ANALYSIS OF *PSEUDOMONAS AERUGINOSA* VIRULENCE IN MODELED MICROGRAVITY CONDITIONS.

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

May 2006

APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the Division of Graduate Education.

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Seratna Guadarrama-Beltran

May 2006.

This thesis is dedicated to the memory of my grandfathers: Nico and Humberto (Siete), whose *sui generis* lives, extraordinary determination and outstanding courage have accompanied me every step of the way.

A mis abuelos:

Tome un pedazo del planeta y, más que mi nombre o pensamiento, fue mi sentimiento el que se anclo a el, a su Naturaleza. Ahí, en la Naturaleza donde habitan Sus verdades y energias eternas.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor Dr. Barry Pyle and all the members of my committee: Dr. Patricia Glee, Dr. Timothy Ford, Dr. Linda Hyman, and Dr. Sandra Halonen for their advising and guidance. Thanks to Rachel Neal for sharing her expertise in proteomics and also to Dr. Elinor Pulcini and Sarah Roth for their assistance. Thanks to Susan Broadaway, and Dr. Tresa Goins for the technical advice, and to Ailyn Lenz, Amresh Karmacharya and Dr. Hyen-mi Chang for their emotional support. Thanks to Dr. Jim Robison-Cox and Ms. Yin Chang from the MSU Mathematics Department for the statistical analysis. This project was supported by INBRE-BRIN (Idea Networks of Biomedical Research Excellence and Biomedical Research Infrastructure Network) Fellowship, Montana Space Grant Consortium-NASA EPSCoR, NASA Ames Research Center, and the European Space Agency, ESTEC.

Thanks to my parents, engineers Humberto Guadarrama and Beatriz Beltran for their guidance, love and emotional support. Gracias por empujarme y esterilizarme de dudas; por darme el mundo con amor y paciencia; por heredarme las armas para sonreír con franqueza y llorar con el alma. Gracias a ustedes mis sueños son realidad. I would like to thank my brother Humberto and sisters Charim and Dubhe for their words, laughter, magic and love, which enhanced my life and helped me through the hard times. Gracias por soportarme y continuar dandome ganas de seguir adelante, aun, cuando kilómetros de distancia nos separan. I would also like to thank my entire extended family in México, “Los Beltranes”, for injecting me with perseverance and “chistes morados” to “make it” in another country and finalize my thesis work. Gracias por apoyar a esta “cientifica loca”.

Finally, I want to give special thanks to my future husband Dr. Enrico Marsili for being my Heart, Sun and Coach all along this thesis work and life journey. Grazie per essere il mio cuore amoroso, il mio sole pieno di luce, la mia guida stella del nord, e la mia fortissima roccia di mare. Grazie per la tua constante presenza nella mia mente, cuore, anima e pelle.

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ABSTRACT

Spaceflight conditions may enhance bacterial growth, alter antimicrobial susceptibility and possibly affect virulence. Since spaceflight causes astronauts to experience secondary immunosuppression, increased virulence would increase the risk of infection. Limited opportunities for spaceflight experiments necessitate ground-based simulations. Clinostats and rotating wall vessel bioreactors, e.g. High Aspect Ratio Vessels (HARVs) are used to simulate microgravity effects at 1xg. Clinostat and HARV rotation on the horizontal axis results in the g-vector being time averaged to near zero. In controls rotated on the vertical axis, the g-vector acts on cells, as in static cultures. *Salmonella enterica* serovar Typhimurium virulence genes are up-regulated in modeled microgravity (MMG); a MMG "regulon" has been postulated. We hypothesize that the virulence of *P. aeruginosa* (PA) may also be affected by microgravity, which could be observed in MMG. This study focused on the regulation of Exotoxin A (ETA) in PA during growth in MMG.

PA103 was grown in ETA medium at 37^{o}C in Clinostat-MMG syringes incubated and sampled at intervals up to 24 h at 15 RPM; and also in HARV MMG bioreactors incubated for time zero, 12 h and 24 h at 15 RPM. MMG samples were rotated on the horizontal axis and gravity controls were rotated on the vertical axis or static position. Agar plate counts (PC) were done on R2A. ETA was monitored by ELISA. Protein expression in clinostat and HARV samples was assayed by 2-dimensional gel analyses.

MMG did not affect ETA production in the Clinostat or HARV (horizontal vs. vertical rotation), whereas, PC showed growth in MMG was comparable to controls. Clinostat and HARV experiments indicated that MMG had negligible effects on ETA production. However, HARV cultures gave much lower ETA production in either MMG or horizontal control rotation compared to static controls. In addition, formulation of the growth medium (presence/absence of nitrate) and culture aeration had dramatic effects on ETA production. These differences should be taken into account in future ground and flight experiments. Analysis of 2-D gels from preliminary experiments was inconclusive.

This study indicates that MMG has a minor effect on the growth of *P. aeruginosa* PA103 under these conditions.

INTRODUCTION

Space Missions and Microbes.

Microbial Growth in Space.

The quest for scientific answers to space related life science questions started back in the 1950's when the Soviet satellite "Sputnik" was launched and placed in orbit. A decade later, initial cell studies in space were done in the Soviet space craft Vostok I. These studies revealed that on similar flights in the same orbit as Vostok I, there was no apparent effect from factors capable of disturbing isolated cells (Gmunder & Cogoli, 1988). The space environment of these space biological experiments consisted of a pressurized, thermostatically-controlled capsule, with characteristic reduced gravity and the existence of heavy particle (cosmic) radiation; in theory, scientists observed, reduced gravity was not supposed to have an effect on single cells smaller than 10 microns (Bouloc and D'Ari, 1991). The ability of bacteria to survive in space environments was demonstrated when *Streptococcus mitis* was recovered from the internal components of a Surveyor III television camera left on the surface of the Moon for two and a half years (Lewis et al., 1997).

The gravitational pull or acceleration that is experienced on Earth is defined as G, where G=9.8 cm/s^2. Surprisingly, microgravity (10^{-6} G) does appear to have an appreciable influence on microbial growth according to several investigations in the area of space flight. Living organisms depend on the mode and timing of delivery of nutrients (Klaus, 2002). Availability of nutrients and the removal of metabolic by-products in

suspension cultures largely depend on forces acting on the cell such as sedimentation, diffusion, motility and on the bulk fluid such as diffusion and convection (Klaus, 2002). Gravity, as a result, is a parameter that must be isolated as an independent variable. On Earth, the pull of gravity acts as a "sedimentation force" on particles, according to the differential densities against the environment where the living or non-living systems are present. Although random cell Brownian motion appears to "ignore" the acceleration gravity, there is a cumulative unidirectional force that makes the cells fall towards the bottom of the container (Klaus, 2002). Therefore, in reduced gravity conditions, constant stirring of the culture suspension can provide extra motion but it can also introduce more variables. It is important to categorize the effects on cells due to their response to gravitational loading (Klaus, 2002). The effects may be based on weight (primary), on sedimentation (secondary), and on the surrounding environment (tertiary) (Klaus, 2002).

Escherichia coli and other Microorganisms in Microgravity.

In the 1960's, Mattoni reported that *E. coli* growth in liquid medium was dramatically denser following space flight than identical cultures on Earth (Klaus, 2002). In 1989, Bouloc and D'Ari (1991) grew cultures of *E. coli* on the Soviet space satellite Biocosmos 2044. The main objective was to study cell growth parameters and energy metabolism in the environment of weightlessness and the heavy particle radiation of space (Bouloc & D'Ari, 1991). They found there was no significant change in parameters of the bacterial cell growth experiment conducted in space (Bouloc & D'Ari, 1991). From previous studies, it was known that unicellular organisms such as *Paramecium tetraurelia* and *Bacillus subtilis* grow faster in space (Richoilley et al., 1986). Increased

cell number could be due to the extra energy obtained by not having to express a motile phenotype (Bouloc & D'Ari, 1991). Microorganisms readily adapt to changes in the environment such as nutrient levels, temperature, oxygen concentration, atmospheric pressure, weightlessness, and light intensity (Cioletti et al., 1989); this adaptation is a consequence of various physiological and morphological changes. Bacterial growth is defined as the "coordinated summation of a complex array of processes including chemical synthesis, assembly, polymerization, biosynthesis, fueling, and transport, the consequence of which is the production of new cells" (Cioletti et al., 1989).

Specific effects of space flight on microbial growth kinetics, and lag, exponential, and stationary phases can be singled out for testing (Klaus, 2002). Previous research studies have shown that in space flight there is a shortened lag phase following on-orbit inoculation in *B. subtilis* (Menigmann & Lange, 1986). Klaus (2002) and Thénevet (1996) found that suspended cultures of non-motile *E. coli* had a lag phase that ended four to eight hours earlier than the ground controls. Interestingly enough, on-orbit experiments on motile *E. coli* demonstrated that the lag-phase was not affected (Bouloc & D'Ari, 1991). This is possible because the motility of *E. coli* is two orders of magnitude greater than the cell movement due to sedimentation (Bouloc & D'Ari, 1997). Presumably, microgravity minimally disturbs or influences the motility of the organism according to the investigators.

