2019

Cultivation in Long-term Simulated Microgravity is Detrimental to Biofilm Formation Ability and Pyocyanin Production of Pseudomonas aeruginosa PA14 Wild Type, ΔflgK, and ΔpelA

Linda M. Vu , '19

Cultivation in Long-term Simulated Microgravity is Detrimental to Biofilm Formation Ability and Pyocyanin Production of *Pseudomonas aeruginosa* PA14 Wild Type, Δ*flgK*, and Δ*pelA*

Linda Vu

Advisor: Dr. Amy Cheng Vollmer

Biology Honors Thesis

April 30, 2019

Swarthmore College

Abstract

Pseudomonas aeruginosa can attach to surfaces and form aggregates known as biofilms. It has been previously found that *P. aeruginosa* cultivated in space form thicker and structurally different biofilms than those grown in Earth gravity. The purpose of our study was to investigate how microgravity, simulated in a laboratory setting, would influence the biofilm formation abilities of *P. aeruginosa* PA14 wild type strain as well as mutants Δ*flgK* and Δ*pelA*. While Δ*flgK* is defective in the initialization of biofilm formation, Δ*pelA* is hindered in biofilm growth and maintenance. The bacteria were cultivated in a High Aspect Ratio Vessel (HARV) on a Rotary Cell Culture System (RCCS) that was used to simulate microgravity. For the Earth gravity control cultures, the RCCS was oriented horizontally and cultures were rotated in HARVS around a vertical axis. Incubation time was six days, and, in contrast to studies done by others, the bacteria were allowed to grow into stationary phase without replenishment of culture medium. At the end of the incubation time, the bacteria were extracted and cultured in a 24-well plate under identical conditions in Earth gravity. After 24 hours, the robustness of biofilm formation was compared by removing the soluble culture from the wells, staining with crystal violet, solubilizing the remaining biofilm, and quantifying spectrophotometrically. Additionally, the concentration of pyocyanin produced during cultivation was determined through extraction and spectrophotometry. Cultures grown under simulated microgravity had a lower biofilm formation ability as well as lower pyocyanin production compared to those grown under Earth gravity. This raises the possibility that *P. aeruginosa* experiencing nutritional starvation under long-term simulated microgravity may become less virulent.

Introduction

Numerous species of bacteria are capable of forming structural aggregates known as biofilms, a term popularized in part by Dr. William Costerton's research over three decades ago (1). Biofilm research was initially qualitative and employed the usage of light microscopes (1). The development of quantitative methods of biofilm analysis employing the usage of stains, such as crystal violet by Dr. O'Toole and Dr. Kolter (2), in the last two decades have enabled a more thorough study of biofilms (3). Additionally, the application of confocal microscopy to visualize biofilms have given us a deeper view of the architecture of these multicellular structures (4). The process of biofilm formation begins when a planktonic bacterium contacts a surface. Following contact, the bacterium will begin upregulating synthesis of the second messenger c-di-GMP, which in turn upregulates the production of adhesins for the purpose of attachment (5).

If the bacterium is flagellated, then during this process the flagellum will play an important role (6). Bacteria may encounter repulsive forces as they approach the surface, either due to environmental reasons, such as hydrodynamic forces, or from the chemical properties of the surface, as in the case of electrostatic repulsion (7). Flagellar motility is believed to aid the bacteria in surpassing such forces to reach the surface (8). Flagella also may directly aid in surface adhesion, but this secondary role is not universal to all species. An absence of flagella impaired bacterial adhesion in *Pseudomonas aeruginosa* biofilms but not in *Escherichia coli* biofilms (9).

There is some research suggesting that the flagellum's importance in biofilm formation is nutrient-dependent, as an impairment in flagellar motility compromised biofilm formation of *P. aeruginosa* in glucose medium but not in citrate medium (10). Therefore, flagellar motility is important in many circumstances of biofilm formation but is not strictly mandatory. This idea is supported by the finding that flagellar mutants may display a wild type phenotype in biofilm mass over an extended period of cultivation (11). It is also the case that not all bacterial species capable of forming a biofilm possess flagella, such as *Staphylococcus aureus*, which possesses a different kind of motility referred to as sliding motility (12, 13). The bacterium's pili are involved in initial adhesion to the surface, though this role has also been found to be nutrient-dependent (9, 10).

Following adhesion, the bacteria will begin the production and secretion of an extracellular matrix. This matrix is composed of polysaccharides, DNA, and proteins and gives the biofilms

structural support (5). The matrix enhances attachment to the surface (adhesion) as well as to other bacteria (cohesion). The biofilm will then grow through clonal division and aggregation with other previously planktonic bacteria, although clonal division may be a greater contributor to biofilm growth during initial biofilm formation (10).

The effect of microgravity on the ability to form biofilms is unclear. Kim *et al* (14) observed that *P. aeruginosa* PA14 cultivated in a fluid processing apparatus (FPA) in spaceflight for three days exhibited thicker biofilms than those grown under Earth gravity. However, these differences were only observed in a fluid processing apparatus with a solid insert that prevented gas exchange between the culture and the environment. When gas exchange was made possible using a gas exchange insert, the differences in biofilm thickness between bacteria cultivated in spaceflight and in Earth gravity disappeared.

Wang *et al* (15) suggested that microgravity, or even simulated microgravity, has different effects on biofilm formation depending on the species used and the method of cultivation. The authors found that under simulated microgravity in a rotating cell culture system (RCCS), biofilm formation was enhanced in *Klebsiella pneumoniae*. The simulated microgravity cultures produced twice as much biofilm as the Earth gravity cultures even though the simulated microgravity cultures had a slightly lower cell density, suggesting that biofilm formation ability was enhanced by exposure to simulated microgravity. On the contrary, the authors also noted that in another study by Li et al (16), simulated microgravity cultures of *Klebsiella pneumonia* (for which they did not describe specific cultivation conditions) did not display notable differences in biofilm formation compared to Earth gravity cultures. Perhaps this is not

surprising, as a notable characteristic of *Klebsiella* is that individual cells form a thick capsule (17). These thick capsules and, consequently, thicker biofilms of *Klebsiella* may have affected the bacterium's sensitivity to simulated microgravity. Rosenzweig et al (18) summarized findings by various studies conducted in spaceflight and in simulated microgravity and noted that *Escherichia coli* and *Pseudomonas aeruginosa* were all found to have increased biofilm formation under simulated microgravity.

It appears that there may be some difficulty in determining whether microgravity has a clear, universal impact on the process of biofilm formation. Nonetheless, there is still value researching the connection between microgravity and biofilm formation.

Studies have shown that astronauts' immune systems are compromised during spaceflight (19-21). Astronauts experience physical stress, as indicated by an upregulation of genes involved in DNA repair (22). Leukocyte and T-cell function becomes impaired, leading to inadequate responses against immunological threats (21). Viruses reactivate, and the ratio of *Firmicutes* to *Bacteroidetes* in the gut microbiome, which has been linked to obesity, is increased during spaceflight, although the authors mention that the F/B ratios of their human spaceflight test subject never exceeded the range found in healthy individuals (21-24). The possibility exists that opportunistic pathogens, including those capable of forming biofilms, may pose a greater risk to humans during spaceflight and in low-gravity environments. As the prospects of commercial spaceflight as well as long-term manned missions gain popularity, it is important to study the role of gravity and its influence on bacterial growth and virulence.

One bacterial species of interest is *Pseudomonas aeruginosa*, a biofilm-forming gram negative bacterium (3). It is a clinically relevant species, as it is the leading cause of death for patients with cystic fibrosis, a genetic defect that results in *P. aeruginosa* infections primarily affecting the patients' pulmonary systems (25, 26). In addition, this bacterium has infected some astronauts in the past (27), so there is clinical concern in whether this bacterium becomes more aggressive in biofilm formation and virulence in space.

In particular, *P. aeruginosa* produces pyocyanin, a characteristically blue-green compound closely connected to the bacterium's virulence as well as its biofilm formation, as the compound was linked to the secretion of extracellular DNA (eDNA) into the biofilm matrix (28, 29). The compound plays a key role in the infections of cystic fibrosis patients and other host organisms and was reported to have anti-microbial activity (28-30). On its own, the compound was found capable of inducing symptoms of cystic fibrosis in otherwise healthy mice (28, 31). Pyocyanin's virulence is in part due to its ability to generate oxidative stress through the formation of reactive oxygen species as well as its ability to participate in redox reactions (31).

