PREFERENTIAL GROWTH OF AN AQUATIC BACTERIUM IN LOW-SHEAR MODELED MICROGRAVITY

by

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DEDICATION

I dedicated my thesis research to Lily, also known as Lilbo Baggins, Lillian, Lillith, and Supreme ruler of the house.

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LIST OF ABBREVIATIONS

Abbreviation	Description
LSMMG	Low-Shear Modeled Microgravity
RWV	Rotating Wall Vessel
HARV	High-Aspect Ratio Vessel
RPM	Revolution Per Minute
CFU/mL	Colony Forming Units per milliliter

ABSTRACT

Bacteria are known to respond to a variety of chemical and physical stimuli. Although gravity is universally encountered by all life forms, preferential growth in simulated low gravity has not been previously investigated. Water samples from the San Marcos River were cultured in a low-shear modeled microgravity (LSMMG) environment and three isolates were obtained. Culture studies included monoculture and mixed culture studies in LSMMG and full gravity (1g) conditions. The responses that were observed signify a change of growth when cultured in LSMMG between monoculture and polymicrobial cultures, and a change in motility. In addition to growth studies, whole-genome sequencing was performed on the environmental isolates to identify the species and potential genes that explain the response when grown in LSMMG. This study is the first observing competition and preferential growth of environmental bacteria in LSMMG.

I. INTRODUCTION

Bacteria are ubiquitous throughout the world, inhabiting almost every environment we have tested. If we plan on expanding our reach into space, like the planned expedition to Mars, we need to expand our understanding of bacterial response when in spaceflight conditions: like growth, metabolite production virulence factors, and commensal relationships. We need to understand what may happen with the bacteria in spaceflight conditions before long-term spaceflight missions may be successful. Current research only focuses on clinical bacterial isolate and not environmental isolates. This is the first study solely utilizing LSMMG culturing to enrich for environmental bacteria that would preferentially grow in this environment to understand the potential effects of spaceflight on environmental bacteria.

Enrichment culture is an important experimental concept in microbiology that allows scientists to adjust culture conditions to promote the growth and isolation of organisms normally present in very small numbers in the natural environment. This experimental approach was first developed in the late 19th century by the Dutch microbiologist, Martinus Beijerinck (Chung & Ferris, 1996). Enrichment allows for modification of microbial diversity based on the introduction of a specific chemical or physical condition. One example of physical enrichment conditions includes culturing at varying temperatures to select for psychrophiles or thermophiles. Similarly, chemical enrichment allows for the isolation and identification of microorganisms capable of using an unusual substrate, such as toluene-oxidizing soil organisms (Sawadogo et al., 2014). We utilized enrichment culturing techniques to select for bacteria that may prefer low-shear modeled microgravity conditions.

LSMMG culturing was initially developed to model spaceflight studies, modeling a specific physical condition in space. With the development of this technology, LSMMG culturing was utilized for ground-based studies. Development of ground-based analogs for modeling spaceflight conditions have helped mitigate problems of cost, limited availability of spaceflight launches, and clinical studies of virulent pathogens (Nickerson, Ott, Wilson, Ramamurthy, & Pierson, 2004a). One of the devices used to emulate spaceflight conditions is the Rotating Wall Vessels (RWV), created by NASA's biotechnology group at Johnson Space Center, which produces a low-shear modeled microgravity environment (Schwarz, Goodwin, & Wolf, 1992). Development of spaceflight analogs has allowed us to study the potential effects of spaceflight at a fraction of the cost and provide clinically relevant research.

The RWV works by utilizing centrifugal and Coriolis forces in order to induce solid body rotation of a liquid depending on the device orientation. Fluid shear force is decreased when in rotation in the correct orientation of these devices (Hammond & Hammond, 2001). Shear force occurs when liquid flows over the surface causing cell deformation. The next condition these devices model is microgravity. This condition is produced because the cells are in continuous orbital freefall that create a net-zero gravitational vector (Klaus, 2001). Combination of these two conditions results in a decrease in membrane deformation of the cells and a change in cell growth and response. In order to achieve LSMMG, the RWV orientation must be parallel to Earth's gravitational vectors (Figure 1). The control counterpart is oriented perpendicular to Earth's gravitational vectors allowing for sedimentation. These responses have been

studied with both prokaryotic and eukaryotic cells and applied in numerous clinical and spaceflight studies.

