

Rapid inactivation of seven *Bacillus* spp. under simulated Mars UV irradiation [☆]

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Abstract

Seven *Bacillus* spp. were exposed to simulations of Mars-normal UV fluence rates in order to study the effects of UV irradiation on microbial survival. A UV illumination system was calibrated to deliver 9.78 W m^{-2} ($35.2 \text{ kJ m}^{-2} \text{ h}^{-1}$) of UVC + UVB irradiation (200–320 nm) to microbial samples, thus creating a clear-sky simulation (0.5 optical depth) of equatorial Mars. The *Bacillus* spp. studied were: *B. licheniformis* KL-196, *B. megaterium* KL-197, *B. nealsonii* FO-092, *B. pumilus* FO-36B, *B. pumilus* SAFR-032, *B. subtilis* 42HS1, and *B. subtilis* HA101. The bacteria were prepared as thin monolayers of endospores on aluminum coupons in order to simulate contaminated spacecraft surfaces. Bacterial monolayers were exposed to Mars UV irradiation for time-steps of 0, 0.25, 0.5, 1, 5, 15, 30, 60, 120, or 180 min. The surviving endospores were then assayed with a Most Probable Numbers (MPN) procedure and with a culture-based assay that utilized a bacillus spore germination medium. Results indicated that *B. pumilus* SAFR-032 was the most resistant, and *B. subtilis* 42HS-1 and *B. megaterium* were the most sensitive of the seven strains exposed to martian UV fluence rates. *Bacillus subtilis* 42HS1 and *B. megaterium* were inactivated after 30 min exposure to Mars UV, while *B. pumilus* SAFR-032 required 180 min for full inactivation in both assays. Spores of *B. pumilus* SAFR-032 exhibited significantly different inactivation kinetics suggesting that this wild type isolate also was more resistant than the standard dosimetric strain, *B. subtilis* HA101. Although the various *Bacillus* spp. exhibited diverse levels of UV resistance, none were immune to UV irradiation, and, thus, all species would be expected to be inactivated on Sun-exposed spacecraft surfaces within a few tens-of-minutes to a few hours on sol 1 under clear-sky conditions on equatorial Mars. The inactivation kinetics of all seven *Bacillus* spp. support the conclusion that significant levels of bioload reductions are possible on Sun-exposed spacecraft surfaces in very short time periods under clear-sky conditions on Mars. However, the presence of UV resistant microbes on spacecraft surfaces rapidly covered in dust during landing operations, and non-Sun-exposed surfaces of spacecraft remain concerns that must continue to be addressed through adequate spacecraft sanitizing procedures prior to launch.

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1. Introduction

Mars spacecraft are assembled under strict conditions of sanitation, and components are decontaminated at various times during the payload integration process. Microbial decontamination procedures can include the use of dry-heat, biocidal gases, and/or chemical surface treatments depending on the

spacecraft component and phase of assembly (Venkateswaran et al., 2004). Through these microbial decontamination procedures, the total bioburden of spacecraft at launch is constrained significantly and generally must be below 3×10^5 spores per vehicle for Category IV missions (Barengoltz, 1997; De Vincenzi et al., 1998; Glavin et al., 2004). Category IV planetary missions are defined as those in which spacecraft will be landed on the surface of a planet, and in which there is a risk of contaminating the planetary body (Glavin et al., 2004). Although there will be significant losses of species diversity and viable bioloads during the 6–8-month cruise phase

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to Mars (Schuerger, 2004), there have been several studies that have demonstrated long-term survival of terrestrial microorganisms in interplanetary space (see reviews by Taylor, 1974; Horneck, 1992; Schuerger, 2004), and, thus, it is likely that viable terrestrial microorganisms have and will be successfully landed on the martian surface. Therefore, microbial contamination of robotic spacecraft could pose a significant risk to near-term Mars surface missions by increasing the forward contamination of scientific payloads, local landing sites, or the global martian environment. In order to correctly model this process, the microbial ecologies of spacecraft and their associated environments must be understood from initial assembly of spacecraft components through the operational termination of each mission. In particular, the effects of the martian environment on microbial survival must be better understood in order to predict the nature and severity of forward contamination to Mars.

Although the Mars solar constant is on average only 43% that observed for Earth (Kuhn and Atreya, 1979), the biologically effective dose rate of UV irradiation on the surface of Mars is up to three orders-of-magnitude higher than on the surface of Earth because of the high UVC fluence rates on Mars caused by low concentration of ozone in the significantly thinner atmosphere (Cockell et al., 2000; Patel et al., 2002). Thus, UVC (200–280 nm) irradiation reaches the surface of Mars at relatively high levels compared to Earth. In general, UV irradiation on Earth is attenuated at 290–300 nm by ozone absorption but can penetrate the martian atmosphere down to 190 nm where it is attenuated by CO₂ (Kuhn and Atreya, 1979). The total UV fluence rate (200–400 nm) for low dust scenarios on the surface of equatorial Mars has been modeled by several research teams (Cockell and Andradý, 1999; Cockell et al., 2000; Patel et al., 2002; Schuerger et al., 2003) and ranges between 42 and 55 W m², depending on the model assumptions. The UVC fluence rates were estimated in these studies to be approximately 8–10% of the total UV flux. Based on these models, between 3.2 and 5.5 W m² of UVC irradiation is likely to reach the surface of equatorial Mars under clear-sky conditions (optical depths between 0.1 and 0.5). These UV fluence rates are generally estimated for the mean orbital distance of Mars but can increase or decrease, respectively, by approximately 18% for perihelion and aphelion positions in solar areocentric longitude (Appelbaum and Flood, 1990). These UV fluence rates have been shown to be capable of rapidly inactivating spores of UV resistant microorganisms during Mars simulations (Green et al., 1971; Hagen et al., 1964; Horneck et al., 2001; Mancinelli and Klovstad, 2000; Newcombe et al., 2005; Schuerger et al., 2003, 2005). Thus, UV irradiation on Mars likely will be a dominant biocidal factor against long-term survival of terrestrial microorganisms on spacecraft surfaces.

