

Interactive effects of hypobaria, low temperature, and CO₂ atmospheres inhibit the growth of mesophilic *Bacillus* spp. under simulated martian conditions

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Abstract

Robotic spacecraft are launched with finite levels of terrestrial microorganisms that are similar to the microbial communities within facilities in which spacecraft are assembled. In particular, spores of mesophilic aerobic *Bacillus* species are common spacecraft contaminants considered most likely to survive interplanetary transfer to Mars. During the cruise phase to Mars, and then again during surface operations, microbial bioloads are exposed to a diversity of biocidal factors that are likely to render the microbial species either dead or significantly inhibited from active metabolic activity and replication. We report here, for the first time, that interactive effects of low pressure, low temperature, and high CO₂ atmospheres approaching conditions likely to be encountered on the martian surface strongly inhibit the growth and replication of seven common *Bacillus* spp. isolated from spacecraft. Tests were conducted within a small glass bell-jar system maintained in a low-temperature microbial incubator. Atmospheric pressures were controlled at 1013 (Earth-normal), 100, 50, 35, 25, or 15 mb, and temperatures were maintained at 30, 20, 15, 10, or 5 °C. Experiments were carried out for 48 h or 7 days under either Earth-normal O₂/N₂ or pure CO₂ atmospheres. Results indicated that low pressure, low temperature, and high CO₂ atmospheres, applied separately or in combination, were capable of inhibiting the growth and replication of *B. pumilus* SAFR-032, *B. pumilus* FO-36B, *B. subtilis* HA-101, *B. subtilis* 42HS-1, *B. megaterium* KL-197, *B. licheniformis* KL-196, and *B. nealsonii* FO-092 under simulated martian conditions. Endospores of all seven *Bacillus* spp. strains failed to germinate and grow at 25 mb at 30 °C. Although, vegetative cells of these strains exhibited a slightly greater ability to replicate at lower pressures than did endospores, vegetative cells of these species failed to grow at pressures below 25 mb. Interactive effects of these environmental parameters acted to generally increase the inhibitory nature of the low-pressure conditions on growth and replication of the seven *Bacillus* spp. tested.

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1. Introduction

Robotic spacecraft are launched from Earth with finite levels of microbial contamination that are composed of species similar to the cleanroom environments within which the vehicles are assembled (reviewed by Schuerger, 2004; see also Taylor, 1974; Venkateswaran et al., 2001, 2003). Immediately after launch, spacecraft enter the harsh environment of inter-

planetary space and the microorganisms on the vented surfaces of spacecraft are subjected to biocidal factors (e.g., solar UV irradiation, cosmic rays, extreme desiccating conditions, high vacuum) that immediately begin to reduce the viable bioloads and species diversity of the launched vehicles. Based on published literature (Dose and Klein, 1996; Hagen et al., 1971; Horneck et al., 1994; Koike and Oshima, 1993; Schuerger, 2004; Schuerger et al., 2003, 2005), it is possible that between 50–70% of spore-forming bacteria, and up to 2 orders of magnitude of non-spore forming species, may be inactivated during the 6–8 month cruise phase to Mars. However, the launched bioloads of robotic spacecraft generally range

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between 10^5 to 10^8 viable cells per vehicle (Dillon et al., 1973), including non-spore forming, spore-forming, and non-culturable species (reviewed by Fajardo-Cavazos et al., 2006; Schuerger, 2004). Thus, the interplanetary environment during the cruise phase to Mars will only impart a slight to moderate reduction in the numbers of viable microorganisms per spacecraft prior to landing.

The harsh conditions found on the surface of Mars are only slightly more conducive to the survival of terrestrial microorganisms than those found in interplanetary space. The biocidal conditions on the surface of Mars likely to contribute to inactivation of terrestrial bioloads on landed spacecraft include UV irradiation, low pressure, low temperature, high CO_2 atmosphere, extreme desiccating conditions, oxidizing conditions of the atmosphere and regolith, and increased levels of cosmic rays (Horneck et al., 2003; Nicholson and Schuerger, 2005; Nicholson et al., 2005; Schuerger, 2004). Schuerger et al. (2003, 2006) demonstrated that after spacecraft land on Mars, it is likely that sun-exposed surfaces can receive high enough levels of UV irradiation to reduce the viable bioloads by up to 6 orders of magnitude in as short a span as several tens-of-minutes to a few hours under clear sky conditions (optical depth 0.5). However, a portion of any landed vehicle will contain surfaces that are completely shielded from UV irradiation. These surfaces might include the undersides of landers or rovers, buried landing pads, internal spacecraft components, and surfaces covered with UV attenuating substances.

During the course of these studies, we found no papers published in the primary literature on the effects of low pressures similar to the martian surface on microbial growth and replication. The literature that is available emphasizes the survival of dormant spores or dormant vegetative cells of terrestrial microorganisms under simulated martian conditions (reviewed by Schuerger, 2004; Schuerger et al., 2003). However, several studies (Hagen et al., 1964, 1967; Hawrylewicz et al., 1964) reported that some microorganisms were recovered at higher levels after Mars simulations as compared to original populations, but these studies were run at higher total atmospheric pressures (113 mb) and higher partial pressures of O_2 (up to 10%) than are found on Mars (6–10 mb and 0.13% O_2 , respectively; Tillman et al., 1993; Kieffer et al., 1992). A large body of literature exists on the labeled-release (LR) experiments developed for the Viking Landers (Levin, 1972; Levin and Straat, 1976, 1977, 1979a, 1979b, and the citations within these papers) that demonstrated active microbial growth at low pressures. But the Viking LR experiments were run at 92 mb total pressure for the first injection, and 116 mb total pressure for the second injection of nutrients and gases (Levin and Straat, 1979b). These pressures are well above those found at the surface of Mars (6–10 mb; Tillman et al., 1993). Several papers were found on microbial survival and possibly metabolic activity in suspended dust, hail, and water vapor in clouds (Dimmick et al., 1979; Imshenetsky et al., 1978; Mandrioli et al., 1973; Sattler et al., 2001), but these studies have all been done in air columns residing in or simulating Earth's troposphere which extends only to about 10 km above the ground near the poles and 15 km above the ground in mid and low latitudes. At a

15-km altitude, the total atmospheric pressure is approximately 120–150 mb, and, thus, is still above the pressure limits used in the Viking LR experiments and above the pressures found at the surface of Mars. Only a few papers were found that reported the recovery of viable bacteria and fungi in stratospheric air-samples obtained above 30 km (Imshenetsky et al., 1976, 1978; Wainwright et al., 2003, 2004) in which the air pressure begins to approach martian surface conditions of 6–10 mb, but these studies did not demonstrate microbial growth and replication at these low pressures.