In relation to a shorter lag phase, it has been theorized that proteins or co-factors need to reach a certain concentration within the cells before the cells begin to double (Klaus, 2002). In normal growth conditions, some of these "building-blocks" may

diffuse from the cells and disperse in the bulk fluid due to convection. Thus, they tend to accumulate more slowly in cells grown in normal gravity compared to microgravity conditions. Later studies made by Gasset et al., (1994) on the growth and division of *E. coli* under microgravity conditions also showed, through an optical density increase in peptone cultures, a shorter lag period and an earlier entry into the stationary phase in flight samples. However, viability counts did not reflect such a result. After nine hours, flight and ground population numbers converged. It was concluded that reduced gravity has no effect on the growth rate of exponentially growing *E. coli* cells (Gasset et al., 1994). On the other hand, Gmunder and Cogoli (1988) refer to an investigation done onboard Vostok 4 and 6. The space flight conditions resulted in a minimal increase in phage productivity and slightly higher survival rates of *E. coli*, in a time period from 27 to 30 hours. Moreover, in an experiment done onboard Biosatellite II, *E. coli* was demonstrated to have an increased resistance against high doses of radiation and a minimal higher final viable cell density (Gmunder & Cogoli, 1988). In this case, population increase may have been the result of enhanced survival in response to radiation rather than a difference in growth rate.

Bacterial populations of spacecraft crew members have also been studied. Back in the 1960's, *Staphylococcus aureus* did not exhibit a growth increase in the crew-members of the Apollo capsule while in orbit, (Ferguson et al., 1975). On the other hand, in the same on-orbit capsule the pathogenic yeast *Candida albicans* population thrived considerably due, reportedly, to the reduced population of other fungi that commonly controls the growth of the pathogen. The population shift and reduced microbial

competition resulted in a decreased natural resistance by the crewmember against the pathogen. On Apollo 17, the opportunistic bacterium *Pseudomonas aeruginosa* was found on the toes of the Commander before flight and spread to the toes of the Command and Lunar Module Pilots during flight (Ferguson et al., 1975). This phenomenon did not pose a threat to the crewmembers since no mediated infection was detected, even though this pathogenic bacterium was found in several isolation sites inside the Apollo capsules (Ferguson et al., 1975).

A summary of the most relevant space flight and ground control microbial experiments to date and, their methodology are shown in Table 1. The main objectives of the experiments described in this table were to investigate the influence of spaceflight on the growth cycle of various bacterial species and also on their virulence capacity (*in vivo* microbe-host experiments). According to Bouloc and D'Ari (1991), fast growing cells were larger than slow growing cells and thus, cell volume at a single point in time was a picture reflecting the growth rate. There was no major change in the growth rate, according to the cell volume of *E. coli* cells. Despite this, there was a well-based correlation between non-motile microbes and a shorter lag phase in microgravity, the overall final bacterial population density was found to be the same in the ground controls. The same holds true for MMG, later described in detail, in which the lag and exponential phases are similar to those observed in the ground controls. Other microorganisms such as *Paramecium* and *B. subtilis* have been reported to grow faster in reduced gravity. Nevertheless, it seems that this is not true for all microorganisms, maybe because the actual increase in growth rate is based, ultimately, on the existence of some form of

genetic "switches" that need to be turned on to provide for prolonged and successful survival.

In a shuttle experiment on STS-81 (Shuttle Atlantis) to study the effects of microgravity and spaceflight on growth and biofilm formation by the motile bacterium *Burkholderia cepacia*, Pyle et al (1999; 2001) found that the microorganisms grew and formed biofilms at least as well if not better than they did on Earth, although the results were not significantly different. Their motility, including experiment-specific factors, may have been related to the detection of small and insignificant differences.

Table 1. Summary of Relevant Microbial Spaceflight Experiments.

Type of Culture	Parameter Tested	Methodology in brief	Conclusion	Reference
Suspension	Lag and Log Phases	*E. coli* grown in M63 +glucose, glycerol and kanamycin. Frozen at –70°C. Incubated @ 37°C pre and on flight in CYTOS hardware. Growth stopped by temperature decrease to 4°C.	No change	Bouloc & D'Ari, 1991.
Suspension	Lag and Stationary Phases	*E. coli* grown in glucose minimal medium. Growth stopped at 6 different times by decreasing temperature to 5°C, and at one time point by formaldehyde fixation. Flown in the STS-42 mission of the space shuttle Discovery.	Faster Lag and earlier entry into Stationary phase, but no overall change exponentially.	Gasset et al., 1994.
Suspension	Growth and Biofilm formation on stainless steel and iodine effect.	*Burkholderia cepacia* grown in water, tryptic soy broth, and weak iodine solution. Kept at 5°C from before launch to 19 hours after launch, remainder of flight and landing. Activated and incubated in ESA Biorack system at 22 °C for 6 days.	Biofilm formed as does on earth; increased growth observed but not statistically significant.	Pyle et al., 1999 & 2001.

Microbial Factors Influenced by Spaceflight Conditions.

The continual interdependence among microbes, animals, plants, and spacecraft is a key element when studying responses of living organisms as a result of spaceflight conditions. Focus will be given to microbes, specifically, factors influenced by spaceflight conditions such as, survival and growth rates, physiology and metabolism and, antimicrobial sensitivity. Microorganisms and other cells in general, must be able to adapt to altered gravity conditions in order to survive. The ways in which organisms respond to altered gravity may vary widely. More so than microgravity (10^{-6} G), environment to which living systems are exposed to during spaceflight, the key in adaptation is the *gravitational unloading* or reduced gravity effect that is experienced by living systems (Lewis et al., 1997).

By 1988, evidence suggested that cells of all sizes and organizational complexity can potentially sense gravity or its absence (Gmunder & Cogoli, 1988). Their argument is based on changes in growth physiology. As mentioned before, the lag phase *E. coli* is reduced by 4 to 8 hours under microgravity conditions (Lanning et al., 1988), and stationary phase population density in reduced gravity is two times greater than that of bacteria grown under normal gravity conditions (Klaus, 1997). An experiment was done to determine if increased bacterial growth of *E. coli* and *B. subtilis* in spaceflight were the result of fluid mechanics vs. cellular effects. Organisms for ground control experiments were grown on agar cultures under static, agitated, and rotated conditions in the laboratory, and for the reduced gravity conditions on four space shuttle flights bacterial samples were grown in a fluid processing apparatus (FPA, BioServe, Inc.) (Kacena et al.,

1997). Termination of growth experiments was done using glutaraldehyde, and subsequently, individual cells were assessed by quantitative elution from the agar (Kacena et al., 1997). Spaceflight and ground results indicated that *E. coli* and *B. subtilis* cultures on agar, as opposed to their suspension grown counterparts, did not experience increased final cell concentration when the inertial (i.e. gravity) environment is changed (Kacena et al., 1997).

Physiological adaptations in microbes depend on the availability of carbon nutrients, substrates such as, phosphate, sulfate, nitrogen, etc., and the interaction with environmental factors such as, pH, temperature, and oxygen tension. Therefore, mechanisms regarding adaptation of microbes to space conditions are largely influenced by the gravity effects imposed on those nutrients and environmental factors (Cioletti et al., 1989). The physiology of autolysis is one example. Autolysis is defined as a cellular enzymatic process resulting in cell dissolution due to cell burst (Brock et al., 1994). Kacena et al., (1999a) performed an experiment to examine the role of gravity in the autolysis of *E. coli* and *B. subtilis* by growing cells on Earth and in microgravity on Space Station MIR. The autolysis analysis was done by testing the death phase or exponential decay of cells for about 4 months following the stationary phase. When FPA's were completely loaded (including the glutaraldehyde fixative) they were stored at 4^{o}C to minimize metabolic processes until experimental payload was transferred to the space shuttle. The stationary-phase cell population was 70% for *B. subtilis* cultures and 90% for *E. coli* cultures, both of which were greater in flight than in ground controls (Kacena et al., 1999a). Although both flight autolysis curves started at higher cell

densities than control curves, the rate of autolysis in flight cultures was equal to that of their ground control counterparts (Kacena et al., 1999a). The autolytic process was observed to be identical to that found in the ground experiment. It appears, according to the authors of the experiment, that the rate and duration of cell loss were the same and that the higher stationary phase cell concentration in the flight cultures was responsible for the higher number of countable cells at the end of 120 days of microgravity exposure (Kacena et al., 1999a). Therefore, it is concluded that the autolysis mechanisms in either gram–positive or gram-negative bacteria are not affected by spaceflight conditions (Kacena et al., 1999a).

Considering spacecraft crew health risks, the performance of antibiotics is critical. Studies on antimicrobial sensitivity in spaceflight conditions have been largely based on antibiotic performance (Table 2). Antibiotics have three modes of action: a) interference with enzymes, b) interference with templates, or c) interference with cellular structures (Lewis et al., 1997). The basis of action relies on specific binding of the natural compound with target molecules on or in the cell (Lewis et al., 1997). In spaceflight, antibiotic function and efficiency may be altered. Several investigations on the biology of spaceflight indicate that there is no inverse correlation between antibiotic dosage and bacterial proliferation, but the opposite.

