Research Paper

*Bacillus subtilis* Spore Survival and Expression of Germination-Induced Bioluminescence After Prolonged Incubation Under Simulated Mars Atmospheric Pressure and Composition: Implications for Planetary Protection and Lithopanspermia

WAYNE L. NICHOLSON¹ and ANDREW C. SCHUERGER²

ABSTRACT

Bacterial endospores in the genus *Bacillus* are considered good models for studying interplanetary transfer of microbes by natural or human processes. Although spore survival during transfer itself has been the subject of considerable study, the fate of spores in extraterrestrial environments has received less attention. In this report we subjected spores of a strain of *Bacillus subtilis*, containing luciferase resulting from expression of an *sspB-luxAB* gene fusion, to simulated martian atmospheric pressure (7–18 mbar) and composition (100% CO₂) for up to 19 days in a Mars simulation chamber. We report here that survival was similar between spores exposed to Earth conditions and spores exposed up to 19 days to simulated martian conditions. However, germination-induced bioluminescence was lower in spores exposed to simulated martian atmosphere, which suggests sublethal impairment of some endogenous spore germination processes. Key Words: *Bacillus subtilis* spores—Germination-induced bioluminescence—Lithopanspermia—Simulated martian atmosphere.

Astrobiology 5, 536–544.

INTRODUCTION

A rise of interest in the possibility of the survival, growth, and metabolism of terrestrial bacteria in the Mars environment has resulted from astrobiological studies of (i) interplanetary transfer of microbes in impact ejecta and (ii) study of microbes as potential contaminants of Mars rovers and landers (reviewed extensively in Mileikowsky et al., 2000; Nicholson et al., 2000, 2005; Horneck et al., 2002; Schuerger, 2004, and references therein). Although a variety of microorganisms have been described that could survive the rigors of spaceflight (reviewed in Horneck et al., 2002), spores of the gram-positive bacterium *Bacillus subtilis* are considered the experimental model of choice due to their hardness, reproducibility of their inactivation response, stability, and ease of experimental manipulation (Nicholson et al., 2000).

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²This paper is Journal Series Paper R-10966 of the Florida Agriculture Experiment Station.
Current interplanetary transfer models have calculated survival probabilities of spore populations during launch, cruise phase through space, and landing (Clark, 2001; Horneck et al., 2002; Millickowsky et al., 2000; Nicholson et al., 2000, 2005; Schuerger, 2004). However, for successful Earth-to-Mars transport to be ecologically relevant, terrestrial microorganisms, once deposited on the surface of Mars, would have to survive and proliferate in the martian environment. This possibility has received serious consideration for decades; the major spacefaring nations operate under planetary protection protocols, originally established under the auspices of COSPAR and international treaties that require spacecraft destined to make contact with Mars (or other potentially habitable sites) to be constructed and assembled in ultra-clean spacecraft assembly facilities (SAFs) under stringent hygienic conditions that, as nearly as possible, approach sterility (Rummel, 2001). Interestingly, the extreme environments of SAFs, characterized by very low nutrient levels, strictly controlled humidity, and periodic disinfection, appear to be excellent selective environments for Bacillus spp. whose spores exhibit ultrahigh resistance to various physical insults such as desiccation, oxidizing agents, ultraviolet (UV), and γ-radiation (La Duc et al., 2003; Venkateswaram et al., 2003; Link et al., 2004a); Hill et al. (2004b) have characterized as strain WN648. Spores were produced was deposited in the corresponding author’s collection elsewhere (Jacobs et al., 1994; Nicho-son et al., 2005; Schuerger, 2004), the potential for spore survival and proliferation on Mars has only recently been the subject of experimental research. Environmental conditions at the surface of Mars are characterized by high solar UV fluences, low atmospheric pressure, extreme cold temperatures, and extreme desiccation (reviewed in Schuerger, 2004; Nicholson et al., 2005). To contaminate the martian environment, spores would not only have to survive these conditions, but also germinate and grow vegetatively. Using a Mars simulation chamber (MSC), Schuerger et al. (2003) modeled the survival of B. subtilis spores exposed to robustly simulated martian environmental conditions of solar irradiation, temperature, atmospheric composition and pressure, and desiccation, and found that solar UV was the single most lethal environmental factor; spores were rapidly inactivated (within minutes) when exposed to a UV spectrum and fluences comparable to those present on the martian surface. However, spores buried under thin contiguous layers of dust (up to 500 μm thick) were well protected from solar UV and inactivated at a much slower rate (Schuerger et al., 2003).