McLean et al. (32) first demonstrated that *P.* aeruginosa PA01 was capable of forming a biofilm during spaceflight. Crabbé et al (33, 34) found increased expression of genes involved in anaerobic metabolism in PA01 when cultivated in spaceflight, using an FPA, and in simulated microgravity, using an RCCS. Differences in gene expression were found between spaceflight and simulated microgravity cultures, and the authors suggested this was due to differences in oxygen availability between the FPA and the vessels used for the RCCS. Finally, as mentioned

before, Kim et al (14) found that *P. aeruginosa* PA14 biofilm formation was enhanced during spaceflight, although only during anaerobic conditions.

Despite these various studies, there is a lack of thorough research on the effect of longer-term exposure of *P. aeruginosa* in simulated microgravity (also referred to as low-shear modeled microgravity) and biofilm formation. The McLean et al study (32) did not compare biofilm formation to Earth gravity controls, and neither did the Crabbe et al (34) investigations in simulated microgravity study, which only compared cultures grown in low shear and high shear environments. In addition, the authors' spaceflight study primarily focused on gene expression, rather than biofilm formation. Most prior studies in simulated microgravity used a 24-hour incubation period, and the few studies looking at long-term cultivation in simulated microgravity maintained their cultures in log phase growth by adding fresh media and transferring cultures to a new vessel at periodic intervals. We sought to examine the effect of longer-term exposure to simulated microgravity during stationary phase growth on *P. aeruginosa* PA14's biofilm formation abilities.

Insufficient research exists on the effects of simulated microgravity on mutants deficient in biofilm formation. We decided to pursue how simulated microgravity affects biofilm formation abilities in Δ*flgK* and Δ*pelA,* which would allow us to identify the stage of biofilm formation at which changes in environmental gravity would have the greatest impact. We chose to examine biofilm formation ability rather than direct biofilm formation during cultivation in simulated microgravity in order to investigate only the long-term effects caused by simulated microgravity on microbial function. Simulated microgravity may confer a direct physical benefit or hindrance

to biofilm formation, but we are only concerned with the changes induced within the bacterium. As Tirumalai et al. (35) state, once simulated microgravity cultures are returned to Earth gravity, temporary phenotypic changes should disappear while genetic-based changes will remain.

The mutants used in our study were previously characterized by O'Toole and Kolter (6) and Friedman and Kolter (36), respectively: Δ*flgK* mutant lacks a hook protein, and as a result, does not express a flagellum. Without motility, it is impaired in the first stage of biofilm formation (6); Δ*pelA* is unable to produce pellicle polysaccharide (Pel), the only polysaccharide present in PA14's extracellular matrix (37, 38). This mutant is able to colonize a surface but is impaired in self-aggregation and stable biofilm maintenance (39).

We hypothesized that simulated microgravity would increase the repulsive forces against the bacteria, hindering the bacterium's attachment to a surface. As a result, the bacterium would be compelled to upregulate genes involved in surface adhesion. We predict that overall biofilm formation ability would become enhanced in *P. aeruginosa* PA14 wild type after exposure to simulated microgravity compared to Earth gravity cultures due to an enhancement in adhesin and matrix production. As Δ*flgK* is impaired in surface adhesion, its biofilm formation under simulated microgravity would be impaired, but its overall biofilm formation abilities are expected to be enhanced after removal from simulated microgravity due to an upregulation in adhesion and matrix production. In contrast, Δ*pelA* is expected to be unaffected by simulated microgravity compared to Earth gravity conditions as its biofilm formation is already impaired by its deficient matrix production.

We also examined the effect of long-term exposure to simulated microgravity during stationary phase growth on pyocyanin production in *P. aeruginosa* PA14, Δ*flgK, and* Δ*pelA*. Pyocyanin itself has many industrial and medical uses, from being utilized as a component of microbial fuel cells to potential applications in cancer treatment, though it is commercially expensive compound (40). It is worthwhile to determine if simulated microgravity could be utilized to enhance production of secondary metabolites in order to reduce costs of pyocyanin production. Such findings could have implications for antibiotic production as well. Prior studies have found an enhancement of antibiotic production in *Streptomyces plicatus* after cultivation on the International Space Station and in *Streptomyces hygroscopicus* after cultivation in simulated microgravity on an RCCS (41), whereas a prior study found that genes involved in pyocyanin biosynthesis were downregulated when *P. aeruginosa* PA01 was cultivated in simulated microgravity (30). We sought to confirm these findings and to determine if simulated microgravity would differently affect pyocyanin biosynthesis in the mutants.

Materials and Methods

Bacterial Strains and Cultivation

The three strains were used (and kindly provided by G. O'Toole): *Pseudomonas aeruginosa* PA14, Δ*flgK*, previously characterized by O'Toole and Kolter (6), and Δ*pelA,* described by Friedman and Kolter (6, 36). Overnight cultures were prepared in 6mL Luria-Bertani medium (LB) (BD/Difco Laboratories, Sparks, MD) and cultivated in a shaking incubator at 37°C, at ~200 rpm. The overnight culture was diluted 1:100 with LB and then 10mL were distributed into a 10mL single-use, disposable High Aspect Ratio Vessel (HARV) (Synthecon Incorporated, Houston, TX) for the simulated microgravity cultures. Gas diffusion was permitted through

permeable membranes on the exterior of the HARV. Each experiment using these vessels was conducted in triplicate. For reasons of simplicity, we will refer to cultures grown in simulated microgravity conditions as 'simulated microgravity cultures' and cultures grown in normal, Earth gravity conditions as 'Earth gravity cultures'.

Cultivation in microgravity

The HARVs were rotated at 30 rpm on a Rotating Cell Culture System bioreactor (RCCS-4D) (Synthecon Incorporated, Houston, TX), positioned in the normal vertical orientation inside a stationary 37°C incubator (Figure 1A). Several prior RCCS studies conducted by other investigators employed a rotational speed of 25 rpm. However, the continuous cultivation in those studies did not exceed a period of 24 hours (15, 30, 34). In order to accommodate the increasing density, and possible aggregation, of cultures over time, a higher rotational speed of 30 rpm was selected. The incubator was kept humid, in order to prevent evaporation of the medium and the consequent formation of air bubbles within the vessels, by the placement of trays of water inside the incubator. Cultures were allowed to go into stationary phase growth and incubated continuously for six days. Cultures were never replenished with fresh media, unlike what was done in other studies (15, 42, 43). Biofilm production ability and cell density were quantified as described below.

Preparation of Earth Gravity Controls

Earth gravity controls were prepared simultaneously with the simulated microgravity cultures in an identical procedure. Due to budget limitations, a second RCCS was not available for simultaneous cultivation of the control cultures, so standard glass culture tubes were used

instead: 10mL of the diluted starter culture were each distributed into triplicate sterile glass culture tubes and incubated in a shaking incubator ~200 rpm.

To determine if the type of vessel used for cultivation would affect biofilm formation or cell density of the controls, overnight cultures of wild type PA14, Δ*flgK*, and Δ*pelA* were prepared and diluted 1:100 in LB. 10mL of each strain was distributed to a HARV and a glass tube. The HARVs were rotated at 30 rpm on the RCCS-4D, positioned horizontally inside the 37°C incubator order to allow for Earth gravity conditions (Figure 1B). The glass culture tubes were cultivated in the shaking incubator at ~200 rpm as described above. After six days of incubation, biofilm production ability and cell density were quantified.

Quantification of Biofilm Production

To evaluate biofilm formation abilities, each of the 6-day old cultures were each diluted 1:100 in LB, and 1mL portions were distributed to separate wells in a sterile polystyrene 24-well plate (Corning Incorporated, Corning, NY). The plate was incubated in Earth gravity at 37°C for 24 hours. The wells were emptied, and the remaining biofilms were stained in 1% crystal violet (Sigma-Aldrich, St. Louis, MO) diluted with water, rinsed, and solubilized in 1mL 95% ethanol, as previously described by O'Toole and Kolter (2). The solubilized biofilm was then diluted 1:10 in 6mL ethanol to be in the range of spectrophotometric detection at 540nm.