More recently, preliminary reports were published by Schuerger and Nicholson (2005a, 2005b) and Schuerger et al. (2006) that indicated that both spore-forming and non-spore forming bacterial species had difficulty growing at pressures below 25 mb at 30 °C. In addition, some of the 13 species tested failed to exhibit growth at pressures significantly higher than 25 mb, and the effects of pressure were sometimes related to the presence or absence of oxygen (O_2) in the headspace above the microbial assays. In addition, a recent report by Kanervo et al. (2005) indicated that a *Synechocystis* sp. was capable of growth and replication at 100 mb if adequate carbon dioxide (CO_2) gas was available for photosynthesis. However, they did not test pressures lower than 100 mb.

The primary objective of the current study was to determine if common spacecraft contaminants in the genus *Bacillus* can replicate under the low pressure, low temperature, and high CO_2 atmospheres that approach those found at the surface of Mars. Two basic assumptions of the current studies included (1) that the growth assays would be conducted at low pressures, low temperatures, and high CO_2 atmospheres in which water and nutrients were not limiting, and (2) the bacteria were not exposed to UV irradiation. *Bacillus* spp. were chosen for this study because they are common contaminants of spacecraft surfaces (Dillon et al., 1973; La Duc et al., 2003; Puleo et al., 1973, 1977; Venkateswaran et al., 2001), they have been used in previous studies under martian conditions (Green et al., 1971; Hagen et al., 1964; Hawrylewicz et al., 1964; Koike et al., 1996; Mancinelli and Klovstad, 2000; Schuerger et al., 2003, 2005, 2006), and because spore-forming bacterial species are generally used as the benchmark for assessing the cleanliness of spacecraft surfaces prior to launch (Anonymous, 2006; De Vincenzi et al., 1998).

2. Materials and methods

2.1. Hypobaric bell-jar system

All temperature experiments were conducted within microbial incubators (range 0–80 °C; model Innova 4230, New Brunswick Scientific, Edison, NJ, USA). All low-pressure experiments were conducted within a 30-cm diameter glass bell-jar placed within a microbial incubator (Fig. 1). The bell-jar system was connected to a low-pressure controller (model PU845, KNF Neuberger, Inc., Trenton, NJ, USA) to accurately hold pressures down to 15 mb (± 1 mb). To create an Earth-normal O_2/N_2 atmosphere, room air was allowed to leak into the bell-jar system and the pressure controlled automatically



Fig. 1. Low-pressure bell-jar system used for testing the microbial activity of terrestrial microorganisms at pressures from 1013 mb down to 15 mb. The bell-jar was placed within a microbiological incubator capable of accurately holding temperature down to 0 °C. Carbon dioxide atmospheres were created by inserting four anaerobic pouches (around the stack of petri dishes used for bacterial growth) into the bell-jar system, immediately sealing the bell-jar, and pumping down to the desired set-point. Also shown are a low-pressure gauge and a set of Earth-normal controls held at 20 °C and 1013 mb.

at specific setpoints by the KNF vacuum controller. To create a pure CO₂ environment within the bell-jar, four anaerobic pouches (Remel, Inc., Lenexa, KS, USA) were inserted into the bell-jar and the system immediately pumped down to the pressures required for each test (Fig. 1). An indicator tablet from Remel, Inc., that was highly sensitive to low O₂, was placed within the bell-jar to confirm that an anaerobic environment was present. The indicator tablets would turn red under ultra-low partial pressures of oxygen (ppO₂), and remain blue under moderate to high ppO₂. To confirm this, the indicator tablets were tested in a second low-pressure chamber (not shown here) in which pure CO₂ or pure N₂ gases could be rapidly passed through the chamber to flush the ppO₂ out of the void spaces within 1–2 min. The indicator tablets would respond quickly to the loss of O₂ from the chamber, and, thus, were believed to accurately indicate anoxic conditions within the bell-jar system. In other preliminary tests, anaerobic indicator tablets were placed within petri dishes to confirm that the internal partial pressure of O₂ would be quickly evacuated from the internal void spaces of the petri dishes. In all tests, the anaerobic tablets placed within petri dishes would mimic the responses of the tablets placed outside of the petri dishes but within the general headspace of the bell-jar. This correlation was accepted as confirmation that the internal void spaces of the petri dishes

equilibrated very quickly with the anaerobic CO₂ atmospheres created within the bell-jar from the Remel anaerobic pouches. The pressures within the bell-jar system were set at either 1013, 100, 50, 35, 25, or 15 mb, and the pressure would stabilize at all set points within 10–15 min.

2.2. Microbiological techniques

A series of experiments were conducted to investigate the interactive effects of low pressure, low temperature, and pure CO₂ atmospheres on the growth and replication of seven *Bacillus* species. The bacteria tested were the following: *B. pumilus* SAFR-032, *B. pumilus* FO-36B, *B. subtilis* HA-101, *B. subtilis* 42HS-1, *B. megaterium* KL-197, *B. nealsonii* FO-092, and *B. licheniformis* KL-196. All of these species, except HA-101, have been recovered from the Odyssey or Mars Exploration Rovers (MER) spacecraft and spacecraft assembly facilities at JPL and KSC (Venkateswaran et al., 2001, 2003). *Bacillus subtilis* strain HA-101 (with *hisA1*, *metB5*; see Okubo and Yanagida, 1968) is a common laboratory strain used extensively in astrobiological studies (Horneck et al., 1994; Schuerger et al., 2003, 2006).

Bacteria were grown on trypticase soy agar (TSA), and were comprised either of vegetative cells streaked onto the TSA plates or endospores applied in a quantitative manner so as to permit estimation of colony numbers and colony diameters during specific tests. Bacteria were grown for either 48 h or 7 days (see tables). A simple rating scheme (see footnotes in tables) was used to score colony size as a measure of robustness of growth of the bacteria under the various conditions. The rating system was a very simple way to gauge whether the *Bacillus* spp. could grow and replicate under various conditions of low pressure, low temperature and pure CO₂ atmospheres in which water and nutrients were not limiting. In addition to the rating system, estimates for colony numbers and colony diameters were determined for endospores grown at different pressures under either O₂/N₂ or CO₂ atmospheres.

Assays with vegetative cells used 16-h cultures incubated at 30 °C on TSA. Cell-preps from the 16-h cultures were examined microscopically to confirm that no endospores had formed during this time period. If endospores were observed, those cultures were discarded. Vegetative cells from the 16-h cultures were streaked onto the upper surfaces of TSA media in 2 or 3 quadrants while flaming the transfer loop between quadrants. The evaluations of microbial growth were estimated only for the 2nd or 3rd quadrants to reduce the possibility of recording as positive growth those vegetative cells transferred over in the first quadrant. Controls were maintained under conditions conducive for growth (1013 mb at 30 °C under O₂/N₂) in parallel to all tests.

