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Growth of 48 Built Environment Bacterial Isolates on Board the International Space Station (ISS)

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Abstract

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Results: Of the 48 strains sent to the ISS, 45 of them showed similar growth in space and on earth. The vast majority of species tested in this experiment have also been found in culture-independent surveys of the ISS. Only one bacterial strain that avoided contamination showed significantly different growth in space. *Bacillus safensis* JPL-MERTA-8-2 grew 60% better in space than on earth.

Conclusions: The majority of bacteria tested were not affected by conditions aboard the ISS in this experiment (e.g., microgravity, cosmic radiation). Further work on *Bacillus safensis* could lead to interesting insights on why this bacteria grew so much better in space.

1 Introduction

From 2012-2014, we conducted a nationwide citizen science project, Project MERCCURI http://spacemicrobes.org/, aimed at raising public awareness of microbiology and research on board the International Space Station (ISS). Project MERCCURI (Microbial Ecology Research Combining Citizen and University Researchers on the ISS) was a collaborative effort involving the "microbiology of the Built Environment network" (microBEnet) group, Science Cheerleader, NanoRacks, Space Florida, and SciStarter. One of the goals of Project MERCCURI was to examine how a number of non-pathogenic bacteria associated with the built environment would grow on board the ISS compared to on earth.

Most previous work growing bacteria in space has focused on species known to contain pathogenic strains (e.g. *Escherichia coli* (17,4) and *Pseudomonas* (8,15), and much less attention has been paid to the "normal" microbes that surround us (i.e., species not known to be pathogenic). An understandable bias towards pathogens and pathogenic pathways is highlighted by work on topics such as biofilm formation (16, 21), antibiotic resistance/production (3, 12, 19 reviewed in 18), and virulence (23, 10).

While concern about pathogens in spacecraft is certainly warranted, it should be emphasized that the ability of a pathogen to survive outside a host and the ability to infect a host are both, at least in part, dependent on the existing community of non-pathogenic microbes in those locations. For example, mechanically ventilated hospital air shows a much higher abundance of potential pathogens (14), presumably due to the lack of inflow or competition from other microbes. Similarly, the infectivity of some pathogens has been shown to be very dependent on

the host microbiome (e.g. 25, 11, 26, 24). Therefore, it is important to understand the entire microbial ecosystem of spacecraft. Indeed, in recent years, several culture-independent studies have examined the microbiome of the ISS (5, 27, 22), including another part of Project MERCCURI (20). These studies have shown, not surprisingly, that the microbiome of the ISS bears a strong resemblance to the microbiome of human-associated built environments on earth. Therefore it is of interest to see how microbes from human-associated environments behave in space.

For this study, samples from human-associated surfaces (e.g. toilets, doorknobs, railings, floors, etc.) were collected at a variety of locations around the United States, usually in collaboration with the public. A wide variety of bacteria were cultured from these samples, and 48 non-pathogenic strains were selected for a growth assay comparing growth in microgravity on the ISS and on earth.

Materials and Methods

Sample collection

Samples were collected from built environment surfaces throughout the United States on cotton swabs (Puritan 25-806 2PC) and mailed (usually overnight) to the University of California Davis where they were transferred to lysogeny broth (LB) plates. Colonies were chosen for further examination based on maximizing morphological variation. Each chosen colony was double-dilution streaked and then the identity determined by direct PCR and Sanger sequencing using the 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1391R (5'-GACGGGCGGTGTGTRCA-3') primers (see 9 for details). Sanger sequences were trimmed and aligned using Geneious (13). The resulting consensus sequence was identified through a

combination of BLAST (1) and building phylogenetic trees using the Ribosomal Database Project (RDP) website (7). The 48 candidates for spaceflight were chosen on the basis of biosafety level (BSL-1 only), taxonomic variety, and human interest. In the absence of international standards, the biosafety level of each organism was determined by searching the American Biological Safety Association (ABSA) risk group database, the American Tissue Culture Collection (ATCC), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), and other public databases. An organism was removed from consideration if it was listed as BSL-2 or higher in any country or collection in the world.