Schuerger et al. (2003) studied the effects of UV irradiation on the survival of the common spore-forming bacterium, *Bacillus subtilis*, under a diversity of martian conditions in order to predict the survival rates of common spacecraft contaminants on Sun-exposed surfaces of spacecraft. They measured the effects of martian UV irradiation (200–400 nm), low pressure

(8.5 mb), low temperature (down to –80 °C), and gas composition (CO₂, O₂/N₂, N₂, or a Mars gas mix) on spore survival. Results indicated that UV irradiation was clearly the dominant biocidal factor for Sun-exposed surfaces producing as much as six orders of magnitude reductions in viable spore counts for short-term exposures of a few tens-of-minutes under martian UV fluence rates modeled for clear-sky conditions (Schuerger et al., 2003). Low pressure was found to have only a minor effect on spore survival for these short-duration experiments, and temperature and gas composition were found to have no effects on the numbers of recovered UV-irradiated spores. Newcombe et al. (2005) extended these studies to include additional *Bacillus* spp. and two different UV simulations for Mars. Results indicated that *B. pumilus* SAFR-032 was significantly more resistant to UV irradiation than all other bacillus strains recovered from spacecraft assembly facilities and the Mars Odyssey spacecraft. Although, *B. pumilus* SAFR-032 exhibited significantly different inactivation kinetics than the other *Bacillus* strains tested (Newcombe et al., 2005), it was not immune to UV irradiation, and, thus, it also exhibited significant reductions in viable spore numbers following exposure to Mars-simulated UVC fluence rates.

The primary objective of the current study was to improve the UV simulations reported by Schuerger et al. (2003) and Newcombe et al. (2005) in order to create a more realistic inactivation model for spore-forming *Bacillus* spp. on Sun-exposed surfaces of spacecraft on Mars. *Bacillus* spp. were chosen for these experiments because spore-forming bacterial species are generally used as the benchmark for assessing the cleanliness of spacecraft surfaces prior to launch (Barengoltz, 1997). In addition, spore-forming *Bacillus* spp. are more resistant than non-spore forming species to the harsh environmental conditions in space (reviewed by Horneck, 1992; Nicholson et al., 2000, 2005; Schuerger, 2004), and *Bacillus* spp. are commonly used in studies exploring the effects of the martian environment on microbial survival (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). A preliminary report of these experiments has been published (Schuerger et al., 2004).

2. Materials and methods

2.1. Mars UV model

A Mars-normal ultraviolet (UV), visible (VIS), and near-infrared (NIR) spectrum was generated using a 450 W xenon-arc UV-enhanced lamp (model 6262, Oriel Instruments, Stratford, CA, USA) (Fig. 1). The UV lighting system and Mars UV model used herein have been described elsewhere (Schuerger et al., 2003). The Mars UV model was based on the work of Appelbaum and Flood (1990), Arvesen et al. (1969), and Kuhn and Atreya (1979), and was similar to the UV models developed by Cockell et al. (2000) and Patel et al. (2002). The experiments below were conducted under a calibrated Mars-normal UV fluence rate, but held at Earth-normal sea-level conditions of pressure (1013 mb), temperature (+23 °C), and

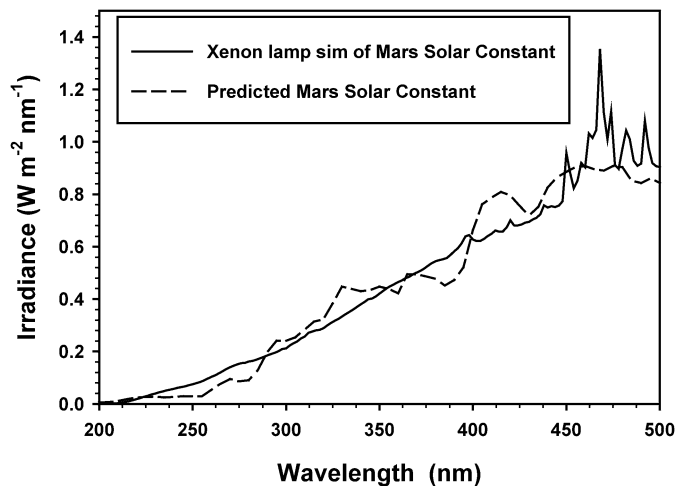


Fig. 1. The predicted Mars solar constant for UV irradiation (200–400 nm) was based on the work of Schuerger et al., 2003 (adapted from Arvesen et al., 1969; Kuhn and Atreya, 1979; Appelbaum and Flood, 1990). The spectrum used for these experiments was generated from a 450 W xenon-arc lamp calibrated to deliver a Mars solar constant of 50.92 W m^{-2} from 200 to 400 nm.

gas composition ($\text{ppO}_2/\text{ppN}_2$). Previous research (Schuerger et al., 2003) demonstrated that these three parameters had either a minor effect (pressure) or no effect (temperature and gas composition) on the survival of *B. subtilis* HA 101 endospores during Mars simulations. The UV-lighting system was assembled on a lab bench and operated manually for all experiments.

The UV fluence rates of the simulated Mars solar constant were 5.15, 8.82, 36.95, and 50.92 W m^{-2} in UVC, UVB, UVA, and total UV, respectively, for a spectral range of 200–400 nm (Fig. 1). However, in order to simulate a Mars-surface UV flux that might be encountered under typical clear-sky conditions, the Mars solar constant was attenuated by passing the UV-enriched light through neutral density filters (optical density of 0.13) to create an optical depth (τ) of 0.5 on the microbial targets (Schuerger et al., 2003). The neutral density filters were composed of fused silica glass coated with thin layers of a nickel–chromium–iron alloy (manufactured by Maier Photonics, Inc., Manchester Center, VT, USA). Viking data (Kahn et al., 1992; Colburn et al., 1989) indicated that the average optical depth of the northern plains under clear-sky conditions was approximately 0.5 (range = 0.3 to 0.7). Thus, the actual down welling direct UV fluence rates that were used to irradiate bacterial monolayers were approximately 70% of the Mars solar constant; i.e., the UV fluence rates for UVC, UVB, UVA, and total UV were 3.61, 6.17, 25.87, and 35.64 W m^{-2} , respectively. The energy flux for UVC + UVB was 9.78 W m^{-2} ($35.2 \text{ kJ m}^{-2} \text{ h}^{-1}$). The VIS and NIR fluence rates of the calibrated lighting system were approximately 240 and 245 W m^{-2} , respectively (Schuerger et al., 2003).

2.2. Microbiological procedures

Monolayers of endospores of each *Bacillus* species were deposited on aluminum coupons according to the procedures of Mancinelli and Klovstad (2000) and Schuerger et al. (2003).

Endospores of the seven *Bacillus* spp. were deposited at densities of approximately 2×10^6 viable spores per aluminum coupon, and dried in monolayers that measured 1 cm in diameter. Monolayers were microscopically inspected with a high-resolution video microscope (model VH-7000, Keyence Corp. of America, Woodcliff Lake, NJ, USA), and only uniform monolayers exhibiting randomly dispersed individual endospores were chosen for the experiments. The species studied were: *B. licheniformis* KL-196, *B. megaterium* KL-197, *B. nealsonii* FO-092, *B. pumilus* FO-36B, *B. pumilus* SAFR-032, *B. subtilis* 42HS1, and *B. subtilis* HA101. All species except HA101 were recovered from spacecraft surfaces (Mars Odyssey) or spacecraft assembly facilities at either the Jet Propulsion Lab (JPL), Pasadena, CA or Kennedy Space Center (KSC), FL (Venkateswaran et al., 2001, 2004; La Duc et al., 2003, 2004). In preliminary tests at JPL, endospores of *B. pumilus* SAFR-032 exhibited the highest UVC resistance to 254 nm UV irradiation (Link et al., 2004; Newcombe et al., 2005), and, thus, SAFR-032 was included in the current study as a high UV-resistant strain. All strains were obtained from K. Venkateswaran (JPL), except *B. subtilis* HA101 which was obtained from R. Mancinelli (NASA Ames Research Center, Moffett Field, CA).