Vegetative cells of the seven *Bacillus* spp. were used in three separate experiments. First, vegetative cells were incubated at 30, 20, 15, 10, or 5 °C for 48 h or 7 days under either O₂/N₂ or CO₂ atmospheres at 1013 mb. Second, vegetative cells were incubated for 48 h at 30 °C at 1013 (Earth-control), 100, 50, 35, or 25 mb total pressure under either O₂/N₂ or CO₂ atmospheres. Third, to test the possibility that bacterial growth inhibition at

low pressures may be due to oxygen starvation, vegetative cells were transferred to TSA plates supplemented with 0.25% glucose and 0.1% nitrate (supplied as KNO_3) and incubated at 1013, 25, or 15 mb total pressure, under pure CO_2 atmospheres, and 20 °C for 48 h. The addition of glucose and nitrate to the TSA media would enhance the ability of bacterial cells to grow via anaerobic pathways such as fermentation or anaerobic respiration using nitrate as the terminal electron acceptor (Nakano and Zuber, 2002).

For endospore experiments, spores were prepared in quantified suspensions at densities of approximately 2×10^6 viable endospores per milliliter. Spores were diluted and then pipetted onto TSA media such that between 200–300 viable spores were dispensed per petri dish. The endospores were then immediately placed within the low-pressure bell-jar system and incubated for 48 h at 30 °C at 1013, 100, 50, 35, or 25 mb total pressure under either O_2/N_2 or CO_2 atmospheres. The numbers of colonies, colony diameters, and ratings of bacterial growth were measured for each species or strain.

In all petri dishes and assays in which “no growth” was observed, the cultures were transferred to a second microbial incubator and maintained an additional 24 h at 30 °C under an Earth-normal O_2/N_2 atmosphere at 1013 mb. This additional incubation period was conducted to determine if the vegetative cells or endospores were killed by the low pressure, low temperature, or CO_2 conditions, or whether they were only inhibited by these environmental conditions.

2.3. Statistical procedures

Statistical analyses were conducted with version 8.0 of the PC-based Statistical Analysis System (SAS) (SAS Institute, Inc., Cary, NC, USA). Data were subjected to analysis of variance procedures (PROC GLM) followed by protected least-squares mean separation tests ($P \leq 0.05$).

3. Results

The temperature minimum for vegetative cell growth after 48 h incubation was observed to be 15 °C for five of the seven *Bacillus* spp. under an Earth-normal O_2/N_2 atmosphere, and 20 °C for six of seven species under a pure CO_2 atmosphere (Table 1). The highest temperature minimum under CO_2 was observed to be 30 °C for *B. pumilus* FO-36B. The interactive effects of CO_2 and low temperature acted to raise the temperature minimum for most of the *Bacillus* spp. from 15 to 20 °C. Furthermore, growth rates under pure CO_2 atmospheres were significantly lower than under O_2/N_2 atmospheres at all temperatures in which growth occurred. For example, at 30 °C, the average growth rating dropped from an average of 4 under O_2/N_2 atmospheres (rating 4 = robust growth with colonies between 5 and 8 mm in diameter) to approximately 1 under a CO_2 atmosphere (rating 1 = colonies \approx 0.5 mm in diameter).

When cultures were incubated under O_2/N_2 atmospheres for 7 days (Table 2), weak growth was observed at 10 °C for *B. pumilus* SAFR-032, *B. pumilus* FO-36B, *B. megaterium*, and *B. nealsonii*, but not for *B. subtilis* HA-101, *B. subtilis*

Table 1

Effects of temperature and gas composition on growth of vegetative cells of seven *Bacillus* spp. at 101 mb for 48 h

<i>Bacillus</i> species	O_2/N_2				
	30 °C	20 °C	15 °C	10 °C	5 °C
<i>B. pumilus</i> (SAFR-032)	4 ^y a ^z	3 b	0.95 c	0 d	0 d
<i>B. pumilus</i> (FO-36B)	4 a	2.8 b	0.65 c	0 d	0 d
<i>B. subtilis</i> (HA-101)	4 a	2.4 b	0.5 b	0 c	0 c
<i>B. subtilis</i> (42HS-1)	4 a	3 b	0.45 c	0 d	0 d
<i>B. megaterium</i> (KL-197)	4 a	2.8 b	1.1 c	0 d	0 d
<i>B. nealsonii</i> (FO-092)	4 a	1.6 b	0 c	0 c	0 c
<i>B. licheniformis</i> (KL-196)	4 a	1.1 b	0 c	0 c	0 c
	CO_2				
<i>B. pumilus</i> (SAFR-032)	0.6 a	0.07 b	0 b	0 b	0 b
<i>B. pumilus</i> (FO-36B)	0.55 a	0 b	0 b	0 b	0 b
<i>B. subtilis</i> (HA-101)	1 a	0.07 b	0 b	0 b	0 b
<i>B. subtilis</i> (42HS-1)	0.75 a	0.09 b	0 b	0 b	0 b
<i>B. megaterium</i> (KL-197)	0.2 a	0.05 a	0 a	0 a	0 a
<i>B. nealsonii</i> (FO-092)	1.3 a	0.09 b	0 b	0 b	0 b
<i>B. licheniformis</i> (KL-196)	1.4 a	0.17 b	0 c	0 c	0 c

^y Rating scale: 4 = large robust colonies > 5 mm in diameter; 3 = colonies 2–4 mm in diameter; 2 = colonies \approx 1 mm in diameter; 1 = colonies \approx 0.5 mm in diameter; 0.50 = colonies < 0.5 mm in diameter; 0.1 = smallest visually discernable colonies (pin-prick sized colonies at \approx 0.1 mm in diameter); 0 = no growth. All bacteria were grown on trypticase soy agar (TSA).

^z Letters in rows designate significant differences among temperature treatments for individual species for each gas composition based on ANOVA and protected least significant difference analyses ($P \leq 0.5$; $n = 5$).

Table 2

Effects of temperature and gas composition on growth of vegetative cells of seven *Bacillus* spp. at 1013 mb for 7 days

<i>Bacillus</i> species	O_2/N_2				
	30 °C	20 °C	15 °C	10 °C	5 °C
<i>B. pumilus</i> (SAFR-032)	4 ^y a ^z	3.1 b	2.7 c	0.6 d	0 e
<i>B. pumilus</i> (FO-36B)	4 a	3 b	2.7 c	0.25 d	0 d
<i>B. subtilis</i> (HA-101)	4 a	2.7 b	2.7 b	0 c	0 c
<i>B. subtilis</i> (42HS-1)	4 a	2.6 b	2.4 b	0 c	0 c
<i>B. megaterium</i> (KL-197)	4 a	2.8 b	2.8 b	0.5 c	0 d
<i>B. nealsonii</i> (FO-092)	4 a	2.2 b	0.7 c	0.15 d	0 d
<i>B. licheniformis</i> (KL-196)	4 a	1.6 b	0.7 c	0 d	0 d
	CO_2				
<i>B. pumilus</i> (SAFR-032)	0.75 a	0.4 ab	0 b	0 b	0 b
<i>B. pumilus</i> (FO-36B)	0.9 a	0.5 b	0 b	0 b	0 b
<i>B. subtilis</i> (HA-101)	0.8 a	0.65 a	0 b	0 b	0 b
<i>B. subtilis</i> (42HS-1)	0.8 a	0.85 a	0 b	0 b	0 b
<i>B. megaterium</i> (KL-197)	0.15 a	0.15 a	0 a	0 a	0 a
<i>B. nealsonii</i> (FO-092)	1.4 a	1.7 a	0 b	0 b	0 b
<i>B. licheniformis</i> (KL-196)	1.4 a	1.3 a	0 b	0 b	0 b

