Research Article

Establishing Standard Protocols for Bacterial Culture in Biological Research in Canisters (BRIC) Hardware

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ABSTRACT

The NASA GeneLab Data System (GLDS) was recently developed to facilitate cross-experiment comparisons in order to understand the response of microorganisms to the human spaceflight environment. However, prior spaceflight experiments have been conducted using a wide variety of different hardware, media, culture conditions, and procedures. Such confounding factors could potentially mask true differences in gene expression between spaceflight and ground control samples. In an attempt to mitigate such confounding factors, we describe here the development of a standardized set of hardware, media, and protocols for liquid cultivation of microbes in Biological Research in Canisters (BRIC) spaceflight hardware, using the model bacteria *Bacillus subtilis* strain 168 and *Staphylococcus aureus* strain UAMS-1 as examples.

Key words: *Bacillus subtilis*; Bacteria; Biological Research in Canisters; BRIC; GeneLab; International Space Station; ISS; Spaceflight; Spores; *Staphylococcus aureus*

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ACRONYMS

BRIC	Biological Research in Canisters
BRIC-DC	Biological Research in Canisters-Dual Chamber
BRIC-PDFU	Biological Research in Canisters-Petri Dish Fixation Unit
BSL	Biological Safety Level
CFU	Colony-Forming Unit
FL	Flight
GAP-FPA	Group Activation Pack-Fluid Processing Apparatus
GC	Ground Control
GLDS	GeneLab Data System
ISS	International Space Station
ISSES	ISS Environmental Simulator
KSC	Kennedy Space Center
MELFI	Minus Eighty-Degree Laboratory Freezer for ISS
NASA	National Aeronautics and Space Administration
OD	Optical Density
PDFU	Petri Dish Fixation Unit
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
SVT	Science Verification Test
TSY	Trypticase Soy Yeast Extract

INTRODUCTION

Microbial cells exposed to the human spaceflight environment respond differently to similarly-treated ground controls, a phenomenon dubbed the "spaceflight syndrome." Over the past four decades, a number of spaceflight experiments have been conducted inside various space vehicles (e.g., Apollo, Spacelab, Space Shuttle, and space stations Mir and ISS) using model organisms in order to understand how microbes sense and respond to the stresses encountered in the human spaceflight environment. Such stresses include, but are not limited to, microgravity and ionizing radiation. In early studies, various phenotypic traits from microbes grown in space were analyzed, such as growth rate and yield, virulence, biofilm formation and architecture, and resistance to antibiotics or abiotic stresses, to name but a few [reviewed in (Horneck et al., 2010; Rosenzweig et al., 2010; Rosenzweig et al., 2014)].

In more recent years, with the advent of the genomics and post-genomics revolutions in biology, there have been initial efforts to understand more fundamental molecular aspects of the spaceflight syndrome by performing global-scale "-omics" analyses of the transcriptome, proteome, metabolome, etc. of microbes exposed to spaceflight. Such studies have yielded valuable insights into the molecular responses of certain species of microbes to the spaceflight environ-ment. For purposes of drawing general conclusions from the results from spaceflight and ground-control experiments, -omics datasets have been collected and deposited into a common data archive called the GeneLab Data System (GLDS; publicly available at http://genelab.nasa.gov/). However, before the advent of the GeneLab concept, spaceflight experiment designs were driven by the needs of each individual investigation, which now presents certain complica-tions for cross-experiment and cross-species comparisons. First, cells were flown in a number of different hardware systems, including (i) simple tape-sealed Petri dishes (Mastroleo et al., 2009), (ii) Group Activation Pack-Fluid Processing Apparatus (GAP-FPA) hardware (Crabbé et al., 2011; Wilson et al., 2007; Wilson et al., 2008), (iii) Biological Research in Canisters (BRIC) hardware (Fajardo-Cavazos and Nicholson, 2016; Nicholson et al., 2015), and (iv) SIMBOX hardware (Huang et al., 2015), to name but a few. Each of these hardware systems imposed different regimes of oxygen availability, presence or absence of fluid/air interfaces, and temperature and relative humidity on samples. Second, pre- and post-flight processing of samples in the flight experiments cited above was accomplished using a variety of different methods. Before flight, cells were stored either refrigerated in buffer or medium, air-dried, or otherwise held in stasis for variable periods before activation in space. After growth, cells were variously stored at ambient temperature, refrigerated, or frozen in the presence or absence of various fixatives, stabilizers, or preservatives.