These results confirmed previous findings indicating an increase in the antibiotic resistance of bacteria in reduced gravity. For example, during the French-Soviet manned flight project “CYTOS 2”, Lapchine tested the antibacterial activity of antibiotics in space. Results indicated an increase in the MIC of *E. coli* cultures developed *in vitro*

during spaceflight (Lapchine et al., 1987). Subsequently, the "ANTIBIO" experiment was performed aboard the space shuttle Challenger in the "Biorack" European program to confirm the results obtained from "CYTOS 2" (Lewis et al., 1997). The antibiotic Colistin, which is a polymyxin that works by binding to the lipopolysaccharides in the outer membrane of the cell causing leakage was investigated during the "ANTIBIO" experiment on Spacelab mission D1 (Lewis et al., 1997). There was an increase in the MIC (Minimal Inhibitory Concentration), which is the minimal quantity of antibiotic required to stop bacterial growth, in cultures developed *in vitro* during spaceflight conditions (Lewis et al., 1997). In fact, these MIC concentrations increased 2 to 4 times in reduced gravity, as opposed to ground based controls. *E. coli* showed an increased resistance in microgravity to the antibiotic Colistin (Lewis et al., 1997). Experiments summarized in Table 2, examined specific microbiological factors that could be affected by the alteration of gravity. Possible reasons for increased antibiotic resistance is, as it was previously mentioned, the shortened lag phase bacteria have in space. This enables the cell to be fortified by not moving away from its by-products, and stimulate cell division and build-up of defense mechanisms to optimal levels as opposed to what would have occurred during sedimentation conditions (Lanning, 1998). The increased *E. coli* resistance to Colistin (Lapchine et al., 1987) is not an acquired characteristic, but rather an adaptation to weightlessness (Lewis et al., 1997). Possible reasons for increased resistance include the finding that bacteria proliferate faster in space and therefore, the concentration of antibiotic may not be sufficient, and there is an altered transport of antibiotics into the cells because of the modification of cellular membrane permeability

(Lewis et al., 1997). This interpretation is supported by the ultra structure modification of cell envelopes found in the CYTOS 2 experiments (Kacena et al., 1999a).

Table 2. Spaceflight Experiments Assessing Microbial Factors.

Type of Culture	Factor Tested	Methodology in brief	Conclusion	Reference
Suspension	Growth rate, final cell density	*E. coli* and *Bacillus subtilis* were grown on agar cultures in FPA, fixed with glutaraldehyde and cell count by quantitative elution.	No change.	Kacena et al., 1997.
Suspension	Antibiotic resistance	*E. coli* cultures developed *in vitro* during spaceflight treated with Colistin antibiotic.	Higher in reduced gravity.	Lapchine et al., 1987.
Suspension	Autolysis	*E. coli* and *Bacillus subtilis* bacterial samples grown in FPA, fixed in glutaraldehyde and cell count on a hemacytometer.	No change.	Kacena et al., 1999a.
Suspension	Antibiotic resistance	Four species of bacteria exposed to microgravity for 4 months on Space Station MIR (Long-term exposure).	Increased growth rate and resistance to some antibiotics, not others.	Juergensmeyer, 1999.

Spacecraft Crew-Microbe Interactions.

Among many other factors, the success of a spaceflight mission depends on the health and safety of the spacecraft crew. Several factors need to be considered in order to safeguard the health within a space vessel and its crew. The list of factors ranges from the space crew-members immunity (weakened system and increased vulnerability to infections) (Leach et al., 1974) to planetary protection strategies (Konstantinova et al., 1993). The immutable and hostile conditions of space may and can cause serious damage

to the crewmembers' immune system and make them easy targets for space craft contaminants. Extensive research has been done on the mechanisms of microgravity effects on the crew's health. There are several studies on the relevance of Earth gravity and stress responses for cellular relationships in an immune response, for processes inside the cell that trigger the activation of lymphocytes and the expression of receptors on cells, and the interactions between the neurohormonal and immune systems. Konstantinova et al. (1993) have investigated cosmonauts during spaceflight and the results obtained showed IgG levels unchanged, in contrast to IgA and IgM levels, which sometimes increased (Konstantinova et al., 1993). Because immunoglobulins A and M are secreted when there is no memory of previous antigen exposure, the immune systems of these cosmonauts reflected first time exposure to the their new spacecraft environment. This was consistent with the finding of post flight decrease in T-lymphocytes, interleukin-2, and cytotoxic activity of natural killer cells (Konstantinova et al., 1993).

Spaceflight experiments concerning crew-microbe interactions include post flight measurement of *in vitro* reactivity of the T cells after contact with PHA (phytohemaglutinin) (Ferguson, 1975). The synthesis of RNA was analyzed based on the incorporation of [^{3}H] uridine, and DNA synthesis was analyzed by the incorporation of [^{3}H] thymidine. The PHA reactivity of lymphocytes which was assessed radio-autographically based on the rate of RNA synthesis, had a higher yield in healthy men (Konstantinova et al., 1993). This was done by observing more than 50 argentum granules per cell after 24 hour of incubation with PHA (Ferguson, 1975). A decreased PHA reactivity was found in 33 of 46 cosmonauts after prolonged spaceflight of 30 to

366 days on Salyut 4, 6, and 7 and the MIR orbital station (Ferguson, 1975). The suppression of PHA reactivity of T lymphocytes on the U.S. Skylab orbital station was related to the increase in blood corticosteroids in these astronauts both during and after flight (Nefedov et al., 1997). Furthermore, according to Lewis et al., (1997) the diffusion of molecules between non-colliding cells will likely be disrupted in space. In other words, the time required for an effector molecule (hormone, or any other messenger molecule) to travel from an effector to a receptor cell in space by diffusion alone (absence of sedimentation and convection) is calculated to need about two hours to cover a distance of 3 mm (Lewis et al., 1997). This situation could affect the communication between cells and the activation of lymphocytes (Lewis et al., 1997).

Studies revealed extensive immunity changes in microgravity for both cosmonauts and astronauts. There seems to be a relationship between microgravity effects and the stress syndrome. There is not a defined line between the two because the conditions are not entirely due to emotional tension but can be triggered by adaptation to the absence of gravitation and by other uncommon or unseen factors (Ferguson, 1975). Nefedov et al. (1997), suggest that the immunity-microbial problem faced by astronauts in long-term space flights can be solved based on various theoretical considerations that reflect the interactions between micro- and macro- organisms, for instance, adaptation of microorganisms to the gastrointestinal tract and methods of stimulating immunocompetence by injections of killed antigens and other substances (Lujan, 1994).

In 1983, during the first voyage of Space Shuttle Columbia an investigation was performed to study the medical microbiology of crewmembers and spacecraft during

OFT (Orbital Flight Test) phase of the missions (Pierson, 1983). Pre-flight and post-flight microbial analyses of the crewmembers and the orbiter were performed for each OFT flight. At the end of each mission, an assessment of the patterns and extent of microbial contamination of the orbiter was completed (Pierson, 1983). Samples were collected from each crewmember for microbial evaluation before 30, 10 and 2 days of launch (F-30, F-10, and F-2, respectively), just before and after launch (L+0 and L+5) The sampled sites were: ears, nose and throat; a fecal specimen (or fecal swab) and a urine sample. According to the results obtained, each member of the crew exhibited normal microbial flora in ears, nose, throat and fecal cultures. Although potential bacterial and fungal pathogens were isolated from the crewmembers during the sampling period, no clinical manifestations resulted (Pierson, 1983). Some examples of these pathogens are: *Staphylococcus aureus* (Pierson, 1997) and *P. aeruginosa* (from nose and throat). No evidence of parasitic infection was found in samples obtained from the OFT phase (Pierson, 1983).

Microbiological monitoring consisted of collecting and analyzing samples from the orbiter's interior surfaces, waste management system, flight hardware, cabin air and potable water supply. Calcium alginate swabs were used to sample 21 surface sites all around the mid and flight decks. Each area in the orbiter, about 25 cm^2, was sampled with two phosphate buffer-moistened swabs (Pierson, 1983). The spacecraft monitoring results showed an increase in the numbers of airborne microorganisms during STS-1 pre-flight sampling. It was believed it was due to the temporary installation of a blower in the cabin prior to launch (Pierson, 1983). A noticeable increase was observed in airborne

microbes during STS-2, though this build up was not observed in STS-3 nor STS-4 flights (Pierson, 1983).

Modeled Microgravity Systems.

The Principle.

Microgravity, a condition achieved during lower earth orbit spaceflight, has been defined as a prolonged "free-fall", where the gravitational force is approximately 10^{-6}G to 10^{-4}G (Lewis et al., 1997). Simulated or modeled microgravity (MMG) has become an integral tool for many biological investigations. In the 1980's and 90's, scientific work on plant gravitropism led to the study of the physiology and behavior of different organisms during spaceflight conditions, specifically, the influence of gravity on the behavior of single cells. MMG equipment has been developed to accommodate a variety of biological experiments. Klaus refers to the Clinostat (Fig.1, A&B) and the Rotating Wall Vessel (RWV) bioreactor as environments created on Earth that model microgravity (Klaus, 2001). Clinostats and RWV's are MMG systems that provide constant reorientation to efficiently eliminate the accumulated sedimentation of particles (Klaus, 2001). However, in reduced gravity, there is an often a lack of structural deformation, displacement of intercellular components and/or reduced mass transfer in the extra-cellular fluid (Klaus, 2001), all of which this equipment cannot fully emulate. There are certain parameters that need to be considered to determine the total gravity dependent effects generated by either a Clinostat or a RWV. These parameters include density, viscosity, and container geometry. Presumably, the Clinostat can produce what is called

"particle motionlessness" relative to the surrounding bulk fluid, which is similar to the extra-cellular fluid (Klaus, 2001). The goal of clinorotation is to effectively cancel out motion in suspension cultures and/or mechanical loading for plants, microbes and other attached systems through the time-averaging of the gravity vector by continuous rotation (Klaus, 2001). On the other hand, the RWV bioreactor maintains cells in suspension as they constantly "fall" through the medium under 1G conditions (Klaus, 2001). In the RWV, cultures can obtain nutrients and discard wastes. The main differences between the Clinostat and the RWV is that the first maintains an unstirred, undisturbed environment that emulates orbital flight, whereas, the RWV may create a low shear but mixed fluid environment ideal for suspension culture and tissue growth (Klaus, 2001). Further techniques utilized to investigate altered gravity like free-fall, neutral buoyancy and electromagnetic levitation can provide additional information on how gravity affects biological systems (Klaus, 2001).