^y Rating scale: 4 = large robust colonies > 5 mm in diameter; 3 = colonies 2–4 mm in diameter; 2 = colonies \approx 1 mm in diameter; 1 = colonies \approx 0.5 mm in diameter; 0.50 = colonies < 0.5 mm in diameter; 0.1 = smallest visually discernable colonies (pin-prick sized colonies at \approx 0.1 mm in diameter); 0 = no growth. All bacteria were grown on trypticase soy agar (TSA).

^z Letters in rows designate significant differences among temperature treatments for individual species for each gas composition based on ANOVA and protected least significant difference analyses ($P \leq 0.5$; $n = 5$).

42HS-1, and *B. licheniformis*. In contrast, incubation under CO_2 atmospheres for 7 days did not change the temperature minimums for most of the seven species; the minimum temperature for positive growth under CO_2 atmospheres remained at

Table 3

Effects of pressure on growth of vegetative cells of seven *Bacillus* spp. under O₂/N₂ or CO₂ atmospheres at 30 °C for 48 h

<i>Bacillus</i> species	O ₂ /N ₂				
	1013 mb	100 mb	50 mb	35 mb	25 mb
<i>B. pumilus</i> (SAFR-032)	4 ^y a ^z	3.5 b	2.2 c	0.42 d	0.07 e
<i>B. pumilus</i> (FO-36B)	4 a	3.5 ab	2.2 c	0.32 d	0.02 e
<i>B. subtilis</i> (HA-101)	4 a	3.1 b	2.3 c	0.9 d	0.05 e
<i>B. subtilis</i> (42HS-1)	4 a	3.1 b	2.2 c	0.57 d	0.07 e
<i>B. megaterium</i> (KL-197)	4 a	3.4 b	2.1 c	0.7 d	0 e
<i>B. nealsonii</i> (FO-092)	4 a	3.4 b	2.8 c	1.5 d	0.1 e
<i>B. licheniformis</i> (KL-196)	4 a	3.4 b	2.8 c	0.65 d	0.05 e
<i>Bacillus</i> species	CO ₂				
	1013 mb	100 mb	50 mb	35 mb	25 mb
<i>B. pumilus</i> (SAFR-032)	0.6 a	0.29 b	0.07 c	0.17 bc	0.05 c
<i>B. pumilus</i> (FO-36B)	0.5 a	0.07 b	0.05 b	0.12 b	0.02 b
<i>B. subtilis</i> (HA-101)	1 a	0.9 a	0.85 a	0.35 b	0.2 b
<i>B. subtilis</i> (42HS-1)	0.55 a	0.1 b	0.02 b	0.2 b	0.12 b
<i>B. megaterium</i> (KL-197)	0.6 a	0.14 b	0.05 b	0.2 b	0 b
<i>B. nealsonii</i> (FO-092)	1.6 a	1.7 a	1.8 a	1.4 a	0.75 b
<i>B. licheniformis</i> (KL-196)	1.3 a	1.2 a	1.3 a	1.2 a	0.6 b

^y Rating scale: 4 = large robust colonies > 5 mm in diameter; 3 = colonies 2–4 mm in diameter; 2 = colonies ≈ 1 mm in diameter; 1 = colonies ≈ 0.5 mm in diameter; 0.50 = colonies < 0.5 mm in diameter; 0.1 = smallest visually discernable colonies (pin-prick sized colonies at ≈ 0.1 mm in diameter); 0 = no growth. All bacteria were grown on trypticase soy agar (TSA).

^z Letters in rows designate significant differences among temperature treatments for individual species for each pressure and gas based on ANOVA and protected least significant difference analyses ($P \leq 0.5$; $n = 5$).

20 °C. However, there was a slight increase in the cumulative ratings of all bacteria at 7 days compared to 48 h, thus indicating that additional growth was possible at 20 °C over 7 days compared to 48 h. All seven bacteria failed to grow at 5 °C under either O₂/N₂ or CO₂ atmospheres at either 48 h or 7 days of incubation.

Vegetative cells of all seven *Bacillus* spp. were then grown in O₂/N₂ or pure CO₂ atmospheres under five pressures at 30 °C for 48 h. The pressures tested were 1013 (Earth-normal control), 100, 50, 35, or 25 mb. Growth under O₂/N₂ atmospheres was relatively robust from 1013 to 50 mb, and then took a sharp decrease at 35 and 25 mb. Weak growth for six of the seven *Bacillus* spp. was observed at 25 mb under O₂/N₂ atmospheres; only *B. megaterium* failed to grow at 25 mb under O₂/N₂ (Table 3).

In contrast, vegetative cells of the seven *Bacillus* spp. exhibited weak growth under CO₂ atmospheres under all of the pressures tested. *Bacillus nealsonii* and *B. licheniformis* exhibited the strongest growth under CO₂ atmospheres at all pressures tested, and in fact, grew significantly better at 25 mb as compared to these species grown under O₂/N₂ atmospheres at 25 mb (Table 3). However, it must be emphasized that the growth at 25 mb under either O₂/N₂ or CO₂ atmospheres for all six species that exhibited growth (*B. megaterium* failed to grow at 25 mb under either gas) was at the lower limit of detection and did not occur consistently in every repetition of the experiment. For example, *B. pumilus* SAFR-032 and *B. pumilus* FO-36B had growth ratings of 0.05 and 0.02, respectively, at 25 mb under CO₂ (Table 3). However, with both of these strains, only one in five repetitions of the experiments showed positive

Table 4

Effects of pressure and glucose/nitrate amendments on growth of vegetative cells of seven *Bacillus* spp. at 20 °C for 48 h under pure CO₂ atmospheres

<i>Bacillus</i> species	TSA media ^y		
	1013 mb	25 mb	15 mb
<i>B. pumilus</i> (SAFR-032)	0 ^z	0	0
<i>B. pumilus</i> (FO-36B)	0	0	0
<i>B. subtilis</i> (HA-101)	0.07	0	0
<i>B. subtilis</i> (42HS-1)	0	0	0
<i>B. megaterium</i> (KL-197)	0.07	0	0
<i>B. nealsonii</i> (FO-092)	0	0	0
<i>B. licheniformis</i> (KL-196)	0	0	0
<i>Bacillus</i> species	TSAGN media ^y		
	1013 mb	25 mb	15 mb
<i>B. pumilus</i> (SAFR-032)	0.03	0	0
<i>B. pumilus</i> (FO-36B)	0	0	0
<i>B. subtilis</i> (HA-101)	0.08	0	0
<i>B. subtilis</i> (42HS-1)	0.28	0.07	0
<i>B. megaterium</i> (KL-197)	0.03	0	0
<i>B. nealsonii</i> (FO-092)	0	0	0
<i>B. licheniformis</i> (KL-196)	0.03	0	0

^y TSA media = trypticase soy agar (Difco); TSAGN = TSA supplemented with 0.25% glucose (G) and 0.1% nitrate (N).

