

1 **Stratosphere Conditions Inactivate Bacterial Endospores from a Mars Spacecraft**
2 **Assembly Facility**

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20

21 **Abstract**

22 Every spacecraft sent to Mars is allowed to land viable microbial bioburden, including
23 hardy endospore-forming bacteria resistant to environmental extremes. Earth's stratosphere is
24 severely cold, dry, irradiated and oligotrophic; it can be used as a stand-in location for predicting
25 how stowaway microbes might respond to the martian surface. We launched E-MIST, a high
26 altitude NASA balloon payload on 10 Oct 2015 carrying known quantities of viable *Bacillus*
27 *pumilus* SAFR-032 (4.07×10^7 spores per sample), a radiation-tolerant strain collected from a
28 spacecraft assembly facility. The payload spent 8 hours at ~32 km above sea level exposing
29 bacterial spores to the stratosphere. We found that within 120 and 240 min, spore viability was
30 significantly reduced by 2 and 4 orders of magnitude, respectively. By 480 min, < 0.001% of
31 spores carried to the stratosphere remained viable. Our balloon flight results predict that most
32 terrestrial bacteria would be inactivated within the first Sol on Mars if contaminated spacecraft
33 surfaces receive direct sunlight. Unfortunately, an instrument malfunction prevented the
34 acquisition of ultraviolet (UV) light measurements during our balloon mission. To make up for
35 the absence of radiometer data, we calculated a stratosphere UV model and also conducted
36 ground tests with a 271.1 nm UVC light source (0.5 W/m^2), observing a similarly rapid
37 inactivation rate when using a lower number of contaminants (640 spores per sample). The
38 starting concentration of spores and micro-configuration on hardware surfaces appeared to
39 influence survivability outcomes in both experiments. With the relatively few spores that
40 survived the stratosphere, we performed a re-sequencing analysis and identified 3 single
41 nucleotide polymorphisms compared to unexposed controls. It is therefore plausible that
42 bacteria enduring radiation-rich environments (e.g., Earth's upper atmosphere, interplanetary
43 space, or the surface of Mars) may be pushed in evolutionarily consequential directions.

44

45 **1. Introduction**

46 Preventing the forward contamination of Mars is required for United States and
47 international space missions (NASA, 2005; Kminek and Rummel, 2015). Yet, spacecraft leaving
48 Earth still carry microorganisms onboard, embedded within surfaces, instruments, electronics,
49 and other inaccessible areas that cannot be readily cleaned (Schuerger *et al.*, 2003; Nicholson *et*
50 *al.*, 2005; Benardini III *et al.*, 2014). Complete spacecraft sterilization has not been enforced
51 since the Viking missions. Currently-allowable microbial bioburden on spacecraft – while
52 relatively low (Benardini III *et al.*, 2014) – makes the pristine Mars environment vulnerable to
53 contamination. Moreover, future life detection missions could be threatened by false positives
54 without a better understanding of which microorganisms are most capable of persistence, growth
55 or replication once delivered to the Red Planet. A recent analysis from the Second MEPAG
56 Special Regions Science Analysis Group (Rummel *et al.*, 2014) identified major knowledge gaps
57 associated with polyextremophiles (microorganisms resistant to more than one environmental
58 stressor), particularly when shielded from ultraviolet (UV) light on Mars by global dust storms,
59 regolith, or overlying dead microorganisms. Nicholson *et al.* (2005) and Horneck *et al.* (2010)
60 reviewed common bacterial adaptations to extreme environments, including sporulation, cell
61 pigmentation, and DNA repair pathways (e.g., homologous recombination and non-homologous
62 end joining). Non-sporulating bacteria also have various mechanisms to resist damaging
63 radiation in the space environment. For example, *Deinococcus radiodurans* can repair its DNA
64 through homologous recombination. Other microbes, such as a halophilic *Synechococcus*
65 species, can shield biomolecules from radiation with exogenous salt crystals.

66 Smith (2013) argued that the Earth's stratosphere would allow multiple Mars-like
67 conditions to be simultaneously tested if polyextremophilic species could be exposed to the
68 upper atmosphere and returned for analysis. Recent missions have demonstrated the feasibility
69 of transporting biological samples to the stratosphere using small, meteorological balloons
70 (Beck-Winchatz and Bramble, 2014) and large scientific balloons (Smith *et al.*, 2014). The
71 pressure of the thin and dry stratospheric air around 25 to 38 km above sea level (ASL) is
72 roughly equivalent to the surface pressure on Mars (0.5 to 1 kPa). The stratosphere is also a cold
73 and extremely dry environment with elevated levels of ionizing and non-ionizing radiation
74 (Adams *et al.*, 2007; Dachev, 2013; Schuerger and Nicholson, 2016). Relative humidity levels
75 can drop below 1%, and temperatures in the lower stratosphere regularly reach -100 °C.
76 Stratospheric radiation is substantially higher (Kylling *et al.*, 2003; Adams *et al.*, 2007; Dachev,
77 2013) than doses at other frequently-visited Mars analog environments, including the McMurdo
78 Dry Valleys of Antarctica (Wynn-Williams and Edwards, 2000) and the Atacama Desert
79 (McKay *et al.*, 2003). Laboratory environmental chamber experiments have been employed in
80 the past to simulate martian conditions (Schuerger *et al.*, 2003; Diaz and Schulze-Makuch, 2006;
81 Tauscher *et al.*, 2006; de la Vega *et al.*, 2007; Moores *et al.*, 2007; Osman *et al.*, 2008; Fendrihan
82 *et al.*, 2009; Smith *et al.*, 2009; Gómez *et al.*, 2010; Peeters *et al.*, 2010; Johnson *et al.*, 2011;
83 Kerney and Schuerger, 2011) but artificial illumination sources do not realistically represent the
84 dynamic nature of sunlight. Moreover, most ground-based simulation studies do not
85 simultaneously create the full range of biological stressors present on Mars (i.e., hypobaria,
86 desiccation, irradiation, nutrient-deprivation, oxidation, and low temperatures). Conveniently,
87 Earth's upper atmosphere produces a natural combination of these extreme conditions.

88 Measuring the response and survival of polyextremophilic species in the stratosphere can
89 therefore be used to test Mars forward contamination scenarios.

90 On 10 October 2015 we flew a balloon experiment to the stratosphere over New Mexico
91 and Texas (United States) reaching an altitude of 31.4 km ASL. The Exposing Microorganisms
92 in the Stratosphere (E-MIST) payload carried known quantities of bacterial endospores (hereafter
93 referred to as ‘spores’) to the Mars-like environment for 2, 4, 6, and 8 h exposures. We used a
94 spacecraft assembly facility-isolated bacterial strain *Bacillus pumilus* SAFR-032 for the balloon
95 flight (and subsequent ground experiments). The Gram-positive, aerobic, endospore-forming
96 bacterium is noteworthy for special resistance to desiccating, UV-intense conditions (Link *et al.*,
97 2004; Kempf *et al.*, 2005; Gioia *et al.*, 2007; Tirumalai *et al.*, 2013). Since the spores used for
98 the balloon experiment were metabolically dormant, exposure to stratospheric conditions
99 resulted in cumulative damage to cellular components that was measurable in the laboratory after
100 the E-MIST payload returned to the ground. Our experimental design was inspired by similar
101 experiments with *B. pumilus* SAFR-032 spores outside the International Space Station (ISS)
102 (Horneck *et al.*, 2012; Moeller *et al.*, 2012; Nicholson *et al.*, 2012; Vaishampayan *et al.*, 2012).

