A Balloon-Based Payload for Exposing Microorganisms in the Stratosphere (E-MIST)

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ABSTRACT

The survival and transit of microorganisms in Earth's upper atmosphere is relevant to terrestrial ecology and astrobiology, but the topic is understudied due to a scarcity of suitable flight systems. We designed, built, and flew a self-contained payload, Exposing Microorganisms in the Stratosphere (E-MIST), on a large scientific balloon launched from New Mexico on 24 August 2014. The payload carried *Bacillus pumilus* SAFR-032, a highly-resilient spore-forming bacterial strain originally isolated from a NASA spacecraft assembly facility. Our test flight evaluated E-MIST functionality in the stratosphere, including microbiological procedures and overall instrument performance. Herein, we summarize features of the E-MIST payload, protocols, and preliminary results that indicate it is possible to conduct a tightly-controlled microbiological experiment in the stratosphere while collecting pertinent environmental data. Additional studies of this nature may permit survival models for microbes traveling through Earth's harsh upper atmosphere. Moreover, measuring the endurance of spacecraft-associated microbes at extreme altitudes may help predict their response on the surface of Mars.

INTRODUCTION

Microorganisms in the upper atmosphere emanate from surface and marine ecosystems (Burrows et al., 2009), and are capable of reaching high altitudes by strong uplifting forces or mixing between the troposphere and stratosphere (Homeyer et al., 2011; Randel and Jensen, 2013). In addition to microbes naturally lofted, landfills, wastewater treatment plants, slash-and-burn agriculture, air traffic, and desertification also contribute to the total amount of bioaerosols in the atmosphere (Smith, 2013). Prevailing winds can connect distant biomes on Earth (Creamean et al., 2013; Smith et al., 2013), and residence time in the upper atmosphere exerts a harsh combination of stresses on microbes outside the range of conditions normally encountered on the surface (e.g., lower pressure, higher irradiation, desiccation, and oxidation). Thus, airborne transport might provoke

exceptional types of cellular damage or mutation. Some bacteria found in previous atmospheric surveys (reviewed by Griffin (2007) and Polymenakou (2012)) have traits that would improve persistence aloft – including cell pigmentation, DNA repair, and the ability to form endospores (hereafter referred to as 'spores'). While microbial survival has been examined using environmental simulation chambers (Smith et al., 2011) and small meteorological balloons (Beck-Winchatz and Bramble, 2014), more comprehensive platforms for controlled, long-duration experiments in the upper atmosphere are needed. Earth's middle stratosphere, about 25 to 40 km above sea level (ASL), resembles the surface conditions of Mars (Kaplan, 1988); thus, the planetary protection community could obtain an improved understanding of the survival of terrestrial microbes on Mars rovers and landers by flying stratospheric experiments. Furthermore, data from survival experiments could contribute to models for bioaerosols carried on globally-circulated winds (Smith et al., 2013; Yu et al., 2013), which is vitally important to global food security (Brown and Hovmøller, 2002). For example, aerially-dispersed wheat fungi threaten a staple calorie and protein source for 4.5 billion people across 94 developing nations (Singh et al., 2011).

Studying microbial survival in the upper atmosphere presents two fundamental challenges: first, removing potential influences from pre-flight, ascent, descent, and landing so as to limit the experiment to targeted altitudes; second, maintaining aseptic conditions within a closed payload system to preserve the integrity of test samples. Large scientific balloons provide unique access to the stratosphere and careful control over exposure experiments. Our aim in this study was to design, construct, and fly a self-contained payload (autonomous avionics, power, environmental sensors) that could attach to the exterior of large balloon gondolas, permitting a survival-based microbiology experiment at desired altitudes. A secondary aim was to collect baseline data and establish microbiology procedures (including ground and negative controls) for enabling future science flights. We worked with *Bacillus pumilus* SAFR-032, originally isolated from a spacecraft assembly facility at the Jet Propulsion Laboratory (Kempf et

al., 2005). The strain was a suitable model microorganism for our study because: (1) the resistance of its spore to environmental extremes is well documented (Gioia et al., 2007; Vaishampayan et al., 2012 and references therein); (2) the full genome is available for transcriptomic and proteomic analysis (Gioia et al., 2007; Tirumalai et al., 2013); (3) the species has no exosporium or extraneous layer associated with spores (Link et al., 2004); and (4) *Bacillus* sp. are commonly found in the upper atmosphere (Smith et al., 2012).