From the above results it is clear that shielding from solar UV at the martian surface is a prerequisite for long-term survival of spores. However, even spores shielded from UV would still be exposed to potentially lethal germination- or growth-inhibiting low temperatures, pressures, and desiccation. How do these Mars parameters affect the ability of spores to survive, germinate and resume vegetative growth, even if conditions become favorable at a later time? To answer this question, we took advantage of a B. subtilis strain that encodes a fusion of the luxAB genes encoding luciferase to the strong sporulation-specific sspE promoter (Hill et al., 1994; Ciarciglini et al., 2000). During sporulation, the sspB-luxAB fusion strain produces luciferase in the developing forespore compartment and retains it within the dormant spores, where it is inactive as spores possess no detectable metabolism, ATP, or electron transport (Hill et al., 1994). Initiation of spore metabolism, and thus luciferase activity, upon germination results in a burst of bioluminescence that can be used as a real-time monitor of spore germination (Hill et al., 1994; Ciarciglini et al., 2000). In this communication we tested the ability of B. subtilis spores carrying an sspB-luxAB gene fusion to survive exposure to Mars-ambient atmospheric pressure and composition, and the possible effects of this exposure on subsequent spore germination.

MATERIALS AND METHODS

B. subtilis strain and cultivation conditions

The B. subtilis strain used was PS832, a Trp+ revertant of strain 168, carrying a cassette consisting of an sspB-luxAB fusion and markers conferring resistance to macrolide-lincosamide-streptogramin B antibiotics and chloramphenicol (Cm), inserted in a single copy in the chromosome. Details of its construction have been published elsewhere (Jacobs et al., 1991; Hill et al., 1994). The strain, a generous gift from P. Setlow, was deposited in the corresponding author’s collection as strain WN648. Spores were produced by incubation of strain WN648 in liquid Schae-
fer Sporulation Medium (SSM) (Schaeffer et al., 1965) containing 5 \( \mu \)g of Cm/ml (SSM+Cm) at 37°C for 48 h in a rotary shaker with vigorous aeration. Cultures were harvested by centrifugation (1,000 g, 10 min, 25°C), and spores were purified using the lysozyme and buffer washing method (Nicholson and Setlow, 1990), heat-shocked (80°C, 10 min), and stored in deionized water at 4°C. All spore preparations were determined by phase-contrast microscopy to be essentially free of vegetative cells and consist of >99.9% phase-bright spores. The viable titer of all spore preparations was determined by serial 10-fold dilution in phosphate-buffered saline (PBS) (10 mM potassium phosphate and 150 mM NaCl, pH 7.4) and plating on SSM+Cm solidified with 1.7% agar.

Exposure of spores to Earth and simulated Mars atmosphere

Spores \((-1 \times 10^8\) in 125 ml of water) were pipetted into several wells of 96-well sterile polystyrene microtiter plates and air-dried overnight at 37°C. One microtiter plate was covered loosely and placed in the laboratory at Earth-normal atmosphere, ambient temperature \((-20°C)\), relative humidity \((-60%)\), and pressure. Daily barometric pressure was taken from the weather data collection at the nearby Cape Canaveral Weather Station. The other plate was covered loosely, placed inside the MSC, and equilibrated to simulated Mars atmosphere (100% CO\(_2\)) at \(-20°C\) and Mars-normal pressure (average 12.5 mbar; range 7–18 mbar; see below). Temperature and pressure within the MSC were measured using an automatic data logging system (model SNAP Ultimate, Opto 22, Temecula, CA). Although a pure CO\(_2\) atmosphere is not exactly like Mars \(95.3\%\) (Owen, 1992), work by Schuerger et al. (2003) indicated that gas composition had no effect on the survival and subsequent germination of endospores of \(B.\ subtilis\), and, thus, the pure CO\(_2\) atmosphere was selected to simplify the experimental procedures. At 7, 12, and 19 days, microtiter plates were removed for viability assays and luciferase assays. Triplicate wells were used for each determination. Spores were rehydrated by addition of 100 \( \mu \)l of sterile deionized water and incubated at room temperature for 60 min. Rehydrated spores were resuspended thoroughly by pipetting and examined under the phase-contrast microscope before further processing. An aliquot of 10 \( \mu \)l was removed from each sample for viability assay, and the remaining 90 \( \mu \)l was used for germination bioluminescence assay.