With these considerations in mind, it was deemed desirable by NASA to develop a standard set of procedures for conducting spaceflight experiments to reduce such variables. The aim of such process standardization would be to minimize variation in the -omics datasets to be archived in the GLDS, due to flight hardware and sample handling. This would in turn increase the likelihood that spaceflight effects observed among different microorganisms were in fact due to spaceflight, and not due to variables introduced during sample preparation and handling. In recent years, we have developed procedures for cultivating bacteria in the human spaceflight environment using Biological Research in Canisters-Petri Dish Fixation Unit (BRIC-PDFU) spaceflight hardware for the BRIC-18 (Fajardo-Cavazos and Nicholson, 2016), BRIC-21 (Nicholson et al., 2015), and BRIC-23 (Fajardo-Cavazos and Nicholson, 2016) missions to the ISS. As an example of how a set of standardized methods could be developed for microbial experiments in space, in this communication we describe our current protocol for cultivation of bacterial cells in BRIC-PDFU hardware.

MATERIALS AND METHODS

BRIC-PDFU Hardware

BRIC spaceflight hardware has been described in detail previously (Paul et al., 2012; Wells et al., 2001) and has an extensive flight heritage. BRIC canisters contain space for six small sub-compartments called dual-chamber Petri Dish Fixation Units (PDFUs). Each PDFU contains a space to accommodate the bottom half

of a single 60 mm diameter Petri dish. In a separate syringe compartment, each PDFU contains a 17 mL dual-septum plunger that permits injection of 2 solutions of 8.5 mL each (Wells et al., 2001). For flight (FL) experiments 4 BRIC canisters were used, each containing five PDFUs and one HOBO temperature data logger (Onset, Inc., Cape Cod, MA). Post-flight asynchronous ground control (GC) experiments were conducted using the same hardware and configuration as in the FL experiment.

Bacterial Strains and Media

Bacteria used were *Bacillus subtilis* strain 168 from the authors' strain collection, *Staphylococcus epidermidis* strain ATCC12228 from the American Type Culture Collection, and *Staphylococcus aureus* strain UAMS-1 obtained from K.C. Rice (Gillaspy et al., 1995). The base medium used throughout was Trypticase Soy Yeast Extract (TSY) medium, containing (g/L): Tryptone, 15; Soytone, 5; NaCl, 5; Yeast Extract, 3; K_2HPO_4, 2.5; glucose, 2.5; final pH 7. For semisolid plates, agar was added to TSY at 15 g/L. For spaceflight and ground control experiments, TSY was supplemented with 10% (v/v) glycerol by mixing equal volumes of separately pre-sterilized solutions of 2xTSY and 20% (v/v) glycerol into sterile glass canning jars (Bormioli Rocco, Fido 500 mL). Glycerol was added as a cryoprotectant to preserve cell viability to freezing and thawing.

Cell Growth and Quantification

Spores of *B. subtilis* strain 168 were prepared and purified as described previously (Nicholson and Setlow, 1990), and were stored at 4˚C in deionized water. Viable spore titers were determined by serial tenfold dilutions and colony counts on TSY plates. Examination by phase-contrast microscopy confirmed that the spore preparation was devoid of vegetative cells and contained >99% phase-bright spores.

S. aureus cells were prepared by overnight growth in TSY li uid medium at 37˚C in a 125 mL sidearm (Klett) flask with moderate rotary shaking (~150 rpm). Optical density of overnight cultures at 660 nm (OD_{660}) was measured in a Klett-Summerson photometer fitted with the No. 66 (660 nm; red) filter. [Note: 1.0 OD_{660} ~100 Klett Units]. Under these conditions, cultures routinely achieved OD_{660} values of ~540 Klett units; viable titers of the overnight cultures were determined by serial tenfold dilutions, and plate counts to be ~$6x10^9$ CFU/ mL.

Sample Preparation

B. subtilis spores

The stock spore suspension was diluted in sterile water to a working suspension of 10^8 CFU/ mL and heat-activated (65˚C, 20 min) before use. From the working suspension, aliquots of 0.1 mL (~10^7 CFU) were applied as scattered droplets to the bottoms of sterile 60 mm diameter Petri dishes (Falcon Cat. No. 1007, Thermo Fisher Scientific) and air-dried for 48-72 h at room temperature, protected from light. All operations were conducted in a disinfected biological containment hood using sterile surgical gloves and aseptic technique, maintaining sterility on both the inside and outside of plates.

S. aureus cells

Overnight cultures of *S. aureus* prepared as described above were diluted 1:60 in TSY to a working concentration of ~10^8 CFU per mL prior to use. Aliquots of 0.1 mL (~10^7 CFU) of the suspension were applied to the bottoms of sterile 60 mm diameter Petri dishes (Falcon Cat. No. 1007, Thermo Fisher Scientific) and air-dried for 48-72 h at room temperature prior to use. All operations were performed using sterile surgical gloves and aseptic technique according to Biological Safety Levels (BSL)-2 procedures in a disinfected biological containment hood, maintaining sterility on both the inside and outside of plates.