MATERIALS AND METHODS

Payload Description

Exposing Microorganisms in the Stratosphere (E-MIST) was built to mount onto the exterior of a high-altitude balloon gondola (Figures 1-2). The payload (83.3 cm x 53.3 cm x 25.4 cm; mass 36 kg) had four independent "skewers" (Figure 3) that rotated 180° for exposing samples to the stratosphere. During ascent or descent, the samples remained enclosed within dark cylinders at ~25°C. Each skewer had an aluminum base plate holding ten separate, rectangular aluminum coupons (M4985, Seton) with spore samples deposited on the surface. Coupon dimensions were 5.40 cm (w) x 1.75 cm (h) x 0.51 cm (thick), including 0.28 cm diameter holes on ends for mounting to skewer base plates. The inside of each cylindrical skewer was a frame laced with Nomex felt to prevent outside light from leaking into the system. A hex shaft through each skewer was attached to a gear system powered by a commercially-purchased motor (SPG30E-300K, Cytron) controlled by a four-channel FD04A motor controller (Brushed DC Moto Controller, Cytron). The motors, gears, and light shield were held together by a frame composed of aluminum cutouts and 3D-printed polycarbonate-ABS components.

T-slotted 80/20 aluminum extrusions formed the framework of the E-MIST payload, with detachable, white powder-coated aluminum panels on each face of the box. Angle brackets on the back plate were used to mount the system onto the balloon gondola and four foldable handles (McMaster-Carr) were attached to the front-facing frame. Multiple sensors, instruments, and computers were embedded within the housing. In
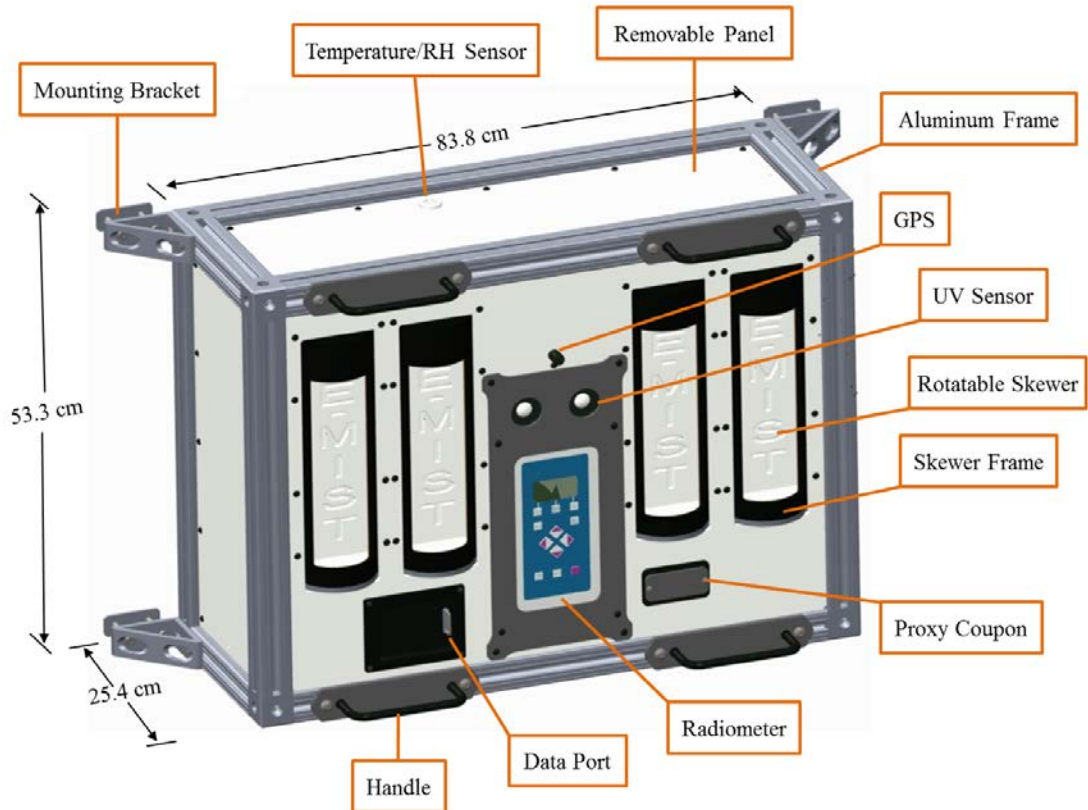
Figure 1. Labeled model of payload. Key design features of the Exposing Microorganisms in the Stratosphere (E-MIST) payload, which mounted onto the exterior of a large high-altitude balloon gondola. See text for details of labeled system and components. The mass of the payload was 36 kg.
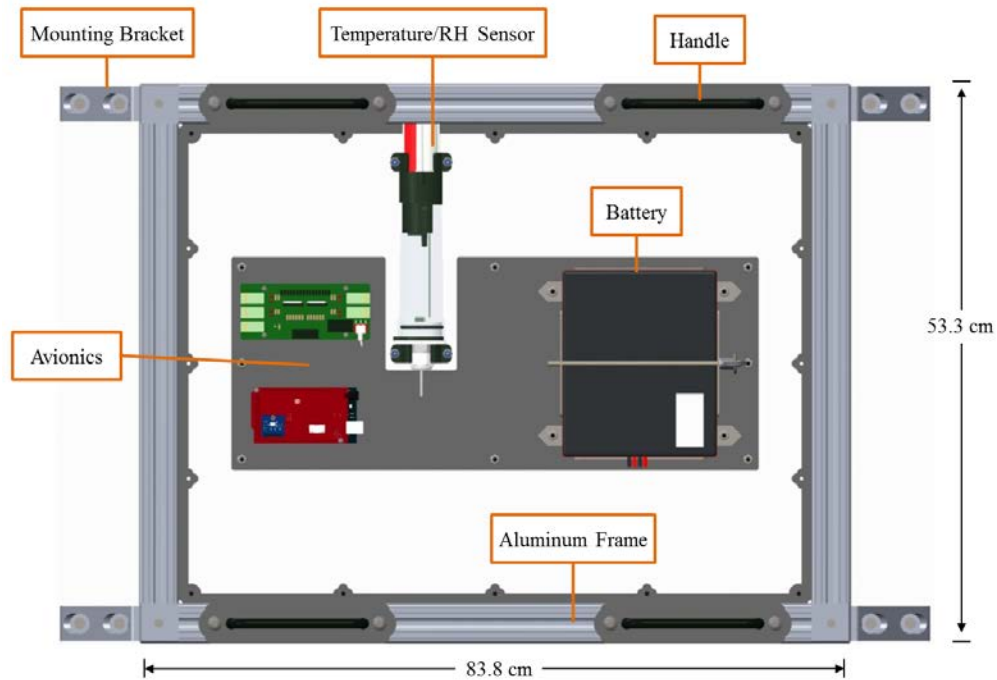
Figure 2. Modeled front view of the E-MIST payload with front panel removed. The key flight computer components, power source, and temperature/relative humidity (RH) sensors are shown.
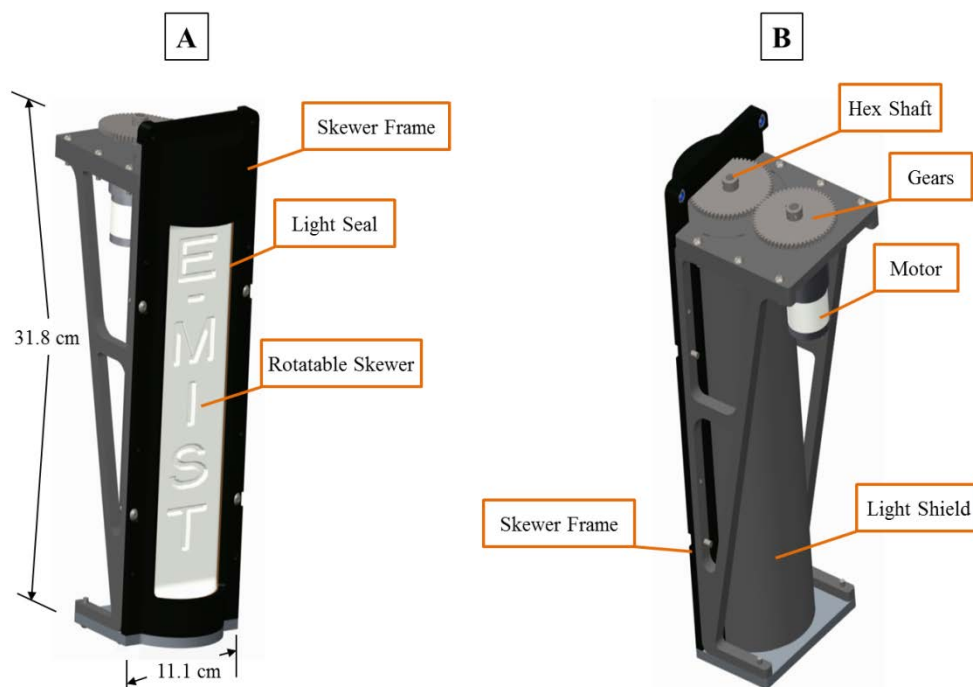
Figure 3. Model of the E-MIST skewers. Four independently-rotating skewers were embedded within the payload and controlled by the flight computer. A light seal and shield on each skewer ensured that the microbe sample exposure was restricted to the target altitudes. This design minimized the influence of the outside environment during other flight activities (e.g., launch, ascent, descent, and recovery). Panel A shows the front face, while Panel B depicts the motor and gear system on the backside of each skewer.