The cell density of the cultures was measured through serial 1:10 dilutions in sterile 0.8% saline solution and spot plating (10μL) on LB agar (BD/Difco Laboratories, Sparks, MD). Individual

serial dilution plates were made for additional verification (100μL). Plates were incubated at 37°C under Earth gravity for 24 hours before reading.

Quantification of Pyocyanin Pigment

A culture of wild-type, Δ*flgK*, and Δ*pelA* were cultivated in an HARV on the RCCS in both simulated microgravity and Earth gravity conditions as described above. After six days of incubation, pyocyanin was extracted from the cultures as described by Essar et al (44) with modifications. 3mL of chloroform, followed by 1mL 0.2M HCl, were added to 5mL of simulated microgravity cultures. The pink layer formed by the addition of HCl, approximately 1mL, was removed for spectroscopic analysis. As there was limited culture available from the HARV Earth gravity controls, 1.5mL of sample, 0.9mL chloroform, and 0.3mL HCl were used. The extracted pink layer was diluted to 1mL with HCl. The absorbance at 520nm was recorded using a spectrophotometer. The concentration of the pyocyanin pigment was calculated via Beer's Law with the extinction coefficient = 17.072 $L\ mol^{-1}\ cm^{-1}$ (44).

Results

Phenotypic differences exist between simulated microgravity and Earth gravity cultures

Simulated microgravity cultures for all strains consistently developed a thick, sticky, mucus-like character (Figure 2A). They were of a dark green color. Earth gravity cultures, in comparison, did not develop such a dramatic mucus-like character and were of a browner color (Figure 2B). Though the cultures were sticky from the production of biofilm matrix, they were never as dense (in terms of cfu/ml) as the simulated microgravity cultures. Difficulties were experienced in pipetting small volumes from the simulated microgravity cultures but less so in the Earth gravity

cultures. After the incubation period, the final volume of cultures grown in glass culture tubes under Earth gravity was close to the initial 10mL, but the simulated microgravity cultures condensed into a final volume of around 6mL. The volume of the HARV Earth gravity cultures condensed as well.

Six days was the limit of continuous long-term incubation in simulated microgravity

Six days was determined to be the optimal incubation time after a series of timepoint testing experiments. Over longer incubation times, the HARV cultures were more susceptible to the formation of air bubbles, despite the humidification of the incubator. It appeared likely that the bubbles were caused not from evaporation, but from bacterial waste gases becoming trapped in culture due to the development of a mucus-like character. Six days was the longest possible incubation time before the appearance of air bubbles, which would disrupt the simulation of microgravity. Because the vessels were single-use, no intermediate timepoints were taken once we settled on the 6-day incubation interval (data not shown).

Cultivation in HARV may affect biofilm formation ability, even in Earth Gravity

It was discovered that cultivation in HARVS led to greater biofilm formation ability than cultivation in culture tubes (Figure 3). There was not a significant difference between Earth gravity cultures grown in a horizontally positioned HARV compared to a those cultured in a culture tube for both biofilm formation ($p = 0.5428$) or cell density ($p = 0.4968$) of the wild type strain. Despite this finding, there was a significant difference in biofilm formation ($p = 0.0001$) and cell density ($p = 0.0001$) for Δ*flgK*. Culture tube cultures had a greater OD_{540} by 0.710 and a greater cell density by 1.56×10^8 cfu/mL. For Δ*pelA*, there was not a statistically significant

difference in biofilm formation ($p = 0.7702$). Glass culture tube cultures had an insignificantly greater OD_{540} of 0.050. There was a significant difference in cell density ($p = 0.0014$), as the culture tube cultures had a greater cell density by $1.98x10^{8}$ cfu/mL. Moving forward, Δ*flgK* and Δ*pelA* simulated microgravity cultures will only be compared to Earth gravity controls cultivated in HARVs. Comparisons between some experiments are limited by the fact that some of the simulated microgravity cultures and the HARV Earth Gravity cultures were prepared from different starter culture inocula. In those comparisons, only OD_{540} per cfu/mL and pigment concentration per cfu/mL will be considered. For PA14 wild type, only HARV Earth gravity cultures will be considered in comparisons against the simulated microgravity cultures to maintain consistency.

It should be noted that even though the glass culture tube cultures had a greater OD_{540} and cell density for Δ*flgK,* when calculating for individual biofilm formation ability of bacteria within the cultures, all strains experienced a greater OD_{540} per cfu/mL after growth in an HARV compared to the glass culture tube cultures (Figure 3).

Biofilm formation ability is hindered in PA14 wild type after cultivation in simulated microgravity

PA14 wild type cultures subjected to simulated microgravity were deficient in biofilm formation ability compared to HARV Earth gravity cultures (Table 1). Simulated microgravity cultures produced $6.13x10^{-9}$ OD_{540} per cfu/mL while HARV Earth Gravity cultures produced $8.22x10^{-8}$ OD_{540} per cfu/mL.

Biofilm formation ability is hindered in Δ*flgK* after cultivation in simulated microgravity

Δ*flgK* cultures subjected to simulated microgravity were in deficient biofilm formation ability compared to Δ*flgK* HARV Earth gravity cultures by three magnitudes (Table 2). Simulated microgravity cultures produced 5.80×10^{-10} OD_{540} per cfu/mL while HARV Earth Gravity cultures produced 1.88×10^{-7} OD_{540} per cfu/mL.

Simulated Microgravity is less detrimental to Δ*pelA* biofilm formation

Δ*pelA* simulated microgravity cultures formed less biofilm than HARV Earth gravity cultures, but the difference was about 7.23×10^{-9} OD_{540} per cfu/mL, less than one magnitude (Table 3). Simulated microgravity cultures produced 1.85×10^{-8} OD_{540} per cfu/mL while HARV Earth gravity cultures produced 2.58×10^{-8} OD_{540} per cfu/mL.

Pigment Production

Pyocyanin concentration was determined for simulated microgravity and Earth gravity samples (Table 4). For all strains, simulated microgravity cultures produced less pyocyanin than Earth gravity cultures. However, it was discovered that cultivation in the HARV under Earth gravity conferred over a two-fold enhancement in production compared to cultivation in the culture tube under Earth gravity.

Discussion

Longer-term (6-day) cultivation in simulated microgravity impaired biofilm formation in wild type and Δ*flgK*. Δ*pelA* simulated microgravity cultures appeared to produce less biofilm than Earth gravity cultures, but the difference is less pronounced (Figure 4). More data will need to be

collected before the significance of this difference for Δ*pelA* can be determined. Regardless, it is apparent that simulated microgravity had a greater detrimental effect on the wild type and Δ*flgK* strain, which does not support our hypothesis. Even after returning to Earth gravity, the simulated microgravity cultures' phenotype remained distinct from the Earth gravity cultures' (at least for the length of our experiment), suggesting that long-term epigenetic changes had occurred within the bacterium during the six-day cultivation period.

Oxygen availability affects biofilm development (14). While the HARVs contain permeable membranes, biofilm formation on the membrane during cultivation will reduce oxygen availability (15). To gauge general biofilm growth within the HARV, a HARV containing Δ*pelA* was stained with crystal violet after the culture was removed following cultivation. The entire membrane was stained (Figure 5), supporting the notion that biofilm had grown along the membrane during cultivation. This may have led to a reduction in oxygen availability. While *P. aeruginosa* PA14 cultivated under anaerobic conditions in spaceflight experienced greater biofilm formation, our cultures may have been hindered by the depletion of oxygen because medium was not replenished during our 6-day cultivation. The simulated microgravity cultures may have experienced starvation due to depletion of nutritional sources, and the limitation of oxygen may have led to additional stress that encouraged the downregulation of genes related to growth and biofilm formation, which would explain the persistent impairment in biofilm formation observed for the simulated microgravity cultures in the 24-well plate despite having been removed from simulated microgravity.