103 Sending known quantities of viable, monolayered spores into the Earth’s stratosphere and
104 making comparisons with unexposed controls allowed us to: (1) determine the survival of spore
105 populations using culture-based enumeration methods; and (2) assess genomic alterations
106 through a re-sequencing analysis of surviving spores. We also collected environmental data
107 during the balloon flight and performed supplemental ground UV experiments, with an overall
108 goal of assessing the survivability of *B. pumilus* SAFR-032 spores in stratospheric conditions
109 that closely resemble the surface of Mars.

110

111 2. Materials and Methods

112 2.1 Bacterial Strain Description and Sample Preparation

113 *Bacillus pumilus* SAFR-032 spores were also used for the first E-MIST balloon test flight
114 (Smith *et al.*, 2014). A full genome map was available (NCBI, GCA000017885.4 ASM1788v4)
115 with a total of 3819 genes previously identified for the species (Gioia *et al.*, 2007). The strain
116 was safe to work with in the field (Biosafety level 1; no hazard posed to balloon personnel or the
117 environment) and prepared spores were stable in stasis allowing for simplified mission logistics.
118 Moreover, there was no exosporium or extraneous biofilms/layers associated with spores
119 resulting in straightforward post-flight molecular assays (Link *et al.*, 2004; Gioia *et al.*, 2007;
120 Vaishampayan *et al.*, 2012; Tirumalai *et al.*, 2013). Testing SAFR-032 spores also enabled
121 comparisons with past experiments that used the model microorganism (Horneck *et al.*, 2012;
122 Moeller *et al.*, 2012; Nicholson *et al.*, 2012; Vaishampayan *et al.*, 2012; Smith *et al.*, 2014).

123 We established a spore stock based on previously-established methods (Schaeffer *et al.*,
124 1965; Nicholson and Setlow, 1990; Vaishampayan *et al.*, 2012) by culturing the original isolate
125 of *B. pumilus* SAFR-032 in Difco nutrient broth and incubating at 35° C, 140 rpm for 16 h.
126 Germinated cells were then transferred to sterile sporulation media with the following nutrients
127 per 1 L sterile nanopure water: 8 g Difco nutrient broth, 1 g potassium chloride, and 0.25 g
128 magnesium sulfate heptahydrate autoclaved together followed by the addition, of 1 ml sterile
129 calcium chloride (7.35 g/100 ml), manganese chloride (0.2 g/100 ml), and ferrous sulfate (0.0278
130 g/100 ml). The culture was incubated for 124 h at 35 °C with shaking at 140 rpm. Resultant
131 spores were then divided evenly into 50 ml tubes (containing ~40 ml of culture each) and
132 submerged into a water bath at 80 °C for 15 min to destroy any remaining vegetative cells,
133 followed by centrifugation for 20 min at 9400 RCF. Next, heat-treated spores were washed by

134 re-suspension with sterile molecular-grade water, centrifugation, and removal of supernatant.
135 This process was repeated 4 times. After the final wash, spores were re-suspended in 8 ml of
136 sterile molecular-grade water and stored overnight in a cold incubator at 4 °C with shaking at 90
137 rpm. To remove any cellular debris we repeated the cold incubation and wash cycle 3
138 consecutive times. Once completed, the supernatant was removed and the pellet containing
139 purified spores was transferred to a sterile glass test tube containing 0.5 ml sterile molecular-
140 grade water.

141 Spore stock concentration was determined to be approximately 6.29×10^9 spores per 1 ml
142 through the Most Probable Number (MPN) method and heterotrophic plate counts. Dilutions
143 from the original stock (20 μ l aliquots) were then seeded onto sterile aluminum coupons
144 (M4985, Seton) and dried for 4 h in a dark laminar flow hood at 25 °C, creating a layer of 4.07
145 $\times 10^7$ spores adhering to coupon surfaces for each individual aliquot. A total of 14 separate 20 μ l
146 aliquots were deposited onto any single experimental coupon (5.40 x 1.75 x 0.51 cm) (Fig. 1A).
147 Experimental coupons were created in the same batch, then stored in sterile, dark containers.
148 Dried coupons were imaged with a scanning electron microscope (SEM) (JSM-7500F, JEOL) to
149 assess the distribution of spores on the aluminum coupon surface (Fig. 1B). Recovery of spores
150 from experimental coupons was achieved through polyvinyl alcohol (PVA) film peels (Horneck
151 *et al.*, 2001; Moeller *et al.*, 2012). Twenty microliters of sterile 10% PVA prepared in water was
152 applied in a thin layer over the dried spores previously deposited onto each coupon. After drying
153 in an incubator for 1 h at 37 °C, the PVA film contained embedded spores and was peeled off the
154 coupon using sterile forceps. The PVA film was dropped into a glass test tube containing sterile
155 molecular-grade water and re-suspended with a vortexer.

156

157 2.2. Enumeration and Re-Sequencing Assays

158 In general, microbial coupons from flight experiments and ground simulations were
159 assayed to (1) determine the number of viable surviving spores (compared to starting quantities);
160 and (2) assess the extent of non-lethal genetic mutations through re-sequencing (compared to
161 controls and the reference *B. pumilus* SAFR-032 genome). The MPN enumeration method
162 originally described by Mancinelli and Klovstad (2000) and modified by Schuerger *et al.* (2003)
163 was used for this study. After incubation at 30 °C for 36 to 42 h, plate wells were scored for
164 growth and calculated to MPN values. To analyze the genome of surviving spores, we followed
165 a protocol from Nicholson *et al.* (2012). After the PVA peel step, spore suspensions were
166 distributed into 5 ml of recovery media (1:9 ratio of 10 mM L-alanine:2X LB) and then
167 incubated at 37 °C with shaking at 250 rpm for 60 min allowing approximately one cell
168 replication cycle. Next, germinated cultures were centrifuged at 5000 rpm for 10 min. The
169 supernatant was removed by pipette and pellets were resuspended in 1.8 ml of sterile molecular-
170 grade water for DNA extraction using the UltraClean[®] Microbial DNA Isolation Kit (MoBio).
171 Tubes were centrifuged at 14,000 rpm for 3 min with the exception of the microbead and filter
172 tubes which were spun at 10,500 rpm. Rather than vortexing the microbead tubes for 10 min, the
173 tubes were placed in a Biospec benchtop mini-bead beater for 1 min. The extracted DNA was
174 then quantified with a Qubit 2.0 Fluorometer and a high sensitivity DNA assay (Invitrogen). All
175 DNA samples were stored at -20 °C. Purities of the spore stock and batch-produced
176 experimental coupons were monitored throughout the study with heterotrophic plate counts and
177 DNA sequencing – no contaminating species were identified.

178 Sequencing was conducted using the Illumina Nexterra XT kit (Illumina). First, 1 ng of
179 DNA was tagged with indices followed by a 5 min temperature cycling of 55 °C and 10 °C to

180 fragment the DNA. Amplification followed, cycling at 72 °C for 3 min, 95 °C for 30 s, and 12
181 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. A final elongation step at 72 °C for 5
182 min completed the cycle. Amplified DNA was cleaned using the MinElute PCR cleanup kit
183 (Qiagen) and denatured with 0.2 N NaOH and heating per Illumina Protocol #15044223 Rev B.
184 Sequencing reads were run on the Illumina MiSeq and prepared using the Illumina V2 300 kit.
185 To identify and assemble contigs, data were analyzed using CLC Workbench v 8.0.2 (Qiagen
186 Bioinformatics). The software located single nucleotide polymorphisms in samples compared to
187 the *B. pumilus* SAFR-032 reference genome. Nucleotide variant type (i.e., deletion, insertion, or
188 substitution) and the frequency across samples were mapped to known coding regions for the
189 strain.