the center of the system was a stand-alone radiometer (PMA2100, Solar Light) with two ultraviolet (UV) sensors (PMA2107 and PMA2180, Solar Light) that measured UV levels (400 to 230 nm) every five min. The front panel data port contained two Universal Serial Bus (USB) ports, two light emitting diodes (LEDs), a Secure Digital (SD) card module, and two key switches. One key switch was used to power on the system and the other was used to manually rotate the skewers (for loading and removing samples). A sliding door on the front port assembly was held in place by two small magnets. One of the USB ports was used to start, stop, and retrieve data from the HOBO, a stand-alone external humidity and temperature sensor (U23-001, Onset) that collected data every ten s. The other USB port was used to retrieve data from the radiometer. LEDs illuminated when the global positioning system (GPS) (SPK-GPS-GS4O7A, S.P.K. Electronics Co.) had a lock on location (altitude, time, latitude, and longitude) and when the flight computer was on. The SD card

(uDRIVE-uSD-G1, 4D Systems) module used a universal asynchronous receiver/transmitter to poll a microcontroller (Mega2560, Arduino) and the SD card.

Other major payload components included: an altimeter (MS5607, Parallax), three 8.5 W heaters, three resistance temperature detector (RTDs) (SA1-RTD-B, Omega), and a temperature and humidity sensor (RHT03/DHT22, Aosong Electronics Co.). Power was generated by a 14.8 v 25.2 Ah lithium-ion polymer battery (CU-J141, BatterySpace) fastened in place with a stainless steel battery holder. The power circuit used a DC-DC converter for stepping down 14.8v to 5v. Thermal performance for the payload during tropospheric ascent was modeled using Thermal Desktop (C&R Technologies). Heating pads (5V Heating Pad 5x10 cm, WireKinetics) were included in the payload so that in-flight heat pulses could keep sensors and instruments within desired operating temperature ranges. This regulation system was controlled by the flight computer and RTDs on the battery, radiometer,

and sample base plate. An additional RTD was placed on a proxy coupon located on the front E-MIST panel.

Bacterial Preparation and Processing Techniques

Bacillus pumilus SAFR-032 was initially grown on tryptic soy agar and incubated at 32°C for 24 h. A nutrient broth sporulation medium induced sporulation, followed by a harvest and purification as previously described (Vaishampayan et al., 2012). The spore stock was re-suspended in sterile deionized water, heat-shocked (80°C for 15 min), and stored at a concentration of 10^8 cfu (colony forming units) ml^{-1} in glass tubes at 4°C. To create a uniform spore monolayer on the aluminum sample coupons, 100 µl aliquots diluted from the stock were spotted onto the surfaces and allowed to dry for 4 h in a dark laminar flow hood (NuAIRE Biological Safety Cabinet, Class II Type A/B3, Model NU-602-400) at standard room temperature (25°C) and pressure (1,013 mb). All coupons had been sterilized overnight with a dry-heat oven at 130°C and cooled to 24°C before deposition of the stock solution. After drying, coupons were stored in sterile, dark containers. We examined a random coupon with a scanning electron microscope (SEM) (JSM-7500F, JEOL Ltd.) to assess the distribution of spores on the surface (Figure 4).
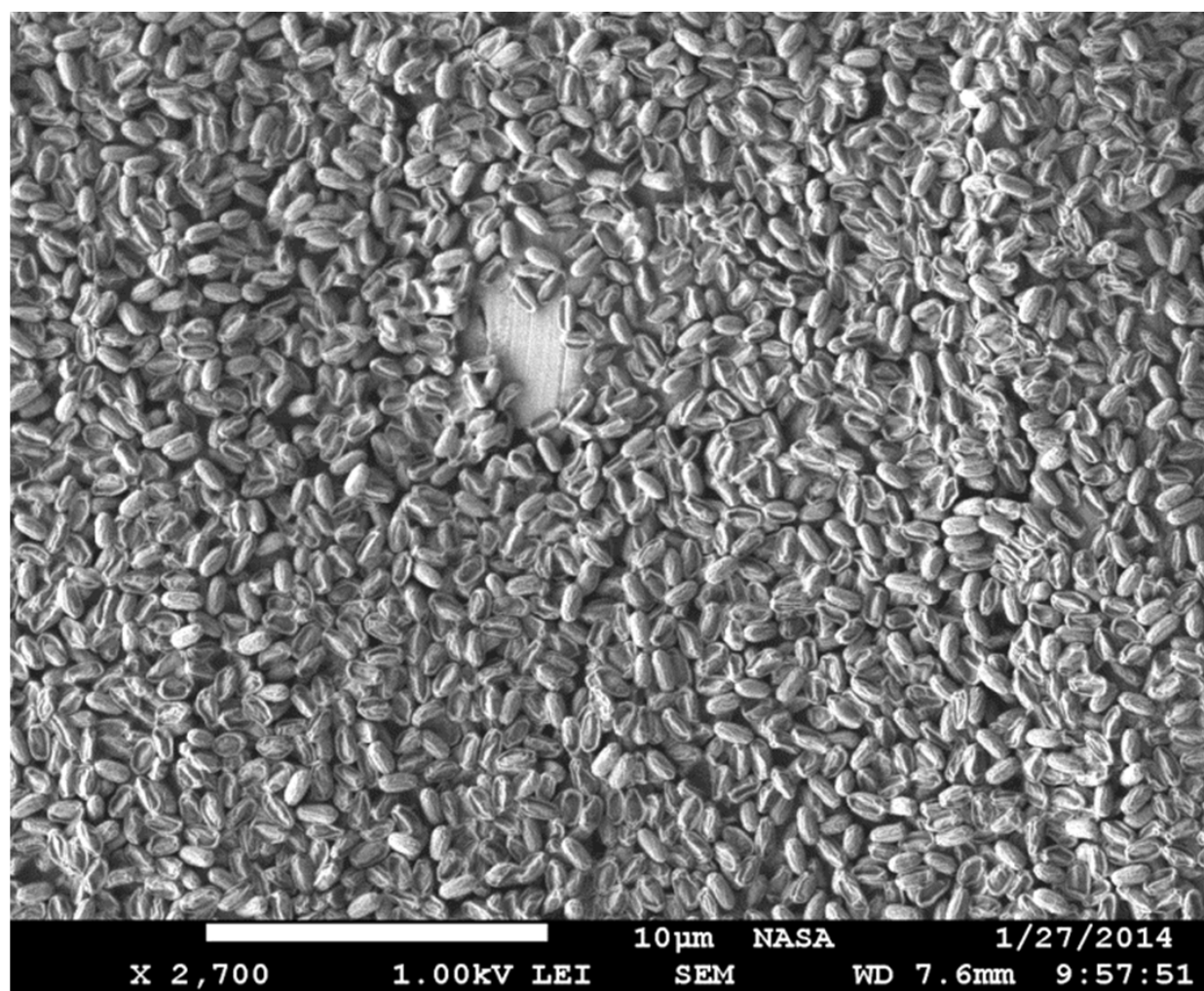
Figure 4. Distribution of *Bacillus pumilus* SAFR-032 spores on an E-MIST sample coupon. Each coupon contained approximately 1 x 10^6 spores and the aluminum coupon surface is visible in the circular gap of spores. The scale bar on the scanning electron micrograph is 10 µm.

