UV Shielding of *Bacillus pumilus* SAFR-032 Endospores by Martian Regolith Simulants

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INTRODUCTION

Planetary Protection

- As exploration of the solar system continues with life detection missions on the horizon, the concern for planetary protection is relevant and necessary.
- Current sterilization procedures are not perfect, so particularly hardy microbes can tag along on interplanetary voyages. For example, it is estimated that the Mars Science Laboratory carried >10^4 bacterial endospores to the Red Planet[1].
- Spacecraft bioburdens must be reduced as much as possible to mitigate contamination and avoid false positives in future life detection missions.

Figure 1. The Sojourner rover in a JPL spacecraft assembly facility[11].

Exposing Microorganisms in the Stratosphere (E-MIST)

- The E-MIST project seeks to determine how resilient terrestrial microbes can survive the stressors of the Earth's middle stratosphere (30-38 km). The stratosphere is a good analog for the surface of Mars due to similarly low temperature and pressure, as well as high ultraviolet (UV) radiation and desiccation[2,3].
- *Bacillus pumilus* SAFR-032 is an endospore-forming bacterium isolated from a spacecraft assembly facility. Its endospores are polyextremophilic, resistant to extreme temperature, high pH, the vacuum of space, perchlorates, extreme desiccation, and ultraviolet radiation[5].
- In a previous E-MIST flight, 99.99% of endospores exposed to UV radiation in the middle stratosphere (31 km) were inactivated, while there was no significant endospore inactivation for samples exposed to stratospheric low temperature, pressure, and moisture with UV radiation shielding[6]. This indicates that UV radiation may be the limiting factor for SAFR-032 endospore survivorship, which corresponds with ground testing results of *Bacillus* species[7].
- Because Earth's ozone layer is mostly contained to the lower stratosphere and Mars has no ozone layer, these two environments receive comparable spectra of solar UV radiation. Thus, microbes on E-MIST's balloon-based payloads are exposed to all wavelengths of UVA, UVB, and UVC radiation, much like they would be on the surface of Mars.

Figure 2. UV radiation permeability in the Earth's atmosphere[2].

Martian Regolith

- Dust storms, such as the nearly global storm of summer 2018[8], constantly reshape the dynamic surface of Mars.
- With continual missions, dust is an expected environmental concern that frequently coats landers and rovers. Wind-deposited regolith could provide sufficient UV radiation shielding to prevent endospore inactivation.
- UV radiation shielding by regolith on *Bacillus* spp. has been studied before[7]; however, no significant shielding was detected. This could possibly be attributed to too low of a dust concentration, which may have resulted in UV radiation scattering through the regolith particles and inactivating the endospores.
- This project seeks to further study the relationship between regolith shielding from UVC radiation and endospore survivorship, using the Martian regolith simulants JSC MARS-1 and JPL MRS-1. These simulants were isolated from volcanic basalt near the Mauna Kea volcano in Hawaii and the Mojave Desert in California, respectively. Both were found to have chemically similar compositions to Martian regolith as found by previous rover expeditions[9, 10], and were both milled and heat-sterilized to mimic sterile Martian dust.
- This project investigated the hypothesis that these Martian regolith simulants provide UV radiation protection for *B. pumilus SAFR-032* spores, but only at a sufficiently high regolith concentration.
- Results from these ground-based irradiation studies will feed into experimental designs for the next E-MIST flight launched by NASA.

MATERIALS & METHODS

Spore Preparation

Bacillus pumilus SAFR-032 endospores were prepared per the "Spore Harvest, Purification, and Dilution" protocol described by Khodadad et al. 2017. As per the protocol, vegetative SAFR-032 cells were grown in Difco nutrient broth, then transferred into a base media to induce sporulation. Once the sporulation media had been sufficiently incubated, spores were purified through a series of water washing and centrifugation. This procedure yielded approximately 15 mL of endospore stock, which was stored in a glass tube at 4°C.

10^{-1} to 10^{-6} dilutions were prepared in sterile deionized water (SDIW). Then, scanning electron microscopy (SEM) imaging was used to assess sample purity and determine whether successful sporulation had occurred. 20 µL aliquots of diluted samples were pipetted onto aluminum coupons and left to dry in the biosafety cabinet (BSC) overnight. The next day, coupons were sputter coated and imaged with a Hitachi S-4800 field emission scanning electron microscope.

MATERIALS & METHODS continued

Regolith Preparation

Regolith simulants JSC MARS-1 and JPL MRS-1 (**Figure 3**) were heat-sterilized at 130°C for about two weeks, then SEM imaged to determine approximate particle size. To create finer dust particles, both simulants were milled with a mortar and pestle. JPL MRS-1 was milled for approximately 30 minutes, while JSC MARS-1 was milled for approximately 15 minutes. The new particle sizes were determined using a Microtrac S2500 Series Particle Size Analyzer. To ensure continued sterility, simulants were stored at 130°C during and following this preparation process.