***S. aureus* Longevity Testing**

Air-dried samples of *S. aureus* deposited on Petri dishes were incubated under ambient laboratory conditions protected from light for up to 28 days. At 7-day intervals, 8.5 mL of TSY+10% glycerol was added to duplicate plates. Cells were removed from each Petri dish bottom and resuspended using a sterile disposable cell scraper (Thermo Scientific/Nunc #179693, 23 cm, rubber blade turned perpendicular to handle). The liquid was transferred to a sterile disposable 15 mL conical centrifuge tube and mixed by vortexing, then viable counts determined as described above.

Pre-Science Verification Test (Pre-SVT)

Samples of *B. subtilis* and *S. aureus* cells were air-dried in the bottoms of 60 mm Petri plates as described above. Growth was initiated by addition of 8.5 mL of liquid TSY+10% glycerol, and plates were incubated without agitation at the indicated temperature. At various times, usually at 4-hour intervals, duplicate plates were removed, and cells suspended off plates using cell scrapers as described above. Cultures were transferred to sterile 15 mL tubes, vortex mixed, diluted, and plated for viable counts as described above.

Process for BRIC-PDFU Integration and Deintegration

Integration

In the assembly process known as "integration," each PDFU was loaded with one Petri dish containing air-dried spores of *B. subtilis* or cells of *S. aureus*. Into the separate media reservoir, 17 mL of sterile TSY+10% glycerol medium were loaded. To prevent contamination, all reagents and equipment used were sterilized prior to use and PDFUs were assembled using aseptic technique within a BSL-2-rated Biological Safety Cabinet. Growth was initiated by injection of 8.5 mL of medium into each 60 mm Petri dish, a process dubbed "actuation." Cultures were incubated at the indicated temperature for the indicated times. Growth was terminated by transfer of BRIC units to a -80˚C freezer.

Deintegration

Samples were recovered in the frozen state using a process known as "deintegration," as follows. BRIC units were removed from the -80˚C freezer and allowed to partially warm at room temperature for ~30 min, allowing for disassembly and recovery of frozen PDFUs and their transfer to a sterile Biological Safety Cabinet. PDFUs were then disassembled in the frozen state. Petri plates containing frozen cultures were recovered from the PDFUs and immediately placed on dry ice for transport to the laboratory, where they were either processed immediately or stored at -80˚C to await further processing. Samples remained solidly frozen throughout this procedure.

Science Verification Test (SVT)

S. aureus cells were air-dried in the bottoms of 60 mm Petri plates and integrated into BRIC-PDFU hardware as described above. The SVT followed a simulated mission scenario to ISS, according to the schedule presented in Table 1. F frozen samples in Petri plates were transferred from -80˚C into a sterilized Biological Safety Cabinet. Samples were allowed to thaw partially until they became "slushy." Cells were removed from the plate surface using a cell scraper and the cold, nearly thawed slush was transferred to sterile graduated 15 mL conical centrifuge tubes and placed immediately on ice to complete thawing. From the graduations on the tube, the volume

Table 1. Schedule of activities for BRIC-23 Science Verification Test (SVT), Flight (FL), and Ground Control (GC) experiments*.

	SVT		FL		GC	
Actual or Simulated Activity	**Day**	**Date**	**Day**	**Date**	**Day**	**Date**
Integration/Handover	0	03/29	0	07/15	0	09/13
Launch	8	04/06	3	07/18	3	09/16
Docking	11	04/09	5	07/20	5	09/18
Unpacking/stowage	12	04/10	6	07/21	6	09/19
Actuation	14	04/12	7	07/22	7	09/20
Transfer to -80˚C (B. subtilis)	15	04/13	8	07/23	8	09/21
Transfer to -80˚C (S. aureus)	16	04/14	9	07/24	9	09/22
Undocking and splashdown			42	08/26		
Arrival at KSC			46	08/30		
Deintegration	20	04/18	54	09/07	21	10/04

*All dates are in the year 2016.

recovered was estimated and recorded. Cultures were vortex mixed, diluted, and plated for viable counts as described above.

Sample Assembly for Spaceflight

Samples of ~10^7 *B. subtilis* spores or *S. aureus* cells were deposited into the bottoms of sterile 60 mm Petri plates and air-dried as described above. Samples and media were integrated into dual-chamber PDFUs as described above. BRIC canisters A and C each contained five PDFUs with *B. subtilis* spores, and canisters B and D each contained five PDFUs with *S. aureus* cells. The sixth space in each PDFU was occupied by a HOBO temperature data logger.

Spaceflight Timeline

The payload described above was the 23rd BRIC mission to space, designated BRIC-23. The flight timeline is presented in Table 1. The BRIC-23 payload was launched on July 18, 2016 on the 9th SpaceX cargo resupply mission to the ISS (SpaceX CRS-9) using the Falcon 9 rocket and Dragon capsule configuration. Upon actuation, samples were inoculated with 8.5 mL TSY liquid media containing 10% (v/v) glycerol (Figure 4A) and incubated at ambient ISS temperature for 36 hours (*B. subtilis*) or 48 hours (*S. aureus*) (Figure 4B). Growth was terminated by transfer of the BRIC canisters to the onboard -80˚C freezer (MELFI). Canisters were kept frozen for return to Earth in the Dragon capsule and were maintained in the frozen state on dry ice until return to Kennedy Space Center (KSC). Upon delivery at KSC, canisters were transferred to a secure -80˚C freezer and stored until deintegration.