Bacteria are known to respond to mechanical stimuli, known as mechanosensing. One physical stimulus detected by bacteria is fluid shear. There are three meachnisms that explain how bacteria detect fluid shear: the "catch-bond" model, cytoskeletal stretching, and gated channel proteins (Nickerson, Ott, Wilson, Ramamurthy, & Pierson, 2004b). The "catch-bond" method utilizes hydrogen bonding located on cell-surface molecules. Low shear regulated genes are only expressed when the hydrogen bonds are intact. These low-shear genes are down-regulated when the bonds are broken.

The next two methods are through cell membrane deformation. The cytoskeleton of bacteria remains relaxed in a low-shear environment. When shear force is applied, the cytoskeleton is stretched causing cell deformation which transports signal molecules. The last proposed method is through gated channel proteins located on the lipid bilayer. When in a low-shear environment the channels are closed, but when placed in a high-shear environment the channels are opened. Genes associated with low shear are expressed when the channels are closed.

Among the previously studied responses of LSMMG culturing, the two that are crucial to this research is the change in growth as well as the genes responsible for these changes. Previously observed changes in bacterial growth have shown that there is an increase in cell density, increased length of exponential growth, and a decreased lag phase: although this is not a ubiquitous response for all bacteria (J. W. Wilson et al., 2007). There is no all-encompassing study observing the changes in growth based on bacterial genera. Studies have also been performed in order to understand gene

regulation. Full genome microarrays were performed on *Salmonella enteric*a serovar Typhimurium strain χ^{3338} that found an alteration in *RpoS* gene expression (James W. Wilson, Ott, et al., 2002). Further studies found that there were changes in the RNA binding protein, Hfq, utilizing gene knockouts and complements to observe the change in the response with and without the gene. This identified *Hfq* as a novel gene associated with an unknown mechanism that affects growth in spaceflight (J. W. Wilson et al., 2007). Studies have also observed the gene homologs in relation to *Hfq* causes a decrease in virulence in *Yersinia pesis* in LSMMG conditions (Geng et al., 2009).

LSMMG culturing is applicable to environments other than spaceflight research. There are low-shear environments in aquatic systems that could be modeled using similar LSMMG culturing methods. Laminar flow occurs in aquatic systems, like the Ekman layer in the ocean and microenvironments of rivers (Malverti, Lajeunesse, & Métivier, 2008; Woods, 2002). When in laminar flow, the objects are suspended along the path of the system. Because these environments are so complex, they present significant challenges to modeling and experimental design. RWVs have the potential to provide a missing physical stimulus that may be necessary for culturing certain bacteria.

One clinically relevant environment that experiences low-shear is the brush border of the microvilli in the small intestines (Guo, Weinstein, & Weinbaum, 2000). It has been shown that there is a change in virulence (increased or decreased) when in a low-shear environment. The use of the RWVs on eukaryotic cells allows for three-dimensional structures to be formed. These structures, coined as organoids, have led to the discovery of novel virulence genes and a better understanding of pathogen colonization of *in vitro* infections (Dutta, Heo, & Clevers, 2017). Building on these

interactions will lead to further understanding of bacterial virulence, modes of infection, and a more representative method of tissue culturing for disease studies.

There is a huge deficit in the understanding of bacterial responses to spaceflight and ground based analogs. We have seen huge disparity in responses from virulence to gene regulation. Virulence factors in bacteria vary when in LSMMG or spaceflight conditions, with *Y. pestis* and *S. enterica* serovar Typhimurium showing down and up regulated virulence factors respectively (Lawal, Jejelowo, & Rosenzweig, 2010; Rosenzweig, Ahmed, Eunson, & Chopra, 2014). We need broader studies observing bacterial responses to LSMMG and spaceflight conditions because of the extreme disparity in responses.

II. MATERIALS AND METHODS

Sample Collection

Environmental samples from the San Marcos River were collected at the Lion's Gate Park (29.8864, -97.9356) using a sterile 50mL conical tubes. The samples were collected with as much care to prevent collector contamination of the sample. The sample was then placed in 4°C until the sample could be used, approximately 18 hours.

Low-Shear Modeled Microgravity Enrichment

Once collected, the sample was then enriched with glucose to create a 5mM concentration in the sample. Once enriched the sample was then placed into their respective High Aspect Ratio Vessels (HARVs). The first HARV was then placed in the low-shear modeled microgravity orientation, while the other was then placed into the normal gravity orientation and used according to the manufacturer's protocols (Synthecon, Houston TX).

Once the sample was placed in the HARV, it was were incubated for 24 hours in their corresponding orientation, rotated at 22 rpm, and in 30°C (Figure 1). Once done, the sample was pulled, the biofilm was isolated, and serial dilutions were performed.