Figure 3. Martian regolith simulants JSC MARS-1 (left) and JPL MRS-1 (right), prior to milling.

Once the desired particle size was reached, 3 weighed quantities of each regolith simulant were mixed with 10 mL of SDIW, yielding 6 regolith solutions (**Figure 4**). To quantify concentration, the optical density of each was measured with an I3 SpectraMax. Following preparation, these solutions were stored at 4°C.

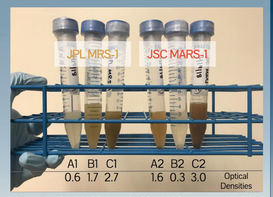

Figure 4. JPL MARS-1 and JSC MARS-1 suspended in SDIW with optical densities of each solution.

Figure 5. Radiometer and sample setup in the BSC prior to turning on the UV light.

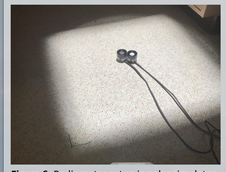

Figure 6. Radiometer setup in solar simulator.

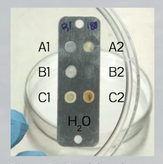

Figure 7. Regolith-endospore solutions on aluminum coupon.

UV Radiometer Testing

Throughout the experiments, a SolarLight PMA2100 Dual-Input Data Logging Radiometer was used to measure UVA/B and UVC irradiation. It was tested in full sunlight (on a table outside on a cloudless day around 2:40pm), in general lab conditions, inside the BSC with the UV light on (**Figure 5)**, and in the solar simulator (**Figure 6**).

Toxicity Assessment

To determine whether the regolith simulants had any significant effects on bacterial survivorship, equal amounts of a 10^{-2} dilution of spore stock and regolith solutions were combined, vortexed, then plated on trypticase soy agar (TSA) in triplicate. Plates were incubated at 30°C overnight, then survivorship was assessed the next day by counting the colony-forming units (CFUs) on each plate.

Coupon Preparation

In preparation for the irradiation trials, the aluminum coupons were sonicated for 50 minutes then heat-sterilized at 130°C for 24 hours. 900 µL of each regolith solution was combined with 100 µL of 10-2 diluted endospore stock, to make 10^{-3} dilutions of SAFR- 032 with regolith. The solutions were vortexed, then 20 µL aliquots of these endospore-regolith solutions were pipetted on the sterile coupons (**Figure 7**). A solution of 900 µL SDIW and 100 µL of 10^{-2} endospore stock was similarly prepared for a control, labeled H_2O. Using this dilution scheme, each aliquot contained approximately $2.6*10^5$ endospores.

Irradiation Trails

Prepared coupons were placed in sterile petri dishes in the BSC (**Figure 5**), uncovered, then irradiated for 360 minutes. Plates were transferred from the BSC to the bench at time steps of 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 minutes.

For the first set of irradiation trials, there were 7 solutions per coupon (**Figure 7**). To remove the samples from the coupons following irradiation, 20 µL of 10% polyvinyl alcohol (PVA) was pipetted onto each sample and left to dry overnight. The next day, each PVA aliquot was peeled from the coupons using autoclaved sterile forceps, then dissolved in 0.5 mL SDIW. From this, 100 µL of each solution was pipetted on TSA. The plates were incubated for at 30°C, then CFUs were counted the next day to evaluate survivorship.

During the first trials, it was observed that the PVA was not removing all the regolith from the coupons. To ensure that the full samples were being removed for analysis, a new method was used. 1, instead of 7, samples were aliquoted per coupon. As per the protocol for the first set of irradiation trials, coupons were left to dry overnight in the BSC, then irradiated. Following irradiation, each coupon was placed into 10 mL SDIW with 1 scoop (approximately 0.5 g) of sand that was heat-sterilized at 130°C for 24 hours (**Figure 8**). Next, each tube was vortexed for 1 minute, then 100 µL was pipetted on TSA. The plates were incubated at 30°C, then CFUs were counted the next day to evaluate survivorship.

Figure 8. Coupon from second round of irradiation trials in 10 mL SDIW + 0.5 g heat-sterilized sand.

PRELIMINARY RESULTS

Spore Preparation

10^{-1} to 10^{-6} dilutions of the endospore stock from the first round of sporulation were prepared and imaged (**Figures 9**). An unexpected crystal-like debris was observed, so spores were prepared a second time. The debris was not observed in the second round of imaging (**Figure 10)**, so the second spore stock was used in the rest of the experimentation. It was determined that sporulation had successfully occurred in both stocks, due to ovoid shape of the cells and a raisin-like appearance. Differences in resolution between the two rounds of sporulation can be attributed to variation in the sputter coat between the different days.