The RWV is a specialized application of a more generic concept of a Clinostat (Klaus, 2001). The RWV is similar to a clinorotator in that it is a cylindrical culture vessel but it rotates about its horizontal axis and the suspended particles fall through circular paths within the medium as a function of rotational velocity (Klaus, 2001). The terminal velocity in the bioreactor is determined by density, viscosity and geometry. As opposed to the Clinostat, the bioreactor ensures that the particles move freely within the culture medium enough to facilitate the diffusion of nutrients, wastes and dissolved gases through permeable membranes and to enable cell-to-cell contacts that lead to aggregation (Klaus, 2001). This vessel was designed to minimize shear stress on the suspended cells

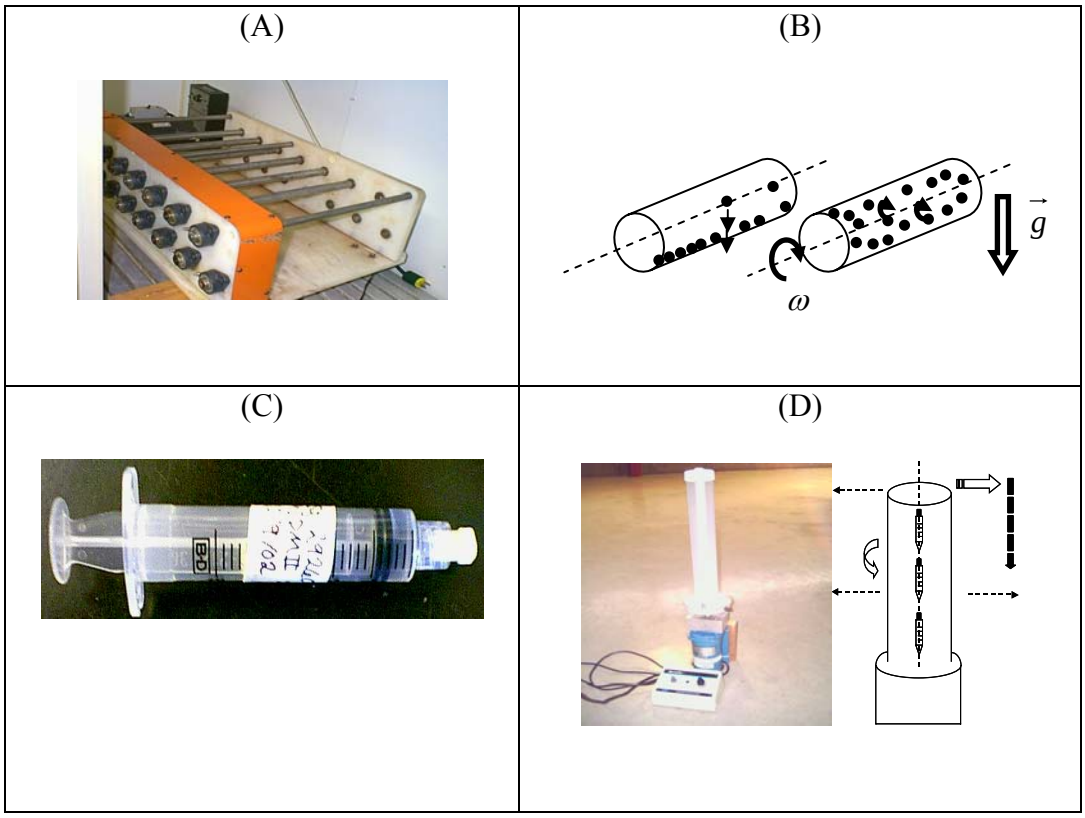


Figure 1. The Rotating Wall Vessel or Clinostat. (A) Clinostat apparatus. (B) Rotation and stationary orientation vectors. (C) Syringe or sample container. (D) Horizontal rotation control. Pictures are not set to scale.

and tissues, while still maintaining a uniformly mixed and oxygenated fluid environment (Klaus, 2001). Cells can aggregate, grow in three dimensions and differentiate, in the case of tissue, very similar to *in vivo*. The rotation speed can be adjusted as the aggregates grow in size to compensate for increased settling rates of larger particles. Earth-based bioreactor cultures may maintain cell growth for up to 60 days (Klaus et al., 1998). The bioreactor is considered optimal for culturing cells or tissues. The environment created in the bioreactor is based on particle behavior in reduced gravity, but

as Klaus claims, the environment simulated is not that of microgravity per se (Klaus, 2001).

One example of a RWV system is the High-Aspect-Ratio Rotating-Wall Vessel (HARV) bioreactor (Fig. 2, i and ii A and B) which is part of the Rotary Cell Culture System (RCCS, Synthecon, Inc.). This RWV was developed to model microgravity effects on cells. The bioreactor vessel is composed of a clear shell through which cell growth can be observed and which has a cylindrical filter that transfers oxygen and nutrients in, and carbon dioxide and wastes out (Klaus, 1998). The HARV produces an

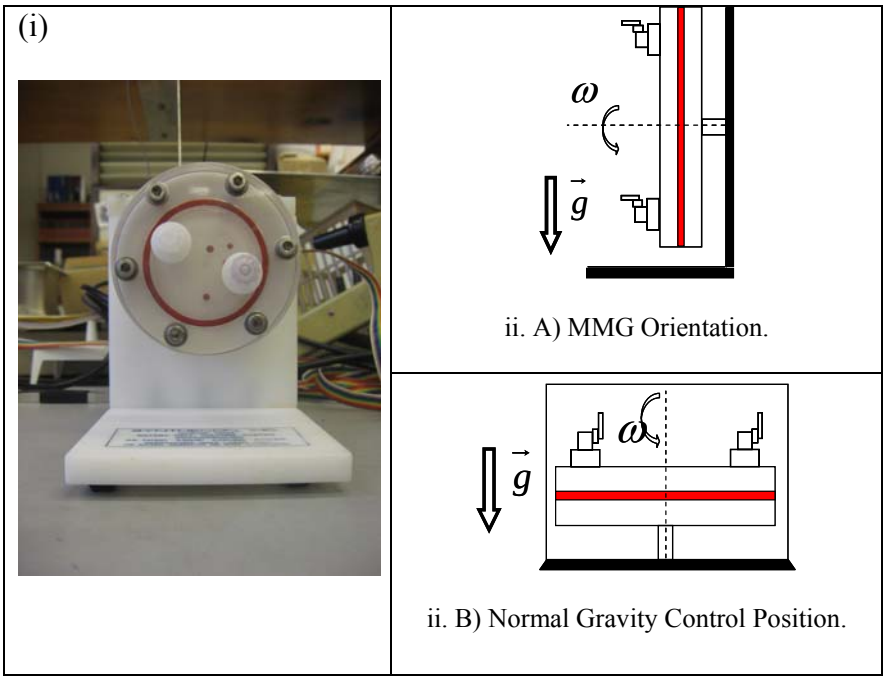


Figure 2. The High-Aspect-Ratio-Vessel (HARV). A HARV bioreactor in the MMG orientation (i. and ii.A) and in the normal gravity "control" position (ii.B) is shown. The vessel is completely filled with liquid so that gas bubbles cannot cause turbulence. When the HARV axis of rotation is perpendicular to gravity, microgravity is modeled by nullifying the downward gravity vector. When the HARV is placed in a horizontal "control" position the axis of rotation is parallel to the gravity vector, hence the gravity vector is no longer nullified (Klaus, 2001 and Nickerson et al., 2000).

environmental condition in which, presumably, the gravitational vectors are randomized over the surface of the cells, resulting in an average of 10^{-2} x G (Nickerson et al., 2000). The reduction of gravity is said to create a *sustained* low-shear environment for cell growth (Nickerson et al., 2000). The HARV may rotate in two orientations. In the control (1 G) position or horizontal (HZN) position (Fig. 2 ii B), the axis of rotation is parallel to the gravity vector, thereby "re-constituting" gravity and allowing the gravity vector to affect the cells in the bioreactor (Klaus, 2001). On the other hand, to achieve MMG, the HARV has its axis of rotation perpendicular to the gravity vector thus, modeling microgravity by "nullifying" the downward gravity vector (Nickerson et al., 2000). Studies done by Hammond and Hammond (2001), report that in order to minimize rotational shear in the vessel the recommended speed need to be on the lower range from 10 to 60 RPM (revolutions per minute), where 25 RPM has been adopted the average the optimized rotational speed.

Experiments in MMG Systems.