Growth Experiment

A set of bacterial plates were created for each aspect of the study: growth in microgravity on the ISS (space plates), or growth on earth (ground plates). The plates were created using clear agar to facilitate optical density (OD) measurements. 1.5 g of Gelzan™ CM agar (Sigma-Aldrich) was added to 1 liter of lysogeny broth (LB). Each well of a flat-bottomed 96-well plate (Costar) was plated with 200 µl of agar. The plates were flamed to remove bubbles and incubated for 48-72 hours at room temperature (~20 °C) to ensure sterility before adding bacteria. Fresh overnights of each bacterial isolate were diluted to .01 OD600 and made into 8% glycerol stocks. For plating, 10 µl of each thawed stock dilution was added to each of the 12 wells (2 replicates per plate x 6 plates). The bacteria were placed into different locations on each plate in order to account for drying at the edges or any other positional effects on the plates. The plates were then sealed with adhesive polypropylene film (VWR #60941-072), into which a grid of micron-diameter holes were cut with a laser to allow for airflow. The ground plates were stored at -80 °C at UC Davis, and the space plates were mailed on dry ice to the National Aeronautics and Space

Administration (NASA) Johnson Space Center in Houston, TX before transfer (at -80 °C) to Cape Canaveral, FL for launch.

This payload was flown on the CRS-3 launch of the Space Exploration Technologies (SpaceX) Dragon spacecraft, on a Falcon 9 v1.1 rocket which successfully launched April 18, 2014. After six days, the space plates were removed from the MELFI (Minus Eighty Lab Freezer For ISS) and partially thawed. However, technical problems arose and the space plates were placed back into the MELFI until December 8, 2014. At that time, all three plates were thawed and the OD600 of each well (3x3 grid) was measured at time 0 (60 minutes after removal from the freezer) and then every 24 hours for 4 days. Measurements were performed in a Molecular Devices SpectraMax M5e plate reader which was modified for integration onto the ISS. On these same days, equivalent measurements of the ground plates were taken in a Molecular Devices SpectraMax M5e plate reader at UC Davis. After the experiment, the ground plates were placed back at -80 °C and the space plates were placed back into the MELFI. In February 2015, the space plates were transferred to a -95 °C freezer on board a Dragon spacecraft. The vehicle splashed down in the Pacific Ocean on Feb 10, 2015. The space plates were then mailed to UC Davis on dry ice and were transferred to -80 °C when received.

Once the plates arrived, we thawed all six plates and performed a high-density measurement in a Tecan M200 plate reader. OD600 readings were taken in a 5x5 grid covering the entire well, these 25 measurements were then averaged within each well.

Analysis

For each sample, the averages of the six space replicates and six ground replicates were compared using a Student's t-test. To correct for multiple hypothesis testing, the p-values were

adjusted using the False Discovery Rate (FDR) method (2). All raw data, analyses and scripts can be found at https://zenodo.org/record/44661.

Confirmation

In order to confirm that the observed results were not due to contamination of the wells, each of the 12 replicates (six space, six ground) for the three bacteria showing statistically different growth between the ISS and earth were cultured after the experiment. Bacteria were struck from the wells onto LB-agar plates, then single colonies were grown into overnight cultures. DNA was extracted using a Wizard Genomic DNA Purification kit (Promega) from each of the 36 cultures (3 bacteria X 12 replicates) and the identity was confirmed with PCR and Sanger sequencing using the 27F and 1391R primers as described above.

Comparison to ISS swab data

The bacterial community on the ISS was recently surveyed by PCR amplification and sequencing of 16S rRNA genes from swabs (20). We compared the 16S sequence of each of our bacterial isolates to the "representative sequence" from each operational taxonomic unit (OTU) generated from the survey data. A local BLAST was performed, limiting the results to 97% identity over at least 250 bp of the rRNA sequence (the amplified fragment is 253 bp).

Results and Discussion

Growth experiments are typically undertaken in liquid media, in part because measuring the optical density of a liquid culture is straightforward. However, liquid cultures present a number of problems in microgravity. Most organisms that passed our screening did not grow well under anaerobic conditions, and thus required some sort of gas exchange with the surrounding air. On the ground, aerobic conditions are easily created by incubating in open or loosely capped vessels.