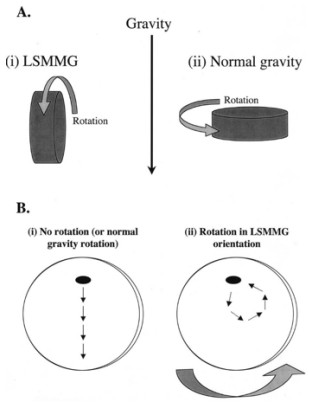

Figure 1: Model for HARV Orientations. Depicting orientations necessary for LSMMG (left) and Normal Gravity (right) (Nickerson et al., 2004a).

Colonies were then isolated by abundance of colony morphology, including the bacteria in the biofilm. Isolated bacteria were then designated with identifiers, A through H. Once isolated and labeled, each sample was gram-stained and observed under microscopy to determine gram nature and cell morphology. Through this procedure, strain A was required to be further isolated in the identifiers A1 and A2.

Growth Studies in Low-Shear Modeled Microgravity

Each bacterium was observed under both LSMMG and Normal Gravity growth conditions for 24 hours. In order to inoculate the HARVs, overnight samples of bacteria were grown at 30°C in R2A media. Once the bacteria have reached stationary phase, they were then diluted to an OD value of 0.20. Then 0.5mL of the diluted culture was placed into each HARV along with the R2A broth media and rotated at its designated orientation. This method was the standard inoculation method with using the HARV. After 24 hours, the sample was pulled and serially diluted to calculate CFU/mL to estimate the bacteria's growth in each condition. These studies were done in triplicate for each isolate.

After each sample was studied individually, the top isolates that grew best in either LSMMG or Normal Gravity conditions (A1, A2, and C) were then further studied by growing each culture in each condition for 72 hours, pulling a sample every 24 hours. Every sample pull was then serially diluted to estimate CFU/mL.

Competition Studies in Low-Shear Modeled Microgravity

The three isolates were then subjected to competition studies. Each HARV was inoculated with two of the isolates and incubated for 72 hours, pulling every 24 hours, similar to the previous inoculation methods. The samples were then serially diluted and plated on selective media in order to accurately estimate bacterial growth of each isolates. The following combinations of bacteria were tested: A1 and A2, A1 and C, A2 and C. Selective media were found by screening the growth of each isolate on varied antibiotic with varied concentrations.

Growth Rates in Low-Shear Modeled Microgravity and Normal Gravity

Growth curves were measured in both LSMMG and 1G conditions in order to observe a change in growth rates in each condition. For the LSMMG conditions, eight HARVs were inoculated with 0.5mL of each inoculum at a ±0.2 optical density. All eight HARVs were then placed in orientation, rotated, and inoculated at 30°C. Every three hours a HARV was pulled and the sample read on a plate reader and serially diluted to obtain CFU/mL counts. The protocol was then performed for all three bacterial isolates. The plate was read at and optical density of 600nm.

A growth curve was also performed in Normal Gravity as a control. This was done by utilizing a 96 well plate and adding 198µL of media and 2µL of overnight culture to obtain a 1:100 dilution factor. The plate reader was then programed to take a read every 15 minutes for 72 hours.

Motility

Isolates A1 and A2 were grown in either LSMMG or Normal Gravity for 24 hours. The samples were then stabbed on R2A plates with 0.4% agar concentration on their own and separated by the following distances: 2mm, 10mm, 20mm, and 50mm. Motility was measured after 24 and 168 hours.

DNA Extraction and Quantification

For sequencing, each isolate's DNA was extracted using ThermoFischer's GeneJET Genomic Isolation Kit (Thermo Fisher), using the manufacturer's protocol. The protocol that was included with the kit was followed, with the only change being the Gram-Positive lysis solution when required. The buffer was prepared and used according to the GeneJET kit protocol. Quantification was done on each extraction using the Qubit, with overall estimation of the total quantity of DNA in each extraction.

Sequencing

For the first sequencing run, the DNA was first sheared using the Bioruptor Pico from Diagenode. Using Diagenode's protocol for shearing DNA, we estimated 8 cycles with 15 sec on and 90 sec off to get fragment lengths of approximately 400 base pairs.

After shearing the DNA we then size selected using the e-gel from Invitrogen, which allows for elution of the sample at designated fragment lengths. The DNA was then cleaned and quantified using AMPure XP (Beckman Coulter) and Qubit, respectively. Once the DNA was cleaned and quantified, library preparation was done using the TruSeq DNA kit from Illumina. The samples were then normalized and pooled so they could be sequenced with Illumina MiSeq.