On the contrary, this argument fails to explain why HARV Earth gravity cultures had a two-fold enhancement, rather than a deficiency, in biofilm formation compared to those grown in culture tubes under Earth gravity that were prepared from the same starter culture inocula (Figure 3). In fact, all strains experienced better biofilm formation abilities after growth in an HARV under Earth gravity than in a culture tube. This argument also does not explain why the simulated microgravity cultures were deficient in biofilm formation by one magnitude compared to HARV Earth gravity cultures, which should have also experienced a similar degree of oxygen depletion under this claim. Simulated microgravity itself likely led to a detrimental effect on biofilm formation, as the simulated microgravity and HARV Earth gravity cultures should have been cultivated in otherwise near identical conditions. In this case, it is possible that the simulated microgravity created a stress upon the cultures that, in conjunction with the stress of nutrient depletion, led to a lasting downregulation of biofilm formation.

The alternative explanation is that the differences in starter culture inocula inherently influenced biofilm formation ability and that we were unable to remove these differences by taking cell density into account when calculating biofilm formation ability. The usage of a second HARV for simultaneous cultivation of simulated microgravity and Earth gravity cultures prepared from the same starter culture inoculum could clear up such concern, but again, this was not possible due to budgetary limitations. Regardless, it cannot be denied that biofilm formation ability is lower in simulated microgravity cultures amongst most strains. It is unlikely that differences in starter culture inocula alone are able to explain such a trend.

Of the three strains, Δ*flgK* experienced the most impairment in biofilm formation after cultivation in simulated microgravity with a three magnitude difference compared to HARV Earth gravity cultures, while Δ*pelA* experienced the least with a less than one magnitude difference (Figure 6). This suggests that absence of motility is detrimental to biofilm formation after exposure to simulated microgravity while absence of Pel production is comparatively less influential. It is possible that non-motile Δ*flgK* bacteria are more susceptible to stress caused by the low-shear forces present in simulated microgravity than PA14 wild type, leading to a greater downregulation of genes involved in biofilm formation in comparison. Thus, motility may mitigate the detrimental effect of simulated microgravity on the bacterium. It is important to differentiate the low-shear forces experienced under microgravity from the forces caused by the cycling of culture experienced in the HARV that was present in the Earth gravity conditions.

Δ*pelA* fared better than the wild type under simulated microgravity, suggesting that Pel production may be connected to detrimental effects of simulated microgravity. Potentially, this may indicate that gravity plays an important role during Pel production or in the formation of the biofilm matrix under regular Earth gravity conditions and that a change to a weaker gravity environment led to lasting limitations in the wild type and Δ*flgK*'s Pel production abilities. This could result in the expression of the Δ*pelA* phenotype by the wild type and Δ*flgK* strains, which would explain the weakened biofilm formation ability. Alternatively, it is possible that Pel production has a directly detrimental effect to the bacterium under simulated microgravity. Pel production may be linked to the mucus-like viscosity observed in the simulated microgravity cultures. Over time, the wild type and Δ*flgK* cultures may be forming larger clumps that are more susceptible to shear stress. The accumulation of Pel may be contributing to oxygen

limitation as well. These additional stressors, absent in the Pel deficient Δ*pelA*, may have led to a downregulation of genes involved in biofilm formation in the wild type and Δ*flgK* cultures. It is not possible to distinguish the two possibilities without a more in-depth genetic analysis with specific focus on genes involved in the formation of the biofilm matrix.

Simulated microgravity hindered pyocyanin production in all strains, consistent with the study by Sheet et al (30). Interestingly, the Earth gravity wild type cultures had the greatest pyocyanin production of the three strains, but under simulated microgravity, the wild type had the lowest pyocyanin production. Δ*flgK* had the lowest levels of pyocyanin production under Earth gravity but the highest under simulated microgravity. It appears that simulated microgravity hinders pyocyanin production differentially between strains.

Δ*flgK* was the most detrimentally affected by simulated microgravity in terms of biofilm formation ability, but wild type was the most detrimentally affected for pyocyanin production. Considering that pyocyanin is involved in eDNA release as previously mentioned (29), it is reasonable to expect that the strain most inhibited in biofilm formation would have also been the most inhibited in pyocyanin production due to its involvement in the formation of the biofilm matrix. The results suggest that the mechanisms of biofilm formation abilities and pyocyanin production are not directly linked as there is a differential effect on them by simulated microgravity. The results also suggest that the aspect of biofilm formation susceptible to simulated microgravity is not directly connected to eDNA release.

For the wild type strain, cultivation in an HARV under Earth gravity resulted in a two-fold increase in pyocyanin production compared those grown in a culture tube, and it is unknown whether this is linked to the observed increase in biofilm formation ability in the same HARV Earth gravity culture. Δ*flgK* and Δ*pelA* grown in culture tubes under Earth gravity were not available for pyocyanin analysis, but it is possible that an RCCS under Earth gravity conditions may be used to enhance pyocyanin production in *P. aeruginosa*.

In summary, it was found that long-term simulated microgravity has a detrimental effect on biofilm formation ability and pyocyanin production for *P. aeruginosa* PA14, *ΔflgK*, and *ΔpelA*, although the degree of this effect varies upon the strain. This may indicate that bacteria experiencing long-term microgravity while in stationary phase growth will become less virulent as long as their nutrients are not replenished and they return to log phase growth. The exact mechanisms behind the impairment of biofilm formation after cultivation in simulated microgravity is unknown. Tests on gene expression and stress responses of simulated microgravity cultures may prove enlightening. Due to time constraints, there were inadequate data for Δ*flgK* and Δ*pelA* HARV Earth gravity cultures to conduct a test for significance in biofilm formation ability. There were also inadequate data for the testing of pyocyanin production. Moving forward, it will be necessary to revisit these experiments to confirm the results. In spite of these concerns, our results suggested a promising potential for the usage of an RCCS under Earth gravity conditions for enhancing pyocyanin pigment and, potentially, other secondary metabolites such as antibiotics.

Table 1. Biofilm formation from *P. aeruginosa* PA14 cultured under different gravity conditions

Strain: PA14 Wild Type		
Gravity Condition	Simulated Microgravity[a]	Earth Gravity[b]
Vessel	HARV	HARV
OD_{540}[c]	0.490	1.423
Cfu/mL[d]	$1.04x10^{8}$	$1.73x10^{7}$
OD_{540} per cfu/mL[e]	$6.13x10^{-9}$	$8.22x10^{-8}$

[a]Simulated microgravity cultures and [b]HARV Earth gravity cultures originated from different starter inocula.

Measurements for [c]biofilm formation (absorption at 540nm), [d]cell density (cfu/mL), and [e]specific biofilm formation ability as defined by OD_{540} per cfu/mL for *Pseudomonas aeruginosa* PA14 wild type.

Table 2. Biofilm formation from *P. aeruginosa* Δ*flgK* cultured under different gravity conditions

Strain: Δ*flgK*		
Gravity Condition	Simulated Microgravity[a]	Earth Gravity[b]
Vessel	HARV	HARV
OD_{540}	1.170	0.503
Cfu/mL	$3.00x10^{9}$	$2.68x10^{6}$
OD_{540} per cfu/mL	$5.81x10^{-10}$	$1.88x10^{-7}$

[a]Simulated microgravity and [b]HARV Earth gravity Δ*flgK* cultures originated from different starter culture inocula.

Table 3. Biofilm formation from *P. aeruginosa* Δ*pelA* cultured under different gravity conditions

Strain: Δ*pelA*		
Gravity Condition	Simulated Microgravity[a]	Earth Gravity[b]
Vessel	HARV	HARV
OD_{540}	0.490	1.423
Cfu/mL	$1.04x10^{8}$	$1.73x10^{7}$
OD_{540} per cfu/mL	$6.13x10^{-9}$	$8.22x10^{-8}$

[a]Simulated microgravity and [b]HARV Earth gravity Δ*pelA* cultures originated from different starter culture inocula.