^z Rating scale: 4 = large robust colonies > 5 mm in diameter; 3 = colonies 2–4 mm in diameter; 2 = colonies ≈ 1 mm in diameter; 1 = colonies ≈ 0.5 mm in diameter; 0.50 = colonies < 0.5 mm in diameter; 0.1 = smallest visually discernable colonies (pin-prick sized colonies at ≈ 0.1 mm in diameter); 0 = no growth. All bacteria were grown on trypticase soy agar (TSA) ($n = 3$).

growth. Thus, although positive ratings were logged in Table 3 for these two species, the evidence for growth at 25 mb for both strains of *B. pumilus* was tentative, at best. Furthermore, all growth ratings below 0.1 must be considered tentative at this time because at least one of the five repetitions of the experiments yielded negative results. Thus, very low growth ratings of below 0.1 suggest that the *Bacillus* spp. were at the very lower limit of growth, and that sometimes the responses were equivocal. All ratings above 0.1 were consistently positive throughout all tests.

The lower limit of pressure that was possible with the bell-jar system at 30 °C was 25 mb. Below this level there were serious problems with the desiccation of the TSA growth medium over the 48 h of each test. Thus, in order to probe bacterial growth at lower pressures, there must be a concomitant decrease in temperature. All seven *Bacillus* spp. grew at 20 °C and 1013 mb under CO₂ atmospheres (Tables 1 and 2), thus, a test was designed to determine if pressure and temperature could be lowered just slightly and still retain the capability to support growth of any of the seven *Bacillus* spp. In addition, two separate media were used in this test in which the basal TSA medium was also amended with glucose and nitrate (TSAGN) in order to enhance anaerobic metabolism. Growth of the *Bacillus* spp. on the basal TSA was compared to growth on TSAGN under pure CO₂ atmospheres maintained at 20 °C for 48 h. Results indicated that on the standard TSA medium, all seven *Bacillus* spp. were unable to grow at 25 or 15 mb at 20 °C under pure CO₂ atmospheres (Table 4). In fact, only two species, *B. subtilis* HA-101 and *B. megaterium* (KL-197), exhibited any growth under an Earth-normal pressure of 1013 mb under the conditions of pure CO₂ and 20 °C. Although the TSAGN media did increase

Table 5

Germination and growth of endospores of seven *Bacillus* spp. at low pressures under an Earth-normal O₂/N₂ atmosphere and grown at 30 °C for 48 h

<i>Bacillus</i> species	Colony number				
	1013 mb	100 mb	50 mb	35 mb	25 mb
<i>B. pumilus</i> (SAFR-032)	317 a ^y	270 b	215 c	0 d	0 d
<i>B. pumilus</i> (FO-36B)	330 a	315 a	264 b	0 c	0 c
<i>B. subtilis</i> (HA-101)	224 a	221 a	120 b	75 c	0 d
<i>B. subtilis</i> (42HS-1)	254 a	244 a	231 a	0 b	0 b
<i>B. megaterium</i> (KL-197)	140 a	114 a	131 a	0 b	0 b
<i>B. nealsonii</i> (FO-092)	152 a	147 a	146 a	0 b	0 b
<i>B. licheniformis</i> (KL-196)	216 a	207 a	220 a	0 b	0 b
Colony diameter (mm)					
<i>B. pumilus</i> (SAFR-032)	3.7 a	2.9 b	1.0 c	0 d	0 d
<i>B. pumilus</i> (FO-36B)	3.7 a	2.9 b	1.0 c	0 d	0 d
<i>B. subtilis</i> (HA-101)	7.2 a	5.2 b	3.3 c	0.4 d	0 e
<i>B. subtilis</i> (42HS-1)	6.2 a	3.5 b	1.9 c	0 d	0 d
<i>B. megaterium</i> (KL-197)	6.7 a	5.2 b	2.0 c	0 d	0 d
<i>B. nealsonii</i> (FO-092)	5.8 a	5.8 a	4.2 b	0 c	0 c
<i>B. licheniformis</i> (KL-196)	4.2 a	4.1 a	3.7 b	0 c	0 c
Growth rating ^z					
<i>B. pumilus</i> (SAFR-032)	4.0 a	3.0 b	1.8 c	0 d	0 d
<i>B. pumilus</i> (FO-36B)	4.0 a	3.0 b	1.8 c	0 d	0 d
<i>B. subtilis</i> (HA-101)	4.0 a	3.3 b	2.3 c	0.5 d	0 e
<i>B. subtilis</i> (42HS-1)	4.0 a	3.0 b	2.0 c	0 d	0 d
<i>B. megaterium</i> (KL-197)	4.0 a	3.7 a	2.3 b	0 c	0 c
<i>B. nealsonii</i> (FO-092)	4.0 a	4.0 a	3.0 b	0 c	0 c
<i>B. licheniformis</i> (KL-196)	4.0 a	4.0 a	3.0 b	0 c	0 c

^y Letters in rows designate significant differences among pressure treatments for individual species based on ANOVA and protected least significant difference analyses ($P \leq 0.5$; $n = 3$).

^z Rating scale: 4 = large robust colonies > 5 mm in diameter; 3 = colonies 2–4 mm in diameter; 2 = colonies \approx 1 mm in diameter; 1 = colonies \approx 0.5 mm in diameter; 0.50 = colonies < 0.5 mm in diameter; 0.1 = smallest visually discernable colonies (pin-prick sized colonies at \approx 0.1 mm in diameter); 0 = no growth. All bacteria were grown on trypticase soy agar (TSA).

the growth of several *Bacillus* spp. at 1013 mb, only *B. subtilis* 42HS-1 exhibited any positive growth at 25 mb and 20 °C. No bacteria grew on either TSA or TSAGN media at 15 mb and 20 °C.

A series of tests at low pressures were conducted at 30 °C for 48 h in which endospores from all seven *Bacillus* spp. were maintained under either O₂/N₂ or CO₂ atmospheres. In contrast to vegetative cells, endospores from all seven *Bacillus* spp. had significant difficulty in germinating and then growing at low pressures (Tables 5 and 6). In all tests, no endospores were observed to germinate and grow on TSA at 25 mb under either O₂/N₂ or CO₂ atmospheres. This is in contrast to the weak growth of vegetative cells in six of seven *Bacillus* spp. at 25 mb observed under O₂/N₂ or CO₂ atmospheres (Table 3). At 35 mb under O₂/N₂ atmospheres, only endospores of *B. subtilis* HA-101 were observed to germinate and grow (Table 5); all other species exhibited pressure minima of 50 mb in these tests.