This is impractical and unsafe in microgravity; there is no "safe" orientation in which the liquid will remain in place. We explored several unsuccessful approaches to this problem. For example, we found that gas-permeable plate seals leak when inverted, and their adhesion failed completely after freezing. We also fabricated custom plates with seals made from hydrophobic polydimethylsiloxane (PDMS) with micron-diameter vent holes, but these also leaked slightly when inverted.

We eventually concluded that the design requirements were mutually exclusive; either we could achieve containment for liquid cultures at the expense of aerobic conditions, or we could achieve aerobic conditions at the expense of liquid culture containment. We chose the latter, so our plates were prepared with solid media. Solid media is not traditionally used for OD measurements, and so our results need to be interpreted differently from OD in liquid culture. Using clear agar to maximize transparency, we programed the plate reader to take OD measurements at nine different locations in each well, each of which was measured twenty five times per observation. The plates were inoculated in a manner intended to create many small colonies (see Materials and Methods). As these colonies grow, their edges intersect with reading points, and the OD for that point increases in a stepwise fashion. As the colony thickens, the OD gradually increases. OD in liquid media is thought to correspond to scattering of light by individual cells, whereas our measurements correspond to the number, diameter, and thickness of the colonies. The intervals elapsed between occultations of the reading points decrease exponentially, and so the average OD across each well behaves very similarly to traditional observations of log-phase growth in liquid media. The data from the different plate readers (Tecan and Molecular Dynamics) was compared at 96 hours by plotting the OD600 values against each other. While the concordance was not perfect, there was a very strong relationship

between the two machines which provided validation of the data from both Molecular Dynamics machines (ground and space).

By this measure, the vast majority of the bacteria (45/48) behaved very similarly in space and on earth (Table 1). Only three bacteria showed a significant difference in the two conditions; *Bacillus safensis*, *Bacillus methylotrophicus*, and *Microbacterium oleivorans*. However, upon Sanger sequencing the 16S rRNA gene from cultures obtained from the wells on the space plates and the ground plates, we inferred contamination of the *B. methylotrophicus* and *M. oleivorans* wells and therefore discarded those data. Some wells showed a mixed Sanger sequence, suggesting the presence of more than one organism in the well, while others gave a clear identification as a contaminating organism. The remaining candidate was *Bacillus safensis*, collected at the Jet Propulsion Laboratory (JPL-NASA) on a Mars Exploration Rover before launch in 2004. As part of standard Planetary Protection protocols, all surface-bound spacecraft are sampled during the assembly process and those strains are then saved for further analysis. We obtained this strain as part of a collection of JPL-NASA strains to send to the ISS (Table 1).

In this experiment, *Bacillus safensis* grew to a final density of ~60% higher in space than on the ground, with very little variation between replicates (Figure 1). The genome sequence of this strain, *Bacillus safensis* JPL-MERTA-8-2 has just been published (6) and may contain clues as to why this strain behaved so differently in space.

It is perhaps no surprise that most built environment-associated bacteria behave very similarly on the ISS as on earth. After all, the ISS is a home and office of sorts, with environmental conditions very similar to a building on earth with the exception of gravity. The ISS is maintained at around 22 °C with a relative humidity of around 60%. Additionally, this

experiment didn't provide enough time to study the long-term adaptation of bacteria to the environment on board the ISS.

A related project from our lab has examined the microbial community already present on the ISS (20). Given that the ISS appears to harbor similar microbes to built environments on earth, we also asked if there were close relatives to our 48 bacteria already present on the ISS. The vast majority (39/48) of our bacterial species were found in the existing microbial community data which is not surprising given the built environment origins of the isolates. This suggests that our data showing these species growing with similar kinetics on space and on earth is potentially relevant to the biology of the microbial communities already present on the ISS.

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Growth (OD600) over time of *Bacillus safensis* JPL-MERTA-8-2

Growth (OD600) over time of *Bacillus safensis* JPL-MERTA-8-2 in space (green) and on earth (brown). Values represent the mean of 6 wells, +/- the standard deviation.