190

191 **2.3 E-MIST Hardware Description**

192 Several noteworthy hardware modifications (Supplementary Figs. S1 and S2;
193 Supplementary Figures are available at www.liebertonline.com/ast) were made after the first E-
194 MIST test flight described by Smith *et al.* (2014). Four independently rotating skewers fitted
195 with an adjustable aluminum sample base plate allowed an exposure time series to be established
196 in the stratosphere. Each sample plate held 10 separate aluminum coupons (see Section 2.1).
197 The plates were enclosed within Nomex-lined cylinders to prevent sunlight from entering during
198 the balloon gondola ascent/descent, and when the skewers rotated to a closed position for the
199 experimental time series. Each skewer was motor-controlled (SPG30E-300K, Cytron) by a 4-
200 channel motor driver (FD04A, Cytron) held together by an aluminum and polycarbonate frame.
201 Multiple instruments were inside the payload housing, including a GPS unit (SPK-GPS-
202 GS407A, S.P.K. Electronics Co.), a radiometer with UV sensors (PMA2100, PMA2107, and

203 PMA2180, Solar Light), and an external humidity and temperature sensor (HOBO, U23-001,
204 Onset). Instrument temperatures were regulated inside the payload with heating pads (5V,
205 WireKinetics). The avionics system (chipKIT Max32, Digilent) used a serial peripheral interface
206 connection to communicate with a micro-SD card (BOB-00544, microSD Transflash
207 Breakout, SparkFun) and a micro DB-9 port (1200-1183-MIL, Digi-Key). A 1080P HackHD
208 camera was controlled by the avionics and recorded imagery throughout the flight. Other major
209 hardware components included an altimeter (MS5607, Parallax), 8.5 W heaters (Omegalux
210 Kapton Insulated Flexible Heater, Omega), and multiple resistance temperature detectors (SA1-
211 RTD-B, Omega). Power was generated by a 14.8v 25.2 Ah lithium-ion polymer battery (CU-
212 J141, BatterySpace). Before flying, the E-MIST payload was subjected to vibration and
213 hypobaric chamber testing to validate system performance. Instruments were re-calibrated after
214 testing, then the payload was shipped to the launch site at Ft. Sumner, New Mexico, where it was
215 mounted onto the uppermost gondola portion of Columbia Scientific Balloon Facility (CSBF)
216 long-duration balloon (LDB) Test Flight II (#667NT). Pre-launch, landing, and recovery
217 procedures described by Smith *et al.* (2014) were repeated for this experiment. Prior to installing
218 base plates onto the skewers with *B. pumilus* SAFR-032 coupons, the inside of skewer canisters
219 were sprayed with sterile air and payload surfaces were wiped with isopropyl alcohol.

220

221 **2.4 Experimental Design**

222 Ten experimental coupons were attached (in random order) to 4 separate base plates on
223 the rotatable E-MIST skewers. Each coupon contained 14 identically-prepared 20 μ l aliquots of
224 *B. pumilus* SAFR-032 deposited at a concentration of 4.07×10^7 spores per aliquot. This
225 arrangement allowed for a high number of potential replicates per skewer ($N = 140$). All

226 skewers were opened simultaneously in the stratosphere, then closed sequentially: Skewer 1
227 exposed samples for 2 h; Skewer 2 exposed samples for 4 h; Skewer 3 exposed samples for 6 h;
228 and Skewer 4 exposed samples for 8 h. Two different sample coupon orientations were
229 established on each base plate to determine the effect of stratospheric conditions with and
230 without sunlight: (1) 9 coupons were mounted upright and exposed directly to sunlight; and (2) 1
231 coupon was mounted upside-down (inverted) to prevent illumination. A blank negative control
232 coupon was also located on the payload and used for monitoring potential contamination
233 associated with launch and landing. To measure the possible influence of transportation to the
234 field site and/or delays associated with balloon flight operations, we included two sets of ground
235 controls – coupons that traveled to Ft. Sumner, New Mexico, but were not flown; and another set
236 that remained in the laboratory at Kennedy Space Center (KSC), Florida.

237

238 **2.5 Ground Experiments**

239 To supplement the balloon flight experiment, we used another group of coupons to
240 evaluate *B. pumilus* SAFR-032 spore resistance to artificially-generated UVC conditions in the
241 laboratory. Within a biological safety cabinet (Labgard Class II, Type A/B3, Model NU-600
242 Series 24, NuAire, Inc), a 3D-printed acrylic UV light emitting diode (LED) test stand (12.0 cm
243 x 7.5 cm x 14.1 cm) held experimental coupons at vertical distances of 1 to 5 cm from the light
244 source (Supplementary Fig. S3; Supplementary Figures are available at
245 www.liebertonline.com/ast). The test stand bridge could vertically move the LED at half-
246 centimeter intervals and change the angle of illumination 45 to 90° relative to the sample base
247 plate below. A UVC LED (Part # UVTOP270TO39FW, QPhotonics, LLC) with 5.668 V and a
248 maximum current setting at 20.00 mA generated a peak wavelength of 271.1 nm and a spectral

249 width of 10.3 nm. A Light Meter (HHUV254SD, Omega) was used to measure maximum UVC
250 intensities of 0.50, 0.17, 0.090, 0.040, and 0.030 W/m² for coupons located 1, 2, 3, 4, and 5 cm
251 from the LED, respectively. The first ground experiment exposed bacterial coupons (4.07×10^7
252 spores per aliquot) to UVC at distances of 1-1.5 cm (resulting in 0.50-0.27 W/m²) for durations
253 of 1.3, 6.7, 50, 100, and 240 min. For each run, the angle of incidence for coupon illumination
254 was 90° from the plane of the UVC LED. Samples were enumerated after the UVC exposure
255 using the MPN procedure described in Section 2.2. A second ground experiment was also
256 conducted with a lower starting concentration of *B. pumilus* SAFR-032 spores (640 spores per
257 aliquot). Samples were exposed for 15 min at 90° from the UVC LED plane in distances of 1-5
258 cm (0.50-0.030 W/m²). Next, samples at a distance of 5 cm (0.030 W/m²) were exposed for 0-25
259 min at 90° from the UVC LED. The final portion of the experiment exposed samples for 15 min
260 at a distance of 5 cm from the UVC LED, but the orientation of the light source was changed to
261 create 45, 60, 75, and 90° angles of incidence relative to the plane of the coupon.

262

263 **2.6 Statistical Analyses**

264 Means and standard errors were calculated for samples from each flight experiment
265 treatment (2, 4, 6, and 8 h). For every group we sampled 3 random coupons, with 3 separate
266 bacterial aliquots processed from each coupon; providing a total of $N = 9$ replicates per group.
267 Fewer inverted test coupons were flown (only 1 coupon per skewer) so samples were enumerated
268 in triplicate. Our UVC ground experiment had 5 time treatments with $N = 9$ replicates from each
269 group. The second UVC experiment (with a lower starting concentration of spores) processed
270 samples in triplicate and had independent treatments for time (0-25 min), distance (1-5 cm), and
271 angles of incidence (45-90°). To analyze values from both flight and ground experiments, we

272 ran one-way ANOVA analyses, producing *P*-values at 95% confidence levels to determine if
273 viability numbers were changing significantly compared to initial quantities. *F*-values were also
274 calculated to estimate the variance of MPN values between and within groups. Finally, a Tukey
275 HSD Test compared individual sample group means using the same confidence level applied to
276 *F*- and *P*-tests.