Spore viability was measured using the Most Probable Number (MPN) enumeration technique that has been described elsewhere (Mancinelli and Klovstad, 2000; Smith et al., 2011). In brief, spores were dislodged from coupons by vortexing in sterile deionized water and sand for 2 min, and then processed through 6 consecutive serial dilutions. Using 96-well plates, 20 µl from each serial dilution, and 180 µl of sterile media (Per 1 L: 16 g Difco nutrient broth, 5 g KCl, 0.22 g $CaCl_2$, 1.6 g $FeCl_3$, 3.4 mg $MnSO_4$, 12 mg $MgSO_4$, 1 g D-glucose) were loaded into 16 wells per dilution and scored (presence or absence of bacterial growth, assessed by turbidity) after incubation for 36 h at 30°C.

We followed the ribonucleic acid (RNA) recovery procedure described by Moeller et al. (2012) to demonstrate the feasibility of this method with our flight coupons. Briefly, spores were lifted from coupons using a 1 ml polyvinyl alcohol solution. Air bubbles were removed by running a sterile glass slide coverslip across the polyvinyl alcohol aliquot, which was then dried overnight inside a petri dish and re-suspended in 1 ml of sterile molecular grade H_2O. Next, spores were germinated using the protocol established by Nicholson et al. (2012), with each centrifugation step at 5,000 rpm for 10 min, except for the final culture volume that was spun down at 14,000 rpm for 5 min. Samples were processed with RNeasy® Protect Bacteria Kit (Cat. No. 74524, Qiagen Inc.) using protocols supplied by the manufacturer. Sample processing then continued at Step 3 Part 1 of the RNeasy® Mini Kit (Cat. No. 74104, Qiagen), followed by an on-column DNase digestion in Part 2 of the RNeasy® Mini Kit (Steps 1 to 4). Finally, the RNA cleanup in Part 2 of the RNeasy® Mini Kit (Steps 5 to 7) were followed, including the optional spin after Step 5 and elution in a volume of 30 µl RNase-free H_2O. RNA yield was measured using the Qubit® 2.0 Fluorometer and standards from the Qubit® RNA HS Assay Kit (Cat. No. Q32855, Life Technologies).