The second sequencing was done by preparing the sample Nextera XT kit by Illumina and following the manufacturer's protocol. After the following the Nextera Protocol, the DNA was then cleaned, quantified, normalized and pooled as before then placed on Illumina's MiSeq.

Bioinformatics

After sequencing, the data needed to be assembled. After sequencing, the reads provided fasta files that were separated based on isolate. Each file was then quality checked using FastQC. Once the quality was assessed Trimmomatic (version 0.36) was then used to trim the low-quality reads. Trimming was perfomed on the sequences, with variations in quality thresholds and length requirements. Each sequencing read was treated as both paired end and single ended reads. Each variation of trimming parameters were then asses for quality through FastQC. The pipeline then continued to error-correction followed by the assembly using SPADES. The output files that were generated were then used for identification of the isolates. Assembly quality was assessed through the use of PRINSEQ which generates statistical analysis of the assembly. The contigs for each assembly were then run through BLAST and AmphoraNet (Altschul, Gish, Miller,

Myers, & Lipman, 1990; Kerepesi, Bánky, & Grolmusz, 2014). Annotation of the assembly was done through RAST, BASys, and NCBI's GenBank(Aziz et al., 2008; Tatusova et al., 2016; Van Domselaar et al., 2005). Submission of the samples accessioned into GenBank with the following submission IDs: SUB6377864 (A1), SUB6378142 (A2), and SUB6378727 (C).

Data Analysis

All data was analyzed through R, using R Studio v. 1.2.1335. All figures were generated through ggplot in R. Statistical analysis was done through a Welch Two-Sample T-test.

III. RESULTS

Enrichment

There was a promotion of growth when the environmental samples were cultured in LSMMG and spiked with glucose. From this trial, eight isolates were obtained with two accounting for the free-floating biofilm was observed. The free-floating biofilm was large and formed from a polymicrobial community but when isolated and serially diluted there was only an abundance of isolate A1 and A2 (Figure 2).

Figure 2: River Sample Enrichment with LSMMG. Biofilm growth in LSMMG exhibiting a large, non-surface adhering, free-floating biofilm.

Growth in LSMMG

Comparing the growth of the strains in both Normal Gravity and LSMMG, the growth of isolate A1 preferentially grew in LSMMG compared to its 1XG counterpart. Both isolate A2 and C had no change when cultured in either LSMMG or 1XG (Figure 3 and Figure 4).

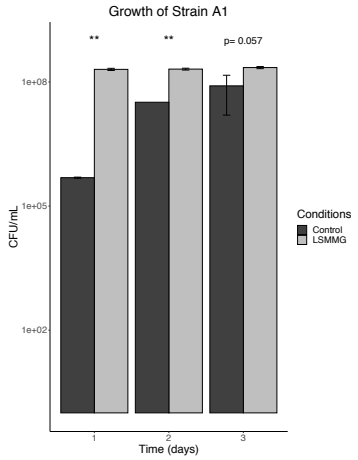

Figure 3: Growth of Isolate A1. Growth of A1 in both LSMMG and 1XG over three days. Statistical significance was observed on Day 1 and 2.

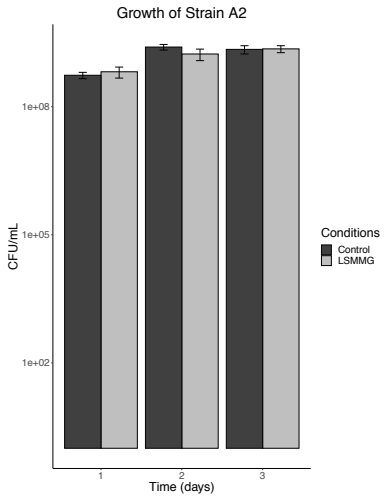

Figure 4: Growth of Isolate A2. Growth of A2 was observed in both LSMMG and 1XG over three days.

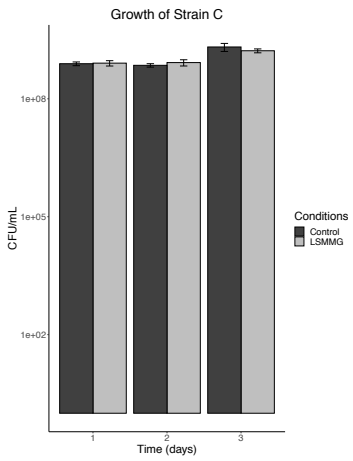

Figure 5: Growth of Isolate C. Growth of C was observed in LSMMG and 1XG over three days.