Table 4. Pyocyanin production by P. aeruginosa cultured under different gravity conditions

Gravity Condition	Earth Gravity				Simulated Microgravity		
Strain	PA14 Wild Type		Δ*flgK*	Δ*pelA*	PA14 Wild Type	Δ*flgK*	Δ*pelA*
Vessel	Culture Tube	HARV	HARV	HARV	HARV	HARV	HARV
OD_{520}	0.030	0.052	0.071	0.087	0.221	0.450	0.110
Dilution factor	3.33	3.33	3.33	1	1	1	2
[Pyocyanin] (mol/L)[a]	0.0059	0.0101	0.0138	0.0051	0.0129	0.0264	0.0129
[Pyocyanin] (mol/L) per cfu/mL	1.90×10^{-10}	5.86×10^{-10}	5.18×10^{-9}	2.51×10^{-10}	1.25×10^{-10}	8.80×10^{-12}	2.47×10^{-10}

[a]Pyocyanin concentration was calculated from OD_{520} using Beer's Law. Extinction coefficient is 17.072 L mol^{-1} cm^{-1} (44) and path length difference was 1 cm.

(A) (B)

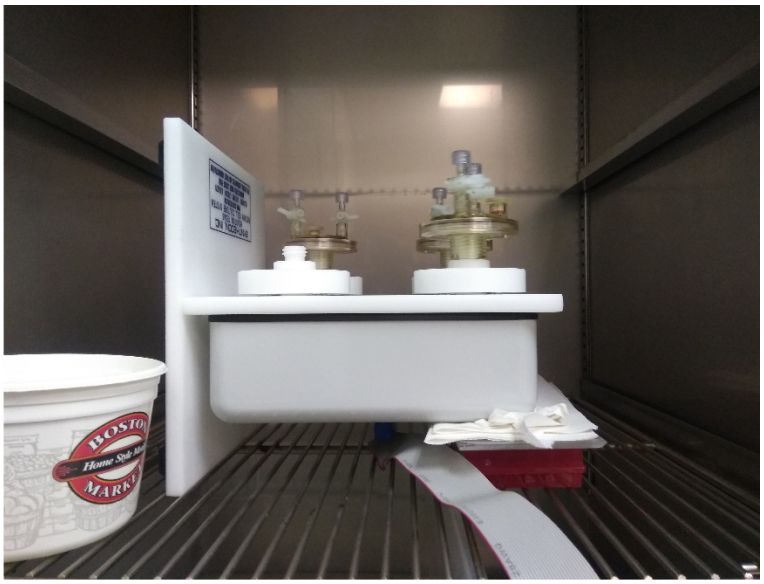

Figure 1 RCCS orientation for different gravity conditions inside the laboratory incubator.

1a. Experimental set-up of RCCS for simulated microgravity cultures. For scale, the compartment within the HARV used for cultivation is approximately 4.2cm in diameter.

1b. Experimental set-up of RCCS for HARV Earth gravity cultures.

(A) (B)

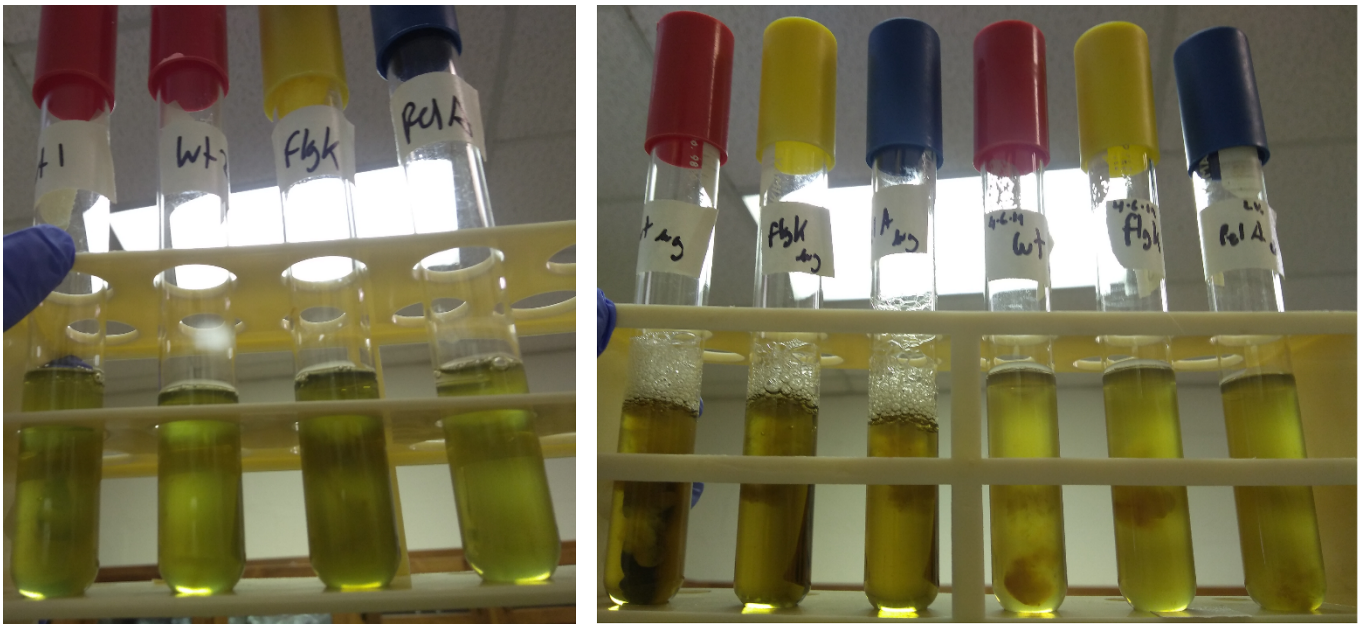

Figure 2. Appearance of cultures after culture in different gravity conditions

2a. Simulated microgravity cultures transferred from the HARV after 6-day incubation. Strains are as follows from left to right: wild type 1, wild type 2 (prepared in case issues were encountered with the first wild type culture; was never used in data collection), Δ*flgK*, and Δ*pelA*. Cultures appeared to have a greenish tint and were generally dense.

2b. Earth gravity cultures prepared when comparing growth in an HARV versus in a glass culture tube. Cultures are as follows: HARV wild type, HARV Δ*flgK*, HARV Δ*pelA,* culture tube wild type, culture tube Δ*flgK,* culture tube Δ*pelA*. HARV Earth gravity cultures appeared to have a browner tint. Cultures from the culture tubes had a less intense, yellow tint and had a low viscosity.