Viability assay

Spore samples were diluted serially 1:10 in PBS, aliquots were plated on SSM+Cm and incubated for 18–24 h at 37°C, colonies were counted, and spore titer was calculated. Further incubation of plates for 2 days yielded no further increase in colony-forming units (cfu).

Germination bioluminescence assay

To each well of a 96-well microtiter plate containing 90 \( \mu \)l of spores was added 100 \( \mu \)l of liquid SSM containing a final concentration of 10 mM L-alanine and 0.003% (vol/vol) n-decanal, freshly prepared for each experiment. Microtiter plates were placed in a luminometer (Harta Instruments, Gaithersburg, MD), and at the indicated times readings were taken in “glow” mode with a dwell time of 1 s. Data are expressed in terms of arbitrary relative light units (RLU). Cumulative bioluminescence was determined by computing a running total of RLU measurements during the course of germination of each sample.

Statistical treatment of data

All viability and germination bioluminescence assays were performed on triplicate samples. Basic statistical data and analysis of variance (ANOVA) were computed using the statistical package supplied with KaleidaGraph version 3.6.2 (Synergy Software, Reading, PA).

RESULTS

Survival and germination of \(B.\ subtilis\) spores exposed to simulated Mars atmosphere

\(B.\ subtilis\) strain WN648 spores were exposed as dried films to either Earth-ambient or simulated Mars-ambient atmospheric composition and pressure. Over the 19-day course of the experiment, from 11 August 2004 to 30 August 2004, the barometric pressure measured from the nearby Cape Canaveral Weather Station averaged 1,017.5 mbar and varied between 1,014 and 1,021 mbar. In the MSC, the simulated Mars atmospheric pressure averaged 12.5 mbar, and var-
ied from a minimum of 7 mbar to a maximum of 18 mbar. Spores were assayed for viability at 0, 7, 12, and 19 days (Table 1). Spores survived well under both Earth or simulated Mars atmospheres; indeed, a slight increase in viable titer was noted on Days 12 and 19 under both atmospheres (Table 1). Because of this slight day-to-day variation noted, comparisons were only made between spore titers measured on the same day of exposure (Table 1). Differences in spore numbers measured after exposure to Earth- or simulated Mars atmospheric conditions were not statistically significant at the \( P \leq 0.05 \) confidence level, as measured by ANOVA (Table 1). Therefore it was concluded that exposure to simulated martian atmosphere and low pressure in the MSC for 19 days did not significantly affect viability (i.e., ability of spores to form colonies) upon return to Earth atmosphere and nutrient conditions. Because colony formation is necessarily dependent upon spore germination, it can be inferred that spores exposed in the MSC for 19 days retained their ability to sense the return of a favorable (Earth) environment and respond by germination and subsequent growth.

**Kinetics of germination luminescence of B. subtilis spores exposed to simulated Mars atmosphere**

Before placement in the MSC at Day 0, triplicate spore samples from both sets of plates were assayed for germination bioluminescence (Fig. 1A). As previously reported (Hill et al., 1994; Ciarcelliaglini et al., 2000), germinating spores of strain WN648 produced a burst of luminescence, which peaked at 90 min. The initial rise in luminescence was due to the activity of luciferase, which had been produced in the forespore during sporulation by expression of \( \text{sspB-luxAB} \) and retained within the dormant spore. After 90 min, luminescence declined, presumably resulting from proteolytic degradation of luciferase in the germinating spores and subsequent vegetatively growing cells. Because expression of \( \text{luxAB} \) is driven by the sporulation-specific \( \text{sspB} \) promoter, which is inactive during germination and vegetative growth, luciferase was not replaced during these developmental periods, hence the decline in activity. There was no apparent difference in either the kinetics or absolute level of germination bioluminescence between the two sets of spores at Day 0; both spore populations exhibited maximum bioluminescence at 90 min, and the kinetic curves were superimposable (Fig. 1A). At 7 days (Fig. 1B), 12 days (Fig. 1C), and 19 days (Fig. 1D) of exposure to Earth- or simulated Mars atmosphere, triplicate spore samples were again removed and assayed for germination-induced bioluminescence. Spores exposed to simulated Mars atmosphere in the MSC for 7, 12, and 19 days exhibited lower levels of germination bioluminescence than did the parallel spore samples exposed to Earth-normal conditions for the same number of days (Fig. 1B–D). In addition, spores exposed in the MSC for 7 and 12 days exhibited a slight delay in maximum bioluminescence, from 90 to 120 min (Fig. 1B and C).