Under CO₂ atmospheres, only endospores of *B. nealsonii* and *B. licheniformis* appeared capable of germination and growth as pressure was reduced from 1013 to 35 mb (Table 6). All other species did not appear capable of germinating and growing at 100, 50, 35, or 25 mb under pure CO₂ atmospheres. In addition, the colony numbers, colony diameters, and growth

Table 6

Germination and growth of endospores of seven *Bacillus* spp. at low pressures under a simulated martian atmosphere (pure CO₂) and grown at 30 °C for 48 h

<i>Bacillus</i> species	Colony number				
	1013 mb	100 mb	50 mb	35 mb	25 mb
<i>B. pumilus</i> (SAFR-032)	0	0	0	0	0
<i>B. pumilus</i> (FO-36B)	0	0	0	0	0
<i>B. subtilis</i> (HA-101)	91 a ^y	0 b	0 b	0 b	0 b
<i>B. subtilis</i> (42HS-1)	3 a	0 a	0 a	0 a	0 a
<i>B. megaterium</i> (KL-197)	0	0	0	0	0
<i>B. nealsonii</i> (FO-092)	60 a	66 a	50 a	54 a	0 b
<i>B. licheniformis</i> (KL-196)	135 a	141 a	147 a	138 a	0 b
Colony diameter (mm)					
<i>B. pumilus</i> (SAFR-032)	0	0	0	0	0
<i>B. pumilus</i> (FO-36B)	0	0	0	0	0
<i>B. subtilis</i> (HA-101)	0.7 a	0 b	0 b	0 b	0 b
<i>B. subtilis</i> (42HS-1)	0.2 a	0 a	0 a	0 a	0 a
<i>B. megaterium</i> (KL-197)	0	0	0	0	0
<i>B. nealsonii</i> (FO-092)	3.1 a	2.7 b	2.5 b	1.7 b	0 c
<i>B. licheniformis</i> (KL-196)	1.4 a	1.4 a	1.3 a	0.7 b	0 c
Growth rating ^z					
<i>B. pumilus</i> (SAFR-032)	0	0	0	0	0
<i>B. pumilus</i> (FO-36B)	0	0	0	0	0
<i>B. subtilis</i> (HA-101)	0.5 a	0 b	0 b	0 b	0 b
<i>B. subtilis</i> (42HS-1)	0.03 a	0 a	0 a	0 a	0 a
<i>B. megaterium</i> (KL-197)	0	0	0	0	0
<i>B. nealsonii</i> (FO-092)	1.9 a	1.3 b	1.3 b	1.0 b	0 c
<i>B. licheniformis</i> (KL-196)	1.0 a	1.0 a	0.8 a	0.7 a	0 b

^y Letters in rows designate significant differences among pressure treatments for individual species based on ANOVA and protected least significant difference analyses ($P \leq 0.5$; $n = 3$).

^z Rating scale: 4 = large robust colonies > 5 mm in diameter; 3 = colonies 2–4 mm in diameter; 2 = colonies \approx 1 mm in diameter; 1 = colonies \approx 0.5 mm in diameter; 0.50 = colonies < 0.5 mm in diameter; 0.1 = smallest visually discernable colonies (pin-prick sized colonies at \approx 0.1 mm in diameter); 0 = no growth. All bacteria were grown on trypticase soy agar (TSA).

ratings for *B. nealsonii* and *B. licheniformis* appeared to be relatively insensitive to decreasing pressure from 1013 to 35 mb. In most cases there were no statistical differences in these parameters as the endospores of *B. nealsonii* and *B. licheniformis* were incubated at lower pressures, but between 35 and 25 mb under CO₂, all growth activity ceased for *B. nealsonii* and *B. licheniformis*. Thus, the thresholds for growth with *B. nealsonii* and *B. licheniformis* appeared to be very sharp and exist somewhere between 35 and 25 mb. Endospores from both strains of *B. pumilus* and from *B. subtilis* 42HS-1 were unable to germinate and grow at an Earth-normal pressure of 1013 mb under pure CO₂ atmospheres.

The complete lack of visible colony growth of several *Bacillus* spp. in preliminary experiments at low pressures prompted us to ask whether low pressure was bactericidal or bacteriostatic under these conditions. To test this question, cultures exhibiting no growth after 48 h or 7 days under all test conditions (i.e., low pressure, low temperature, or high CO₂ atmosphere) were transferred to conditions conducive to growth; namely, incubation for an additional 24 h under an O₂/N₂ atmosphere at 1013 mb and 30 °C. In all cases, bacteria were able to grow nominally when returned to Earth-normal conditions (data not shown). Thus, incubation at low pressure, low temperature, and

high CO₂ environments did not irreversibly inactivate the bacteria, at least over the time periods tested (48 h or 7 days).

4. Discussion

Landers or rovers sent to Mars must undergo significant levels of spacecraft cleaning and sterilization activities in order to reduce the bioloads prior to launch to at least 3×10^5 total viable spore-forming bacteria per vehicle (Anonymous, 2006). However, the total microbial bioloads on spacecraft likely will be one to two decades higher than this level based on models for the contamination of the Moon by unmanned robotic landers (Dillon et al., 1973) and recent progress on characterizing non-culturable microorganisms on spacecraft (Venkateswaran et al., 2001, 2003). Although there will be significant losses of species diversity and bioload during the 6–8 month cruise phase to Mars (Schuerger, 2004), it is likely that viable terrestrial microorganisms have and will be successfully landed on the martian surface. The risk of contaminating scientific payloads, the local terrain, or the global Mars environment will depend on four key factors: (i) survival of viable microorganisms from an Earth launch to Mars landing, (ii) dispersal of viable microorganisms away from the landed or crashed vehicles, (iii) long-term survival of the dispersed bioload, and (iv) the ability of the dispersed bioload to undergo replicative growth in the martian surface environment. Of these factors, the one of greatest concern for the forward contamination of Mars is whether terrestrial microorganisms can undergo replicative growth on the surface of Mars. If microbes cannot grow at Mars surface conditions, then the risks to the forward contamination of Mars would be limited to the distance of dispersal away from the landed or crashed vehicle and the length of time that the cells or spores remain viable. Furthermore, if replicative growth of terrestrial microbes on Mars cannot occur, then the level of contamination at each landing site would steadily decrease due to the action of various biocidal factors (e.g., solar UV, soil oxidants, cosmic rays, etc.) (Schuerger, 2004; Horneck et al., 2003). If the answer is yes for replicative growth of terrestrial microorganisms on Mars, then the bioloads on landed or crashed spacecraft constitute significant risks to the long-term viability of scientific exploration of Mars and the search for an extant microbiota on Mars.