Studies in Low-Shear Modeled Microgravity

Proportions of each bacteria were used to find asses the dominant species in the co-culture systems. We found that there was no change in the proportions of strains A1 and A2 when cultured in 1XG, but a decrease in the proportion of A1 over the three days in LSMMG (Figure 6). Co-culture growth of strain A1 and C, showed an increase in the proportion of A1 in normal gravity conditions and a decrease in the proportion of A1 in LSMMG (Figure 7). Co-culture growth of strains A2 and C showed that strain A2 remained the predominant strain. There was a minor increase in the proportion of strain C at day three in 1XG conditions (Figure 8).

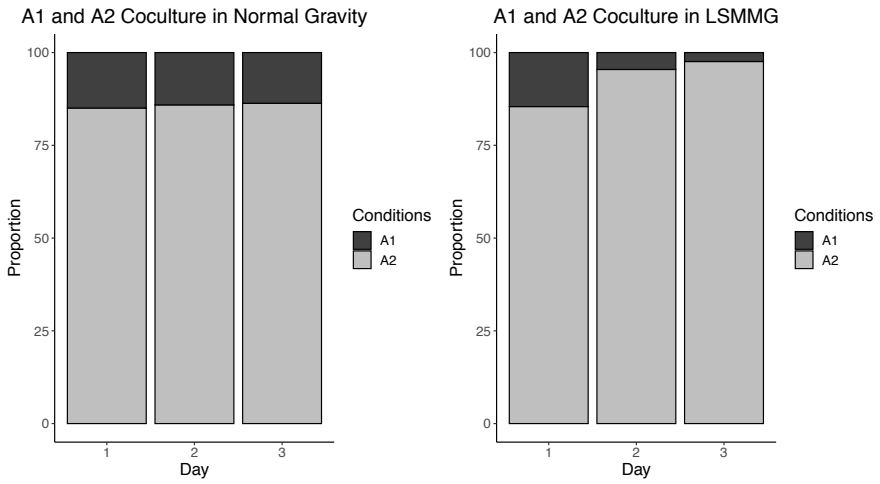

Figure 6: Population Shift of Strains A1 and A2. Strains A1 and A2 were grown in co-culture showing that there was a decrease in proportion of A1.

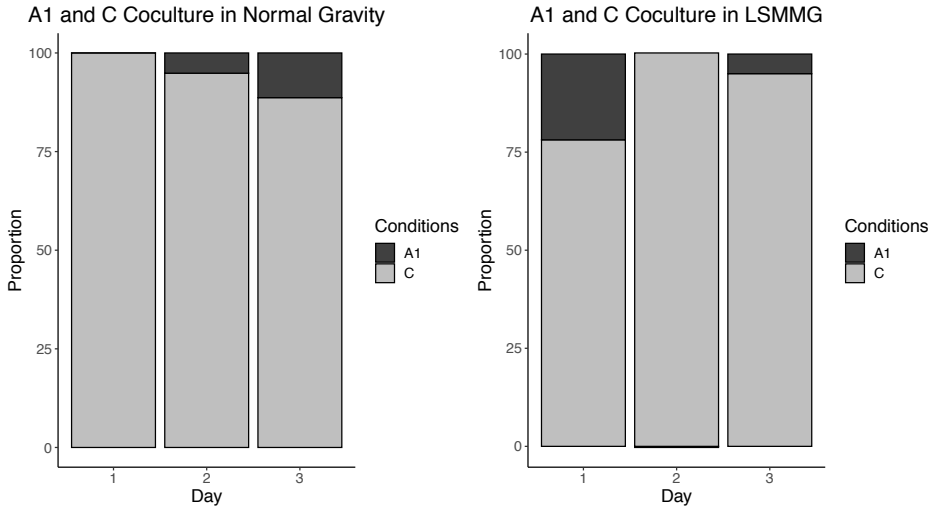

Figure 7: Population Shift of Strains A1 and C. Strain C had an overall higher population percentage than Strain A1 in both growth conditions.