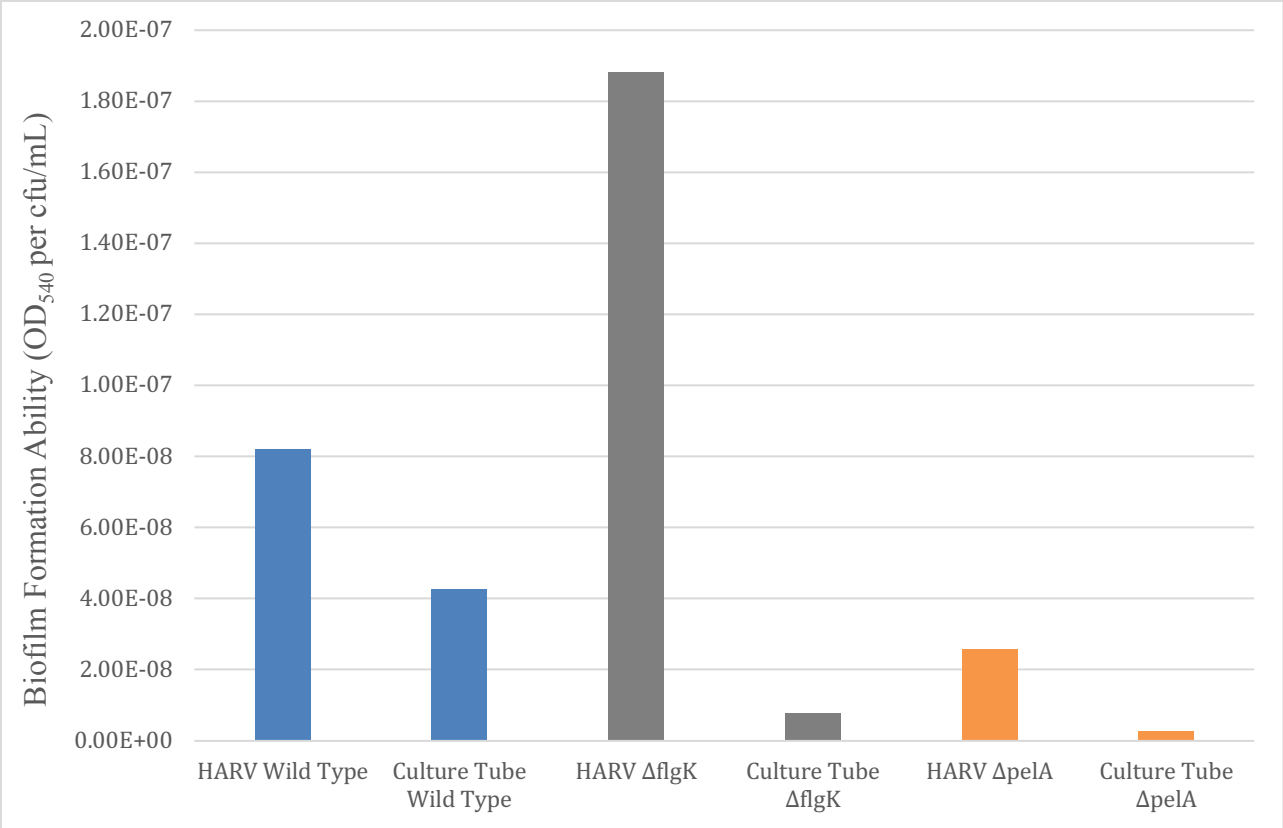

Figure 3. Comparison of biofilm formation ability (OD_{540} per cfu/mL) of wild type, Δ*flgK,* and Δ*pelA* after cultivation under Earth gravity in HARVs versus glass culture tubes. For all strains, there was a greater biofilm formation ability after cultivation in an HARV. Not enough data were available to determine error on these values.

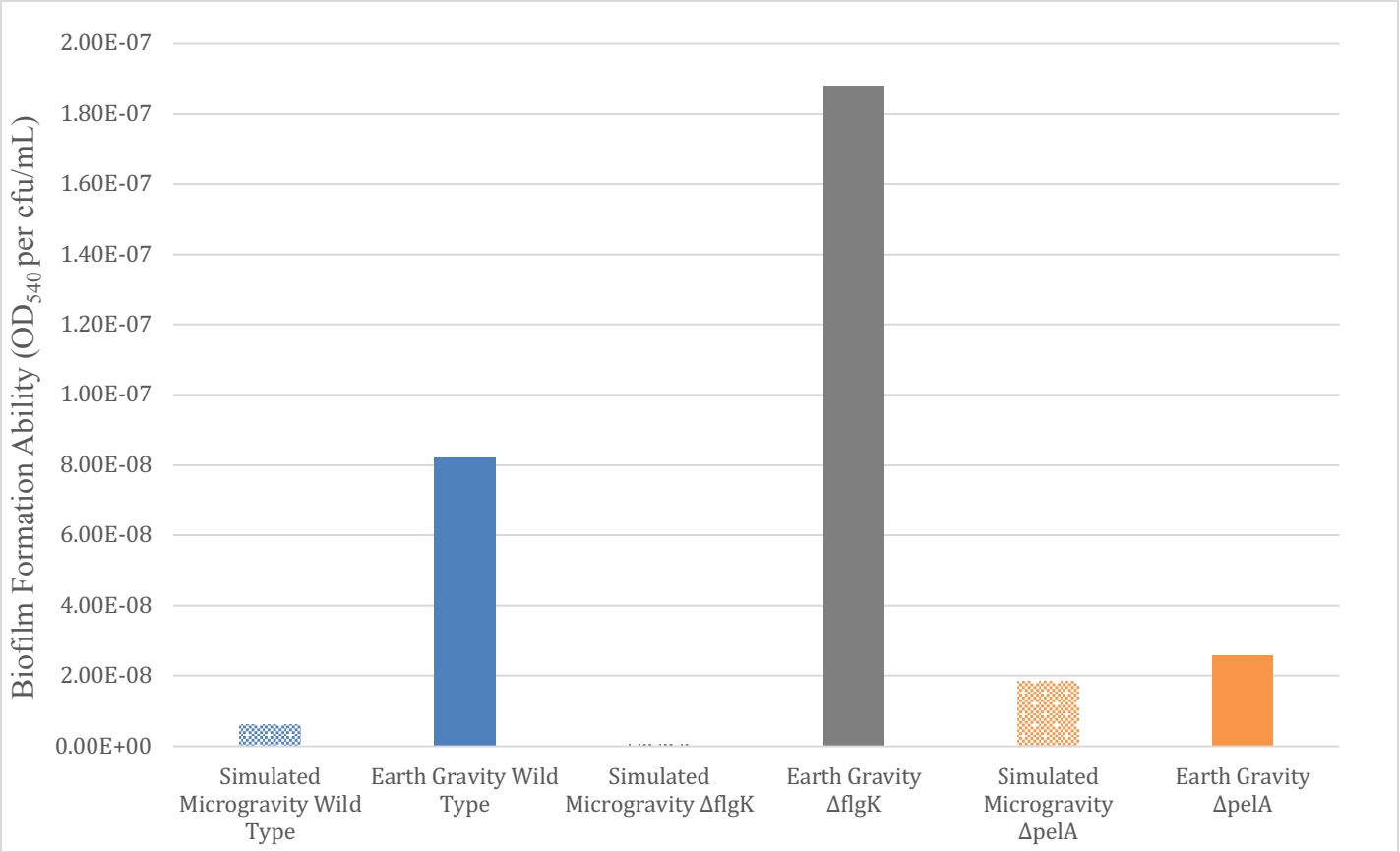

Figure 4. A comparison of biofilm formation ability (OD_{540} per cfu/mL) of wild type, Δ*flgK,* and Δ*pelA* cultivated under simulated microgravity versus cultivation under Earth gravity in an HARV. Cultivation period was 6 days and culture media were not replenished. Earth gravity cultures showed a greater biofilm formation ability for wild type and Δ*flgK*. Δ*pelA* Earth gravity also demonstrated a greater biofilm formation ability, but not enough data was available to determine if this result was significant. Not enough data were available to determine error on these values.

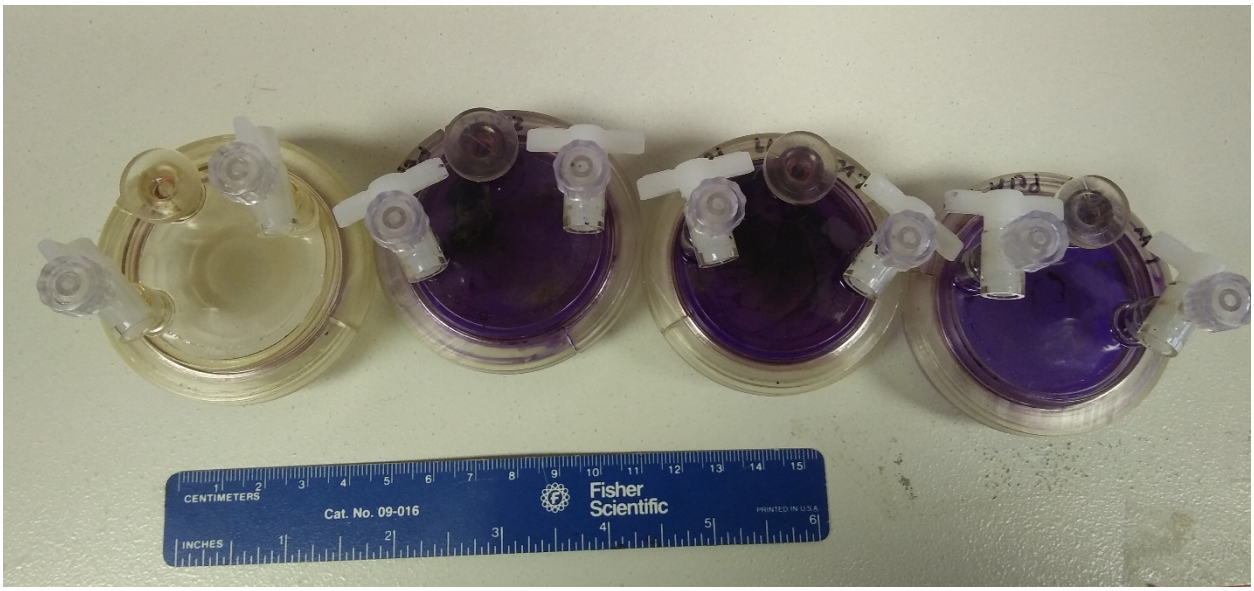

Figure 5. Comparison of a clean, unused and unstained HARV (left) against three that were used for the cultivation of Δ*pelA* under simulated microgravity. Culture had been removed from the three HARVs prior to staining with crystal violet and rinsing with deionized water. After six-days of incubation, a biofilm appears to have formed against the permeable membrane on the exterior of the HARV, supporting the argument for oxygen limitation during the later stages of the incubation period.