Monolayers of endospores for each species were exposed to a simulated Mars UV spectrum for time-steps of 0, 0.25, 0.5, 1, 5, 15, 30, 60, 120, or 180 min. After UV exposure, each aluminum coupon was processed separately using a Most Probable Numbers (MPN) procedure described previously (Mancinelli and Klovstad, 2000; Schuerger et al., 2003). In brief, each coupon was placed in a separate 50-cc centrifuge tube with 20 ml sterile deionized water (SDIW), vortexed for 2 min, serially diluted through six ten-fold dilutions, and 20 μl of each dilution was pipetted into 16 individual wells of a 96-well plate each containing 180 μl of a bacillus spore medium (Schuerger et al., 2003). The MPN procedure as described by Schuerger et al. (2003) had a detection limit of 180 viable spores per coupon if the endospores used for the assays were recently prepared and no older than 4–6 weeks. Three modifications to this MPN procedure were developed to increase the detection limit to a theoretical level of one viable endospore per coupon. First, 1 g of previously heat-sterilized silica sand (24 h at 130°C) was added to each MPN assay tube to increase the recovery of endospores from the aluminum coupons. The silica sand acted as a mild abrasive and significantly increased the removal of endospores from aluminum coupons. The recovery efficiency with the silica sand extraction procedure was approximately 90% of the initial viable numbers of endospores applied to aluminum coupons (Schuerger, unpublished). In preliminary experiments there was no evidence that the silica sand extraction procedure killed or precipitated endospores of several *Bacillus* spp. tested. Second, in samples exposed to UV irradiation for 15 min or longer, serial dilutions were not generated from the original extraction tubes because it was anticipated that very few viable endospores would be present in the longer exposures. Instead, these coupons were extracted in the 50-cc tubes as described above, and then all 96 wells of MPN assay plates received 20 μl each of the original extraction

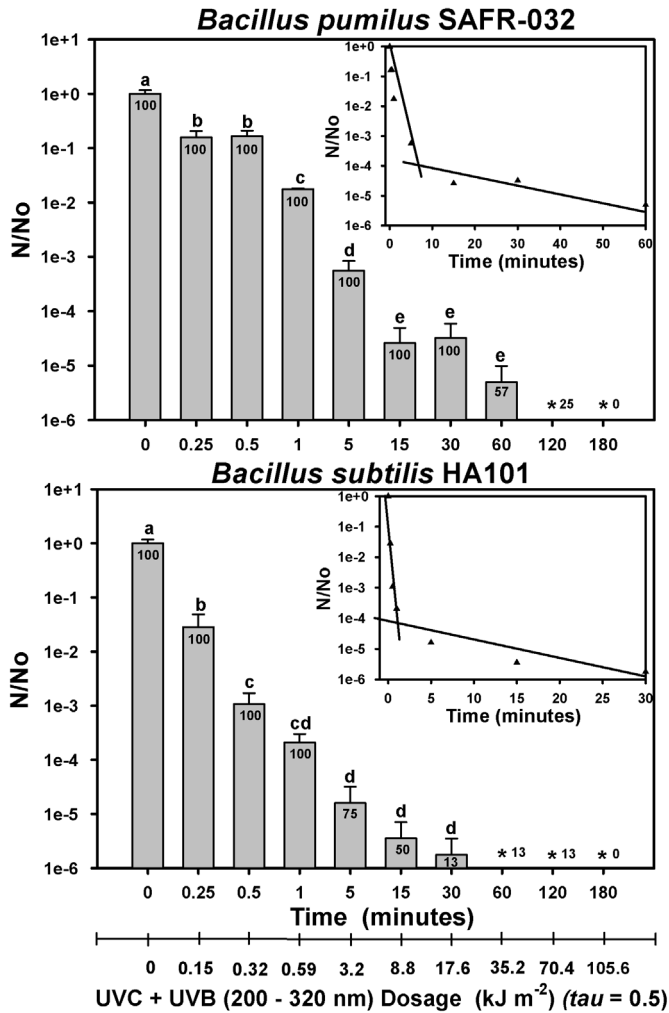


Fig. 2. Numbers of recovered endospores of *Bacillus pumilus* SAFR-032 and *Bacillus subtilis* HA101 exposed up to 180 min to Mars-normal UV–VIS–NIR irradiation. Different letters indicate significant differences in the MPN assays based on ANOVA and protected least-squares mean separation tests ($P \leq 0.05$; $n = 8$); bars = standard errors. Asterisks indicate MPN assays in which no viable bacteria were recovered. Numbers within or to the right of the bars represent the percentages of coupons exhibiting positive growth in spore-medium assays after incubation for 24 h at 37 °C.

fluid. This process increased the assayed volume from 0.32 to 1.96 ml of the original extraction fluid per sample. At this stage of processing, the detection limit of the MPN procedure was lowered to 10 viable endospores per coupon. Third, after the MPN assay was completed for each coupon, 20 ml of the bacillus spore medium was added to each extraction tube in which the aluminum coupon, silica sand, and residual SDIW were still present. The spore-medium assay tubes were then incubated at 37 °C for 24 h. Any bacterial growth could be detected by a visual increase in turbidity of the spore medium and was recorded as a (+) in the assay. The MPN assay was best suited for estimating the lethality of the UV irradiation during the earlier time-steps of these experiments, and the spore-medium assay was best suited for the later time-steps when the MPN assays were yielding zeroes. Thus, combining the MPN and spore-medium extraction procedures yielded a total theoretical detection limit of approximately 1 viable endospore per aluminum

coupon. Data of both assays are presented separately in Figs. 2 and 3.

2.3. Statistical procedures

Statistical analyses were conducted with version 9.0 of the PC-based Statistical Analysis System (SAS) (SAS Institute, Inc., Cary, NC, USA). For most experiments, 0.25 power transformations were used to induce homogeneity of variances of individual treatments; all data are presented as untransformed data. Transformed data from the MPN assay were subjected to analysis of variance procedures (PROC GLM) followed by protected least-squares mean separation tests ($P \leq 0.05$). Linear regression models were generated with PROC REG ($P \leq 0.05$). The MPN data in Figs. 2 and 3 are presented as the log-reductions in viable numbers of spores in which N equals the number of survivors per UV treatment and N_0 equals the number of viable endospores recovered from non-UV irradiated control coupons ($T = 0$ time-step). The spore-medium assay data are presented in Figs. 2 and 3 as the numbers within each MPN histogram bar.