Clinostat Experiments. Space flight has been reported to affect microorganisms in different ways. The effects include changes in bacterial growth, and antibiotic production and resistance (Table 2). According to Nickerson, there have been no studies regarding the effect of spaceflight on bacterial virulence (Nickerson et al., 2000). Nevertheless, the issue is becoming increasingly important because of the risk of infectious diseases in space craft crewmembers. Klaus et al. studied functional weightlessness during clinorotation of *E. coli* cell suspensions (Klaus et al., 1998). The objective of the study was to determine the appropriate speed of rotation for suspended cells. Particle behavior

factors that are prevalent during clinorotation such as gravity, diffusion and centrifugation were assessed mathematically. The experimental results of bacterial growth studies using a Clinostat were consistent with previous space flight results. Brownian motion, or diffusion of particles as a result of their thermal energy, functions independently of gravity (Klaus et al., 1998). It was believed that even in absolute weightlessness, particles will continue to move about randomly at a rate that depends on their size and the viscosity of the fluid in which they are suspended (Klaus et al., 1998). On Earth, cells constantly sediment downward at the same time that Brownian motion is attempting to randomly distribute them. Upper and lower limits on clinorotation speed are necessary to keep suspended particles in a state of "relative motionlessness" (Klaus et al., 1998). It was observed that particles "fall" due to gravity during the period of one revolution when the rotation is too slow. On the contrary, if the rotation is too fast, the particle will be centrifuged towards the container wall. Brownian motion or random movement of the cell is considered in this case to be noise.

The population dynamics behind the functioning of the Clinostat were determined using *E. coli* suspensions and Clinostat results were compared to previous experiments done in space. The clinorotated *E. coli* samples with their respective static controls were compared to the relative differences observed between spaceflight samples and their matched static ground controls. Surprisingly, there was a statistically significant increase of almost two fold in the final cell numbers for both spaceflight and ground control conditions (Klaus et al., 1998). Moreover, the lag phase was shortened in both clinorotation and spaceflight conditions, although this was not conclusive statistically

(Klaus et al., 1998). These results were consistent with those obtained by spaceflight experiments with *E. coli* (Bouloc & D'Ari, 1991). The Clinostat bacterial suspensions showed an average lag phase when the cultures were grown in pre-conditioned medium rather than fresh medium, and air bubbles were removed for not to interfere in the cell growth process (Klaus, 1998).

HARV Experiments.

In 2000, Fang and Pierson studied the growth of *Streptomyces hygroscopicus* in a non-HARV RVW. They found that the bacteria grew in a pellet form, it had a lowered dry cell weight and that its antimicrobial rapamycin production was inhibited (Fang et al., 2000). Following the addition of Teflon beads to the bioreactor growth became much less pelleted, the dry cell weight increased but the rapamycin production was still inhibited. It was assumed that the beads disturbed the low-shear environment, causing shear effects. Simulated microgravity appeared to stimulate extra-cellular production of rapamycin as opposed to the intracellular one observed in normal gravity conditions (Fang et al., 2000).

Similar results were observed in another investigation done on 1997 by Fang and Pierson in which a HARV was used instead of a plain RWV. They tested whether gravity affects microbial production of secondary metabolites by utilizing High-aspect rotating vessels (HARVs). Specifically, the antibacterial polypeptide Microcin B17 (MccB17) production by *Escherichia coli* ZK650 was tested in MMG (Fang et al., 1997). It was found that although MccB17 production by *E. coli* was significantly inhibited by MMG, bacterial cell growth was still stimulated. However, it was reported that the antibacterial

agent was found extra-cellularly when the culture was grown in the rotating bioreactors, where shear is very low. This change in the location of the peptide was not expected (Fang et al., 1997). In later work in duplicate HARVs it was shown that the change did not depend on MMG (Fang et al., 1997). Rather, the excretion of extra-cellular fluid into the medium appeared to be a function of the low shear stress condition of the HARV. Addition of beads (Teflon PTFE solid balls) to a 50 ml medium in the HARV under MMG considerably changed the MccB17 accumulation from 91% extra-cellular to 98% cellular. Nevertheless, there was no net effect difference between 1 bead versus that of 25 beads. Apparently, the MccB17 was kept from being secreted to the extra-cellular medium by the shear stress created by even only one bead (Fang et al., 1997). On the other hand, the addition of beads to the control HARV incubated to allow exposure to normal gravity (NG) resulted only in a partial effect on the change of extra-cellular to cellular production. The addition of 25 beads resulted in about 41% of MccB17 in cells and the other 58.6% in the supernatant. It seems that there is no significant shear stress in the environment of the HARV when in the NG position. It is thought that this happens because the beads remain at the walls or periphery of the vessel as opposed to the free movement they have throughout the liquid in the MMG position (Fang et al., 1997).

Nickerson et al used the low shear environment of the HARV to study *Salmonella enterica* serovar Typhimurium stress response and virulence (Nickerson et al., 2000 & 2004, Wilson et al., 2000a and Wilson et al., 2000b). It was reported that when identical normal gravity bacterial growth conditions are compared to its "LSMMG" (low-shear MMG) counterparts, the latter differentially regulates certain transcriptional regulators,

virulence factors, lipopolysaccharide biosynthetic enzymes, iron-utilization enzymes, and proteins of unknown function. It was proposed that a "LSMMG-regulon" is globally distributed on the chromosome and that certain genetic loci may be target of the "LSMMG" response (Wilson et al., 2000b). It appears that the number of down-regulated genes is slightly larger than the up-regulated number of genes in "LSMMG". These investigators claim their results are consistent with previous ones showing that the proteins down-regulated by "LSMMG" were higher than the number of up-regulated proteins as detected by two-dimensional gel protein analysis. Furthermore, by utilizing gene microarray analyses, results indicated that several genes involved in iron metabolism (*fep*D, STM1537, *hsc*B, *feo*B, *yli*G, *suf*C, *suf*S) are up-regulated by "LSMMG" whereas, type (III) three secretion systems (*orgA, prgH, sipD, invI, invA, pigB, sseB, ssaL, ssaV and sseJ*) are down-regulated (Wilson et al., 2000b). Additionally, the Fur-binding site has previously been reported to regulate a number of processes other than iron metabolism in *E. coli* and *Salmonella* (i.e. acid resistance and succinate utilization). Interestingly, a number of potential Fur-binding sites were found to be located upstream of several different "LSMMG"-regulated genes (Wilson et al., 2000b).

These results led to the identification of "LSMMG "regulon" gene groups. This regulon is believed to be involved in transcription regulation, LPS, and cell wall synthesis, iron metabolism, ribosomal structure and membrane structure (Wilson et al., 2000b). The "LSMMG regulon" has been defined as the signaling pathway within the *Salmonella* response to "LSMMG". Studies done on this signaling pathway have indicated that "LSMMG" may be one of the growth conditions that can serve to pre-adapt

an *rpo*S mutant for resistance to diverse environmental stresses (Wilson et al., 2000a). The investigators claim that RpoS would be a likely regulator of the *Salmonella* response to "LSMMG" because there is, apparently, a relationship between this regulator and the bacteria acid stress response and enhanced virulence (Wilson et al., 2000a & b). However, since the regulator has not yet been identified conclusively, it is more appropriate to use the term "stimulon" rather than "regulon" in reference to the "LSMMG" phenomenon.

The pathogenicity and protein synthesis of *S. enterica* serovar Typhimurium strain *x3339* in MMG was also investigated. HARV bioreactors were used to examine the effects of MMG on pathogenic bacteria. Bacterial virulence was studied on mice models. *S. enterica* was grown under "LSMMG" and NG conditions and then inoculated into mice to study the level of virulence. It was found that the lethal dose to kill 50 % of the animals (LD_{50}) with "LSMMG" grown serovar Typhimurium bacterial cells was 5.2 times lower than the LD_{50} for the same strain under normal gravity (Nickerson et al., 2000). Mice inoculated with "LSMMG" grown cells died faster (average time) than the mice inoculated with the same number of bacterial cells but grown in NG (Nickerson et al., 2000). Apparently, *S. enterica* serovar Typhimurium "LSMMG" grown cells are more virulent than NG-grown ones. Furthermore, this enhanced virulence displayed by "LSMMG" cultured serovar cells was thought to be partly due to increased acid stress resistance of macrophage exposed *Salmonella*. The survival rate observed in "LSMMG" grown cells was threefold greater than the NG-grown ones (Nickerson et al., 2000). Although data was not shown, it was found that there was no relationship between

virulence and the ability to adhere to and invade tissue culture cells similar to those encountered in a systemic infection, such as CaCo (human colon cell line). H-MMG-grown serovar cells produced flagella and displayed similar adherence and invasion patterns into tissue culture cells compared to NG-cultures. It seems that the ability of the bacterial cells to colonize and penetrate epithelial cells of the murine gastrointestinal tract is not enhanced by increased virulence (Nickerson et al., 2000). *S. enterica* serovar Typhimurium was also analyzed for proteins synthesized in response to MMG. Two-dimensional gel electrophoresis was utilized to study the total protein synthesis during growth of this strain under MMG (Nickerson et al., 2000). Results revealed significant differences between proteins synthesized by these bacteria in MMG as compared to the ones in NG. About 38 proteins were down-regulated threefold or more during growth in MMG versus NG (Nickerson et al., 2000). Relevant MMG experiments are described in Table 3.

