470.
- Gómez F., Mateo-Martí, E., Prieto-Ballesteros, O., Martín-Gago, J., and Amils, R. (2010) Protection of chemolithoautotrophic bacteria exposed to simulated Mars environmental conditions. *Icarus* 209:482–487.
- Grotzinger, J.P., Sumner, D.Y., Kah, L.C., Stack, K., Gupta, S., Edgar, L., Rubin, D., Lewis, K., Schieber, J., Mangold, N., Milliken, R., Conrad, P.G., Des Marais, D., Farmer, J., Siebach, K., Calef, F., III, Hurowitz, J., McLennan, S.M., Ming, D., Vaniman, D., Crisp, J., Vasavada, A., Edgett, K.S., Malin, M., Blake, D., Gellert, R., Mahaffy, P., Wiens, R.C., Maurice, S., Grant, J.A., Wilson, S., Anderson, R.C., Beegle, L., Arvidson, R., Hallet, B., Sletten, R.S., Rice, M., Bell, J., III, Griffes, J., Ehlmann, B., Anderson, R.B., Bristow, T.F., Dietrich, W.E., Dromart, G., Eigenbrode, J., Fraeman, A., Hardgrove, C., Herkenhoff, K., Jandura, L., Kocurek, G., Lee, S., Leshin, L.A., Leveille, R., Limonadi, D., Maki, J., McCloskey, S., Meyer, M., Minitti, M., Newsom, H., Oehler, D., Okon, A., Palucis, M., Parker, T., Rowland, S., Schmidt, M., Squyres, S., Steele, A., Stolper, E., Summons, R., Treiman, A., Williams, R., Yingst, A.; MSL Science Team. (2014) A habitable fluvo-lacustrine environment at Yellowknife Bay, Gale Crater, Mars. *Science* 343, doi:10.1126/science.1242777.
- Hecht, M.H., Kounaves, S.P., Quinn, R.C., West, S.J., Young, S.M.M., Ming, D.W., Catling, D.C., Clark, B.C., Boynton, W.V., Hoffman, J., DeFlores, L.P., Gospodinova, K., Kapit, J., and Smith, P.H. (2009) Detection of perchlorate and the soluble chemistry of martian soil at the Phoenix lander site. *Science* 325:64–67.
- Horneck, G. (1993) Responses of *Bacillus subtilis* spores to space environment: results from experiments in space. *Orig Life Evol Biosph* 23:37–52.
- Johnson, R.E., Quickenden, T.I., Cooper, P.D., McKinley, A.J., and Freeman, C.G. (2003) Europa and the production of oxidants in Europa's surface. *Astrobiology* 3:823–850.
- Keller, L.C., Thompson, T.L., and Maxcy, R.B. (1982) UV light-induced survival response in a highly radiation-resistant isolate of the *Moraxella-Acinetobacter* group. *Appl Environ Microbiol* 43:424–429.
- Kempf, M.J., Chen, F., Kern, R., and Venkateswaran, K. (2005) Recurrent isolation of hydrogen peroxide-resistant spores of *Bacillus pumilus* from a spacecraft assembly facility. *Astrobiology* 5:391–405.
- La Duc, M.T., Satomi, M., and Venkateswaran, K. (2004) *Bacillus odyssey* sp. nov., a round spore forming bacillus isolated from the Mars Odyssey spacecraft. *Int J Syst Evol Microbiol* 54:195–201.
- La Duc, M.T., Benardini, J.N., Kempf, M.J., Newcombe, D.A., Lubarsky, M., and Venkateswaran, K. (2007a) Microbial diversity of Indian Ocean hydrothermal vent plumes: microbes tolerant of desiccation, peroxide exposure and ultraviolet and γ -irradiation. *Astrobiology* 7:416–431.