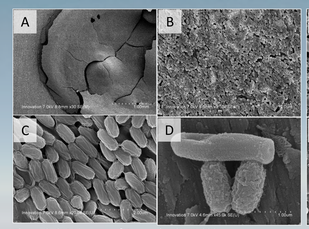

Figure 9. SEM images of B. pumilus SAFR-032.
Scale: (A) 1.00 mm, (B) 10.0 µm, (C) 2.0 µm, (D) 1.00 µm

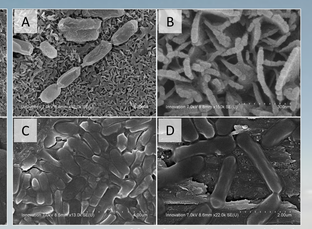

Figure 10. SEM images of from first (A, B) and second (C, D) rounds of sporulation. Scale: (A) 3.00 µm, (B) 300 nm

Regolith Preparation

Regolith simulants were SEM imaged to get an approximate idea of particle size (**Figure 11**). Large variation in particle size was observed, ranging from above 0.5 mm to below 1 µm – 0.5 mm in length. Following milling, a smaller and more uniform particle size distribution was observed, with the upper bound of the length range now about 400 µm (**Figure 12**).

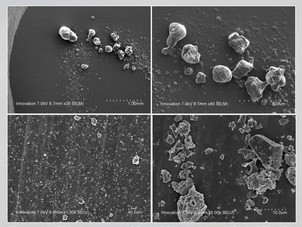

Figure 11. SEM images of JSC MARS-1 (A, B) and JPL MRS-1 (C, D) prior to milling.
Scale: (A) 1.00 mm, (B) 500 µm, (C) 40.0 µm, (D) 10.0 µm

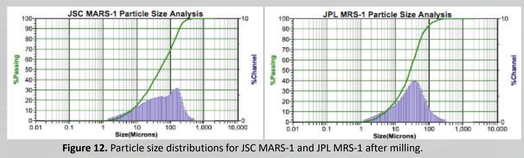

Figure 12. Particle size distributions for JSC MARS-1 and JPL MRS-1 after milling.

Location	UVA/B (W/m²)	UVC (µW/cm²)
Martian surface	47.4*	318*
Terrestrial middle stratosphere	94.9*	260*
Terrestrial surface	46.0	0.53
Biosafety cabinet	0.06	22.5
Solar simulator	21.7	56.8

Table 2. UVA/B and UVC irradiation data for various relevant locations.
*Modeled data[6] given for comparison

UV Radiometer Testing

The radiometer was used to quantify the UVA/B and UVC irradiation in different locations (**Table 2**).

Toxicity Assessment

CFUs on the triplicate plates from the toxicity assessment were counted and averaged (**Figure 13**), then a single-factor ANOVA was performed to compare the three treatments (JPL MRS-1, JSC MARS-1, and H_2O). The results between treatment were not significant (p>0.05), indicating no negative effect of regolith on survivorship.

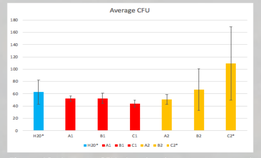

Figure 13. Average CFUs for the three treatments of no regolith (blue), JPL MRS-1 (red), and JSC MARS-1 (yellow).
*n=2, for all else n=3

Irradiation Trials

Throughout the irradiation trials, some troubleshooting had to be done with the procedure to ensure that the samples were being fully removed from the coupons. Vortexing with water and sand visibly removed the samples from the coupons, but only 1 of the 3 replicates exhibited any growth on the TSA plates, even in the non-irradiated control plates. Even then, variation in growth was observed that did not correspond with exposure conditions. Thus, further experimentation is necessary to refine the procedure and quantify a relationship between regolith concentration and SAFR-032 survivorship.

DISCUSSION

In this project, preliminary work for a future E-MIST flight was done to begin investigating the role of regolith shielding from UV radiation in survivorship of bacterial endospores. *B. pumilus* SAFR-032 endospore stocks were assessed for purity by SEM imaging. Martian regolith simulants JSC MARS-1 and JPL MRS-1 were prepared, heat-sterilized, imaged, analyzed, and made into suspensions for testing. Toxicity of the simulants on SAFR-032 was analyzed, and a protocol for sample irradiation and survivorship assay was refined.

Figure 14. E-MIST payload during a stratospheric flight[13].

Future Directions

Following this preliminary experimentation, UV shielding by these regolith simulants on the prepared stock of *B. pumilus* SAFR-032 endospores will continue to be studied, with the goal of determining the optimal concentration of regolith to prevent endospore inactivation by UV radiation. Once this is determined, samples will be prepared for a future E-MIST long-duration polar flight, which will occur in late 2019 or 2020.

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Background image : NASA Ames Research Center Space Life Sciences Training Program 2018 cohort