To summarize, a Clinostat can reproduce an almost undisturbed fluid environment similar to the one observed within an unstirred container in reduced microgravity (Klaus, 2001). The RWV bioreactor, conversely, simulates microgravity to a lesser extent while other factors such as relative cell motionlessness and reduced extra-cellular mass transport are considered (Klaus, 2001). The RWV is an ideal system for cell or tissue growth where cells are induced to settle at terminal velocity. At the same time, the cell suspension is under low shear and facilitates necessary extra-cellular medium and gas mixing (Klaus, 2001). Bacteria in suspension can be clinorotated because their small size and the movement due to gravity and centrifugation is not significant compared to the

Brownian "noise" the cells experience (Klaus et al., 1998). The similarities reflected in *E. coli* suspension culture kinetics under microgravity and clinorotation indicate that the cell's physical interaction with its external medium may be the principal mechanism responsible for changes due to spaceflight (Klaus et al., 1998). A Clinostat can provide an environment almost like real microgravity in many instances and thus is useful in evaluating spaceflight experiments (Klaus et al., 1998). Presumably, there are factors that can favor the ability of a Clinostat to simulate reduced gravity. These include: small particle size and highly viscous medium, minimal density difference between particle and medium, rotation speed sufficiently fast to keep particle within Brownian "noise", rotation speed slow enough to avoid introducing a noticeable amount of centrifugal force and a container with a practical small diameter to lower centrifugal acceleration (Klaus et al., 1998).

An engineering investigation showed that at higher rotation rates and equilibrium positions closer to the center of the bioreactor, tissue and cells would experience lower shear stress levels (Ramirez et al., 2003), while tissue and cells closer to the outer perimeter of the bioreactor would experience higher shear stress levels. There are certain physical properties that are altered by microgravity. These include those from fluids, like convective movements and surface tension (Ramirez et al., 2003). Evidence suggests that cells of all sizes and organizational complexity under certain situations can sense gravity (Klaus et al., 1998).

The data indicate that specific microbiological processes could potentially be affected by the alteration of gravity or intrinsic factors. In MMG and in actual MG the lag

phase is shortened because the cell is not moving away from its by-products but instead surrounded by a boundary layer of metabolites. Therefore, bacterial exponential growth and stationary phase may be reached earlier in MMG conditions, as they are during spaceflight. Experimental results for final cell density confirm this phenomenon. Fluid dynamics (i.e. shear stress) and extra-cellular transport phenomena and not cellular dynamics are the most probable cause of the reported increases in bacterial growth in actual microgravity and microgravity models (Klaus et al., 1998). Moreover, increased resistance of *E. coli* to the antibiotic Colistin during spaceflight conditions was found not to be an acquired characteristic, but rather an adaptation to weightlessness (Lewis et al., 1997). Possible reasons for increased resistance include: bacteria proliferate faster in space and therefore, the concentration of antibiotics may not be sufficient and also, there may be a defective transport of antibiotics into the cells because of the modification of cellular membrane permeability (Lewis et al., 1997). It would be interesting to determine if the same antibiotic resistance scenario holds true for MMG experiments with the same bacterium, since this would help to confirm the validity of MMG vs. spaceflight experiments.

Results from HARV experiments suggest that in the MMG condition a variable that affects bacterial virulence is the level of shear stress in the bioreactor vessels. If gravity is at all altered in these vessels, it does *not* appear to affect bacterial cells as much, but instead, surrounding environmental physical factors that could also be intrinsic (but not limited to the altered gravity condition), such as flow shear and density may be responsible for bacterial changes in MMG. The MMG experiments reported by the

Nickerson group suggest that the "low shear" of the HARV bioreactor could be a novel regulator of gene expression in *S. enterica* serovar Typhimurium. If indeed there is a specific genetic "regulation" they claim it may contribute to bacterial pathogenicity. This is particularly important for long-term space missions that will use regenerative life support systems. The International Space Station space crew needs to be protected of the potential changes in bacterial pathogenicity caused by prolonged conditions in microgravity. The next step would be to determine if changes in gravity and/or shear modulate virulence only in Salmonellae or also in a diversity of other microbial pathogens. Studies are needed to corroborate if low shear is indeed the influential factor of the HARV. To remain objective, HARV MMG (H-MMG) will be used instead of "LSMMG".

In addition to microgravity, it is important to consider that factors such as cosmic radiation, changes in fluid convection, vibration, and cabin conditions (e.g., temperature, artificial light, etc.) which might contribute to changes in microbial physiology during spaceflight (Klaus, 2001). These factors, interestingly enough, are not necessarily considered in MMG experiments.

Table 3. Summary of Relevant Microbial Ground Experiments.

Type of Culture	Factor Tested	Methodology in brief	Conclusion	Reference
Suspension	Lag and Log Phases	*Pseudomonas nitroreducens* grown in 1:2 Tryptic Soy Broth in Modeled Microgravity or Control (1.005xG) conditions in a HARV @ 30°C and 15 RPM for 10 days.	No change between MMG and control.	Bouma & Pierson, 1997.
Suspension	Lag and Mid-log Phases	*Salmonella enterica* serovar Typhimurium grown in Lennox broth, in Modeled Microgravity or Control (1.005xG) conditions in a HARV @ 37°C and 25 RPM for 10h.	Mice infected with MMG-cultured *Salmonella enterica* serovar Typhimurium show organism colonizes liver and spleen more efficiently; and exhibit increased resistance to thermal osmotic and acid stresses.	Nickerson et al., 2000; Wilson et al. 2002.
Suspension	Mid-log and Stationary Phases	*E. coli* grown in M9 + glucose and kanamycin, in Modeled Microgravity or Control (1.005xG) conditions in a HARV @ 37°C and 25 RPM for 4 and 24 h.	No change in growth rate between MMG and control. MMG enhanced *E. coli* resistance to both osmotic and acid stresses.	Lynch et al., 2004.

The Bacterium *Pseudomonas aeruginosa.*

P. aeruginosa, a ubiquitous, water-borne gram-negative rod bacterium and opportunistic pathogen, may thrive in water and other environments. It may form a biofilm of cells in an extra-cellular polymeric matrix on the mucous membranes of the

lungs in cystic fibrosis patients. This bacterium also produces yellow/green and blue/green pigments called pyochelin and pyocyanin, respectively (Table 4) which have been postulated to contribute to the infection capacity of PA (Sorensen and Joseph, 1993). An important virulence protein of *P. aeruginosa* is Exotoxin A (ETA) (Galloway, 1993). The present investigation focuses on how ETA production and other virulence factors of *P. aeruginosa* may be affected by altered gravity using MMG systems. It is anticipated that in the future a similar experiment will be performed in spaceflight.

Exotoxin A was discovered and purified 30 years ago and has been identified as the major virulence-associated factor produced by *P. aeruginosa* (Galloway, 1993). In 1986, its three-dimensional structure was determined and since then has been the model upon which other similar bacterial toxins are being studied (Galloway, 1993). ETA is composed by a single-chain polypeptide of 613 amino acids, with a molecular weight of 66,583 (Galloway, 1993). It is also part of the adenosine-diphosphate-ribosylating toxin (ADPRT) group to which the diphtheria, cholera, pertussis toxins and *E. coli* heat-labile enterotoxin belong, (Galloway, 1993). The mechanism through which ETA impairs its target, like all other ADPRT toxins, is based on its capacity to bind to NAD (nicotine adenosine) and covalently link the Adenosine Diphosphate-Ribose moeity to a specified target protein, preventing normal function of this protein (Galloway, 1993). DT and ETA, in particular, link to target protein eukaryotic elongation factor 2 (EEF-2) causing the loss of protein synthesis in the target cell (Galloway, 1993).

Table 4. Virulence Factors Present in *P. aeruginosa* PA103. (Based on *http://textbookofbacteriology.net/pseudomonas.html* website).

Surface Bound	Periplasmic Enzymes	Toxins and Pigments
Fimbriae	Elastase	Endotoxin
Alginate	Phospholipase C	Pyocyanin
Pyochelin	Collagenase	Pyorubin
	Gelatinase	α-oxyphenazine
	Lecithinase	Fluorescein
	Alkaline protease	Pyocins
	Neutral protease	Exotoxin A
	Cytotoxin	Exotoxin S

Previous studies on *S. enterica* serovar Typhimurium have shown up-regulation of virulence genes in modeled microgravity (MMG), for which a "LSMMG regulon" has been postulated (Nickerson et al., 2004). Furthermore, past proteomic analyses show that in MMG there is variation in the protein expression of *P. aeruginosa*. These changes are said to reflect alterations in metabolic and physiological functions, and also in putative pathways responsible for the production of virulence factors (Pulcini et al., 2004).

P. aeruginosa has been of particular importance to space flight missions. For instance, during the Apollo 13 space mission, a crew-member developed a urinary tract infection with PA (Sonnenfeld, 2005). The psychological and physical stress that space flight imposes on astronauts may cause changes in the immune systems of astronauts and thus contribute to the development of a serious infection given the opportunistic nature of this bacterium.

Thesis Statement and Hypothesis.

The growth and physiology of *P. aeruginosa* has not been well studied in MMG systems. The goal of this study was to establish the growth patterns of this microorganism in MMG systems, and to determine how Exotoxin A production, a virulence factor, is related to different stages of growth. Virulence in *P. aeruginosa* may be enhanced under MMG conditions.

MATERIALS AND METHODS

Clinostat Experiments.

Bacterial Strain and Preparation of Inocula.