277

278 **3. Results**

279 **3.1 Description of Balloon Flight**

280 The E-MIST payload was launched on a high altitude scientific balloon at 1441 UTC on
281 10 October 2015 from Fort Sumner, New Mexico (34°29'30" N; 104°13'36" W) traveling 335
282 km to the northeast for ~11 h, landing just beyond Amarillo, Texas (36°06'30" N; 100°29'30"
283 W). Samples of *B. pumilus* SAFR-032 spores remained sealed inside the payload until reaching
284 21.3 km ASL in the lower stratosphere at 1619 UTC, at which point the flight computer rotated
285 the skewers open into the air (Fig. 2). By 1715 UTC, a stable float altitude was achieved and the
286 payload remained 29.7 to 31.4 km ASL for a total of 8 h 20 min. The flight computer rotated
287 one skewer to its closed position every 2 h: Skewer 1 closed at 1819 UTC (Fig. 2A); Skewer 2
288 closed at 2019 UTC (Fig. 2B); Skewer 3 closed at 2219 UTC (Fig. 2C); and Skewer 4 closed at
289 0019 UTC (Fig. 2D). Environmental data from sensors located inside and outside of the E-MIST
290 payload are summarized in Table 1 and Fig. 3. At 0133 UTC (11 October 2015), the gondola
291 was jettisoned from the balloon, returning the payload to the ground on a parachute during a 20
292 min descent. Personnel from CSBF recovered the payload and transported it back to the launch
293 site facility inside a climate-controlled vehicle. Two days later, flight samples were removed

294 from the E-MIST payload and shipped to the laboratory at KSC (along with unflown ground
295 controls) inside sterile containers at ambient conditions.

296 Upon landing, a command to the payload's UV radiometer malfunctioned, preventing the
297 avionics from automatically powering off the instrument. A similar failure occurred during the
298 previous E-MIST test flight (Smith *et al.*, 2014). Briefly, the flight computer received a false
299 indication that the radiometer was shut down; however, the instrument continued to record data
300 until the payload was manually powered down 6 d later at KSC. The radiometer instrument
301 could only store 72 h of measurements. Consequently, the flight UV data were overwritten and
302 unrecoverable. Since a subsequent experiment could not be flown, we remained reliant upon
303 modeling from previously-acquired UV measurements in the stratosphere to understand the
304 likely dose of irradiation. To provide a range of expected UV values for the 21.3 to 31.4 km
305 ASL mission float profile, we constructed a model framed between 20 and 50 km ASL altitudes,
306 based on previous calculations (Smith *et al.*, 2011). Model inputs used the E-MIST balloon
307 flight path latitude (34-36 °N) and UV values measured by the Nimbus-7 Solar Backscatter UV
308 instrument that took solar spectra while ascending through the stratosphere in January 1979
309 (McPeters *et al.*, 1984; MCPeters *et al.*, 1996). Ultraviolet attenuation was determined by using
310 previously-established ozone concentration values acquired in the 30-40 °N latitude range by
311 monthly satellite observations (McPeters *et al.*, 2007). Ozone thickness measurements averaged
312 out to 303.1 DU, 214.1 DU, and 0.858 DU for ground, 20 km ASL and 50 km ASL, respectively,
313 and were converted to absorption coefficient factors for the model. Dosages were calculated to
314 provide a total, instantaneous flux rate (in W/m²) for UVA (315-400 nm), UVB (280-315 nm),
315 and UVC (100-280 nm) using two simplifying assumptions: (1) direct irradiation (i.e., no
316 scattering) and (2) a fixed solar zenith angle of 30°. For the 20 km ASL estimation, we used a

317 temperature of -45 °C with atmospheric pressure at 5.57 kPa; for the 50 km ASL estimation, we
318 used a temperature of 0 °C with atmospheric pressure at 0.101 kPa. Modeled quantities are
319 summarized in Table 2 and Fig. 4.

320

321 **3.2 Survival of *Bacillus pumilus* SAFR-032**

322 Coupons that were flown to the stratosphere but inverted (shielding bacterial spores from
323 sunlight) did not change significantly over the course of the 8 h experiment (Fig. 5). MPN
324 estimates for inverted flight coupons ranged from 2.00×10^7 to 5.17×10^7 spores per aliquot
325 which were values similar to transport and ground control coupons (4.02×10^7 and 4.12×10^7
326 viable spores per aliquot, respectively). In contrast, spores flown to the stratosphere and exposed
327 to sunlight changed significantly across the experiment compared to controls ($F = 11.52$, $P <$
328 0.0001). Coupons exposed for 2 h (Skewer 1) dropped by 2 orders of magnitude to 2.04×10^5
329 viable spores ($N = 9$). Rapid inactivation continued with viable spores declining another 2 orders
330 of magnitude to 1.76×10^3 ($N = 9$) by the 4 h time step (Skewer 2). The difference between
331 Skewer 1 and 2 samples was significant ($P < 0.01$). Another 2 h in the stratosphere resulted in a
332 more gradual but still significant ($P < 0.01$) inactivation rate with viability values at the 6 h
333 exposure group (Skewer 3) dropping an additional order of magnitude to 353 spores ($N = 9$).
334 Only 267 viable spores ($N = 8$), or 0.0007% of the *B. pumilus* SAFR-032 spore quantity initially
335 seeded onto coupons, were recovered from the final sample set exposed to the stratosphere for 8
336 h (Skewer 4). One outlier from the 8 h group (MPN value of 8.60×10^3) was discarded due to a
337 suspected MPN processing error. The overall decline (5-orders of magnitude) between control
338 samples and Skewer 4 was strongly significant ($P < 0.01$); however, the smaller difference
339 between Skewer 3 and 4 groups (86 viable spores, on average) was not significant based on a

340 Tukey HSD Test. Notably, none of the sample coupons exposed to the stratosphere for 8 h were
341 completely sterilized. In fact, the lowest viability estimate from a single aliquot processed was
342 200 spores. To forecast the amount of time in the stratosphere needed for complete inactivation,
343 a trend line was calculated using the survivability decay rate from exposed flight coupons.
344 Based on this projection, no viable spores would remain if flight samples had an additional 150
345 min of sun exposure in the stratosphere (630 min total time).

346 Unchanged survival of spores harvested from the inverted stratosphere coupons
347 compared to the sun-exposed stratosphere coupons revealed UV irradiation was responsible for
348 bacterial inactivation (i.e., not extreme cold, dryness, or hypobarica). Thus, we conducted a series
349 of ground-based UV studies in the laboratory to determine how the initial starting concentration
350 of bacterial spores might influence survivability. Our tests varied the intensity and duration of
351 UVC illumination by changing exposure time, distance from the light source and incidence angle
352 to coupon surfaces. Samples that were identical to flight coupons (prepared at the same time
353 with a concentration of 4.52×10^7 spores) did not change significantly ($F = 3.69$, $P > 0.01$) when
354 illuminated with 0.27 to 0.5 W/m² of 271.1 nm UVC for up to 4 h. Most probable number
355 estimates ($N = 9$ with each treatment) for exposure times of 1.3, 6.7, 50, 100, and 240 min were
356 stable and generally within standard error ranges at 2.82×10^7 , 3.96×10^7 , 6.96×10^7 , 1.78×10^7 ,
357 and 1.13×10^7 spores, respectively.