In the current study, we tested the abilities of seven *Bacillus* spp., known to be common contaminants of spacecraft, to survive and grow under Mars surface conditions if shielded from solar UV. The results presented here indicate that of the *Bacillus* spp. tested, all strains had significant difficulties growing at pressures, temperatures, and gas compositions that began to approach those found on the surface of Mars. First, vegetative cells of the seven *Bacillus* spp. failed to grow under Earth-normal pressures of 1013 mb after 48 h at 5 or 10 °C regardless of the gas composition used. However, when exposed to low temperatures, the vegetative cells of all bacteria grew under O₂/N₂ but not under CO₂ at 15 °C indicating that low temperatures and CO₂ atmospheres acted in concert to inhibit growth. Even though there was a slight increase in growth noted at 10 °C for the *Bacillus* spp. exposed to 7 d under O₂/N₂ at-

mospheres, there was no apparent change in the temperature minima of these strains grown for 7 d at 15 °C under CO₂. Data collected by the infrared thermal mappers (IRTM) on both Viking orbiters (Kieffer, 1976; Kieffer et al., 1977, 1992) indicated daily highs for the Viking landing (VL) sites ranged between –85 °C for VL2 in the winter to –10 °C for VL1 in the summer. Although the southern summer day-time highs on Mars can reach 20 °C for short periods of time near local noon (Kieffer et al., 1977), the average surface temperature on Mars remains a chilly –60 °C (Kieffer et al., 1992). Thus, even if viable endospores of the *Bacillus* spp. tested here were present on the Viking landers, temperature alone would have made it very difficult to carry out active metabolism and replication on Mars during the nominal missions, and the CO₂ atmosphere on Mars would have accentuated the inhibitory effects of low temperature. The temperature profiles of both the Mars Pathfinder landing site (Schofield et al., 1997) and the Mars Exploration Rovers (MER) Opportunity and Spirit (Smith et al., 2004; Squyres et al., 2004) are similar to the Viking data, and support the conclusion that temperature alone can be inhibitory to the growth of common *Bacillus* spp. on Mars.

Second, vegetative cells of all seven *Bacillus* spp. exhibited significant reductions in growth when exposed to reduced pressures in either O₂/N₂ or CO₂ atmospheres. Similar to the interactive effects of low temperature and CO₂ atmospheres on microbial activity, the interactions of low pressure and CO₂ atmospheres acted together to increase the inhibitory nature of the simulated martian conditions. For example, when comparing growth in O₂/N₂ to growth in CO₂, atmospheres the growth-ratings were always significantly lower for CO₂ assays. Other than three preliminary reports of the current study (Schuerger and Nicholson, 2005a, 2005b; Schuerger et al., 2006), no other study in the literature was found that described microbial activity and replication at pressures below 92 mb (1st injection of the nutrients in the Viking LR assay; Levin and Straat, 1979b). We establish here for the first time that vegetative cells of *Bacillus* spp. appear to be able to carry out active metabolism and replication down to at least 35 mb total atmospheric pressure in O₂/N₂ or CO₂ atmospheres, and that six of the seven species appear able to replicate at 25 mb total pressure in O₂/N₂ or CO₂. Only the strain *B. megaterium* KL-197 failed to replicate at 25 mb in either gas. These results support the conclusion that high CO₂ atmospheres alone are likely to be inhibitory to a diversity of terrestrial microorganisms typically found on spacecraft. This conclusion is further supported by a review of the literature on microbial sampling of spacecraft (see Schuerger, 2004; Taylor, 1974) in which most of the microbial species recovered from spacecraft surfaces are mesophilic aerobic species of bacteria, fungi, and yeasts that are likely to be inhibited by the high CO₂ anaerobic atmosphere on Mars.

Third, the propagule types of *Bacillus* spp. most likely present on spacecraft surfaces are not actively replicating vegetative cells but rather dormant endospores. Endospores in the genus *Bacillus* are significantly more resistant to the harsh conditions encountered in space than are vegetative cells of the same species (Horneck et al., 1994; Nicholson et al., 2000, 2005). Thus, the effects of low pressure and gas composition

on the germination and growth of endospores were studied in order to determine if the inhibitory effects of these parameters were different as compared to vegetative cells. In all low-pressure assays in either O₂/N₂ or CO₂ atmospheres, dormant endospores of all seven *Bacillus* spp. failed to germinate and grow at 25 mb. In fact, endospores from several of the *Bacillus* spp. (both *B. pumilus* strains and *B. megaterium*) failed to germinate at 1013 mb under pure CO₂ atmospheres; and five of seven *Bacillus* spp. failed to germinate and grow at 100 mb of CO₂ or lower. One intriguing observation was that at 35 mb of total pressure, *B. subtilis* HA-101 was able to germinate under O₂/N₂ but not CO₂ atmospheres, and conversely, *B. nealsonii* FO-092 and *B. licheniformis* KL-196 were able to germinate and grow under CO₂ but not O₂/N₂ atmospheres. At first, we thought these anomalies were random fluctuations in the responses of these bacteria to the low-pressure environments, but two observations now lead us to conclude that they accurately represent the responses of these species to low-pressure O₂/N₂ and CO₂ atmospheres. First, the responses mentioned above were very consistent over the course of three experiments. Second, we have observed this phenomenon with at least six non-spore forming species (two strains of *Escherichia coli*, *Enterococcus faecalis*, *Proteus mirabilis*, *Staphylococcus aureus*, *Paenibacillus pabuli*) in other ongoing hypobaric experiments on microbial growth under martian conditions (Schuerger and Nicholson, 2005a, 2005b; Schuerger et al., 2006). These results support the conclusion that low pressure alone can be strongly inhibitory to the germination and growth of common bacterial species on Mars.

It is interesting to note that in general, low pressure exerted a greater inhibitory effect on spore germination than it did on vegetative growth. While most vegetative cells could still grow at an extremely low level of activity at 25 mb in either O₂/N₂ or CO₂ atmospheres (Table 3), spore germination was completely inhibited in all strains at 25 mb, or below, regardless of the atmosphere or growth medium used (Tables 4, 5, and 6). Are the inhibitory effects on growth and germination truly exerted by low pressure itself, or is it a secondary effect of lowering the amount of oxygen available to cells? Certainly in some cases a lack of O₂ may be involved. For example, spores of the two *B. pumilus* strains and the *B. megaterium* strain were unable to germinate in CO₂ atmosphere even at 1013 mb (Table 6) even though these same strains could grow vegetatively under the same conditions (Table 3). In contrast, *B. nealsonii* and *B. licheniformis*, two facultatively anaerobic strains, were clearly able to both grow vegetatively and germinate endospores at 1013 mb in the absence of oxygen (Tables 3 and 6), yet germination was still inhibited at 25 mb (Table 6), which argues in favor of an effect of low pressure independent of oxygen concentration. At present the molecular or biophysical mechanisms underlying these hypobaric effects are unclear, and we are currently exploring several possible mechanisms that might directly affect microbial activity at low pressures.