3. Results

Under the martian simulations examined here, the most UV-resistant bacterium was *B. pumilus* SAFR-032 (Fig. 2). All other species were significantly more sensitive to UV irradiation than SAFR-032 (Figs. 2 and 3; Tables 1 and 2). For example, the time required for a 99.9% reduction (three orders of magnitude) in recovered endospores was at least 5 min for *B. pumilus* SAFR-032 while all other species achieved this level of bioload reduction between 30 and 60 s. The times required to render the surviving numbers of endospores below the detection limits in both the MPN and spore-medium assays were: *B. pumilus* SAFR-032 (180 min), *B. subtilis* HA101 (180 min), *B. pumilus* FO-36B (120 min), *B. licheniformis* KL-196 (60 min), *B. nealsonii* FO-092 (30 min), *B. megaterium* KL-197 (30 min), and *B. subtilis* 42HS1 (30 min) (Figs. 2 and 3). *Bacillus subtilis* 42HS1 was the most UV sensitive strain tested and exhibited six orders of magnitude reductions in the MPN assay after only 5 min exposure to the UV simulation. The UVC + UVB fluence rate required to achieve zero detectable endospores in both assays for the most UV-resistant bacterium, *B. pumilus* SAFR-032, was 105.6 kJ m^{-2} (Fig. 2), which equates to approximately 3 h on the surface of equatorial Mars on a clear sol (based on the UV models of Schuerger et al., 2003).

Although *B. pumilus* SAFR-032 was significantly more UV resistant than all other strains tested, the time required to reach non-detectable numbers of endospores in both assays was similar for *B. pumilus* SAFR-032 and *B. subtilis* HA101, namely 180 min for both strains. This may be explicable on the basis that (a) the time-course study did not have the time-step resolution to differentiate between these two strains between 120 and 180 min, and (b) at the very low levels of detection, only a single viable spore is required to yield a positive response in the

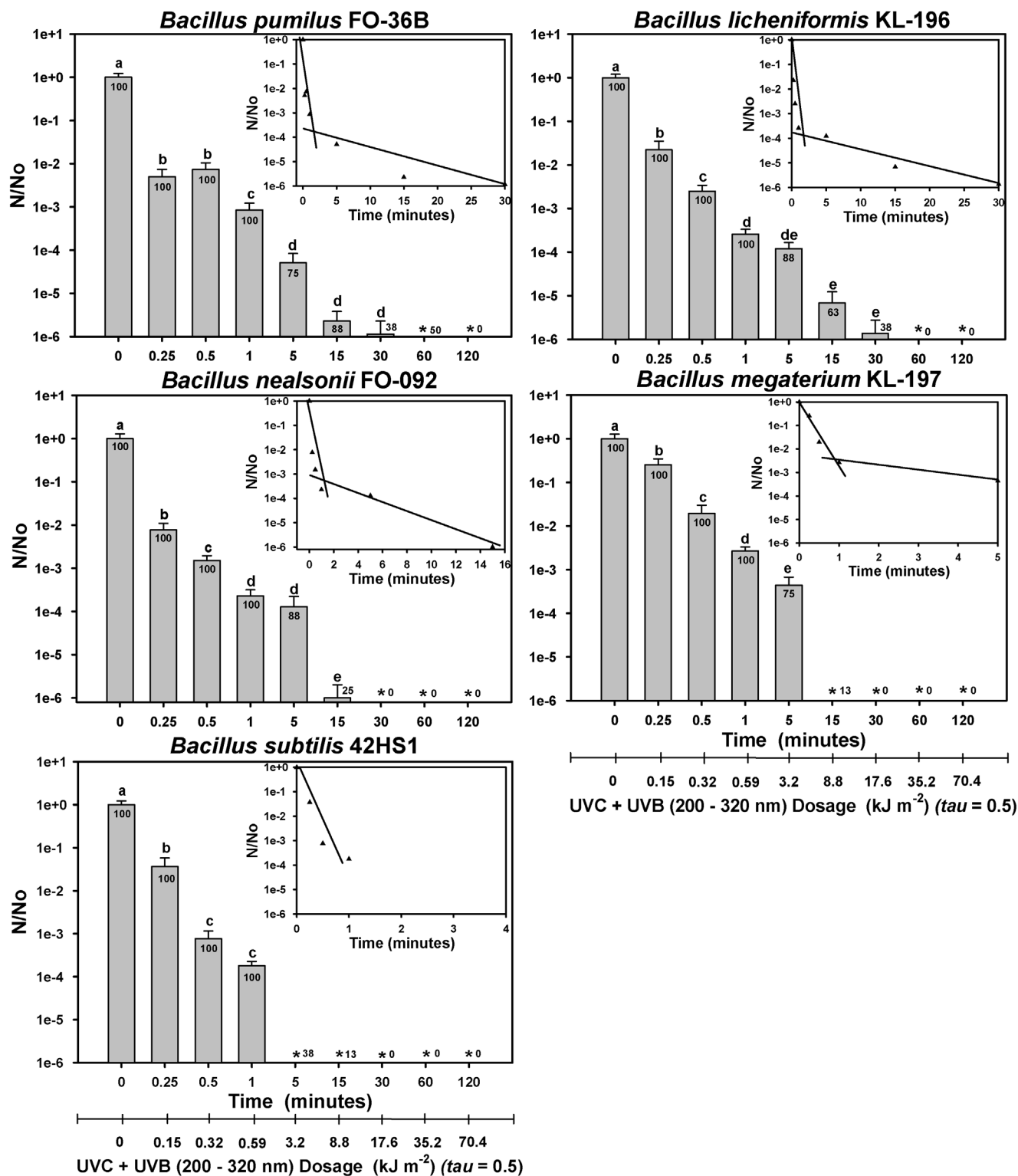


Fig. 3. Numbers of recovered endospores of *Bacillus pumilus* FO-36B, *B. licheniformis* KL-196, *B. nealsonii* FO-092, *B. megaterium* KL-197, and *B. subtilis* 42HS1 exposed up to 120 min to Mars-normal UV–VIS–NIR irradiation. Different letters indicate significant differences in the MPN assays based on ANOVA and protected least-squares mean separation tests ($P \leq 0.05$; $n = 8$); bars = standard errors. Asterisks indicate MPN assays in which no viable bacteria were recovered. Numbers within or to the right of the bars represent the percentages of coupons exhibiting positive growth in spore-medium assays after incubation for 24 h at 37 °C.

spore-medium assay. Thus, HA101 may have been fully inactivated closer to 120 min than to 180 min, but unless there were intermediate time-steps in the assay, this response would not have been measured. Furthermore, if one or 100 viable spores

remained on the aluminum coupons after 120 min for HA101 or SAFR-032, respectively, the spore-medium assay would not have been able to resolve this difference. Therefore, although both strains SAFR-032 and HA101, with different intrinsic lev-

els of UV resistance, were inactivated after 180 min under the simulated martian UV flux, it seems reasonable to maintain the conclusion that inactivation of spore-forming bacterial species on Sun-exposed spacecraft surfaces can occur very quickly over the course of several hours on sol 1 on Mars.