- La Duc, M.T., Dekas, A., Osman, S., Moissl, C., Newcombe, D., and Venkateswaran, K. (2007b) Isolation and characterization of bacteria capable of tolerating the extreme conditions of clean room environments. *Appl Environ Microbiol* 73:2600–2611.
- La Duc, M.T., Osman, S., Vaishampayan, P., Piceno, Y., Andersen, G., Spry, J.A., and Venkateswaran, K. (2009) Comprehensive census of bacteria in clean rooms by using DNA microarray and cloning methods. *Appl Environ Microbiol* 75:6559–6567.
- La Duc, M.T., Vaishampayan, P., Nilsson, H.R., Torok, T., and Venkateswaran, K. (2012) Pyrosequencing-derived bacterial, archaeal, and fungal diversity of spacecraft hardware destined for Mars. *Appl Environ Microbiol* 78:5912–5922.
- La Duc, M.T., Venkateswaran, K., and Conley, C.A. (2014) A genetic inventory of spacecraft and associated surfaces. *Astrobiology* 14:15–23.
- Lane, D.J. (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, edited by E. Stackebrandt and M. Goodfellow, John Wiley & Sons, New York, pp 115–175.
- Leshin, L.A., Mahaffy, P.R., Webster, C.R., Cabane, M., Coll, P., Conrad, P.G., Archer, P.D., Jr., Atreya, S.K., Brunner, A.E., Buch, A., Eigenbrode, J.L., Flesch, G.J., Franz, H.B., Freissinet, C., Glavin, D.P., McAdam, A.C., Miller, K.E., Ming, D.W., Morris, R.V., Navarro-González, R., Niles, P.B., Owen, T., Pepin, R.O., Squyres, S., Steele, A., Stern, J.C., Summons, R.E., Sumner, D.Y., Sutter, B., Szopa, C., Teinturier, S., Trainer, M.G., Wray, J.J., Grotzinger, J.P.; MSL Science Team. (2013) Volatile, isotope, and organic analysis of martian fines with the Mars Curiosity rover. *Science* 341, doi:10.1126/science.1238937.
- Lim, J.M., Yoon, J.H., Lee, J.C., Jeon, C.O., Park, D.J., and Kim, C.J. (2004) *Halomonas koreensis* sp. nov., a novel moderately halophilic bacterium isolated from a solar saltern in Korea. *Int J Syst Evol Microbiol* 54:2037–2042.
- Link, L., Sawyer, J., Venkateswaran, K., and Nicholson, W. (2004) Extreme spore UV resistance of *Bacillus pumilus* isolates obtained from an ultraclean spacecraft assembly facility. *Microb Ecol* 47:159–163.
- Malin, M.C. and Edgett, K.S. (2000) Evidence for recent groundwater seepage and surface runoff on Mars. *Science* 288:2330–2335.
- Malin, M.C. and Edgett, K.S. (2003) Evidence for persistent flow and aqueous sedimentation on early Mars. *Science* 302:1931–1934.
- Marshall, M.M., Hayes, S., Moffett, J., Sterling, C.R., and Nicholson, W.L. (2003) Comparison of UV inactivation of spores of three encephalitozoon species with that of spores of two DNA repair-deficient *Bacillus subtilis* biosimetry strains. *Appl Environ Microbiol* 69:683–685.
- McKay, C.P. (2004) What is life—and how do we search for it in other worlds? *PLoS Biol* 2:1260–1263.
- Ming, D.W., Archer, P.D., Jr., Glavin, D.P., Eigenbrode, J.L., Franz, H.B., Sutter, B., Brunner, A.E., Stern, J.C., Freissinet, C., McAdam, A.C., Mahaffy, P.R., Cabane, M., Coll, P., Campbell, J.L., Atreya, S.K., Niles, P.B., Bell, J.F., III, Bish,