Post-Flight Sample Processing

To ensure that samples remained frozen during deintegration, BRIC canisters were processed sequentially. Each canister was removed from the -80˚C freezer and placed at lab-ambient temperature for ~30 minutes. BRIC canisters were then transferred to the interior of a sterile Biological Safety Cabinet, disassembled, and PDFUs removed. The still-frozen PDFUs were allowed to warm at lab-ambient temperature for a further ~5-10 minutes to allow each PDFU to be disassembled. Culture samples were removed in their 60 mm Petri plate bottoms, sterile labeled lids were placed on the samples, plates were wrapped individually in aluminum foil and labeled, then placed on dry ice for transfer to a -80˚C freezer for storage. Culture samples remained solidly frozen throughout the deintegration procedure.

RESULTS

Pre-Flight Survival of Air-Dried *S. aureus* Cells

In spaceflight experiments, a significant amount of time can elapse between the time that a biological sample is prepared and when it is actually cultivated in space. Such pre-experiment activities include: (i) integration of samples into spaceflight hardware, (ii) loading of samples into the space vehicle, (iii) launch countdown, (iv) potential launch scrub and rescheduling, (v) transit time to the ISS, (vi) docking and cargo transfer activities, and (vii) astronaut scheduling for experiment initiation. These activities can take up to several days to accomplish, and can affect the response of living organisms. In addition, space launch and landing themselves present unique environmental challenges to biological systems, such as high and variable accelerations, shock, vibration, or changes in ambient temperature/oxygen availability (Baert et al., 2006; Tjandrawinata et al., 1997; Wehland et al., 2016).

Many of these problems can be circumvented in microbial systems by using cells in metabolic stasis. For example, some bacteria, such as *B. subtilis*, form metabolically dormant endospores that can remain stable for indefinite periods of time, at least months to years [reviewed in (Nicholson, 2004)]. Other bacteria can be stabilized by air-drying or lyophilization, either in the presence or absence of protectant molecules, resulting in metabolically arrested cells that quickly resuscitate upon hydration [reviewed in (Potts, 1994; Potts et al., 2005)]. The genus *Staphylococcus* is noted for its resistance to desiccation (Nocker et al., 2012; Ryan, 1994), and thus was deemed suitable for these experiments.

Previously, as part of the pre-flight testing for the BRIC-18 mission to the ISS (Fajardo-Cavazos and Nicholson, 2016), we performed a viability time course for air-dried *S. epidermidis* cell samples. A series of samples consisting of air-dried *S. epidermidis* cells in 60 mm Petri plate

bottoms was prepared, incubated at ambient laboratory conditions protected from light for various times, then the air-dried cells were suspended in 8.5 mL of TSY+10% glycerol and viable counts determined. It was observed that the inactivation of air-dried *S. epidermidis* cells exhibited biphasic kinetics; during the first 14 days cells retained essentially full viability, then afterwards, viability was lost exponentially at a rate of 1 log every ~21 days (Figure 1).

As part of BRIC-23 pre-flight testing, we repeated this experiment using *S. aureus* cells and observed slightly different inactivation kinetics (Figure 1). As seen with *S. epidermidis*, during the first 14 days air-dried *S. aureus* cells also retained essentially full viability (Figure 1). However, the subsequent rate of exponential viability loss with *S. aureus* cells occurred at a faster rate of 1 log every ~13 days (Figure 1). As expected, air-dried *B. subtilis* spores retained full viability for the entire course of the experiment (data not shown). In all cases, the data indicated that *Bacillus* spp. spores and at least two different species of *Staphylococcus* could be stored for up to two weeks at ambient conditions before actuation without significant loss of viability.

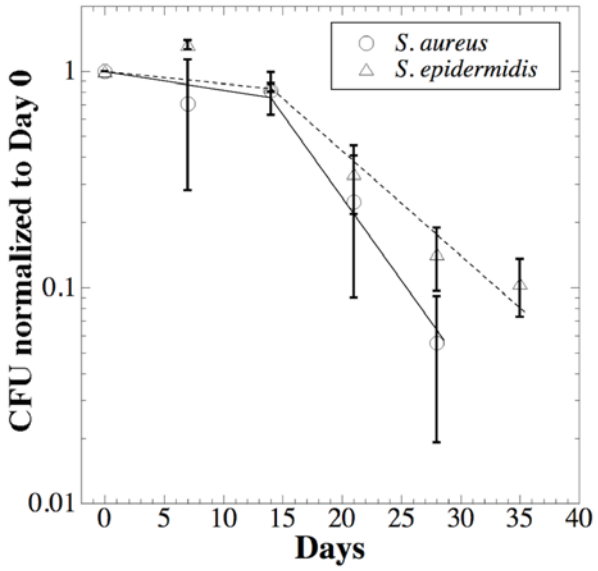

Figure 1. Survival of air-dried cells of *S. aureus* (circles) and *S. epidermidis* (triangles) as a function of time. Data points are averages ± standard deviations of duplicate samples.