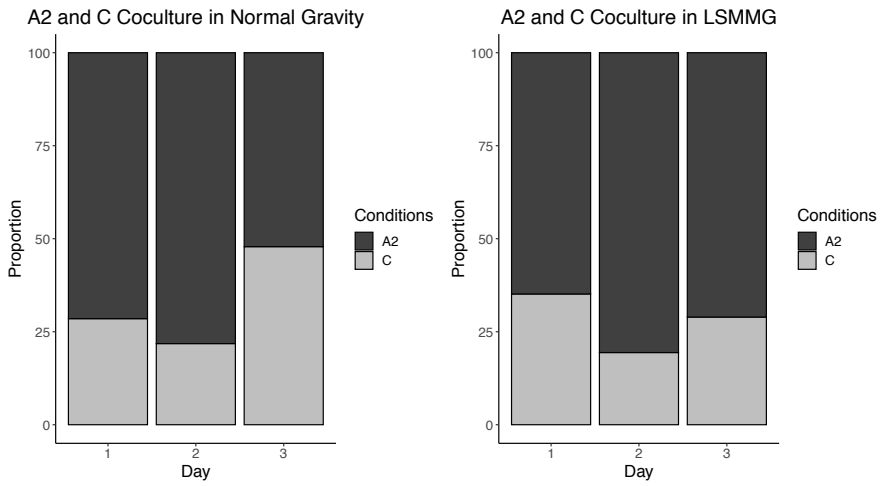

Figure 8: Population Shift of Strain A2 and C. Both strains grown in co-culture show population shifts depending on environmental condition.

Growth Rates in Low-Shear Modeled Microgravity and Normal Gravity

Studying the growth rates of bacteria allowed for observation on the change of growth rate between LSMMG and Normal Gravity (Figure 9). A2 reached the highest optical density in both LSMMG and Normal Gravity compared to all three isolates but grew best in Normal Gravity. A1 achieved a higher cell density and an increased exponential growth phase in LSMMG than Normal Gravity.

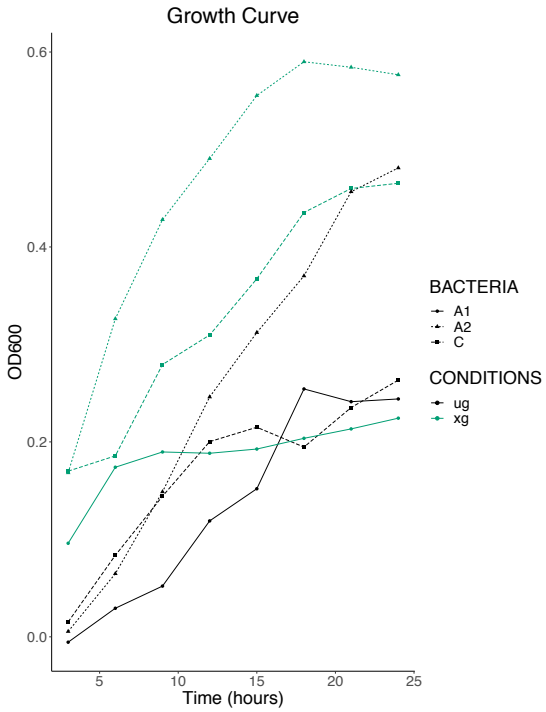

Figure 9: Growth Curve of Isolates in LSMMG and Normal Gravity. Growth rate of LSMMG and Normal Gravity were observed for 24 hours.

Motility

We tested motility due to the high affinity between strains *Exiguobacter* (A1) and *A. soli* (A2) from previous data on isolation and culturing methods of both isolates (data not shown). Motility was found to decrease in LSMMG compared to its Normal Gravity counterpart. When stabbed near each other in LSMMG there was no preferential growth of A2 towards A1 unlike what is seen in the control. After approximately 7 days, both isolates motility was reduced in LSMMG compared to the Normal Gravity counterpart. Motility was quantified in order to compare the changes between the control and LSMMG conditions (Table 1).

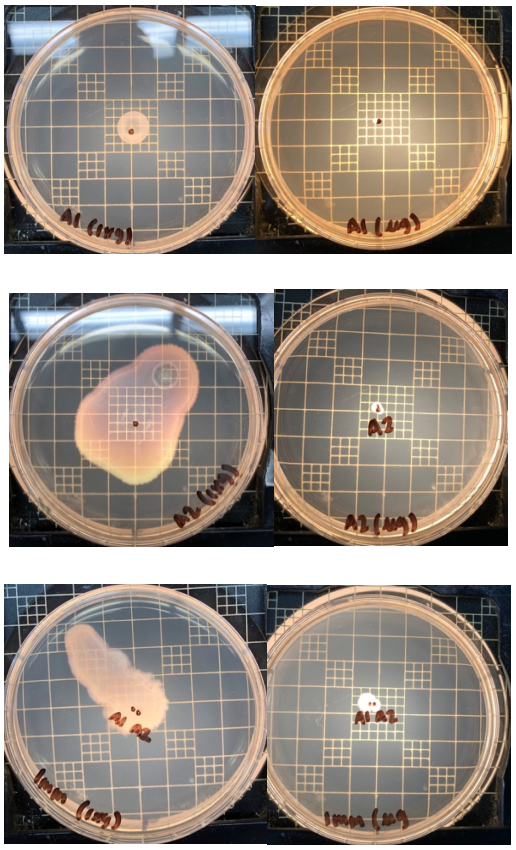

Figure 10: Stabs of All Isolates in Motility Agar. Top row is *Exiguobacter* (A1), middle is *A. soli* (A2), and bottom is the two isolates stabbed 1mm apart.