- D.L., Brinckerhoff, W.B., Buch, A., Conrad, P.G., Des Marais, D.J., Ehlmann, B.L., Fairén, A.G., Farley, K., Flesch, G.J., Francois, P., Gellert, R., Grant, J.A., Grotzinger, J.P., Gupta, S., Herkenhoff, K.E., Hurowitz, J.A., Leshin, L.A., Lewis, K.W., McLennan, S.M., Miller, K.E., Moersch, J., Morris, R.V., Navarro-González, R., Pavlov, A.A., Perrett, G.M., Pradler, I., Squyres, S.W., Summons, R.E., Steele, A., Stolper, E.M., Sumner, D.Y., Szopa, C., Teinturier, S., Trainer, M.G., Treiman, A.H., Vaniman, D.T., Vasavada, A.R., Webster, C.R., Wray, J.J., Yingst, R.A.; MSL Science Team. (2014) Volatile and organic compositions of sedimentary rocks in Yellowknife Bay, Gale Crater, Mars. *Science* 343, doi:10.1126/science.1245267.
- Moissl, C., Osman, S., La Duc, M.T., Dekas, A., Brodie, E., DeSantis, T., and Venkateswaran, K. (2007) Molecular bacterial community analysis of clean rooms where spacecraft are assembled. *FEMS Microbiol Ecol* 61:509–521.
- Moissl, C., Bruckner, J.C., and Venkateswaran, K. (2008) Archaeal diversity analysis of spacecraft assembly clean rooms. *ISME J* 2:115–119.
- Morozova, D., Möhlmann, D., and Wagner, D. (2007) Survival of methanogenic archaea from Siberian permafrost under simulated martian thermal conditions. *Orig Life Evol Biosph* 37:189–200.
- Muyzer, G., de Waal, E.C., and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59:695–700.
- Nanchaiah, Y.V. and Lens, P.N. (2015) Ecology and biotechnology of selenium-respiring bacteria. *Microbiol Mol Biol Rev* 79:61–80.
- NASA. (2010) *Handbook for the Microbial Examination of Space Hardware*, Publication NASA-HDBK-6022, National Aeronautics and Space Administration, Washington, DC.
- Newcombe, D.A., Schuerger, A.C., Benardini, J.N., Dickinson, D., Tanner, R., and Venkateswaran, K. (2005) Survival of spacecraft-associated microorganisms under simulated martian UV irradiation. *Appl Environ Microbiol* 71:8147–8156.
- Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P. (2000) Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev* 64:548–572.
- Nicholson, W.L., Fajardo-Cavazos, P., Rebeil, R., Slieman, T.A., Riesenman, P.J., Law, J.F., and Xue, Y. (2002) Bacterial endospores and their significance in stress resistance. *Antonie Van Leeuwenhoek* 81:27–32.
- Nielsen, P., Fritze, D., and Priest, F.G. (1995) Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. *Microbiology* 141:1745–1761.
- Osman, S., Peeters, Z., La Duc, M.T., Mancinelli, R., Ehrenfreund, P., and Venkateswaran, K. (2008) Effect of shadowing on survival of bacteria under conditions simulating the martian atmosphere and UV radiation. *Appl Environ Microbiol* 74:959–970.
- Puleo, J.R., Fields, N.D., Bergstrom, S.L., Oxborrow, G.S., Stabekis, P.D., and Koukol, R.C. (1977) Microbiological profiles of the Viking spacecraft. *Appl Environ Microbiol* 33:379–384.
- Rascovan, N., Maldonado, J., Vazquez, M.P., and Farías, M.E. (2016) Metagenomic study of red biofilms from Diamante Lake reveals ancient arsenic bioenergetics in haloarchaea. *ISME J* 10:299–309.
- Rasmussen, B., Blake, T.S., Fletcher, I.R., and Kilburn, M.R. (2009) Evidence for microbial life in synsedimentary cavities from 2.75 Ga terrestrial environments. *Geology* 37:423–426.
- Reysenbach, A.L., Wickham, G.S., and Pace, N.R. (1994) Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. *Appl Environ Microbiol* 60:2113–2119.
- Riesenman, P.J. and Nicholson, W.L. (2000) Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar radiation. *Appl Environ Microbiol* 66:620–626.