The data in Figs. 2 and 3 were used to estimate the lethal dose rates of UV irradiation to inactivate 90 (LD₉₀) and 99 (LD₉₉) percent of the initial populations of the bacteria tested (Table 1). Results indicated that the highest LD₉₀ and LD₉₉ rates were observed for *B. pumilus* SAFR-032 at 50 and 120 s, respectively. All other species were significantly lower (Table 1). The LD₉₀ and LD₉₉ rates for *B. subtilis* HA101 were observed to be 12 and 20 s, thus rendering *B. pumilus* SAFR-032 approximately four to six times more resistant to UV irradiation than *B. subtilis* HA101. The overall lethal dose rate (LD₁₀₀) required to reduce the numbers of surviving endospores of all species below the minimum detection limits of both the MPN and spore-medium assays was 180 min of Mars UV simulation (UVC + UVB at 105.6 kJ m⁻²).

Table 1

LD₉₀ and LD₉₉ values for seven *Bacillus* spp. exposed to a UV fluence rate calibrated for equatorial Mars at an optical depth of 0.5

<i>Bacillus</i> spp.	LD ₉₀ values (s)	LD ₉₉ values (s)
<i>B. pumilus</i> SAFR-032	50 ^a	120
<i>B. megaterium</i> KL-197	18	30
<i>B. subtilis</i> HA101	12	20
<i>B. subtilis</i> 42HS1	12	18
<i>B. licheniformis</i> KL-196	8	22
<i>B. pumilus</i> FO-36B	5	30
<i>B. nealsonii</i> FO-092	5	15

^a LD₉₀ and LD₉₉ values were estimated from the data presented in Figs. 2 and 3. Species are listed (top-down) in the order of decreasing LD₉₀ values.

Six of the seven *Bacillus* spp. exhibited a bi-phasic response to UV irradiation in which rapid decreases in surviving spores were observed within the first few time-steps of the experiments, followed by a much slower reduction in survivors before dropping below detectable limits with the MPN assay (Figs. 2 and 3). Only *B. subtilis* 42HS1 failed to exhibit the bi-phasic response. Because it was so sensitive to UV irradiation, the kill curve for 42HS1 was composed of a single regression model ending at 1 min. During the 1st-phase portions of these tests, surviving endospores were reduced between 3 and 4 orders of magnitude for all species except *B. subtilis* 42HS1. It is possible that the 2nd phase is composed of either UV-resistant spores, endospores aggregated in small groups in which some UV protection is provided by peripheral spores, and endospores entrapped in small pits or cracks within the aluminum coupon surfaces. In any case, longer time intervals were required in the 2nd phase to achieve relatively minor additional reductions in surviving spores. But in all cases, the simulated martian UV irradiation was able to reduce the numbers of viable endospores to below detection limits for two assays over relatively short periods of time.

Linear regression models for both portions of the bi-phasic kill curves are given in Table 2. The criterion for delimiting the 1st-phase portion of the kill curve for each species was the time-step interval required to achieve a 3–4-decade reduction in surviving endospores. For all species except SAFR-032, this occurred from 0 to 1 min. Thus, the regression models for the 1st-phase portions of the kill curves were very similar for all species except *B. pumilus* SAFR-032. The overall averaged model for the 1st-phase responses for HA101, FO-36B, KL-196, FO-092, KL-197, and 42HS1 was $y = -0.82x + 0.625$. The model that best described the 1st-phase portion of the

Table 2

Regression models for the bi-phasic responses of seven *Bacillus* spp. to UV irradiation

<i>Bacillus</i> spp.	Linear models ^a	<i>P</i> values	<i>r</i> ²
1st phase ^b			
<i>B. pumilus</i> SAFR-032	$y = -0.097x + 0.399$	0.0067	0.178
<i>B. subtilis</i> HA101	$y = -0.809x + 0.611$	0.0002	0.376
<i>B. pumilus</i> FO-36B	$y = -0.800x + 0.603$	0.0009	0.312
<i>B. licheniformis</i> KL-196	$y = -0.807x + 0.609$	0.0003	0.355
<i>B. nealsonii</i> FO-092	$y = -0.802x + 0.603$	0.003	0.258
<i>B. megaterium</i> KL-197	$y = -0.889x + 0.710$	0.0004	0.341
<i>B. subtilis</i> 42HS1	$y = -0.812x + 0.615$	0.0006	0.330
2nd phase ^c			
<i>B. pumilus</i> SAFR-032	$y = -0.0000072x + 0.00036$	0.0691	0.113
<i>B. subtilis</i> HA101	$y = -0.000051x + 0.00012$	0.037	0.137
<i>B. pumilus</i> FO-36B	$y = -0.000021x + 0.00049$	0.036	0.138
<i>B. licheniformis</i> KL-196	$y = -0.0000078x + 0.00020$	0.0009	0.310
<i>B. nealsonii</i> FO-092	$y = -0.000016x + 0.00023$	0.04	0.178
<i>B. megaterium</i> KL-197	$y = -0.00056x + 0.0032$	0.0056	0.432
<i>B. subtilis</i> 42HS1	NA	NA	NA

^a Linear models were estimated with PROC REG using SAS 9.0 PC version. Species are listed (top-down) in the rank order given in the text for overall UV resistance.

^b Time range used for the 1st phase was 0–1 min for the MPN data, except *B. pumilus* SAFR-032 which was tested from 0 to 5 min due to the greater UV resistance of this strain. The 1st-phase time range limits were based on a reduction in surviving numbers of endospores of between 3 and 4 orders of magnitude for each species.

^c Time range used for 2nd-phase analyses were from 1 min to the maximum in which endospores were recovered, except *B. pumilus* SAFR-032 which was tested from 5 to 60 min due to the greater UV resistance of this strain.

SAFR-032 kill curve was $y = -0.097x + 0.399$. The slope value for SAFR-032 was nearly one order of magnitude lower than the average slope value for all other species combined, thus indicating that the rate of UV inactivation for SAFR-032 was significantly slower than the other *Bacillus* spp. In addition, the linear regression models for the 2nd-phase portions of the kill curves for all species, except 42HS1, were significantly different than the 1st-phase models. The slopes were all dramatically lower, indicating that the rate of change of surviving spores over time in the 2nd phase was significantly slower than the loss of viable spores in the 1st phase.