Procedures for Ground and Balloon Flight Operations

A fully integrated test flight (all payload components powered on and *Bacillus pumilus* SAFR-032 samples loaded) was conducted on a NASA Balloon Program Office large scientific balloon, launched from Ft. Sumner, New Mexico,

on 24 August 2014. E-MIST was one of several payloads on the Long Duration Balloon Tech Flight 651N. The gondola was carried into the stratosphere by an 8 x 10^5 m^3 helium balloon. Prior to flight (in a launch site hangar), sample coupons were installed onto skewer base plates working inside a portable hood with sterile screws, a screwdriver, and forceps. The hood established an 8-fold reduction in airborne particles (Model 229 Particle Counter, Met One). Loaded E-MIST base plates were sealed until mounting on the flight-line at 1230 Coordinated Universal Time (UTC), about 90 min prior to balloon lift-off. The installation procedure was done at dawn in 10 min, limiting the exposure of samples to light and open air. Sterile tools were carried inside 50 ml Falcon tubes and the entire payload surface was wiped down with isopropyl alcohol before installation. Prior to mounting E-MIST base plates, the inside of each skewer was sprayed with sterile air from a canister. Once installed, the samples were rotated back to the closed position and the other instruments within E-MIST were powered on. Thus, *Bacillus pumilus* SAFR-032 samples remained in the sealed position until the payload reached the lower stratosphere (~20 km ASL), at which point the flight computer rotated the skewers into the outside air (Figure 5). After a short rotation (2 s), all skewers reverted to the closed position for the remainder of the flight. The gondola stayed at a float altitude of 37.6 km for almost 4 h before beginning a 23 min descent on parachute at 1956 UTC. It landed 294 km southwest (33°55'42.6" N; 107°21'46.8" W) of the launch site (34°29'30.1" N; 104°13'36.1" W) and was recovered by Columbia Scientific Balloon Facility (CSBF) personnel. E-MIST was powered off, removed from the gondola, and kept at ambient conditions inside an air-conditioned vehicle. One week later, samples were shipped back to NASA Kennedy Space Center (KSC) still inside the payload.

Experimental Design

Temperature, relative humidity (RH), atmospheric pressure, and UV levels were measured across the test flight by our team and additional CSBF instruments. Each E-MIST skewer base plate carried 10 *Bacillus pumilus* SAFR-032 coupons: 9 facing up and 1 inverted, since this will be the configuration for future
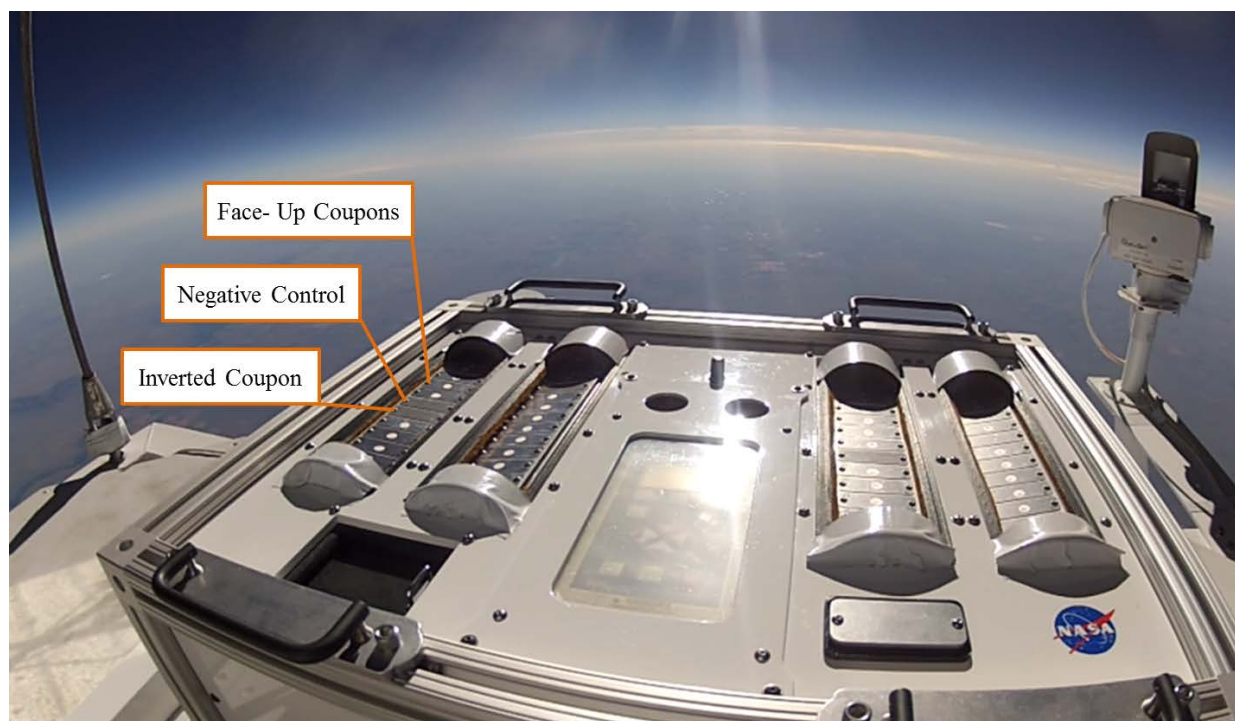
Figure 5. Test flight deployment on 24 August 2014 in the lower stratosphere over New Mexico. E-MIST payload in flight, with the four skewers rotated open, briefly (2 s) exposing the *Bacillus pumilus* SAFR-032 samples and other control coupons to the stratosphere. On Face-Up Coupons, the spore aliquots are visible as white dots in the center of each sample. One Inverted Coupon (protecting spores from sunlight) was included on each skewer base plate as well. A Negative Control (blank, sterile coupon) was also flown to verify payload seals prevented outside contamination.