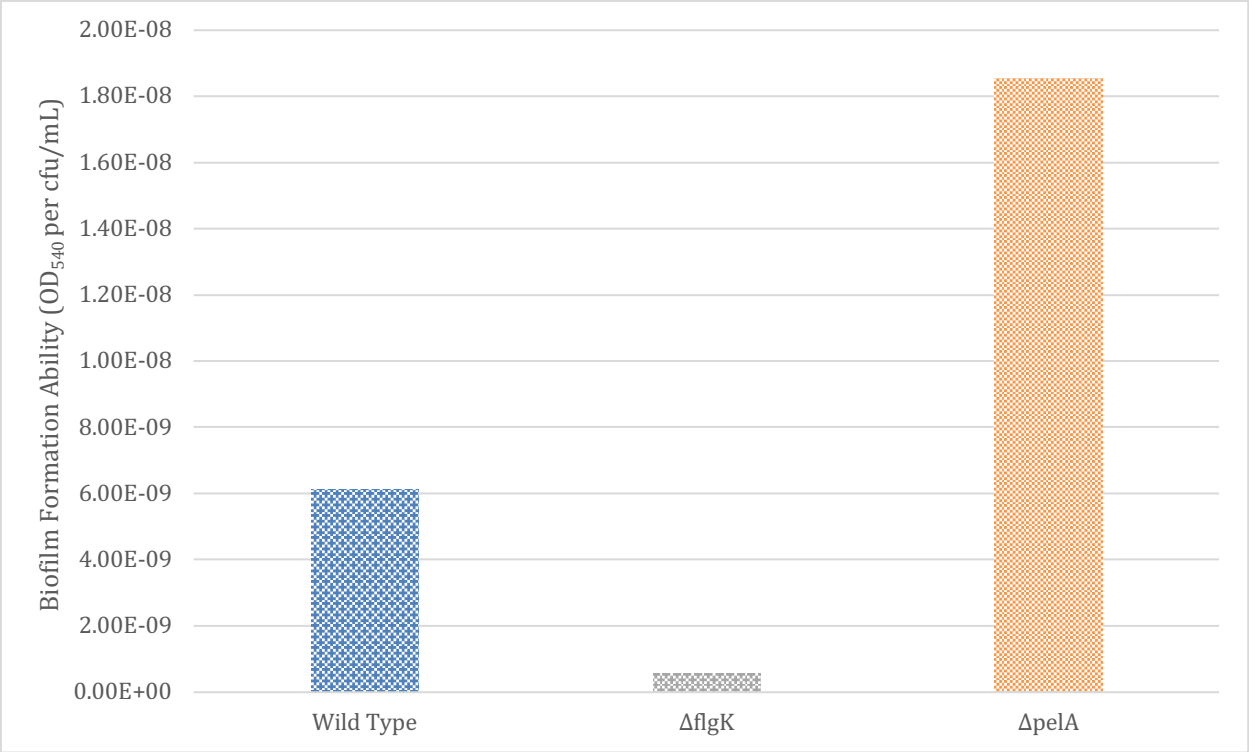

Figure 6. A comparison of biofilm formation ability (OD_{540} per cfu/mL) between wild type, Δ*flgK,* and Δ*pelA* cultivated under simulated. , Δ*flgK* fared the worst under simulated microgravity as its biofilm formation ability was the most detrimentally affected. Δ*pelA* was the least detrimentally affected. Not enough data were available to determine error on these values.

Acknowledgements

We would like to thank the following people whose contributions have made this study possible: laboratory manager Gwen Kannapel and members of the Vollmer lab: Calla Bush, Evangeline Adjei-Danquah, and Ryan Stanton for their technical assistance within the lab as well as moral support; Dr. George O'Toole for supplying the mutants; the other biology honors students: William Colgan, Serena Sung-Clark, Anthony Velleca as well as their mentors: Dr. Dawn Carone, Dr. Brad Davidson, and Dr. Liz Vallen for their consultation during this project; Laela Ezra and Julia Morriss for their assistance with collecting pyocyanin absorption data; and the Francis Velay Womens' Science Research Fellowship Program and the Swarthmore College Biology Department for funding this project.

Literature Cited

1. Hoiby N. 2014. A personal history of research on microbial biofilms and biofilm infections. Pathogens and Disease 70:205-211.
2. O'Toole GA, Kolter R. 1998. Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Molecular Microbiology 28:449-461.
3. O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm formation as microbial development. Annual Review of Microbiology 54:49-79.
4. Schlafer S, Meyer RL. 2017. Confocal microscopy imaging of the biofilm matrix. Journal of Microbiological Methods 138:50-59.
5. Tolker-Nielsen T. 2015. Biofilm Development, p 51-66. *In* Ghannoum M, Parsek M, Whiteley M, Mukherjee PK (ed), Microbial Biofilms, 2nd ed. ASM Press, Washington DC, USA.
6. O'Toole GA, Kolter R. 1998. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Molecular Microbiology 30:295-304.
7. Dunne WM. 2002. Bacterial adhesion: seen any good biofilms lately? Clinical microbiology reviews 15:155-166.
8. Toutain CM, Caizza NC, Zegans ME, O'Toole GA. 2007. Roles for flagellar stators in biofilm formation by Pseudomonas aeruginosa. Research in Microbiology 158:471-477.
9. Pratt LA, Kolter R. 1998. Genetic analysis of Escherichia coli biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Molecular Microbiology 30:285-293.
10. Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jorgensen A, Molin S, Tolker-Nielsen T. 2003. Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Molecular Microbiology 48:1511-1524.
11. Leid JG, Kerr M, Selgado C, Johnson C, Moreno G, Smith A, Shirtliff ME, O'Toole GA, Cope EK. 2009. Flagellum-Mediated Biofilm Defense Mechanisms of Pseudomonas aeruginosa against Host-Derived Lactoferrin. Infection and Immunity 77:4559-4566.
12. Das M, Paul S, Gupta P, Tribedi P, Sarkar S, Manna D, Bhattacharjee S. 2016. 3-Amino-4-aminoximidofurazan derivatives: small molecules possessing antimicrobial and antibiofilm activity against Staphylococcus aureus and Pseudomonas aeruginosa. Journal of applied microbiology 120:842-859.
13. Das T, Das MC, Das A, Bhowmik S, Sandhu P, Akhter Y, Bhattacharjee S, De UC. 2018. Modulation of S-aureus and P-aeruginosa biofilm: an in vitro study with new coumarin derivatives. World Journal of Microbiology & Biotechnology 34:18.
14. Kim W, Tengra FK, Young Z, Shong J, Marchand N, Chan HK, Pangule RC, Parra M, Dordick JS, Plawsky JL, Collins CH. 2013. Spaceflight Promotes Biofilm Formation by Pseudomonas aeruginosa. Plos One 8:8.
15. Wang HL, Yan YF, Rong D, Wang J, Wang HD, Liu ZZ, Wang JP, Yang RF, Han YP. 2016. Increased biofilm formation ability in Klebsiella pneumoniae after short-term exposure to a simulated microgravity environment. Microbiologyopen 5:793-801.
16. Li J, Liu F, Wang Q, Ge PP, Woo PCY, Yan JH, Zhao YL, Gao GF, Liu CH, Liu CT. 2014. Genomic and transcriptomic analysis of NDM-1 Klebsiella pneumoniae in spaceflight reveal mechanisms underlying environmental adaptability. Scientific Reports 4:11.
17. Huang T-W, Lam I, Chang H-Y, Tsai S-F, Palsson BO, Charusanti P. 2014. Capsule deletion via a λ-Red knockout system perturbs biofilm formation and fimbriae expression in Klebsiella pneumoniae MGH 78578. BMC research notes 7:13.