4. Discussion

The UV fluence and spectral characteristics used herein created a simulation of clear-sky conditions on equatorial Mars approximating an optical depth (τ) of 0.5. Similar conditions in atmospheric opacity were reported for clear skies during the Viking (Kahn et al., 1992), Mars Pathfinder (Smith and Lemmon, 1999), and MER missions (Lemmon et al., 2004). Based on the LD₉₀ and LD₉₉ rates, the times required for full inactivation of endospores reducing their populations below detection limits in the MPN and spore-medium assays, and the slope values for the 1st-phase inactivation kinetics of each species, the following decreasing order of UV resistance was observed: *B. pumilus* SAFR-032, *B. subtilis* HA101, *B. pumilus* FO-36B, *B. licheniformis* KL-196, *B. nealsonii* FO-092, *B. megaterium* KL-197, and *B. subtilis* 42HS1. These results confirm the work of Newcombe et al. (2005) that clearly demonstrated that *Bacillus pumilus* SAFR-032 was significantly more resistant to UV irradiation than all other *Bacillus* spp. tested. In addition, these new data extend the studies of Schuerger et al. (2003) and Newcombe et al. (2005) to include a much more accurate simulation of spore survival of various *Bacillus* spp. on simulations of Sun-exposed spacecraft surfaces under clear-sky conditions on equatorial Mars.

The exact mechanisms in the *Bacillus* spp. tested here that contributed to their inherent UV resistance are not known. Several factors may have been involved including cellular protective mechanisms (e.g., cell wall thickness, accumulation of UV-absorbing pigments, spore water content), DNA photochemistry (e.g., presence of spore photoproducts like cyclobutane pyrimidine dimers and 5-thymine-5,6-dihydrothymine, spore concentrations of dipicolinic acid and α/β -type small acid-soluble proteins, formation of reactive oxygen species), and DNA repair mechanisms in spores (Nicholson et al., 2000, 2005). Although it was beyond the scope of the current study, new experiments are warranted in order to characterize the most important mechanisms of UV resistance in bacterial spores under martian conditions. Furthermore, it is essential that research be targeted at discerning the interactions of various environmental conditions on Mars (e.g., low pressure, low temperature, high desiccating conditions, UV irradiation, etc.), taken in combination or alone, on both DNA damage and DNA repair for a wide range of spore and non-spore forming species. Much work has been published on the effects of extreme environmental conditions on DNA damage and DNA repair in

Bacillus spp. (see reviews by Nicholson et al., 2000, 2005), but only a few studies have begun to ask specific questions related to the effects of the martian environment on spore survival (Mancinelli and Klovstad, 2000; Newcombe et al., 2005; Schuerger et al., 2003, 2005). However, no papers were found in the literature that specifically addressed the issue of DNA damage and DNA repair in terrestrial microorganisms under martian conditions.

Although *B. pumilus* SAFR-032 was significantly more UV resistant than the other *Bacillus* spp. tested, the general inactivation kinetics were similar among the seven *Bacillus* spp. in which >99.9% (>3 orders of magnitude) of the populations of endospores applied to aluminum coupons were inactivated within 5 min under a simulated Mars-normal UV fluence rate calibrated for equatorial Mars at an optical depth of 0.5. Furthermore, the numbers of surviving endospores of all seven *Bacillus* spp. were reduced greater than six orders of magnitude within 30 to 180 min of UV exposures. The bi-phasic response first reported for *B. subtilis* HA101 (Schuerger et al., 2003) also was observed for five additional *Bacillus* spp. tested here. This result supports the conclusions by Schuerger et al. (2003) that viable bioloads on Sun-exposed surfaces of spacecraft on Mars can undergo rapid inactivation on the first sol after landing, and extends the rapid inactivation kinetics to additional species of the spore-forming *Bacillus*.

Bacillus pumilus SAFR-032 was the most UV resistant bacilli in the current study requiring 180 min of UV irradiation (UVC + UVB flux equal to 105.6 kJ m^{-2}) to drop below the detection limits of the MPN and spore-medium assays (defined as the lethal dose rate [LD₁₀₀] for the current study). Although *B. subtilis* HA101 was significantly more sensitive to UV irradiation compared to SAFR-032, it too exhibited a LD₁₀₀ of 105.6 kJ m^{-2} for UVC + UVB (180 min). The other five *Bacillus* spp. exhibited significantly greater sensitivities to UV irradiation and the average LD₁₀₀ was 54 min (UVC + UVB equal to 31.7 kJ m^{-2}) for *B. pumilus* FO-36B, *B. licheniformis* KL-196, *B. nealsonii* FO-092, *B. megaterium* KL-197, and *B. subtilis* 42HS1. These results are consistent with other studies that reported *B. pumilus* SAFR-032 as the strongest UV-resistant bacilli recovered from spacecraft assembly facilities (Link et al., 2004; Newcombe et al., 2005). These lethal dose rates are estimated for equatorial Mars under an optical depth of 0.5 at the mean orbital distance of Mars to the Sun. But the UV fluence rates on Mars increase or decrease approximately 18% at the perihelion or aphelion positions in solar areocentric longitude, respectively (Appelbaum and Flood, 1990). Thus, under clear-sky conditions, the lethal dose rates might be expected to shift slightly throughout the 668 sol martian year. Furthermore, the times required to accumulate lethal dose rates on Mars would be expected to increase under (a) global dust storm conditions, (b) on spacecraft surfaces partially shielded from direct UV irradiation, and (c) at high latitudes on Mars due to lower Sun angles, and, thus, lower UV fluence rates.

The times required to accumulate these lethal dose rates would increase under global dust storm conditions in which the total UV fluence rates might be reduced significantly. For example, if atmospheric conditions on Mars were to reduce the

total UV irradiation by 75% (global dust storm conditions as modeled by Appelbaum and Flood, 1990 and Schuerger et al., 2003), then instead of 180 min to accumulate the lethal dose rate for *B. pumilus* SAFR-032, as described above for an optical depth of 0.5, it might require 720 min of UV irradiation at an optical depth of 3.3. Thus, 720 min of UV irradiation at an optical depth of 3.3 would be the equivalent of approximately 1.3 sols on Mars if the daily illumination period was approximately 12 h per day and the first and last 1.5 h of each sol (3 h) were subtracted from the total due to extremely high UV attenuation caused by high solar zenith angles at sunrise and sunset (Appelbaum and Flood, 1990). Under this first-order approximation of UV fluence rates under clear-sky and global dust storm conditions, the inactivation kinetics of *Bacillus* spp. are extremely fast, and are likely to result in significant reductions in viable bioloads on Sun-exposed spacecraft surfaces on Mars over short periods of time.