Growth Kinetics of Cells in 60 mm Petri Plates: Pre-SVT

The next task we performed was to define the growth kinetics of static (i.e., not shaken or agitated for aeration) *B. subtilis* and *S. aureus* cultures under conditions similar to those encountered within BRIC-PDFUs. In batch culture, bacterial growth typically progresses through several well-defined phases: (i) the lag phase, during which the cells adjust to their new environment; (ii) the logarithmic growth phase (also known as the exponential or "log" phase), during which cells grow by binary fission with a constant exponential doubling time; (iii) the transition phase, when the growth rate of the cells slows due to limitation of some critical growth factor; (iv) the stationary phase, at which the culture has usually achieved its maximum cell density and growth ceases; and (v) the death phase, during which cells lose viability (Drew, 1981).

To examine the growth kinetics of cultures grown without agitation in 60 mm Petri plates, cells of *S. aureus* or spores of *B. subtilis* were air-dried into sets of 60 mm Petri plates. Plates were inoculated with 8.5 mL of TSY+10% glycerol and incubated at 25˚C (*S. aureus*) or 23˚C (*B. subtilis*). These temperatures were chosen to simulate the actual temperatures recorded in BRIC canisters on the ISS during the previous BRIC-18 (25.1±0.1˚C) and BRIC-21 (22.8±0.5˚C) spaceflight experiments. At various time points, duplicate sets of plates were processed as described and plated on TSY for viable counts (Figure 2).

In static culture at 25˚C, *S. aureus* cultures grew with a doubling time of ~6.7 hours and reached transition phase at ~40 hours (Figure 2A). Cultures grew to high densities, reaching 1-2x10^{10} total viable CFU per culture by 48 hours (Figure 2A). In comparison, *B. subtilis* cultures behaved quite differently than *S. aureus* cultures. In static culture at 23˚C, *B. subtilis* cells exhibited a substantial lag period (12 hours), likely due in part to time required for spore germination. However, *B. subtilis* cultures subsequently grew logarithmically more quickly than *S. aureus*, with a doubling time of ~2 hours, and reached transition phase earlier at ~28 hours (Figure 2B).