- Roth, L., Saur, J., Retherford, K.D., Strobel, D.F., Feldman, P.D., McGrath, M.A., and Nimmo, F. (2014) Transient water vapor at Europa's south pole. *Science* 343, doi:10.1126/science.1247051.
- Rummel, J.D., Beaty, D.W., Jones, M.A., Bakermans, C., Barlow, N.G., Boston, P.J., Chevrier, V.F., Clark, B.C., de Vera, J.P., Gough, R.V., Hallsworth, J.E., Head, J.W., Hipkin, V.J., Kieft, T.L., McEwen, A.S., Mellon, M.T., Mikucki, J.A., Nicholson, W.L., Omelon, C.R., Peterson, R., Roden, E.E., Sherwood Lollar, B., Tanaka, K.L., Viola, D., and Wray, J.J. (2014) A new analysis of Mars "Special Regions": findings of the Second MEPAG Special Regions Science Analysis Group (SR-SAG2). *Astrobiology* 14:887–968.
- Schofield, J.T., Barnes, J.R., Crisp, D., Haberle, R.M., Larsen, S., Magalhães, J.A., Murphy, J.R., Seiff, A., and Wilson, G. (1997) The Mars Pathfinder Atmospheric Structure Investigation/Meteorology (ASI/MET) experiment. *Science* 278:1752–1758.
- Schuerger, A.C., Mancinelli, R.L., Kern, R.G., Rothschild, L.J., and McKay, C.P. (2003) Survival of endospores of *Bacillus subtilis* on spacecraft surfaces under simulated martian environments: implications for the forward contamination of Mars. *Icarus* 165:253–276.
- Science Mission Directorate. (2011) *Planetary Protection Provisions for Robotic Extraterrestrial Missions, NASA Procedural Requirements*, NPR 8020.12D, NASA, Washington, DC.
- Setlow, P. (2001) Resistance of spores of *Bacillus* species to ultraviolet light. *Environ Mol Mutagen* 38:97–104.
- Setlow, P. (2006) Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J Appl Microbiol* 101:514–525.
- Smith, S.A., Benardini, J.N., III, Strap, J.L., and Crawford, R.L. (2009) Diversity of aerobic and facultative alkalitolerant and halotolerant endospore formers in soil from the Alvord Basin, Oregon. *Syst Appl Microbiol* 32:233–244.
- Stolz, J.F. and Oremland, R.S. (1999) Bacterial respiration of arsenic and selenium. *FEMS Microbiol Rev* 23:615–627.
- Suzuki, M.T. and Giovannoni, S.J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* 62:625–630.
- Tauscher, C., Schuerger, A.C., and Nicholson, W.L. (2006) Survival and germinability of *Bacillus subtilis* spores exposed to simulated Mars solar radiation: implications for life detection and planetary protection. *Astrobiology* 6:592–605.
- Vaishampayan, P., Osman, S., Andersen, G., and Venkateswaran, K. (2010) High-density 16S microarray and clone library-based microbial community composition of the Phoenix spacecraft assembly clean room. *Astrobiology* 10:499–508.
- Vaishampayan, P., Probst, A.J., La Duc, M.T., Bargoma, E., Benardini, J.N., Andersen, G.L., and Venkateswaran, K.

- (2013) New perspectives on viable microbial communities in low-biomass cleanroom environments. *ISME J* 7:312–324.
- Venkateswaran, K., Satomi, M., Chung, S., Kern, R., Koukol, R., Basic, C., and White, D. (2001) Molecular microbial diversity of a spacecraft assembly facility. *Syst Appl Microbiol* 24:311–320.
- Welch, A.B. and Maxcy, R.B. (1975) Characterization of radiation-resistant vegetative bacteria in beef. *Appl Microbiol* 30:242–250.
- Zenoff, V.F., Heredia, J., Ferrero, M., Siñeriz, F., and Farías, M.E. (2006) Diverse UV-B resistance of culturable bacterial community from high-altitude wetland water. *Curr Microbiol* 52:359–362.
- Zhu, Y.G., Yoshinaga, M., Zhao, F.J., and Rosen, B.P. (2014) Earth abides arsenic biotransformations. *Annu Rev Earth Planet Sci* 42:443–467.

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Abbreviations Used

JPL = Jet Propulsion Laboratory

MSL = Mars Science Laboratory

PBS = phosphate-buffered saline

TSA = tryptic soy agar

TSB = tryptic soy broth