In previous work, Schuerger et al. (2003) reported that martian pressure had a slight effect on the survival of endospores of *B. subtilis* HA101. Numbers of viable endospores exposed to martian conditions of pressure (8.5 mb), temperature -10°C , and gas composition (pure CO_2) for up to 12 h were reduced 35% compared to endospores maintained at Earth-normal pressure (1013 mb) and temperature ($+23^{\circ}\text{C}$) under an O_2/N_2 atmosphere. Thus, even though pressure had only a minor effect on spore survival compared to UV irradiation, the low martian pressure did slightly impact the survival of spores during Mars UV simulations and would be expected to accelerate the inactivation kinetics described here for UV irradiation by 20–30%.

There are three key caveats to this model of rapid inactivation kinetics of terrestrial microorganisms on Sun-exposed spacecraft surfaces on Mars. First, a robust UV model for the surface of Mars has not been developed. Although several models have been published (Appelbaum and Flood, 1990; Cockell et al., 2000; Patel et al., 2002; Schuerger et al., 2003), no direct UVC, UVB, and UVA measurements have been made from the surface of Mars. Such measurements are critical to developing a truly robust model of microbial inactivation on the surface of Mars, and are recommended for near-term lander missions.

Second, the precise nature of the surface topography of spacecraft materials may play an important part in determining the effects of UV irradiation on microbial survival on Mars. In a recent study (Schuerger et al., 2005) reported that bacterial spores embedded within shallow cracks and pits on metal surfaces could still be inactivated with slightly longer UV exposures than bacterial spores present on horizontal surfaces. The authors concluded that UV irradiation could penetrate into shallow cracks and pits on some spacecraft materials leading to reductions in viable bioloads. In addition, they reported that non-metal surfaces enhanced the aggregation of bacterial spores into large multi-layered colonies, thus, enhancing the survival of spores under high UV dosages. The work of Schuerger et al. (2005) is consistent with the work of Mancinelli and Klovstad (2000) who reported that multi-layered aggregates of bacillus spores can act to shield the underlying spores from UV irradiation by the UV-absorption of overlying dead cells. Some

work has been published on the nature of microbial bioloads on spacecraft surfaces (La Duc et al., 2003, 2004; Venkateswaran et al., 2001, 2004), but much more research is required to answer at least the following three questions. (1) What are the actual characteristics of microbial cells and colonies on authentic spacecraft materials, including colonial forms, adhesive properties, and encasing materials? If viable spores are present on spacecraft materials as single spores attached to superficial dusts and particulates, then UV irradiation on Mars might impact their survival more rapidly than if they are present as multi-layered colonies. (2) Following sterilization of spacecraft components during assembly, how deep can recontamination actually penetrate spacecraft surfaces? If recontamination does not penetrate deeply into spacecraft materials after initial sterilization, then UV irradiation might reduce viable bioloads more quickly than if the recontamination is more deeply embedded. (3) Are there UV-attenuating materials present on spacecraft components (e.g., greases, lubricants, Kapton tape) that could dramatically reduce the UV flux on spacecraft surfaces, and, thus, prolong the survival of terrestrial microorganisms on Mars? For example, recent work in one of our labs (Schuerger) has revealed that Kapton tape dramatically absorbs UV irradiation. Transmission of irradiation over the entire UV range (200–400 nm) was reduced to $<0.0001\%$ of the downwelling UV irradiation (Schuerger, unpublished). Thus, spacecraft components wrapped in Kapton tape and directly exposed to solar UV still might retain their viable bioloads for significantly longer time periods than fully exposed surfaces.

And third, how will dust settling on spacecraft components after landing alter the biocidal effects of UV irradiation in reducing viable bioloads on spacecraft surfaces? Schuerger et al. (2003) demonstrated that individual dust particles measuring up to $50\text{ }\mu\text{m}$ in diameter had only a minor protective effect on the survival of *B. subtilis* endospores when exposed to UV irradiation under simulated martian conditions. This was in sharp contrast with the literature in which a number of papers have reported that analog Mars dusts can attenuate UV irradiation and enhance survival of bacterial spores under simulated martian conditions (Green et al., 1971; Hagen et al., 1970; Horneck et al., 2001; Packer et al., 1963). However, in these reports, the dust layers were composed of soil particles placed on coupons in thick contiguous layers. In contrast, Schuerger et al. (2003) used individual dust particles deposited in thin light coatings on bacterial monolayers of *B. subtilis* spores. The approach by Schuerger et al. (2003) more accurately simulated the deposition of aeolian dust onto spacecraft surfaces in which individual dust particles in the martian atmosphere measure only $1\text{--}2\text{ }\mu\text{m}$ in diameter (Tomasko et al., 1999). Furthermore, based on the reduction in solar cell power outputs on the Spirit rover (Arvidson et al., 2004) and Sojourner rover (Landis and Jenkins, 2000), between 0.2 and 0.28% of spacecraft surfaces may be covered by aeolian dust per day. Thus, the process in which UV irradiation inactivates terrestrial bioloads on Sun-exposed spacecraft surfaces on Mars is actually a race against time in which UV lethal dosages must be accumulated by terrestrial microorganisms faster than the daily dust load can cover and protect the adhered or embedded microbes.