An original bacterial stock culture of *P. aeruginosa* PA103 (ATCC 29260) was inoculated on an R2A plate. After incubation at 30 ° C for 24 hours, green rough type colonies were isolated from the entire type smooth ones. Four colonies of only rough colony type were streaked onto each of 2 R2A plates. Incubation was again done for 24 hours but at 25° C. Under the laminar flow hood, 2 ml of Peptone Glycerol Frozen Stock Broth (see Reagents section) was aseptically pipetted onto the surface of each plate. Colonies were harvested using a sterile cotton swab. Broth was aspirated from agar surfaces with a sterile transfer pipette, placing the broth into a sterile glass vial and overtaxing for 30 seconds. To reduce clumping, culture suspensions were filtered through sterile 5 μm pore size nylon filters into a sterile glass vial. The amount of broth was measured, recovered and diluted 1:150 in peptone glycerol broth, and vortexed for 30 seconds. It was then dispensed in 100 μl aliquots into sterile microcentriffuge tubes. Each tube was labeled with date and strain name and stored at –80°C.

The growth protocol used for PA103 to be used to inoculate MMG cultures was as follows: after 100 μl PA103 frozen stock was thawed, 900 μl of Simple Modified Media 1 (SDM1) (Table 5) broth was added and the mixture was vortexed for 30 seconds. A volume of 100 μl of this bacterial suspension was mixed with 99.9 ml of SDM1 in a side armed flask. The flask was incubated at 25^{o}C on a horizontal shaker at

150 RPM. Klett readings were monitored and after 27 hours the bacterial population had reached 100 Klett Units (~10^9 CFU (colony forming units)/ml). Twenty ml of the culture was dispensed into each of two 50 ml polycarbonate Oakridge screw-cap centrifuge tubes. The samples were centrifuged for 5 minutes at 10,000 RPM in Sorvall Centrifuge (RC2-B with SS-34 rotor) with refrigeration. Supernatants were discarded and the pellet in each tube was washed and resuspended with 20 ml of phosphate buffered saline glucose (PBS+G) (see Reagents section). The centrifugation step, washing and resuspension of pellet were repeated another two times. In the final step, the pellet in each tube was resuspended in l0 ml of FAMQ (Filtered-Autoclaved-Milli-Q) water (see Reagents section). All suspensions were combined in one sterile bottle and after vigorous shaking 91 µl were diluted using the drop plate technique on R2A plates; the suspension was stored in a refrigerator. After overnight incubation at 30^{o}C, CFU's per ml were calculated and determined for the suspension. The inoculum suspension was adjusted to ca. 5x10^8 CFU/ml before use.

Growth of PA103 (ATCC 29260) in the lag, log and stationary growth phases was examined to assess MMG effects (Guadarrama et al., 2005a & b). For the clinostat experiments, inoculum was prepared in two different ways. If the inoculum could be frozen, this would allow for preparation of larger volumes and aliquoting, which would be more efficient for subsequent experiments. Use of refrigerated inoculum requires preparation every few weeks to ensure viability and consistency. The frozen inoculum was suspended in glycerol (20 % v/v final concentration) and frozen at -80^{o}C while the

refrigerated inoculum was suspended in double distilled MilliQ (MQ) water and refrigerated at 4°C.

Reagents.

Sterile Filtered MilliQ Water (FAMQ). Two liters of MQ water were dispensed into clean 2.5 liter bottles. Autoclaved for 60 minutes at 121°C, and stored at 5°C until needed. One liter Wheaton bottles were washed with RBS detergent and rinsed thoroughly using 3 rinses with tap water, 2 rinses with RO water and 2 rinses with MilliQ water. Bottles were sterilized by covering with aluminum foil and placing in the oven at 175°C for 3 or more hours. Caps were covered and autoclaved separately at 121°C for 30 minutes. Prior to use, autoclaved MQ water was filtered through a sterile PES bottle top filter (Nalgene # 2954520 or Millipore #SCGPT05RE or equivalent) into the sterile, particle-free, 1L bottles and cap. Stored at 4° C.

10 mM Phosphate Buffered Saline pH 7.5 and PBS + Glucose (Stock). Concentrated PBS was prepared by adding 12 g of sodium phosphate (Na_2HPO_4), 2.2 g of anhydrous sodium phosphate ($NaH_2PO_4 \cdot H_2O$), and 85 g of sodium chloride (NaCl) to 1 liter of MQ water. pH was adjusted to 7.5 as necessary with 0.1 N HCl or 0.1 NaOH. Autoclaved for 60 minutes.

PBS + Glucose. Stock phosphate buffered saline solution with glucose was added at a concentration of 2.5 g/L. 100 ml of the concentrated PBS was combined with 2.5 g glucose. Brought to 1 liter with MQ water. Adjusted pH to 7.5, and filter-sterilized.

2.4% Formalin Solution. To make the stock solution, 6.5 ml of 37% formalin q.s. to 100 ml with MQ water and filter sterilized. The working solution was prepared by diluting 1:12 for a final concentration of 0.2%.

Peptone Glycerol Frozen Stock Broth. Two grams of Peptone and 40 ml of Glycerol (100%) were mixed in 60 ml of MQ water.

Sample Preparation.

Both inocula were added to Modified Simple Defined Media 2 (MSDM2) (Table 5) at a ratio of 1:11 and used to fill 5 ml syringes each with 2 ml of medium. MSDM2 which stimulates ETA production by iron deprivation also contains sodium nitrate. In non-aerated conditions like in spaceflight and Clinostat experiments, PA utilizes nitrate instead and generates bubbles into the medium (Liu et al., 1998). Sampling times were time zero, 30 min, 3 h, 6 h, 9 h, 12 h, 15 h, 18 h, 21 h, and 24h. For both inocula and every time point, syringes (Fig. 1,C) were placed in the Clinostat (Modeled Micro-Gravity (MMG) environment) (Fig. 1,A&B) with static controls (STAT). Horizontal (HZN) (Fig. 1,D) rotating controls were included for the refrigerated inoculum. MMG and HZN samples were rotated at 15 RPM and all samples incubated at 37^{o}C. All culture samples were diluted, drop plated on R2A agar (Table 5) and incubated for 24 hrs at 30ºC. Half of the remaining suspension was fixed with 2.4% formalin at a dilution ratio of 1:12 (final concentration, 0.2 % formalin) for ETA assay and the other half with no added formalin was frozen at -20ºC for sample processing for proteomic analysis. The volumes and dilution ratios of fixative to inoculum to medium were based on the

Table 5. Media Formulation.

SDM1

Amino Acids

2.0 g/l	L-Aspartic Acid
8.0 g/l	L-Glutamic Acid Monosodium Salt
6.0 g/l	L-Arginine
6.0 g/l	L-Serine

Organic Compounds

1.1 g/l	Citric Acid Monohydrate
5.0 g/l	D(+) Glucose

Salt and Mineral

1.5 g/l	Dipotassium Phosphate (K_2HPO_4) Dibasic Anhydrous

Dissolved in 900 ml MQ water

1.0 µM	$FeSO_4$ Heptahydrate Added 0.1 ml/l of 10 mM stock
1 mM	$MgSO_4$ Anhydrous Powder. Added 1 ml/l of 1 M stock

pH was 7.6. Brought to 1 liter and filter-sterilized. Stored at 4-6°C.

MSDM2

Amino acids

2.0 g/l	L-Aspartic Acid
8.0 g/l	L-Glutamic Acid Monosodium Salt
6.0 g/l	L-Arginine
6.0 g/l	L-Serine

Organic Compounds

1.3 g/l	DL-Malic Acid Disodium Salt
5.0 g/l	D(+) Glucose

Salt and Mineral

1.5 g/l	Dipotassium Phosphate (K_2HPO_4) Dibasic Anhydrous
1.0 g/l	Sodium Nitrate ($NaNO_3$)

Dissolved in 900 ml MQ water

1 mM	Magnesium Sulfate ($MgSO_4$) Anhydrous Powder Added 1ml/l of 1 M stock
35 g/l (28 ml/l)	Glycerol

pH was 8.5, adjusted to pH 7.6 with 5N HCl. Brought to 1 liter, and filter-sterilized. Stored at 4° to 6°C. (Blumentals et al., 1987). **Note: In addition, the medium was not supplemented with sodium nitrate, as required for some HARV cultures.

R2A

9.1 g	R2A Agar (Difco Cat. No. 218263)
500 ml	MQ water

Autoclaved at 121°C, 20 minutes and tempered in a 50°C water bath for 30 minutes prior to plate pouring.

configuration required for ESA Type I experiment containers with Phorbol Cassette inserts which were used for the space shuttle Columbia mission STS-107 experiment.

Exotoxin A Assay.

ETA was quantified by an Enzyme Linked Immunoadsorbent Assay (ELISA) developed for spaceflight experiment BACTER on Space Transportation System STS-107. A 96-microwell plate was coated with a series of standard ETA (CalBioChem Lot # B67153; 0.5 mg/ml) and sample dilutions at a volume of 50 µl/ well, and incubated overnight at room temperature on an anti-static mat. The ETA-coated plate was then washed three times with Tween Buffer Solution (TBS) (Table 6) and subsequently blocked with 200 µl/ well blotto for 2 hours at room temperature. After the plate was again washed three times with TBS, 50 µl/ well of anti-*Pseudomonas* Exotoxin A (Sigma P2318) diluted 1/4000 in blotto (Table 6) was added as the primary antibody and incubated overnight at 4^{o}C. The plate was washed another three times with TBS and treated with 50 µl/ well of the secondary antibody anti-Rabbit-HRP (Horse-Radish Peroxidase) (Sigma A6154), previously diluted 1/2000 in blotto, and incubated overnight at 4^{o}C. After further washing, orthophenylenediamine (OPD), the HRP indicator substrate, was added at a volume of 75 µl/ well, and after 30 minutes, 75 µl/ well of 10% percent sulfuric acid was added to terminate the reaction. Optical density was read at 490 nm and sample concentrations were determined from the standard curve in ng/ml. The optical density readings were multiplied by the dilution factor of the sample, and divided

by the slope of the standard curve to obtain the ETA concentration. The conditions of the assay were optimized for reproducibility and reliability. Samples were done in triplicates.