358 A different pattern was observed for our low concentration ground tests using only ~640
359 *B. pumilus* SAFR-032 spores per coupon ($N = 3$) and exposing samples to 0.03 W/m² of 271.1
360 nm UVC. We measured a significant survivability reduction by 15 min ($F = 5.41$, $P = 0.014$)
361 and an overall negative correlation ($R^2 = 0.911$) between UVC exposure time and spore viability
362 (Fig. 6A). Not all spores were inactivated at the longest exposure period of 25 min;

363 approximately 117 remained. When the incidence angle of UVC illumination (relative to the
364 sample coupons) was tested at 90, 75, 60, and 45° for experiments using 0.03 W/m² of UVC, no
365 significant survivability changes between groups were observed (data not included) – all groups
366 declined at a similar rate. In a final 15 min experiment using the same starting concentration of
367 spores and a 90° incidence angle, bacterial inactivation was greater when the UVC light source
368 was closer to the sample coupon ($R^2 = 0.950$) (Fig. 6B). The decline across groups was
369 significant ($F = 15.4$, $P = 0.00028$) and viable spores were only recovered from 33% of sample
370 coupons located 1 cm away from the LED.

371 Finally, we performed an SEM analysis to better understand the distribution and
372 arrangement of bacterial spores deposited on aluminum test coupons at a concentration of ~ 4.07
373 $\times 10^7$ spores per 20 μ l aliquot (see Fig. 1B). Random areas of the dried bacterial aliquots were
374 surveyed and the micrographs revealed complete sporulation (i.e., no vegetative *B. pumilus*
375 SAFR-032 cells) with a predominantly monolayered arrangement of clustered spores on the
376 coupon surface. Where stacking occurred, it generally appeared about 2-3 spores thick. No
377 layering was noticeable for the ground test coupons prepared at a lower concentration (~ 640
378 spores per aliquot). Since MPN enumeration depended upon the full removal of spores from
379 experimental coupons, a subset of test coupons were examined after PVA peel processing to
380 determine if any spores were left behind; none were observed. In addition, PVA chemistry did
381 not affect the viability of spores – a control test resulted in the same MPN range for identical
382 spore aliquots with and without PVA peels.

383

384 **3.3 Re-Sequencing of *Bacillus pumilus* SAFR-032**

385 Using germinated spores that survived the stratosphere (from exposed and inverted
386 sample groups), we performed a nucleotide variant analysis focusing on known coding regions
387 within the *B. pumilus* SAFR-032 genome. After re-sequencing the samples, we used the CLC
388 Workbench to identify deletions, insertions, and substitutions compared to the reference genome.
389 The mapping analysis was also done for unflown control coupons. Only *B. pumilus* SAFR-032
390 sequences were detected during the sequencing analysis indicating that microbial contamination
391 was not an issue in the laboratory or field. Re-sequencing produced a total of 87 nucleotide
392 variants at sample frequencies ranging from 33 to 100%, most of which were from the same
393 coding region: deletions at ABV62232.1 (A/-), ABV62537.1 (T/-), ABV62702.1 (T/-),
394 ABV62978.2 (G/-), ABV63654.1(A/-); insertions at ABV62978.2 (-/G), ABV63866.1 (-/T); and
395 substitutions at ABV61973.1 (T/G), ABV62475.1 (G/T), ABV62978.2 (C/A), ABV63728.1
396 (T/A), ABV63728.1 (G/A), ABV63728.1 (C/A), ABV60913.1 (C/T), and ABV61863.1 (C/A).
397 Since these variants appeared with both flight and ground control sequences, we removed them
398 from our analysis in order to focus strictly on unique changes for flight samples. The nucleotide
399 variants – all single base pair substitutions – were observed in the 2 h exposed group
400 (ABV61341.1 (A/T) at 23.8% frequency) and the inverted flight coupon group (ABV63490.1
401 (C/T) at 39.3% frequency; ABV63868.1 (C/T) at 27.6% frequency). Upon publication, raw
402 sequencing results will be archived in the NASA GeneLab repository (<http://genelab.nasa.gov/>)
403 for the scientific community to access.

404

405 **4. Discussion**

406 We conducted a mixture of flight and ground experiments examining bacterial spore
407 survival within a Mars analog environment high above the Earth's surface. After enduring

408 intense selective pressures used in spacecraft assembly facilities and with natural
409 polyextremophilic resistance, *B. pumilus* SAFR-032 spores could be pre-adapted to the harsh
410 conditions of spaceflight and capable of reaching Mars unharmed (Link *et al.*, 2004; Ghosal *et*
411 *al.*, 2005; Kempf *et al.*, 2005; Gioia *et al.*, 2007; Vaishampayan *et al.*, 2012; Tirumalai *et al.*,
412 2013). Besides the *B. pumilus* SAFR-032 isolate, numerous planetary protection studies have
413 documented the diversity of microorganisms on spacecraft surfaces and in cleanrooms (Link *et*
414 *al.*, 2004; La Duc *et al.*, 2009; La Duc *et al.*, 2012; Vaishampayan *et al.*, 2013; Benardini III *et*
415 *al.*, 2014). However, without samples returned from Mars it is not possible to measure the
416 survival of unintentionally-introduced terrestrial contamination. Instead, specimens sent to
417 Earth's stratosphere can be analyzed after exposure to a similar environment. Our balloon study
418 challenged *B. pumilus* SAFR-032 spores to conditions reaching about 1 kPa, -73 °C, < 1% RH,
419 and UV levels totaling 86.6-109 W/m² for up to 8 h.

420

421 **4.1 Flight and Ground Experiment Implications**

422 Flight results revealed a rapid inactivation of *B. pumilus* SAFR-032 spores in the
423 stratosphere. Viability was reduced by > 99.9% after only 8 h exposed to the stratospheric
424 conditions with sunlight. Spores subjected to identical conditions without sunlight (i.e., inverted
425 flight coupons) did not decline significantly. Thus, we can conclude that solar radiation was the
426 leading factor influencing *B. pumilus* SAFR-032 spore survival in the stratosphere. Reduced
427 atmospheric pressure, temperature, and water availability were not biocidal to the spores. Our
428 results were consistent with other Mars simulation survivability investigations (Schuerger *et al.*,
429 2003; Cockell *et al.*, 2005; Diaz and Schulze-Makuch, 2006; Schuerger *et al.*, 2006; Tauscher *et*
430 *al.*, 2006; de la Vega *et al.*, 2007; Moores *et al.*, 2007; Osman *et al.*, 2008; Fendrihan *et al.*,

431 2009; Smith *et al.*, 2009; Gómez *et al.*, 2010; Peeters *et al.*, 2010; Johnson *et al.*, 2011; Kerney
432 and Schuerger, 2011). For instance, Schuerger *et al.* (2003) showed that monolayered bacterial
433 spores exposed to simulated martian conditions in an environmental chamber were inactivated
434 after 3 h. Collectively, our flight results and other experiments predict a low probability of
435 bacterial persistence on the surface of Mars – provided that contaminating microbes are directly
436 illuminated by sunlight. Even though the solar constant at Mars is only ~43% of Earth's, under
437 clear-sky conditions the surface of the Red Planet has roughly 3 orders of magnitude higher UV
438 irradiance than the surface of the Earth (Cockell *et al.*, 2000; Nicholson *et al.*, 2005), particularly
439 in biocidal UVC wavelengths (100-280 nm). This is due to the rarified martian atmosphere
440 (~0.7 kPa) with fewer chemical species capable of UV attenuation (Zurek *et al.*, 1992); about
441 95% of the martian atmosphere is CO₂ which leads to solar radiation absorbance more efficiently
442 at wavelengths < 190 nm (Kuhn and Atreya, 1979). Nicholson *et al.* (2005) discussed the
443 resistance and susceptibility of bacteria to UVC and described it as ≥ 300-fold more effective at
444 damaging DNA and killing bacterial spores than UVB and UVA.