18. Rosenzweig JA, Ahmed S, Eunson J, Chopra AK. 2014. Low-shear force associated with modeled microgravity and spaceflight does not similarly impact the virulence of notable bacterial pathogens. Applied Microbiology and Biotechnology 98:8797-8807.
19. Crucian B, Stowe R, Mehta S, Uchakin P, Quiriarte H, Pierson D, Sams C. 2013. Immune System Dysregulation Occurs During Short Duration Spaceflight On Board the Space Shuttle. Journal of Clinical Immunology 33:456-465.
20. Crucian BE, Chouker A, Simpson RJ, Mehta S, Marshall G, Smith SM, Zwart SR, Heer M, Ponomarev S, Whitmire A, Frippiat JP, Douglas G, Lorenzi H, Buchheim JI, Makedonas G, Ginsburg GS, Ott CM, Pierson DL, Krieger SS, Baecker N, Sams C. 2018. Immune System Dysregulation During Spaceflight: Potential Countermeasures for Deep Space Exploration Missions. Frontiers in Immunology 9:21.
21. Rooney BV, Crucian BE, Pierson DL, Laudenslager ML, Mehta SK. 2019. Herpes Virus Reactivation in Astronauts During Spaceflight and Its Application on Earth. Frontiers in Microbiology 10:9.
22. Garrett-Bakelman FE, Darshi M, Green SJ, Gur RC, Lin L, Macias BR, McKenna MJ, Meydan C, Mishra T, Nasrini J, Piening BD, Rizzardi LF, Sharma K, Siamwala JH, Taylor L, Vitaterna MH, Afkarian M, Afshinnekoo E, Ahadi S, Ambati A, Arya M, Bezdan D, Callahan CM, Chen S, Choi AMK, Chlipala GE, Contrepois K, Covington M, Crucian BE, De Vivo I, Dinges DF, Ebert DJ, Feinberg JI, Gandara JA, George KA, Goutsias J, Grills GS, Hargens AR, Heer M, Hillary RP, Hoofnagle AN, Hook VYH, Jenkinson G, Jiang P, Keshavarzian A, Laurie SS, Lee-McMullen B, Lumpkins SB, MacKay M, Maienschein-Cline MG, et al. 2019. The NASA Twins Study: A multidimensional analysis of a year-long human spaceflight. Science 364:eaau8650.
23. Mehta SK, Laudenslager ML, Stowe RP, Crucian BE, Sams CF, Pierson DL. 2014. Multiple latent viruses reactivate in astronauts during Space Shuttle missions. Brain Behavior and Immunity 41:210-217.
24. Koliada A, Syzenko G, Moseiko V, Budovska L, Puchkov K, Perederiy V, Gavalko Y, Dorofeyev A, Romanenko M, Tkach S, Sineok L, Lushchak O, Vaiserman A. 2017. Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. Bmc Microbiology 17:6.
25. Larson JE, Cohen JC. 2000. Cystic fibrosis revisited. Molecular Genetics and Metabolism 71:470-477.
26. Bhagirath AY, Li YQ, Somayajula D, Dadashi M, Badr S, Duan KM. 2016. Cystic fibrosis lung environment and Pseudomonas aeruginosa infection. Bmc Pulmonary Medicine 16:22.
27. Wilson N. 2019. A Microbial Hitchhiker's Guide to the Galaxy: Researchers race to understand effects of deep space on microbiome. Bioscience 69:5-11.
28. Rada B, Leto TL. 2013. Pyocyanin effects on respiratory epithelium: relevance in Pseudomonas aeruginosa airway infections. Trends in Microbiology 21:73-81.
29. Jayaseelan S, Ramaswamy D, Dharmaraj S. 2014. Pyocyanin: production, applications, challenges and new insights. World Journal of Microbiology & Biotechnology 30:1159-1168.
30. Sheet S, Sathishkumar Y, Choi MS, Lee YS. 2019. Insight into Pseudomonas aeruginosa pyocyanin production under low-shear modeled microgravity. Bioprocess and Biosystems Engineering 42:267-277.
31. Hao YH, Kuang ZZ, Walling BE, Bhatia S, Sivaguru M, Chen Y, Gaskins HR, Lau GW. 2012. Pseudomonas aeruginosa pyocyanin causes airway goblet cell hyperplasia and metaplasia and mucus hypersecretion by inactivating the transcriptional factor FoxA2. Cellular Microbiology 14:401-415.
32. McLean RJC, Cassanto JM, Barnes MB, Koo JH. 2001. Bacterial biofilm formation under microgravity conditions. Fems Microbiology Letters 195:115-119.

33. Crabbe A, Schurr MJ, Monsieurs P, Morici L, Schurr J, Wilson JW, Ott CM, Tsaprailis G, Pierson DL, Stefanyshyn-Piper H, Nickerson CA. 2011. Transcriptional and Proteomic Responses of Pseudomonas aeruginosa PAO1 to Spaceflight Conditions Involve Hfq Regulation and Reveal a Role for Oxygen. Applied and Environmental Microbiology 77:1221-1230.
34. Crabbe A, De Boever P, Van Houdt R, Moors H, Mergeay M, Cornelis P. 2008. Use of the rotating wall vessel technology to study the effect of shear stress on growth behaviour of Pseudomonas aeruginosa PA01. Environmental Microbiology 10:2098-2110.
35. Tirumalai MR, Karouia F, Tran Q, Stepanov VG, Bruce RJ, Ott CM, Pierson DL, Fox GE. 2017. The adaptation of Escherichia coli cells grown in simulated microgravity for an extended period is both phenotypic and genomic. npj Microgravity 3:15.
36. Friedman L, Kolter R. 2004. Genes involved in matrix formation in Pseudomonas aeruginosa PA14 biofilms. Molecular Microbiology 51:675-690.
37. Colvin KM, Alnabelseya N, Baker P, Whitney JC, Howell PL, Parsek MR. 2013. PelA Deacetylase Activity Is Required for Pel Polysaccharide Synthesis in Pseudomonas aeruginosa. Journal of Bacteriology 195:2329-2339.
38. Limoli DH, Jones CJ, Wozniak DJ. 2015. Bacterial Extracellular Polysaccharides in Biofilm Formation and Function, p 223-247. *In* Ghannoum M, Parsek M, Whiteley M, Mukherjee PK (ed), Microbial Biofilms, 2nd ed. ASM Press, Washington DC, USA.
39. Vasseur P, Vallet-Gely I, Soscia C, Genin S, Filloux A. 2005. The pel genes of the Pseudomonas aeruginosa PAK strain are involved at early and late stages of biofilm formation. Microbiology-Sgm 151:985-997.
40. El-Fouly MZ, Sharaf AM, Shahin AAM, El-Bialy HA, Omara AMA. 2015. Biosynthesis of pyocyanin pigment by Pseudomonas aeruginosa. Journal of Radiation Research and Applied Sciences 8:36-48.
41. Horneck G, Klaus DM, Mancinelli RL. 2010. Space Microbiology. Microbiology and Molecular Biology Reviews 74:121-+.
42. Jiang WJ, Xu BX, Yi Y, Huang YL, Li XO, Jiang FQ, Zhou JL, Zhang JZ, Cui Y. 2014. Effects of simulated microgravity by RCCS on the biological features of Candida albicans. International Journal of Clinical and Experimental Pathology 7:3781-3790.
43. Tirumalai MR, Karouia F, Tran Q, Stepanov VG, Bruce RJ, Ott CM, Pierson DL, Fox GE. 2017. The adaptation of Escherichia coli cells grown in simulated microgravity for an extended period is both phenotypic and genomic. Npj Microgravity 3:9.
44. Essar DW, Eberly L, Hadero A, Crawford I. 1990. Identification and characterization of genes for a second anthranilate synthase in Pseudomonas aeruginosa: interchangeability of the two anthranilate synthases and evolutionary implications. Journal of bacteriology 172:884-900.