flight experiments. The upright coupons received the full stratospheric exposure, including irradiation from sunlight; whereas the inverted coupons were subjected to all other stratospheric effects, except for sunlight. Since the skewer rotation demonstration lasted only 2 s in our test flight, we anticipated no survival differences between the treatments – unless spores were dislodged on inverted coupons due to possible contact with the skewer base plate. Two sets of positive ground controls were prepared along with the flight coupons. One set was transported, but not flown, and another set remained at KSC. We expected the survivability of the ground controls to match flight coupons if the payload successfully prevented outside environmental influences. One (sterile) negative control coupon was also included to understand if the sample base plates were protected from exterior contamination sources.

RESULTS AND DISCUSSION

Our test flight demonstrated the functionality and reliability of a new payload for exposing microbes in the stratosphere. Baseline data collected can better prepare future research teams using this system. Table 1 summarizes upper and lower limits of key environmental data from the balloon launch, ascent, and float. Internal payload heaters performed nominally, keeping hardware components and sensors within operating limits – particularly in the upper troposphere during the coldest part of the flight. For instance, at 16.4 km ASL when the free air temperature was -67.5°C (measured by an independent, CSBF gondola sensor), the avionics board inside E-MIST remained at -4.10°C. However, a few instruments and components did not function properly. First, the altimeter failed, possibly due to radio frequency interference with other gondola instruments. Second, UV measurements were lost

because the stand-alone radiometer was not powered off during payload recovery (forcing the instrument to eventually overwrite the data stored during float). Obtaining UV data on future experiments will be critical since other survival studies have shown a relationship between irradiation and bacterial inactivation (Smith et al., 2011). Looking forward, modifying the radiometer to store data on the payload flight computer will resolve this issue, and stratospheric UV measurements from other balloon missions (e.g., McPeters et al., 1984) can be used to establish an expected range of irradiation. Finally,

the rotation of the sample skewers was suboptimal. In principle, end-to-end bacterial coupons will receive near-identical sunlight if the skewers open evenly (on the same plane) and no shadows cross the payload face – yet, neither condition was observed in flight. Intermittent gondola shadows passed over the skewers but were transitory since the balloon was constantly rotating. We will build a smart switch system that communicates with the motor controller to ensure skewers open evenly on the next flight opportunity.

Table 1. Launch, Ascent, and Float Profile.

	Max.	Min.	Remarks
Atmospheric Pressure (mb)	837	4.26	Altitude of Ft. Sumner, NM, 1.25 km ASL
Air Temp. (°C)	23.3	-67.5	CSBF Free Air Thermistor
Payload External Temp. (°C)	18.5	-13.7	HOBO measurements
Payload Internal Temp. (°C)			Internal heaters pulsed during acent and descent
Avionics	22.3	-4.10	
Proxy Coupon	46.1	-28.0	
Battery	16.0	-4.65	
Radiometer	37.1	4.15	
Payload Internal RH (%)	65.0	< 3.5	Measurements below sensor sensitivity
Payload External RH (%)	60.5	< 3.5	Measurements below sensor sensitivity
UV (W m^{-2})	N/A	N/A	Data were lost; see text for details