445 Since short UV wavelengths predominantly determine the fate of microbes delivered to
446 Mars, artificial solar radiation light sources used in past survival simulation studies (Schuerger *et al.*
447 *et al.*, 2003; Cockell *et al.*, 2005; Diaz and Schulze-Makuch, 2006; Schuerger *et al.*, 2006;
448 Tauscher *et al.*, 2006; de la Vega *et al.*, 2007; Moores *et al.*, 2007; Osman *et al.*, 2008; Fendrihan
449 *et al.*, 2009; Smith *et al.*, 2009; Gómez *et al.*, 2010; Peeters *et al.*, 2010; Johnson *et al.*, 2011;
450 Kerney and Schuerger, 2011) must be carefully considered. No single lamp or group of lights
451 can fully simulate the spectrum of wavelengths expected at the Martian surface. It is also
452 difficult to mimic a drifting solar zenith and hardware surface scattering effects inside the
453 restrictive confines of an environmental chamber. Thus, most ground survival simulation studies

454 bathe microorganisms continuously to radiation at a fixed angle of incidence within a narrow
455 band of UV. Light does not behave this way in nature. Earth's stratosphere can be used to
456 provide a more dynamic illumination with UV doses roughly equivalent to levels expected on the
457 martian surface, as an alternative approach to the inherent limitations associated with laboratory
458 studies. At its mean orbital distance, Mars is thought to have fluence rates on the surface around
459 3.18 and 8.38 W/m² for UVC and UVB, respectively (Nicholson *et al.*, 2005). In comparison,
460 the stratosphere UV model developed for this study generated similar levels for UVC and UVB
461 at 20-50 km ASL: 0.00550-5.20 and 4.16-17.1 W/m², respectively. Our model was consistent
462 within the range of measured values by Kylling *et al.* (2003) who flew a UV radiometer to 30.5
463 km ASL in the stratosphere over France (~44.5° N), recording measurements at 312 and 340 nm
464 across a solar zenith range of 76-94° in flight.

465

466 **4.2 Significance of Bioburden Configuration and Concentration**

467 Since Mars-bound, robotic spacecraft missions are required to reduce bioburden,
468 lingering contaminants would likely be in low concentrations across hardware components. For
469 instance, the total bioburden on exposed surfaces of the landed Mars Science Laboratory (MSL)
470 hardware was estimated at 5.64 x 10⁴ spores (22 spores/m²); with only 1.57 x 10⁴ spores
471 estimated on the rover itself (Benardini III *et al.*, 2014). Comparatively, our dried 20 µl aliquots
472 contain approximately 41 million spores which is several orders of magnitude higher than typical
473 bioburden for cleaned spacecraft surface. While our concentration could be considered a
474 “nightmare” contamination scenario, we still measured near-complete bacterial inactivation (>
475 99.9%) after 8 h in the stratosphere using one of the most UV-resistant bacterial strains
476 recovered from a spacecraft cleanroom to date (Link *et al.*, 2004; Gioia *et al.*, 2007;

477 Vaishampayan *et al.*, 2012; Tirumalai *et al.*, 2013). With our experimentally derived kill curve,
478 we forecasted a complete spore inactivation in the stratosphere with only 630 min of sun
479 exposure.

480 For our results to be useful for predicting the response of landed Mars bioburden on
481 spacecraft would require a few key assumptions: (1) stratospheric conditions resemble the
482 surface environment on Mars; (2) that a homogenous monolayer of spores with physiology
483 similar to *B. pumilus* SAFR-032 would be distributed across spacecraft external surfaces; and (3)
484 that no dust or hardware components shade bacterial contaminants. It is already known that the
485 biocidal effects from radiation are mostly applicable to surface or shallow subsurface
486 contamination (Nicholson *et al.*, 2005). Both Schuerger *et al.* (2003) and Cockell *et al.* (2005)
487 found that the survival of bacteria increased significantly when shielded from UV irradiation by
488 thin layers of dust or rocks. In fact, *Chroococcidiopsis* sp. 029 retained viability after 8 h under
489 rock coverage only 1 mm thick (Cockell *et al.*, 2005) and *B. subtilis* survived 8 h of UV
490 irradiation when covered by only a 0.5 mm coating of dust (Schuerger *et al.*, 2003). Similarly,
491 when *B. pumilus* SAFR-032 spores were combined with 60 μm Mars regolith soils and incubated
492 in a simulated Mars atmosphere for 24 h, only a 4-log reduction in viability was reported (Osman
493 *et al.*, 2008). Overlying dead biomass can provide another means of UV shielding to
494 microorganisms in layers below. Orbital experiments outside the ISS concluded *B. pumilus*
495 SAFR-032 spores were capable of tolerating Mars-like radiation dosages combined with the
496 vacuum of space for 18 months probably due to such shielding (Horneck *et al.*, 2012; Moeller *et*
497 *al.*, 2012; Nicholson *et al.*, 2012; Vaishampayan *et al.*, 2012). Desiccated layers 2–3 spores
498 thick of *B. pumilus* SAFR-032 were used for the ISS study (Horneck *et al.*, 2012) and we used
499 the same order of magnitude with our balloon mission to the stratosphere. Predictably, the two

500 experiments had similar outcomes for sun-exposed spores. Outside the ISS, the *B. pumilus*
501 SAFR-032 survival rate was less than 10^{-6} (Horneck *et al.*, 2012). A small but noteworthy
502 number of spores persisted in both experiments conducted in the stratosphere and outside the
503 ISS. Either a subset of bacteria were more resistant to biocidal conditions, or more likely, lethal
504 wavelengths of sunlight were attenuated by dead spore layers, clustering, or microscopic pits and
505 cracks in the aluminum coupon surface (Schuerger *et al.*, 2005; Horneck *et al.*, 2012).

506 To further examine the response of individual spores to irradiation, we performed a
507 standalone UVC experiment with a lower concentration of spores to eliminate layering and
508 clustering. Our starting concentration was the same order of magnitude as balloon flight
509 survivors. The ground experiment showed rapid inactivation within 25 min, even at UVC levels
510 ($0.03\text{-}0.5\text{ W/m}^2$) lower than what spores would experience in the stratosphere, outside the ISS, or
511 on the surface of Mars. When the same ground experiment was performed for up to 4 h using a
512 higher concentration (4.52×10^7 spores per aliquot), the UVC had no significant effects on
513 viability compared to controls. From these supplementary ground studies, we concluded that the
514 concentration and layering configuration of bacterial spores on sample surfaces primarily
515 determined survivability. It is also worth emphasizing how efficiently UVC LEDs sterilized
516 small amounts of bacterial bioburden in our ground experiment. This relatively new technology
517 could be a low-mass, low-power solution to the problem of spores buried deep within spacecraft
518 hardware, otherwise inaccessible to cleaning efforts and fully-shielded from sunlight at Mars.
519 Our single UVC LED produced biocidal action ($> 95\%$) within 15 min at distances ranging 1-5
520 cm from illuminated surfaces. Vehicles someday sent to Mars special regions (Rettberg *et al.*,
521 2016) could be designed with larger, more sophisticated UVC LED arrays embedded in
522 spacecraft hardware most likely to touch the martian regolith (e.g., drill bits and wheels).