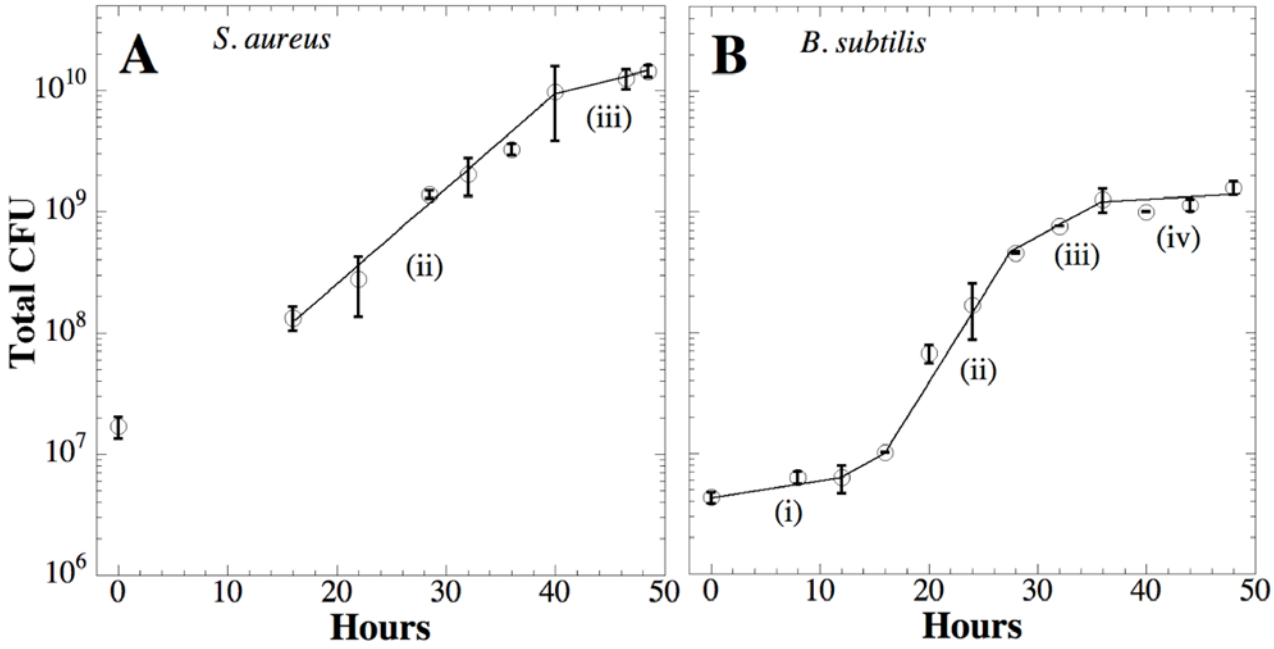

Figure 2. Pre-SVT growth curves of *S. aureus* (A) and *B. subtilis* (B) in 60 mm Petri plates. Data are averages ± standard deviations of duplicate experiments. Growth phases are denoted: (i) lag, (ii) logarithmic, (iii) transition, and (iv) stationary. Data are plotted on the same scales for comparison.

B. subtilis cultures grew to ~10-fold lower final density than *S. aureus* cultures, reaching 1-2x10^9 total viable CFU per culture by 48 hours (Figure 2B). The very different growth kinetics obtained with *B. subtilis* and *S. aureus* cultures indicated that it is critical to establish the growth characteristics of each microorganism before each flight experiment.

SVT of *S. aureus* in BRIC-PDFU Hardware

Because *B. subtilis* had previously been subjected to SVT protocols in preparation for the BRIC-18 and BRIC-21 spaceflights, it was not subjected to SVT. However, *S. aureus* had not previously been cultivated in BRIC-PDFU hardware, so we next performed an SVT to assess the organism's performance under the timeline and conditions of a typical mission to the ISS. Samples of air-dried *S. aureus* cells in 60 mm Petri plates were prepared and integrated into Biological Research in Canisters-Dual Chamber (BRIC-DC) hardware using stringent aseptic protocols developed for the prior BRIC-18 and BRIC-21 missions, and SVT testing proceeded according to the simulated flight timeline presented in Table 1.

For scheduling purposes, five BRIC canisters (designated Canisters A-E) were used in SVT; Canisters A, C, and E each contained a HOBO temperature data logger. SVT testing occurred in the ISS Environmental Simulator (ISSES) chamber at KSC, following the real-time temperature regime as telemetered to Earth from the ISS. At the conclusion of the experiment, temperature data retrieved from the HOBO data loggers revealed that the samples in the ISSES chamber had been subjected to temperatures of 21.4±0.3˚C (Canister A), 21.8±0.3˚C (Canister C), and 21.6±0.3˚C (Canister D). Growth of the *S. aureus* cultures was measured in triplicate at 4-hour intervals from 24 to 48 hours of incubation and is displayed in Figure 3.

BRIC-23 Flight

Onboard the ISS, all PDFUs were actuated within a 30-minute period on July 22, 2016 (Figure 4A). Canisters A and C each contained five PDFUs, in which air-dried *B. subtilis* spores