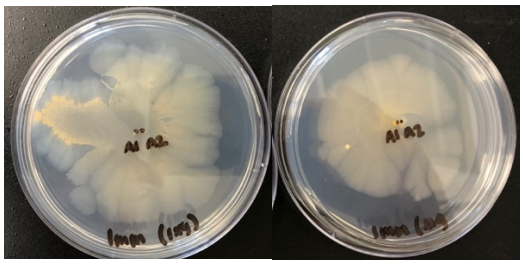

Figure 11: Co-stab of A1 and A2 in Motility Agar with Prolonged Incubation. Continued incubation of seven days from Figure 10, bottom row, showing motility returned.

Table 1: Measurements of Motility Between Culturing Conditions.

Isolate	Control	LSMMG
A1	3.05mm	.37mm
A2	16.47mm	1.88mm
A1 and A2	19.11	2.14
A1 and A2 (Figure 11)	28.67	18.63

Sequencing and Analysis

Full genome assembly using *de novo* methods identified all bacteria to the genus level but only identified one strain, A2. Contigs from each assembly were ran through Amphora and BLAST. Isolate A1 was identified to the genus *Exiguobacterium* and is considered to be a novel species. Isolate A2 was identified as *Acinetobacter soli*. Isolate C was identified as *Enterobacter* sp. 638.

Annotations were performed through RAST, NCBI's annotation pipeline, and through BASys. Annotation provided quantifications of genes in each subsystem and were compared to each other (Table 3). We classified motility, stress response, and defense genes from RAST annotation system. The RAST annotation program on average classified ~30% of the genes present with ~70% of the remaining annotation being genes not in the RAST subsystem and hypothetical proteins.

Through a BLAST we searched each genome for potential spaceflight encoding genes. Through blast we did not find *Hfq*, which aids growth in spaceflight (Table 2). We did find flagella proteins in strains A1, C. Interestingly, there were no flagellar proteins in strain A2, but flagella motility could not be playing a role in this observation. No genes

for pili were searched for, such as the gene found on *Acinetobacter baumanni* (Eijkelkamp et al., 2011).

Table 2: Table Assessing Genes Present.

Genes	*Exiguobacter sp.* (A1)	*A. soli* (A2)	*Enterobacter* sp. 638 (C)
rpoS	Present	Present	Present
flaA	Present	Not Present	Present
hfq	Not Present	Present	Present

Table 3: RAST Genome Annotation

Subsystem Category	*Exiguobacter sp.* (A1)	*A. Soli* (A2)	*Enterobacter* sp. 638 (C)
Motility and Chemotaxis	15	0	22
Stress Response	41	61	119
Virulence, Disease and Defense	46	35	64

IV. DISCUSSION

We have highlighted four key points in this study: the effects of culturing environmental isolates in LSMMG, potential causes for those effects, identification of novel species, and novel culturing methods to enrich for environmental bacteria.

LSMMG culturing affects bacteria differently, causing a wide range of responses in bacteria. Culturing in low-shear environments causes some bacteria to have an increase in final cell density. We saw this trend only in the *Exiguobacter* sp. (A1). The other two strains showed no change in growth when cultured in LSMMG or its control condition. This response could be due to *Exiguobacter* sp. (A1) containing genes that allow for optimal growth in low-shear environments compared to the control. We believe that there is an undescribed gene causing increased growth in low-shear conditions on *Exiguobacter* (A1) because there are no previously described genes associated with growth in LSMMG or spaceflight conditions were found on its genome (Table 2) (Castro, Nelman-Gonzalez, Nickerson, & Ott, 2011).