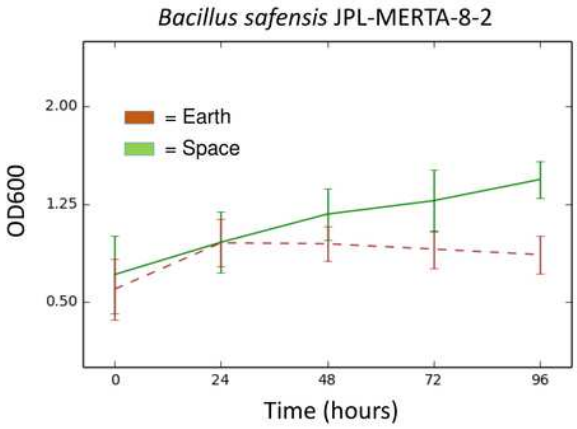

Table 1(on next page)

Growth (OD600) of all 48 strains as measured in space and on earth

Values represent the mean of 6 wells, +/- the standard deviation. Difference between space and earth were determined using a Student's t-test and the p-values were adjusted for multiple hypothesis testing by using the False Discovery Rate (FDR).

Organism	Location	Source	Mean OD (space)	Mean OD (ground)	FDR p-value
Bacillus safensis	**JPL-NASA (CA)**	**Mars Exploration Rover**	**1.44 +/- 0.09**	**0.86 +/- 0.07**	**0**
Bacillus methylotrophicus	**Yuri's Night New York (NY)**	**Doorknob**	**1.54 +/- 0.05**	**1.69 +/- 0.09**	**0**
Microbacterium oleivorans	**St. Joseph's Prep (PA)**	**School mascot**	**1.61 +/- 0.3**	**1.93 +/- 0.14**	**0.04**
Bacillus atrophaeus	JPL-NASA (CA)	Mars Exploration Rover	1.69 +/- 0.05	1.57 +/- 0.14	0.07
Porphyrobacter mercurialis	Pop Warner: Coronado (CA)	Stadium seat	0.85 +/- 0.13	1.03 +/- 0.2	0.07
Bacillus flexus	NFL: Tennesse Titans (TN)	Stadium field	1.47 +/- 0.24	1.72 +/- 0.1	0.07
Bacillus atrophaeus	Denver Museum of Nature and Science (CO)	Antique microscope	1.62 +/- 0.06	1.3 +/- 0.25	0.14
Bacillus altitudinis	Deerfield Academy (MA)	School field	1.23 +/- 0.09	1.22 +/- 0.15	0.14
Macrococcus brunensis	WHYY Radio (PA)	Keyboard	1.06 +/- 0.15	1.29 +/- 0.11	0.14
Bacillus tequilensis	Today Show (NY)	Candy jar	1 +/- 0.21	1.09 +/- 0.1	0.14
Bacillus amyloliquefaciens	NFL: New England Patriots (MA)	Stadium seat	1.41 +/- 0.13	1.53 +/- 0.12	0.14
Bacillus subtilis	JPL-NASA (CA)	Robotic arm (Insight)	1.32 +/- 0.16	1.08 +/- 0.25	0.16
Micrococcus luteus	NBA: Sacramento Kings (CA)	Sweat mop	1.01 +/- 0.08	0.87 +/- 0.16	0.21
Leucobacter chironomi	Davis (CA)	Toilet	1.03 +/- 0.27	1.03 +/- 0.12	0.21
Kocuria kristinae	NBA: San Antonio Spurs (TX)	Court floor	1.93 +/- 0.06	1.85 +/- 0.16	0.21
Kocuria rhizophila	Yuri's Night Los Angeles (CA)	Camera	2.01 +/- 0.14	1.97 +/- 0.19	0.21
Bacillus stratosphericus	Academy of Natural Science (PA)	Water dish	1.34 +/- 0.14	1.1 +/- 0.13	0.21
Bacillus tequilensis	MBA: Philadelphia Phillies (PA)	Dugout	1.41 +/- 0.18	1.03 +/- 0.15	0.21
Micrococcus luteus	Pop Warner: Lake Brantley (FL)	Football goalpost	1.71 +/- 0.03	1.69 +/- 0.06	0.21
Paenibacillus mucilaginosus	Field Museum (IL)	"Sue" the T-rex	1.57 +/- 0.13	1.54 +/- 0.14	0.21
Exiguobacterium sibiricum	AT&T Park (CA)	Second base	1.3 +/- 0.23	1.38 +/- 0.14	0.21
Exiguobacterium indicum	NFL: Team from Washington D.C.	Stadium field	1.26 +/- 0.16	1.17 +/- 0.23	0.21