We observed differences in levels of germination-induced bioluminescence between Earth- and simulated Mars atmosphere exposures, and documented considerable day-to-day variation in the absolute values of germination bioluminescence (note the changes in y-axis scales in each panel of Fig. 1). These differences were attributed to variations in the preparation of the germination solution for each day. We thus concluded that valid statistical comparisons could only be

### Table 1. Survival of B. subtilis Strain WN648 Spores After Exposure to Earth or Simulated Mars Atmosphere

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<th>SD ( \times 10^7 )</th>
<th>( P ) value (ANOVA)</th>
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</table>

Data are averages \( \pm \) standard deviation (SD) of triplicate determinations. NSD, not significantly different.
made between parallel sets of samples assayed on the same day.

In addition, pairwise statistical analysis by ANOVA of particular time points gave mixed results. For example, exposure of spores for 7 days in the MSC (Fig. 1B) resulted in significantly lower germination bioluminescence at 60 min ($P = 0.0048$) and 90 min ($P = 0.0026$), but not at 120 min ($P = 0.257$). We reasoned that the total cumulative bioluminescence generated during the time course of germination could be used as a basis of comparison for parallel spore populations. Examination of plots of total cumulative bioluminescence versus time (Fig. 2) reinforced this notion. As judged visually by overlap in error bars, there was no significant difference in cumulative germination-induced bioluminescence between spore populations at 0 days (Fig. 2A). However, after 19 days in the MSC, cumulative germination-induced bioluminescence was significantly lower than the control kept at Earth atmospheric conditions (Fig. 2B). Guided by the above results (Fig. 2), we calculated the total cumulative germination bioluminescence generated...
during the entire germination time course of each sample, normalized the resulting cumulative values to the spore titer of each suspension, and compared the normalized values by ANOVA (Table 2). The data clearly showed that 7, 12, or 19 days of spore exposure to simulated Mars atmospheric conditions in the MSC resulted in a highly statistically significant decrease in germination-induced bioluminescence, up to a 32% decrease by Day 19 (Table 2).

DISCUSSION

Germination-induced bioluminescence has been considered to be an effective real-time monitor of the germination and outgrowth process, and a measurement of how this process is affected by inhibitory or lethal treatments to spores (Hill et al., 1994; Ciarciaglini et al., 2000). The data presented here indicate that exposure to simulated Mars atmosphere (100% CO₂, average of 12.5 mbar) for 19 days significantly reduced germination-induced bioluminescence of B. subtilis WN648 spores (Figs. 1 and 2 and Table 2), but was insufficient to inactivate spores or completely prevent spore germination, as measured by the viability assay (Table 1). Light production in the bacterial luciferase reaction used in this study depends on the LuxAB protein, long-chain aldehyde, oxygen, and reduced FMN (Hastings et al., 1969; Nealson et al., 1970). Because decanal and oxygen were supplied exogenously in the assay, the observed decrease in germination-induced bioluminescence after prolonged exposure to simulated Mars atmosphere in the MSC could be due to inactivation of LuxAB luciferase activity, to impairment in FMN reduction, or to both. It should be noted that the Mars atmosphere simulation used here was quite rudimentary, in that it lacked trace gases, low temperatures, and UV spectrum and flux characteristic of the true martian atmosphere (Schuerger et al., 2003; Cockell et al., 2005; Nicholson et al., 2005). However, the data are consistent with the hypothesis that days of exposure to the low pressure and high CO₂ components of the Mars atmosphere alone, in the absence of the full suite of harsh martian environmental conditions, slowly degrade the ability of spores to germinate upon return to favorable environmental conditions.