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4.3 Genomic Consequences of Mars-Like Conditions on *B. pumilus* SAFR-032

Spores surviving our flight experiment could have been a stochastic result or beneficiaries of coupon cracks and overlying dead biomass. But another possibility worth examining was if a genomic advantage enabled persistence. To test this hypothesis, we re-sequenced surviving *B. pumilus* SAFR-032 spores to look for non-lethal mutations in coding regions of the genome. Common nucleotide variants from flight and ground samples were identified. After subtracting these variants from the analysis, 3 single nucleotide polymorphisms remained from the 2 h exposed population at ABV61341.1 (A/T) and the inverted flight coupon population at ABV63490.1 (C/T) and ABV63868.1. The first region ABV61341.1 is thought to be associated with producing ABC transporter proteins (Gioia *et al.*, 2007). ABV63490.1 is a well conserved region within Firmicutes and possibly involved with the initiation of sporulation and resistance of bacteria to extreme pH, temperature, and hypersaline conditions. Similarly, ABV63868.1 codes for an amidase protein that may guide sporulation and other metabolic pathways (Gioia *et al.*, 2007; Krulwich *et al.*, 2009). The total number of single nucleotide polymorphisms detected was fairly small considering the genome size surveyed (3.7 Mbp). However, lethal changes to the genome should not have been detected by our assay which targeted sequences from the surviving subset of spores. Moreover, *Bacillus* sp. have cellular systems for repairing UV damage to DNA. Spore photoproduct lyase or recombinatorial and nucleotide excision repairs in germinating spores (reviewed by Nicholson *et al.*, 2005) might explain the relatively few nucleotide variants identified through re-sequencing. While variants were relatively uncommon in our samples, base pair substitutions in 3 coding regions associated with bacterial sporulation and metabolism does warrant further investigation. Selective pressures

546 in Earth's upper atmosphere, in transit to Mars, or on the surface of the Red Planet could feasibly
547 establish non-lethal mutations that alter bacterial gene pools deposited in new environments.
548 Functional gene changes resulting in heightened *B. pumilus* SAFR-032 resilience was not tested
549 in this study, but comparative transcriptomics, proteomics, and metabolomics are targets of
550 future investigation exploring the molecular basis of polyextremophiles. Intriguingly, previous
551 research showed UVC resistance doubled in *B. pumilus* SAFR-032 spores exposed to the ISS
552 space environment for 18 months (Vaishampayan *et al.*, 2012). Induced mutations to rifampicin
553 resistance and sporulation deficiency were also observed to increase by several orders of
554 magnitude for spores exposed outside of the ISS (Moeller *et al.*, 2012).

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556 **4.4 Earth's Stratosphere as a Mars Analog Environment**

557 While our study focused on the forward contamination of Mars, Earth's stratosphere also
558 provides opportunities for multicellular biological investigations focusing on the destructive
559 effects of ionizing radiation (Cucinotta *et al.*, 2001; Schimmerling, 2007). In fact, two weeks
560 before the E-MIST launch, a suite of ionizing radiation instruments were sent to 36.7 km ASL in
561 the stratosphere from Ft. Sumner, New Mexico (Flight #666N). The Radiation Dosimetry
562 Experiment (RaD-X) measured ionizing dose rates of approximately 0.066 mGy/d with a Liulin
563 LET spectrometer (Mertens *et al.*, 2016). For comparison, recent measurements by the MSL
564 rover detected an ionizing radiation flux of approximately 0.18–0.225 mGy/d on the surface of
565 Mars (Hassler *et al.*, 2014). Antarctic balloon flights would provide even greater levels of
566 ionizing radiation due to the window of energy-intense particles penetrating stratospheric
567 latitudes above 70° (Adams *et al.*, 2007). Moreover, circumpolar flights now offer long-duration
568 NASA balloons capable of staying aloft for 100 d (Jones, 2014). Advantages of using the

569 stratosphere as a Mars analog environment instead of ground-based hypobaric chambers, particle
570 accelerator facilities, or orbital experiments in space can be summarized to include: (1) seasonal
571 balloon flight opportunities from multiple locations around the world, (2) logistical simplicity
572 with biological experiments (late loading and a rapid return of samples to the laboratory), (3)
573 relative affordability and rapid development compared to other flight-based investigations, and
574 most importantly (4) a realistic, dynamic radiation spectrum naturally paired with other Mars-
575 like conditions (extreme cold, dryness and hypobaria).

576

577 **4.5 Limitations and Future Directions**

578 An unexpected loss of radiometer data from our test flight made a direct analysis between
579 UV levels in the stratosphere and *B. pumilus* SAFR-032 spore survival unachievable. This
580 includes possible effects of transient shadows cast by the balloon flight train (as the gondola
581 rotated in the stratosphere). Also, total illumination on sample coupons likely decreased as the
582 sun set on the horizon towards the end of the experiment (see Fig.2D). The inactivation rate of
583 the spores decelerated with the final two time steps (6 h, Skewer 3; 8 h, Skewer 4), probably due
584 to the setting sun – but without UV radiometer measurements such direct correlations could not
585 be established in our study. Future missions will aim to fly on a balloon gondola flown in the
586 polar stratosphere at summertime to enable continuous, uninterrupted sunlight and longer
587 duration exposures. To prevent another UV radiometer command malfunction, backup data will
588 be stored off-instrument. The next generation E-MIST payload system will also aim to
589 incorporate a dosimeter for measuring ionizing radiation levels in the stratosphere. Until a re-
590 flight opportunity for the E-MIST payload, independent radiation measurements from the
591 stratosphere can be used for evaluating the fidelity of the UV model developed herein (e.g.,

592 Kylling *et al.*, 2003; Mertens *et al.*, 2016). Our forecast suggested that stratosphere UVB/UVC
593 levels (averaged between 20 and 50 km ASL) would be similar to Mars conditions calculated by
594 Schuerger *et al.* (2003) – approximately 10 and 3 W/m² for UVB and UVC, respectively, from
595 each modeled environment. This agreement for fluence rates between environments is important
596 because short wavelength UV is the most effective biocidal wavelength for spore-forming
597 bacteria (Nicholson *et al.*, 2005). While modeled rates from 315-400 nm were higher for the
598 stratosphere (~84 W/m²) than expected on Mars (~39 W/m²), UVA would not have the same
599 influence on bacterial survival.

600 Our experiment tested only one spacecraft assembly facility isolate, *B. pumilus* SAFR-
601 032, due to its unique resistance to environmental extremes, including radiation, and its use in
602 similarly scoped studies (Horneck *et al.*, 2012; Moeller *et al.*, 2012; Nicholson *et al.*, 2012;
603 Vaishampayan *et al.*, 2012). Subsequent flights evaluating other cleanroom-archived isolates
604 would be useful to determine which contaminants might be problematic if viably landed on
605 Mars. Since the *B. pumilus* SAFR-032 response to the stratosphere environment was unknown
606 prior to our balloon flight, we prepared samples at concentrations much higher than what would
607 be reasonably expected for a Mars spacecraft prepared in a cleanroom. Our results reveal that
608 spore stacking could have prolonged survival for a subset of the stratosphere-flown population.
609 Another limitation of our experimental design was that spores were deposited on a relatively flat
610 metal surface for sun exposure. Actual spacecraft components where terrestrial contaminants
611 could linger would have a more complex configuration, and the effects of martian dust buffering
612 sunlight are unknown. Consequently, the need for evaluating more complicated but realistic
613 bioburden distributions with future E-MIST experiments is a high priority for our team.
614