Curtobacterium pusillum	UC Davis (CA)	Stadium gate	1.28 +/- 0.3	1.49 +/- 0.14	0.21
Kocuria marina	Yuri's Night North Carolina (NC)	Water Fountain	1.77 +/- 0.1	1.73 +/- 0.08	0.26
Bacillus megaterium	The Liberty Bell (PA)	The Liberty Bell	1.38 +/- 0.24	1.46 +/- 0.15	0.34
Bacillus lichenformis	NBA: Philadelphia 76ers (PA)	Practice court	1.18 +/- 0.13	1.07 +/- 0.14	0.34
Bacillus megaterium	JPL-NASA (CA)	Mars Curiosity Rover	1.6 +/- 0.14	1.55 +/- 0.16	0.38
Bacillus subtilus	NBA: Orlando Magic (FL)	Game ball	1.35 +/- 0.08	1.17 +/- 0.19	0.38
Arthrobacter nitroguajacolicus	Chapman Hill Elementary (OR)	Stadium field	1.67 +/- 0.12	1.76 +/- 0.25	0.44
Bacillus aryabhattai	NFL: Oakland Raiders (CA)	Practice field	1.62 +/- 0.3	1.64 +/- 0.13	0.44
Microbacteria arborescens	JPL-NASA (CA)	Viking Mars Orbiter	1.69 +/- 0.26	1.59 +/- 0.47	0.49
Bacillus pumilus	JPL-NASA (CA)	Mars Exploration Rover	0.97 +/- 0.25	1.26 +/- 0.25	0.49
Paenibacillus elgii	JPL-NASA (CA)	Mars Exploration Rover	1.39 +/- 0.3	0.84 +/- 0.14	0.49
Kocuria rosea	JPL-NASA (CA)	Mars Exploration Rover	1.61 +/- 0.26	1.53 +/- 0.18	0.49
Bacillus aryabhattai	Pop Warner: Broncos (FL)	Stadium field	1.65 +/- 0.28	1.54 +/- 0.05	0.49
Micrococcus yunnanensis	Discover Magazine (WI)	Dictionary	1.68 +/- 0.41	1.75 +/- 0.23	0.49
Bacillus amyloliquefaciens	Franklin Institute (PA)	Statue	1.4 +/- 0.09	1.38 +/- 0.14	0.6
Bacillus megaterium	Chemical Heritage Foundation (PA)	Antique pressure vessel	1.57 +/- 0.43	1.56 +/- 0.14	0.61
Exiguobacterium acetylicum	NFL: San Franciso 49ers (CA)	Stadium field	1.57 +/- 0.18	1.53 +/- 0.21	0.61
Bacillus horikoshii	Parkway Middle School (FL)	Banister	1.53 +/- 0.34	1.67 +/- 0.09	0.61
Macrococcus equipercicus	Catholic Montessori School (OH)	Floor	0.99 +/- 0.19	0.94 +/- 0.2	0.64
Streptomyces kanamyceticus	KARE11 Morning News (MN)	Set kitchen	1.11 +/- 0.2	0.92 +/- 0.16	0.66
Pantoea eucrina	Smithsonian Air and Space Museum (D.C.)	Mercury Orbiter	1.57 +/- 0.31	1.57 +/- 0.09	0.76
Bacillus horikoshii	Pop Warner: Saints (NJ)	Stadium field	1.64 +/- 0.2	1.58 +/- 0.07	0.79
Curtobacterium herbarum	Georgia Tech (GA)	Stadium seat	1.42 +/- 0.19	1.5 +/- 0.13	0.79
Bacillus pumilus	Pop Warner: Chittanoga (NY)	Porta-Potty handle	1.17 +/- 0.31	1.35 +/- 0.12	0.82
Micrococcus luteus	Pop Warner: Apopka (FL)	Practice mat	0.99 +/- 0.27	0.86 +/- 0.34	0.82
Bacillus marisflavi	Pop Warner: PeeWee Bengals (NC)	Stadium field	1.66 +/- 0.19	1.61 +/- 0.26	0.82

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