To date, interplanetary transfer models have only considered the probability of survival of endolithic bacteria to forces generated during the transfer process itself (i.e., impact-mediated ejection, transit through space, and entry and deposition on the target planet) (Clark, 2001; Mileikowsky et al., 2000; Nicholson et al., 2000; Horneck et al., 2002). In these models, transfer in either direction between Earth and Mars has been...
considered; natural Earth-to-Mars transfers were considered less probable because of Earth’s denser atmosphere and higher escape velocity (Mileikowsky et al., 2000). For the interplanetary transfer process to be ecologically relevant, the habitabilities of the donor and recipient planets, which constrain both the initial populations subjected to launch and the fate of survivors upon arrival, must be considered. The present results combined with the data and considerations previously discussed (Schuerger et al., 2003; Nichol-son et al., 2004) suggest that, at least for Bacillus spp. spores and currently prevailing environmental conditions, the environment of the Mars surface, characterized by high solar UV, low pressure, low temperature, and predominance of atmospheric CO₂, may present a formidable barrier to the establishment of viable populations resulting from natural Earth-to-Mars interplanetary transfer events.

The implications of these results for planetary protection issues related to Mars exploration are threefold. First, during spacecraft assembly, the use of bioluminescent or fluorescent biomarkers to monitor viable bioloads must be confirmed to directly correlate with cell viability before any one assay is relied upon for microbial detection and quantification. As sterilizing protocols are applied to spacecraft materials during assembly (i.e., heat, chemical, and gaseous treatments), it might be possible that the different sterilizing protocols may degrade viability, bioluminescence, and fluorescent biomarkers to different degrees, thus obscuring the evaluation of the level of cleanliness achieved. Second, the use of bioluminescence in future life-detection experiments on Mars has been proposed for the search for an extant Mars microbiota, as well as any terrestrial contamination on Mars (Venkateswaran et al., 2003a; Steele et al., 2004). The results presented here indicate that spore viability and bioluminescence of terrestrial bacteria might not always match, and that caution should be used when interpreting the intensity of bioluminescence as a

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C-RLU, cumulative RLU; NSD, not significantly different; S, log₁₀ cfu of spores; SD, standard deviation.
biomarker for cell viability. A similar effect was reported by Cockell et al. (2005), who found that the viability of the cyanobacterium *Chroococcidiopsis* sp. 029 did not match several fluorescent biomarkers used to assay cell viability following exposure to martian conditions. This study reported that viability of *Chroococcidiopsis* sp. 029 decreased rapidly under martian conditions, while several fluorescent biomarkers retained their intensity over much longer time frames. Although the effects in the study of Cockell et al. (2005) were opposite those reported here, both studies indicate that bioluminescence or fluorescent biomarkers might not always match the viability of terrestrial microorganisms exposed to martian conditions. Third, the use of bioluminescence or fluorescent biomarkers as a single check for terrestrial back-contamination of returned samples from Mars may not be adequate for confirming either the presence or absence of terrestrial microorganisms. As the results of the current study and the report by Cockell et al. (2005) clearly demonstrate, detection of bioluminescence or fluorescent biomarkers may not always correlate to the viability of the biosoid of a sample.

**ACKNOWLEDGMENTS**

The authors thank Peter Setlow for the generous gift of the *B. subtilis* strain used. This work was supported by a grant (20022023/21988) from the University of Florida/University of Central Florida Space Research Initiative to A.C.S. and W.L.N., and NASA Exobiology grant NNA04- CI35A to W.L.N. The MSC used in this study was supported by a grant (20020023/21988) from the University of Central Florida Space Research Initiative to A.C.S. and W.L.N. and NASA Exobiology grant NNA04-CI35A to W.L.N. The MSC used in this study was supported by a grant (20020023/21988) from the University of Central Florida Space Research Initiative to A.C.S. and W.L.N. The authors thank Peter Setlow for the generous gift of the *B. subtilis* strain used. This work was supported by a grant (20022023/21988) from the University of Florida/University of Central Florida Space Research Initiative to A.C.S. and W.L.N., and NASA Exobiology grant NNA04-CI35A to W.L.N.

**ABBREVIATIONS**

ANOVA, analysis of variance; cfu, colony-forming units; Cm, chloramphenicol; MSC, Mars simulation chamber; PBS, phosphate-buffered saline; RLU, relative light units; SAF, spacecraft assembly facility; SSM, Schaeffer Sporulation Medium; UV, ultraviolet.

**REFERENCES**


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