There are several important conclusions that might be drawn from these experiments. First, dormant endospores of at least seven *Bacillus* spp. were inhibited by incubation at low pressures under CO₂ atmospheres and, in general, failed to germinate

at either 35 or 25 mb. By extrapolation, we presume that endospores would be unable to germinate and grow at 7 mb on Mars if they cannot germinate and grow at 25 mb under CO₂. Second, actively growing vegetative cells of the same species were able to continue replication down to at least 25 mb in either O₂/N₂ or CO₂ atmospheres maintained at 30 °C. However, there was no growth observed at 15 mb when cultures of actively replicating vegetative cells were maintained at 20 °C under CO₂. Even the addition of glucose and nitrate to the TSA media [added to improve anaerobic metabolic activity (Nakano and Zuber, 2002)] failed to significantly improve microbial growth at low pressure. Third, the low-pressure, low temperature, or high CO₂ conditions were not biocidal to either vegetative cells or endospores of the seven *Bacillus* spp. In all cases in which a zero growth rating was observed in these assays (Tables 1–6), all cultures exhibited obvious and robust growth once the cultures were returned to conducive conditions of an Earth-normal O₂/N₂ atmosphere, 1013 mb total pressure, and 30 °C. Fourth, no bacteria were observed to grow and replicate below 25 mb in any of the tests reported here. Fifth, interactive effects of low pressure, low temperature, and high CO₂ atmospheres generally acted to increase the inhibitory nature of the simulated martian conditions, but these data cannot precisely establish if these interactions were additive, synergistic, antagonistic, or independent. For example, the effects of temperature and gas composition (Table 1) appeared to be additive in which there was a slight increase in the temperature minima of the seven *Bacillus* spp. grown under CO₂ at low temperatures. But in contrast, the effects of low pressure and CO₂ may have been either synergistic (for SAFR-032, FO-36B, HA-101, 42HS-1, KL-197) or independent (for FO-092 and KL-196) when endospores were grown under simulated martian conditions. Thus, the data presented herein support the conclusions that interactive effects of low pressure, low temperature, and CO₂ atmospheres can be complex, that there can be species-specific effects, and in general act to increase the inhibitory nature of the conditions found on the surface of Mars.

In recent papers (Clark et al., 2005; Horneck et al., 1994, 2003; Ming et al., 2006; Newsom and Hagerty, 1997, 1999; Schuerger et al., 2003; Schuerger, 2004; Yen et al., 2000), several diverse biocidal conditions were identified that are likely to contribute to the loss of viability of launched bioloads on spacecraft sent to Mars. These included the following (not in priority): (1) solar UV irradiation, (2) low pressure, (3) extreme desiccating conditions in space, (4) extreme diurnal temperature fluctuations, (5) solar particle events, (6) galactic cosmic rays, (7) UV-glow discharge from blowing dust, (8) solar UV-induced volatile oxidants [e.g., O₂⁺, O⁺, H₂O₂, NO_x, O₃], (9) globally distributed oxidizing soils, (10) extremely high salt levels [e.g., MgCl₂, NaCl, FeSO₄, and MgSO₄] in surficial soils at some sites on Mars, (11) high concentrations of heavy metals in martian soils, (12) likely acidic conditions in martian regolith, and (13) high CO₂ concentrations in the global atmosphere. The results of the current study emphasize the importance of considering the interactive effects of these biocidal factors in characterizing the effects of the martian environment on the ability of terrestrial microorganisms to survive,

replicate, and evolve on Mars. In the current study, interactive combinations of low pressure, low temperature, and high CO₂ atmospheres generally increased the inhibitory effects of the Mars simulations. We believe it is then reasonable to expect that the additional environmental factors listed above may impart even greater levels of stress on terrestrial microorganisms found on spacecraft that are landed on the surface of Mars. We conclude that interactive effects of a diversity of biocidal factors on Mars missions are likely to render the launched bioloads of spacecraft either dead or severely inhibited. In either case, it seems reasonable to predict that terrestrial microorganisms on spacecraft are unlikely to find it easy to survive, replicate, and evolve on the surface of Mars. In addition, of all the biocidal factors listed above, the effects of low pressure on the growth and replication of terrestrial microorganisms is the least understood. Supporting this suggestion is the fact that this study, and three preliminary reports (Schuerger and Nicholson, 2005a, 2005b; Schuerger et al., 2006), are the first to indicate that there might be a low-pressure threshold near 25 mb below which common spacecraft contaminants might not be able to replicate on Mars.

5. Conclusions

In summary, these results indicate that of the bacteria tested (seven *Bacillus* spp.), all strains had significant difficulties growing at low pressures, low temperatures, and high CO₂ atmospheres that began to approach those found on the surface of Mars. Similar results have been observed with 29 strains of 20 species of non-spore forming mesophilic bacteria incubated under low-pressure and high CO₂ conditions (Schuerger et al., 2006). Thus, the microorganisms that remain viable on spacecraft surfaces after the 6–8 month cruise-phase to Mars may not be readily capable of growth on the surface. However, in order to broaden this conclusion into a more established paradigm, many additional tests are required. First, additional species of microorganisms typically recovered from spacecraft surfaces need to be tested at pressures down to 7–10 mb under accurately simulated martian conditions. Second, future research should include assays on microbial activity that test interactive effects of potentially biostatic or biocidal factors found on the surface of Mars (see list above). The results presented herein clearly demonstrated that interactive effects can increase inhibition of growth of several bacteria typically found on spacecraft surfaces. Third, only culturable mesophilic bacteria were used in these experiments, and additional work should include psychrophilic, halophilic, acidophilic, and other extremophilic species in order to examine a wide range of terrestrial microorganisms at low pressures. Fourth, non-culturable microorganisms need to be included in low-pressure tests in order to rule out their growth under conditions similar to Mars. Fifth, a wide range of additional test procedures are required to expand upon these results. The assays used in these tests examined bacterial growth on primarily one selective medium (TSA) after primarily 48 h incubation at 30 °C. A few tests with low temperatures were extended to 7 days, and a few tests utilized a glucose/nitrate amended TSA medium. Future experiments are

therefore required to include longer incubation times, more precise measurements of metabolic activity and growth than those used here, and testing with a wider range of carbon sources available for growth. It is possible that the species tested in the current study were metabolically active at low pressures, albeit at extremely low levels, but the assays were not sensitive enough to capture these responses. Thus, the results presented herein should be viewed as intriguing preliminary findings that indicate terrestrial bacteria may not be capable of growth under martian conditions. However, many additional experiments are necessary in order to broaden this preliminary conclusion into a possible paradigm for Mars.

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