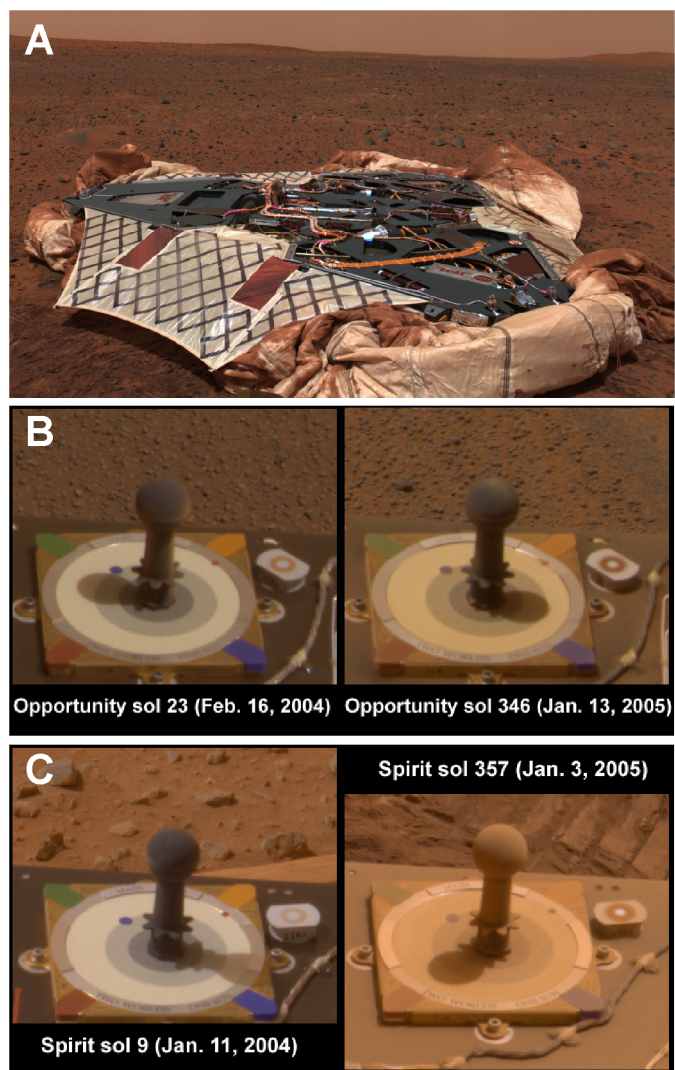


Fig. 4. Spirit and Opportunity rover images were downloaded from <http://marsrovers.jpl.nasa.gov>. (A) Spirit lander on sol 16 after the Spirit rover had departed. (B) Opportunity calibration targets exhibiting minor and moderate dust loads after sols 23 and 346, respectively. (C) Spirit calibration targets exhibiting minor and heavy dust loading after sols 9 and 357, respectively.

For example, consider the Spirit lander (Fig. 4A) in which the Spirit rover rolled off the lander on sol 12. The upper surfaces of the Spirit rover were exposed immediately to martian UV irradiation after petal opening on January 3, 2004 (sol 1), and dust began settling on the rover's upper surface that same day at the rate of approximately 0.2–0.28% per day (Arvidson et al., 2004; Landis and Jenkins, 2000). This process was then repeated on the upper surfaces of the lander following the departure of the rover on sol 12. Based on the results reported here, over six orders of magnitude of bioload reductions on Sun-exposed surfaces may have been achieved on sol 1 for the Spirit rover's upper Sun-exposed surfaces, and the same bioload reductions may have occurred on sol 12 for the Sun-exposed lander surfaces. If dust particles accumulated at the rate of only 0.2–0.28% of the surface per day, it is likely that the dust settling on the rover or lander surfaces may have had no significant impact on protecting viable spores on these Sun-exposed sur-

faces because UV inactivation likely occurred much faster than the dust was capable of protecting spores.

However, if viable spores were present in deep cracks, pits, or void spaces, or as multi-layered colonies (see Mancinelli and Klovstad, 2000; Schuerger et al., 2003, 2005), then long-term dust accumulations on spacecraft may play an important part in protecting the spores from martian UV irradiation because significantly longer times may be required in these protected niches to accumulate biocidal levels of UV. For example, over the course of the extended missions for both the Spirit and Opportunity rovers, dust accumulated at different rates for each rover (Figs. 4B and 4C). Opportunity had significantly lower levels of dust after 346 sols (Fig. 4B) than did the Spirit rover after 357 sols (Fig. 4C). Thus for the Spirit rover, it is possible that protected niches in deep cracks, pits, or void spaces might have accumulated dust layers thick enough to attenuate downwelling UV irradiation, and the accumulated dust may have offered adequate protection against UV irradiation to embedded spores.

Based on our current data and notwithstanding these caveats, we propose that rapid inactivation of terrestrial bioloads on spacecraft components may be possible on Sun-exposed surfaces on Mars, and that this process may occur faster than the aeolian dust can accumulate on these surfaces. And due to the rapid inactivation of terrestrial bioloads on Sun-exposed surfaces of Mars landers, the risk for forward contamination of a landing site may be lower than previously thought. However, we caution that because UV irradiation can only impact the viability of microorganisms on Sun-exposed surfaces, UV irradiation should not be relied upon for the primary sterilization of spacecraft components. For example, the airbag delivery system used for the Spirit lander (Fig. 4A) impacted the soils on Mars within 1–2 min after deployment. This short time period before contacting the martian soil would not have been adequate to achieve dramatic reductions in bioload levels in the airbag material due to UV irradiation, and, thus, the airbags relied upon heat-sterilization prior to launch to reduce terrestrial contamination.

And lastly, it is also important to base spacecraft sanitizing procedures on the most resistant microorganisms present as contaminants on these surfaces. Thus, we advocate that future spacecraft should be monitored for microbial contamination during spacecraft assembly, and the recovered microorganisms be tested for at least UV, vacuum, desiccation, and cosmic ray resistance in order to accurately model the effects of these parameters on the changes of the launched bioloads surviving the cruise phase to Mars and the operational surface mission.

5. Conclusions

The UV environment on Mars covers a wider spectral range than on Earth because of the lack of significant ozone in the martian atmosphere. This results in UVC down to 190–200 nm reaching the surface of Mars under most conditions. The higher UVC flux on Mars compared to Earth increases the biologically effective dose rate for UV irradiation for Mars rendering it significantly more biocidal than the surface of the Earth. Un-

der this high UV flux on Mars, terrestrial microorganisms on Sun-exposed surfaces of spacecraft are likely to accumulate lethal doses over relatively short periods of time. The results presented here support the conclusion that rapid inactivation of terrestrial microorganisms on Mars may be possible resulting in greater than six orders of magnitude reduction of viable bioloads on Sun-exposed surfaces over time periods of a few hours on sol 1. The most UV-resistant species was *B. pumilus* SAFR-032. The most UV-sensitive species were *B. nealsonii* FO-092, *B. megaterium* KL-197, and *B. subtilis* 42HS1. However, this model requires additional research to (a) collect direct UV measurements on the surface of Mars to validate the UV models currently proposed for the surface, (b) characterize the actual colonial forms of microorganisms on spacecraft materials to better understand how terrestrial microorganisms actual colonize spacecraft materials, and (c) clarify the effects of dust loading on spacecraft surfaces in order to predict the rates of inactivation of terrestrial microbes on spacecraft components. The results presented here support the conclusions of previous studies in which rapid inactivation rates are described for *Bacillus* spp. on spacecraft surfaces under martian conditions. But we emphasize that UV irradiation only assists in the bioload reduction on Sun-exposed surfaces on Mars and should not be used as a primary means of sterilizing spacecraft components for Category IV missions to Mars. We have not observed any phenomena in these experiments nor have we found any literature that would support the conclusion that common terrestrial microorganisms typically found on spacecraft are "immune" to the UV environment on Mars. Thus, we predict that typical spacecraft contaminants are likely to be inactivated on Sun-exposed spacecraft surfaces on Mars based on the rate that lethal doses of UV irradiation are accumulated by individual spores. Locations on spacecraft in which spores receive biocidal levels of UV irradiation, even at extremely slow rates of accumulation, are likely to experience significant reductions in viable bioloads over time.

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