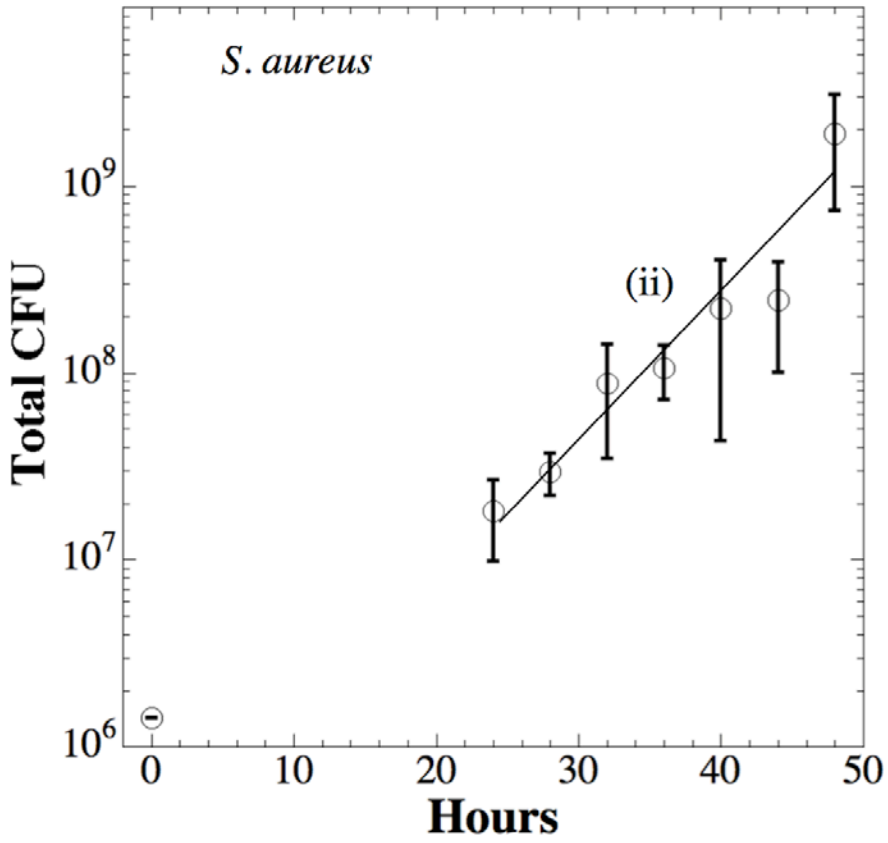

Figure 3. Growth of *S. aureus* cells in BRIC-PDFUs during SVT. Data are averages ± standard deviations of triplicate samples. Logarithmic (ii) phase is denoted. See text for details.

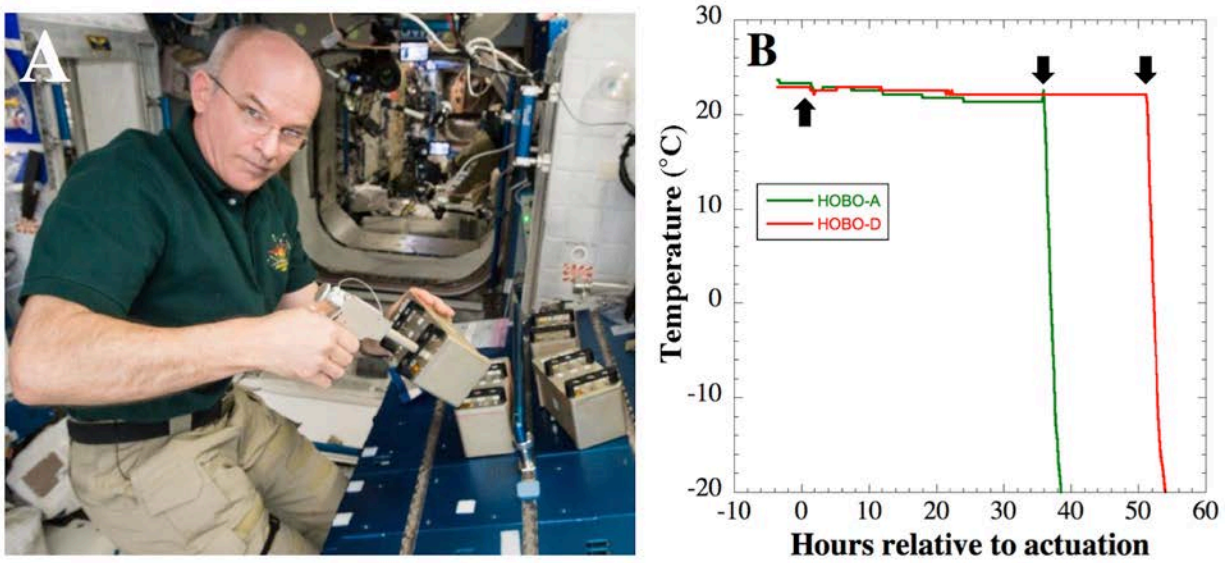

Figure 4. (A) Actuation of BRIC-23 canisters onboard the ISS by astronaut Jeffrey Williams. (B) Temperatures recorded during BRIC-23 flight for *B. subtilis* (HOBO-A, green line) and *S. aureus* (HOBO-D, red line). Time of actuation is denoted by upward arrow, and time of canister transfer to the onboard -80˚C freezer is denoted by the downward arrows.

were placed, and Canisters B and D each contained five PDFUs in which *S. aureus* air-dried cells were placed. Each PDFU received 8.5 mL of TSY+10% glycerol medium, and cells were incubated at ISS ambient temperature until transfer to the onboard -80˚C MELFI freezer after 36 hours (*B. subtilis*) or 48 hours (*S. aureus*) of incubation (Figure 4B).

Each BRIC canister contained its own dedicated HOBO temperature data logger. During the flight, the data logger in Canister B failed; however, complete temperature data was recorded by the other adjacent data loggers as 22.0±0.6˚C (Canister A), 22.5±0.4˚C (Canister C), and 22.3 ±0.3˚C (Canister D). Examination of the data from the HOBO temperature loggers indicated that the samples achieved temperatures at or below 0˚C within 30 minutes after placement in the MELFI freezer. The complete temperature profiles of Canisters A (*B. subtilis*) and D (*S. aureus*) are presented in Figure 4B.

Upon return of BRIC canisters to Kennedy Space Center, canisters and PDFUs were deintegrated as described in "Materials and Methods," and samples were stored in a locked -80˚C freezer provided with emergency power backup.