Concerns about contamination (e.g., external microbes penetrating the payload) or inactivation (e.g., outside biocidal factors besides stratospheric conditions) motivated this methods-focused study. Including *Bacillus pumilus* SAFR-032 on the test flight allowed us to evaluate the success of payload containment, microbiological procedures, and protocols. A properly-controlled stratospheric microbiology survival experiment should only expose samples to the targeted region of the upper atmosphere. Our experimental design assessed unknowns associated with sample transportation, balloon gondola installation, balloon ascent/descent, and time lingering in the hot,

dusty New Mexico desert awaiting launch and recovery. We created a batch of experimental control coupons (each containing approximately 1 x 10^6 spores) used throughout the investigation for ground and flight test purposes. Several treatment categories were evaluated: Lab Ground Coupons (kept in the KSC laboratory); Transported Ground Coupons (traveled to New Mexico and back, but not installed in payload); Face-Up Flight Coupons (flown, experienced internal payload conditions and exposed to stratospheric sunlight for 2 s); Inverted Flight Coupons (flown, experienced internal payload conditions and exposed to stratospheric conditions other than sunlight for 2

s). A subset of coupons from each treatment category was processed, resulting in MPN values reported in Table 2. Averages between coupon groups were compared using one-way permutations (Kruskal-Wallis rank-sum test) at a 95% confidence level, and the differences in the means were not statistically significant. Notably, the brief (2 s) skewer rotation in the lower stratosphere did not reduce the number of spores on the Face-Up Coupons. Also, time spent on the ground did not impact survival numbers.

Temperatures ranging from 80°C to 110°C are generally capable of inactivating spores (Ghosh et al., 2009), but the highest temperatures recorded on the ground (32.4°C for Payload Internal Temperature; 40.1°C for Proxy Coupon) and in flight (46.1°C for Proxy Coupon) were below the threatening range. Taken together, nearly identical numbers from all coupons indicate that balloon flight operations and payload procedures did not influence spore survival.

Table 2. Most Probable Number of Viable Spores.

Sample Category	Treatment	MPN (N, σ)
Lab Ground Coupons	Kept in the laboratory	1.84×10^6 (1, N/A)
Transported Ground Coupons	Transported to launch site but not installed	1.93×10^6 (2, 3.74×10^5)
Face-Up Flight Coupons	Flown, exposed for 2 s	1.91×10^6 (4, 4.30×10^5)
Inverted Flight Coupons	Flown, exposed for 2 s without sunlight	1.66×10^6 (4, 5.96×10^5)

If future flights deployed the same experimental design and exposed microbes for hours at float, we would expect to see a rapid inactivation. Smith et al. (2011) simulated stratospheric conditions and measured a 99.9% loss of viable *Bacillus subtilis* spores after only 6 hours of direct UV irradiation. Beyond investigating viability alone, transcriptome sequencing and differential expression analysis of RNA could provide valuable insight into the functional effects of stratospheric transport on surviving microorganisms. For instance, this approach could help identify genes associated with repairing damage to the cell envelope, genome, and/or core metabolic proteins. Even with complete survival, our coupons yielded only between 0.53 to 0.97 µg total RNA. Generally, about 1 µg RNA per sample is needed for preparing a transcript library from germinated survivors. Recent developments in library preparation may soon allow reproducible single-cell transcriptomics using as little as 10 pg of starting total RNA (Sasagawa et al., 2013). Nevertheless, follow-on stratosphere experiments

may require an order of magnitude increase in the number of spores doped onto coupon surfaces since longer exposures will inactivate a greater portion of samples. Boosting biomass to harvest enough RNA may generate misleading survival data, resulting in a layer of spores that block irradiation effects. Our coupon preparation method using a 100 µl aliquot generated a layer of 1 to 3 spores (Figure 4). Larger coupons (accommodating more spores across the surface) or flying more replicates (to pool samples) may yield enough recoverable RNA without creating unwanted spore layers. The simplicity of the skewer base plate lends itself to customization. Biological requirements can guide the optimal engineering of the skewer base plate. Future investigators can easily reconfigure the base plate to allow for the desired number of replicates and microbe concentrations, or accommodate other categories of microorganisms incompatible with the aluminum coupons.

Earth's stratosphere is extremely dry, cold, irradiated, and hypobaric, and it may be useful for the archive of microorganisms isolated from

NASA spacecraft assembly facilities (e.g., Benardini III et al., 2014) to be evaluated in an environment analogous to Mars. Survival-based studies were recently deployed outside the International Space Station (ISS) (Horneck et al., 2012) and the same category of experiments can be conducted in the stratosphere. We hope the baseline data, procedures, and controls discussed herein can provide a pathway for future investigations using E-MIST. While spore-forming bacteria have remarkable resistance to atmospheric extremes, non-spore-forming bacteria, archaea, fungi, algae, and viruses should also be examined. A species-specific inactivation model that predicts the persistence of microbes in Earth's upper atmosphere (e.g., pathogenic cereal rusts), or even on the surface of Mars, is one of many possible outcomes from stratospheric microbiology research.

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