615 **5. Conclusion**

616 We observed a > 99.9% inactivation for sun-illuminated bacterial spores exposed to
617 Mars-like conditions in the stratosphere for 8 h. Our starting concentration of viable spores was
618 substantially higher than contamination levels typical for Mars-bound spacecraft, and we used
619 one of the most radiation-resistant bacterial strains known to be in cleanrooms; nevertheless, *B.*
620 *pumilus* SAFR-032 spores were rapidly killed by sunlight in the stratosphere. Survivors were
621 most likely lingering below layers of overlying dead spores or within small surface defects on
622 the experimental coupons. Our flight results and supplementary ground experiments suggested
623 that the concentration of spores and their distribution on spacecraft hardware primarily
624 determined survival rates. Based on our experimental observations, it seems unlikely that a
625 fully-exposed, sunlit bioburden sent to Mars could perpetually withstand the effects of UV
626 radiation at the Red Planet's surface. Using this balloon study in the stratosphere as a stand-in
627 for the martian environment, we predict that most sun-exposed bacterial contaminants at a level
628 below ~10,000 spores would be inactivated within 1 Sol. Future balloon-based missions in the
629 stratosphere can be used to continue studying other spacecraft cleanroom isolates, with and
630 without dust coverage, and at lower concentrations more representative of spacecraft bioburden.
631 Outcomes from such experiments could be used to develop species-specific inactivation models
632 and may also reveal genes or cellular mechanisms bestowing exceptional microbial resistance to
633 environmental extremes, including conditions expected on Mars.

634

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643

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645 The authors have no competing financial interest with vendors, reviewers, or
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648

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829 **Tables**

830

831 **TABLE 1. BALLOON FLIGHT ENVIRONMENTAL CONDITIONS**

	Max.	Min.	Remarks
Atmospheric Pressure (kPa)	87.6	0.962	Altitude of Ft. Sumner, NM, 1.25 km ASL
Air Temp. (°C)	16.2	-73.1	Average air temp. at float was -38.5 °C
Payload Internal Temp. (°C)			Internal heaters pulsed during ascent & descent
Avionics	23.1	-13.3	
Proxy Coupon	36.2	-45.9	Average coupon temp. at float was 15.4 °C
Battery	14.4	-7.39	
Radiometer	14.1	-6.39	
Battery Power (V)	16.7	15.7	
RH (%)	100	< 1	Average RH at float altitude was 2.32%
UV (W/m ²)	N/A	N/A	Data were lost; see text for details
Ground speed (m/s)	33.2	0.010	Average speed at float was 13.6 m/s

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833

834 **TABLE 2. ESTIMATED UV RATES (INSTANTANEOUS)**
 835

	UVA Total (W/m²)	UVB Total (W/m²)	UVC Total (W/m²)	Total (W/m²)
	315-400 nm	280-315 nm	100-280 nm	100-400 nm
Ground (Earth)	44.1	1.01	0	45.1
20 km ASL (Earth) ^a	82.4	4.16	0.00550	86.6
50 km ASL (Earth)	86.2	17.1	5.20	109
Ground (Mars) ^b	39.0	8.38	3.18	50.6

836 ^a Modeled stratosphere UV data from Smith *et al.* (2011).

837 ^b Modeled Mars ground UV data from Schuerger *et al.* (2003).

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840 **Figure Legends**

841 **FIG. 1.** (A) An experimental coupon with 14 separate *B. pumilus* SAFR-032 spore aliquots;
842 each aliquot contained approximately 4.07×10^7 spores total. (B) Scanning electron micrograph
843 of dried *B. pumilus* SAFR-032 spores distributed within a single 20 μ l coupon aliquot.

844 **FIG. 2.** E-MIST flight path on NASA Balloon Program Flight 667NT. Spots visible on 9
845 experimental coupons per skewer base plate were *Bacillus pumilus* SAFR-032 dried spore
846 aliquots. Each sample base plate had 1 inverted test coupon carrying spores shielded from
847 sunlight. The proxy coupon (visible on the lower right portion of the payload) served as a
848 negative control and also recorded temperature. The upper panel shows the gondola at float 31.4
849 km ASL and the sequential closure of the payload samples at (A) 1819 UTC (Skewer 1); (B)
850 2019 UTC (Skewer 2); (C) 2219 UTC (Skewer 3); and (D) 0019 UTC (Skewer 4). The lower
851 panel shows ascent, float, and descent trajectory, with skewer closure events labeled across the
852 flight path. The blue arrow marks initial opening of all skewers (at 1619 UTC) and the red arrow
853 marks the start of the gondola descent (11 October 2015 at 0133 UTC). Map credit: "E-MIST
854 Flight Path" 35°20'23.20" N; 102°49'7.15" W. [Google Earth](#). 9 April 2013. 8 December 2015.

855 **FIG. 3.** Environmental data for E-MIST flight, showing payload altitude (dashed black) with
856 corresponding atmospheric temperature (red) and relative humidity values (blue). The average
857 atmospheric pressure at the balloon gondola float altitude (which ranged from 29.7 km to 31.4)
858 was 1.07 kPa.

859 **FIG. 4.** Modeled UV light conditions in Earth's stratosphere (purple and green) closely
860 resemble modeled irradiation levels on the surface of Mars (red) (data from Schuerger *et al.*,
861 2003), particularly in UVB/UVC wavelengths (100-315 nm). For comparison, modeled ground

862 UV conditions on Earth (at sea level) are depicted in orange, much lower in UVB/UVC due to
863 atmospheric absorbance.

864 **FIG. 5.** Inactivation rate of *B. pumilus* SAFR-032 spores in the Earth's stratosphere over the 8 h
865 experiment. Note: average MPN values shown on a logarithmic scale. Experimental control
866 coupons contained approximately 4.07×10^7 spores per aliquot. Black values ($N = 3$ per time
867 group) represent the inverted flight coupons exposed to stratospheric conditions without sunlight;
868 red values ($N = 8-9$ per time step) show the exposed flight coupons ($P < 0.0001$). Dashed trend
869 lines were added to the data series, depicting the relative stability of inverted samples and the
870 estimated 630 min required for total inactivation of exposed samples (red asterisk).

871 **FIG. 6. (A)** Survivability of *B. pumilus* SAFR-032 spores exposed to 0.03 W/m^2 of 271.1 nm
872 UVC for 0-25 min at 5 cm and 90° angle of incidence ($R^2 = 0.911$; $N = 3$). **(B)** Survivability of
873 *B. pumilus* SAFR-032 spores exposed to $0.03-0.5 \text{ W/m}^2$ of 271.1 nm UVC for 15 min at 90°
874 angle of incidence and distances 1-5 cm from LED source ($R^2 = 0.950$; $N = 3$).

875 **FIG. S1.** Labeled isometric model of the re-designed Exposing Microorganisms in the
876 Stratosphere (E-MIST) payload with a mass of 36 kg. The system mounts to NASA scientific
877 balloon gondolas and has 4 independently-rotating sample canisters ("skewers") controlled by a
878 flight computer.

879 **FIG. S2.** Labeled model of internal components for the re-designed E-MIST payload, showing
880 power supply, instrument and computer configuration.

881 **FIG. S3.** Custom-built test stand for performing ground UVC irradiation studies. Experimental
882 coupons with quantities of *B. pumilus* SAFR-032 spores were mounted on the base plate below

883 the test stand bridge. The distance and angle of the UVC LED could be modulated as depicted in
884 the right panel.

885