BRIC-23 Ground Control

After return of BRIC-23 flight samples from the ISS, an asynchronous ground control experiment was performed following the schedule (Table 1) and temperature profiles (Figure 4B) recovered from the flight experiment. Again, canisters A and C each contained five PDFUs in which air-dried *B. subtilis* spores were placed, and Canisters B and D each contained five PDFUs in which *S. aureus* air-dried cells were placed. Upon actuation, each PDFU received 8.5 mL of TSY+10% glycerol medium and cells were incubated in the ISSES chamber programmed to play back the ISS temperature profile recorded during the flight. After 36 hours (*B. subtilis*) or 48 hours (*S. aureus*) of incubation, samples were transferred to a secure -80˚C freezer.

During the ground control experiment, each BRIC canister contained its own dedicated HOBO temperature data logger. Temperatures recorded during the ground control experiment were 22.6 ±0.3˚C (Canister A), 22.2±0.4 (Canister B), 22.4 ±0.3˚C (Canister C), and 22.2±0.4˚C (Canister D). Upon conclusion of the ground control experiment, canisters and PDFUs were deintegrated as described in "Materials and Methods," and samples were stored in a locked -80˚C freezer provided with emergency power backup.

DISCUSSION

In conducting spaceflight vs. ground control experiments, it is desirable to minimize confounding factors such as pre- and post-flight culture handling and media effects. As an example of efforts towards this goal, in the present communication we report the development of a standardized protocol to be used for growth and handling of microorganisms in BRIC-PDFU spaceflight hardware for the purpose of obtaining various -omics datasets for deposition in the GLDS. The timeline for establishment of the standardized protocol to be used in preparation for the BRIC-23 mission was rather limited, from initial development of the concept in late October 2015 to launch on July 18, 2016. To expedite protocol development, we chose to use with minimal modification the flight hardware (dual-chamber BRIC-PDFUs), bacterial strain (*B. subtilis*), and media (TSY+10% glycerol) that had previously been flight tested on the BRIC-18 (Fajardo-Cavazos and Nicholson, 2016) and BRIC-21 (Nicholson et al., 2015) missions to the ISS. In addition, because of its greater relevance for astronaut health and disease, we chose to replace the harmless skin commensal bacterium *S. epidermidis* used in previous flight experiments, with the closely related common human pathogen *S. aureus*. Because *S. aureus* is designated as a BSL-2 organism, its inclusion necessitated development of stringent BSL-2 level pre-flight protocols that will prove to be important in future spaceflight experiments utilizing organisms with potential to become human pathogens.

An important step in pre-flight sample preparation is the preparation of cells that can survive in stasis from sample preparation through pre- and post-launch activities. This was accomplished by the technically simple expedient of air-drying the microbial cell samples. However, not all microbes are as resistant to drying as bacterial spores or *Staphylococcus* spp. cells. Several techniques can be used to prepare viable

cells at high titer in a dried state, such as lyophilization, fluidized bed drying, and/or inclusion of xeroprotective agents in the drying process (Narváez-Reinaldo et al., 2010; Strasser et al., 2009). Therefore in future experiments using different microbes in BRIC-PDFU hardware, the drying protocol must be optimized empirically for each microorganism. Because of the long and variable time lags between sample preparation and final actuation in space, another important parameter to consider is the loss of viability upon storage. This also must be determined empirically as we demonstrated for *S. aureus* and *S. epidermidis* (Figure 1) and *B. subtilis* spores, which are inherently stable for indefinite periods of time.

Gene expression of bacterial cells varies as a function of many parameters, including the growth medium. The medium we chose for cell cultivation in this study was TSY containing 10% glycerol. TSY is a rich medium suitable for growth of most non-fastidious microorganisms. It should be noted that TSY+10% glycerol may not be an optimum medium for all test microorganisms, as media effects can alter the results of spaceflight experiments (Wilson et al., 2008).

Optimum preservation of labile macromolecules for subsequent -omics studies is a high priority for spaceflight experiments. In prior testing we attempted to use the product RNAlater™ II (Life Technologies, Carlsbad, CA) as a preservative, but found that its addition provoked extensive cell lysis of *B. subtilis* and *S. aureus* cultures (data not shown). In the present study, glycerol was added directly to TSY as a cryoprotectant to preserve the viability of cultures to freezing and thawing. In the prior BRIC-18 and BRIC-21 experiments, we observed that cells retained essentially complete viability upon freezing and thawing in TSY+10% glycerol, and furthermore that total RNA extracted from these samples exhibited high RNA Integrity Number (RIN) values of 9.5-10.0 (Morrison, Fajardo-Cavazos, Nicholson, manuscript in preparation).

In conclusion, understanding the "spaceflight syndrome" in microbial systems will require performance of experiments in the human spaceflight environment and comparison of the results among different organisms. Standardization of hardware and protocols for conducting spaceflight experiments is therefore considered of high importance to reduce extraneous variables and enhance reliability for cross-experiment comparisons in -omics experiments.

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