No other study has focused on culturing environmental isolates in LSMMG and includes co-culture studies. This prompted the question "Would we find a bacteria that preferred to grow in LSMMG and be the predominant strain in LSMMG co-culturing?". *Exiguobacter* sp. (A1) decreased in proportion over time in LSMMG in co-culture (Figure 6 & 7). Reasons for the decrease could be that there are fewer stress response and defense genes that allow for increased competition against other bacteria (Figure 14). Another explanation for the decrease in growth is that *Exiguobacter* (A1) has a slower rate of growth and reaches stationary phase at a lower optical density than the other

isolates (Figure 9). The slower rate of growth could lead to a decrease in its ability to compete with other bacteria. These are only a few potential reasons for the decrease in growth of strain A1 (*Exiguobacter* sp.) when co-cultured in LSMMG.

We found that *A. soli* (A2) was a predominant strain in both LSMMG and normal gravity conditions. *A. soli* (A2) contains previously described LSMMG-associated genes and stress response factors that could cause an increase in its ability to grow in both conditions (Table 2). Preferential growth in LSMMG conditions is not indicative of the potential outcome in co-culture (Figures 3, 6, & 8). LSMMG studies, in turn spaceflight studies, need to expand from monoculture to co-culture studies.

Two potential modification to this study are observing the change in growth with different medias that have higher nutrient concentrations and inoculation with equal cell numbers. One potential concern to this study is the media that was used. Previous literature have used medias that have higher nutrient concentrations (Luria broth) or more clinically relevant media (Modified Artificial Urine Media) which play a role in bacterial growth (Lynch, Mukundakrishnan, Benoit, Ayyaswamy, & Matin, 2006; Tucker et al., 2007; James W. Wilson, Ramamurthy, et al., 2002). The next change would be in the inoculation method. We found, based on an OD600 calibration curve (data not shown), there was a difference in number of cells that were inoculated in the RWV. We could minimize inoculum variation by utilizing the OD600 calibration curve to calculate cell numbers. Applying these modifications could lead to a change in the data generated and interpretation of our results.

We also found differing data when we obtained a growth curve of each isolate over 24 hours in both culturing conditions. *Exiguobacter* (A1) reached a higher optical

density in LSMMG conditions compared to its normal gravity control in monoculture (Figure 9). *A. soli* (A2) and *Enterobacter* sp. 638 (C) both decreased in optical density when cultured in LSMMG. This growth curve must be replicated because our control was done in a plate reader that does not accurately model our previous controls.

Motility of *Exiguobacter* (A1) and *A. soli* (A2) decreased when cultured in LSMMG (Figures 10). Interestingly, prolonged incubation after LSMMG culturing recovered bacterial motility (Figure 11). Potential causes for this response could be due to an acclimation period necessary after LSMMG culturing. Cells could be lagging in growth although, cells grow similarly when plated on normal agar after culturing in LSMMG compared to our control condition (anectodical evidence seen from our CFU/mL counts). Another potential cause is that laminar flow could deform flagella formation. *A. soli* (A2) does not show filamentous growth like *Streptomyces* when plated normally (Flärdh & Buttner, 2009). If there is a change in motility with LSMMG culturing, we know that the factor causing the change is laminar flow due to motility occurring in microgravity. Bacterial motility has been seen in spaceflight as a requirement for biofilm formation (Kim et al., 2013). Further studies are needed definitively assert the effect LSMMG culturing has on motility. To do this, gene expression, microscopy, and protein expression should all be performed. This change in motility leads to a question of the effect that laminar flow has on bacteria. We know that laminar flow occurs in different environments, like the brush border of the microvilli (Guo et al., 2000). Further exploration would be required to understand if laminar flow affects how bacteria colonize host cells, as well as if laminar flow causes a ubiquitous response to changes in motility.

Two novel species have been identified in this study, isolate A1 (*Exiguobacter*) and C (*Enterobacter* sp. 638). These bacteria have been a sequenced and submitted to NCBI. We propose isolate A1 to be Candidatus "*Exiguobacter astronautus"*. Phenotypic characterization still needs to be continued for these isolates.

The final outcome of this study is the use of LSMMG culturing as an enrichment method. We only identified one of our three isolates from genome sequencing and assembly (*A. soli*). Two of the isolates are believed to be novel. LSMMG culturing could be a bridge in providing the necessary conditions required for previously unculturable bacteria. Additional studies are needed in order to validate the application of this potential enrichment technique.

This is the first study that asks what the effects LSMMG culturing has on environmental isolates and acknowledges potential results of long-term spaceflight missions. Future studies are necessary to answer what caused the increased growth of *Exiguobacter* (A1) in LSMMG, the potential decrease in motility after LSMMG culturing, the potential of LSMMG enrichment to culture other novel bacteria. Additional studies like this one need to be performed on a larger proportion of environmental bacteria to fully understand the potential effects of long-term spaceflight studies, like the future Mars expedition.

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