Table 6. ELISA Reagents and Solutions.

TBS Buffer
6.61 g/l Trizma HCl
0.97 g/l Trizma Base

Once dissolved, pH reached 7.4 at room temperature (25^{o}C).
Subsequently added:
8.77 g/l NaCl (Sodium Chloride)
Filtered sterilized using 0.2 µm pore size filter apparatus.

TBS-T
Added to TBS:
0.1 % w/v Tween 20

Blotto
5% w/v Carnation instant milk
1% w/v Bovine Serum Albumin
Dissolved in TBS-T

Sodium Citrate
4.8 g Citric Acid
1.5 ml 5 N Sodium Hydroxide
484 ml MQ water

OPD
25 ml Sodium Citrate (pH 5.0)
25 mg O-phenylenediamine
10 µl 30% Hydrogen Peroxide

Sulfuric Acid (10% Solution)
10 ml Concentrated Sulfuric Acid (H_2SO_4)
90 ml MQ water.

Statistical Analysis.

Data was statistically analyzed using the R statistical software (www.R-Program.org) by the non-linear mixed-effects model package. The data were fit using a model of fixed effects designed by Dr. Robison-Cox from the MSU Math Dept. for

incubation time and treatment (STAT, MMG and HZN). Statistical analyses were obtained by calculating the random mean of the optical density readings for each ELISA 96-well plate, and by a weighted regression. ETA concentration (ng/ml) was calibrated according to an inverse-proportional-weight function from the variance (OD reading estimates).

HARV Experiments.

Bacterial Strain and Preparation of Inocula.

Original bacterial stock and PA growth were prepared as described previously in the Clinostat experiments section.

Growth of PA103 (ATCC 29260) in the mid-log and stationary growth phases was examined to assess model microgravity effects by using the High-Aspect-Ratio-Vessel (HARV) bioreactor (Fig. 2). Inoculum was prepared by MQ water suspension and refrigerated at 4^{o}C. The inoculum suspension was adjusted to ca. $5x10^{8}$ CFU/ml prior to sample preparation.

Sample Preparation.

MSDM2 was mixed with the inoculum suspension at a 11:1 ratio. Subsequently, HARVs were each filled with 10 ml of inoculated medium. Sampling times were 0h, 12h and 24h. For every time point, separate HARVs were set-up for Modeled Micro-Gravity (H-MMG) environment (horizontal axis results in the G-vector being time averaged to near zero, (Klaus, 2000) with static controls (H-STAT) and Horizontal (H-HZN) rotating controls (the G-vector acts on cells, as in static cultures). In the first experiment, a

number of HARVs were aerated and non-aerated. In the second experiment, HARVs were all aerated, and a number of them were added MSDM2 suspension with no nitrate (labeled $-NO_3$) (Tables 6&7). In addition, for the non-aerated vessels, a rubber membrane and plastic washer were held tight to the back side of the vessel by alligator clips (Fig. 7 A). Samples were rotated at 15 RPM, where appropriate, and incubated at 37^{o}C. Duplications were only done for H-STAT in order to confirm consistency of results. All culture samples were diluted, drop plated on R2A agar and incubated for 24 hrs at 30ºC. Remaining suspension was fixed with 2.4 % formalin at a dilution ratio of 1:12, for ETA assay and the other half with no added formalin was frozen at -20ºC for sample processing for proteomic analysis.

Table 7. HARV Experiments Set-Up. (X= 10 ml of sample loading).

Set-up Exp. 1 *	**H-MMG**		**H-HZN**		**H-STAT**	
Panel letter & condition	12h	24h	12h	24h	12h	24h
A) Non- aerated	X	X	X	X	X	X
B) Aerated	X	X			X	X
*The sample medium was supplemented with nitrate unless stated otherwise.						
Set-up Exp. 2 *	**H-MMG**		**H-HZN**		**H-STAT**	
Panel letter & condition	12h	24h	12h	24h	12h	24h
C) Aerated and no nitrate	X	X	X	X	X	X
D) Aerated			X	X	X	X

Exotoxin A Assay.

ETA was quantified by an Enzyme Linked Immunoadsorbent Assay (ELISA), as previously described in the Clinostat Experiments section.

Proteomics.

Protein expression in samples was assessed by 2-Dimensional Gel Electrophoresis. The major steps of the procedure were as follows: protein determination, precipitation and solubilization; two-dimension gel electrophoresis and gel staining.

Protein Determination.

The Modified Lowry protein assay was used to determine protein concentration in samples. A standard curve was created by diluting BSA (Bovine Serum Albumin) to 1 mg/ml in MQ water (see Table 8). Aliquots of 1ml were frozen at -80˚C.

Table 8. BSA Standard Curve and Sample Volumes.

BSA (1mg/ml) in µl	Volume H2O in µl
0	100
10	90
20	80
30	70
40	60
50	50

Samples were diluted as follows:

Sample (µl)	H2O (µl)
10	90

To every 100 µl of sample and BSA standard, a volume of 1.1 ml Biuret solution (Sigma) was added. After 20 minutes, 50 µl of Folin's (Sigma) solution was also added.

During another subsequent 20 minute period, standard and samples were pipetted in triplicates onto an ELISA micro-well plate, at a volume of 200 µl per well. Optical density (OD) was read at 550 nm and sample concentrations were determined from the standard curve set by BSA in mg/ml. An Excel spreadsheet calculation was used to determine protein concentration from the BSA linear proportion graph using the following formula:

$$x=(y-b/m)/10$$

Where "*x*" is the protein concentration in µg, "y" is the average OD reading of the sample, "b" is the intercept and "m" is the slope of the BSA standard linear graph. The result was divided by 10, since 10 µl of sample were originally diluted in 90 µl of MQ water and we wanted to obtain our result in µg per µl.

Protein concentration adjustment calculations were used to standardize loading of Iso-Electric Focusing (IEF) strips. In the first Clinostat experiments, sample protein load varied, since a constant volume of 500 µl was assessed from every sample regardless of protein weight. In later HARV experiments, protein calculations were improved and more consistent sample protein loads were used. There were 150 µg of sample protein assessed consistently for all samples. The volume analyzed for that set weight of protein varied according to the calculated protein concentration for each sample.

Protein Precipitation and Solubilization.

Protein samples were treated with cell lysis buffer, lysozyme, and TCA/Acetone for precipitation and B-9 Buffer for solubilization (see Table 9 for formulations). Using the protein concentration determinations, to every 500 µl of protein, cell lysis buffer was

added at 20% of sample volume (i.e. to 500 µl sample 100 µl buffer added). Samples were treated with lysozyme at 2% of sample volume. Protein was precipitated by adding TCA/Acetone at a concentration of 0.10% of sample volume. B-9 buffer was added for the solubilization step (2 hours min.). Centrifugation in between steps allowed removal of non-soluble particles. Supernatant was retained to re-hydrate overnight Immobiline, pH range 3-10, Drystrips (Amersham BioSciences) in preparation for IEF.

Table 9. Protein Precipitation Solutions.

Cell Lysis Buffer	250 µl TE (Tris and EDTA) 250 µl PMSF (Phenylmethylsulfonyl and ETOH) 5 µl DTT (Dithiothreitol) at a concentration of 0.2%)
Lysozyme	10 mg Lysozyme 1 ml MQ water. Sonicated and left to dissolve for one hour.
TCA/Acetone	1 ml 40% TCA (trichloroacetic acid) 3 ml Acetone
B-9 Buffer	
	0.420 g/ml 7M Urea 0.152 g/ml 2M Thiourea 0.02 g/ml 0.115M CHAPS 2 µl /ml 0.002% Bromophenol Blue
Add to buffer right before use:	
	20 µl/ml Pharmalytes or 31 µl/ml Ampholytes and 100 µl/ml 65mM DTT

Two-Dimension Gel Electrophoresis.

Solubilized proteins were re-hydrated on IEF strips (Immobiline, Amersham Pharmacia Biotech) to prepare for the first dimension, prior to molecular weight separation (second

dimension) (Sonawane et al., 2003). Re-hydrated strips were subjected to IEF (Multiphor II, Amersham Pharmacia Biotech) for a period of 27 hours with alternating voltage currents (Table 10). Individual strips were then immediately and sequentially incubated in two different equilibration buffer solutions (Table 11) for 10 minutes each. In the second dimension, known as molecular weight separation, proteins were separated by preparing and using 12 % SDS-polyacrylamide gels (Table 12) using the Hoefer DALT Tank System (Amersham Pharmacia Biotech). IEF strips were placed on top of these gels and fixed on place using 0.5% agarose prepared with SDS running buffer (Table 11) and a few grains of bromophenol blue. Twenty liters of SDS running buffer was also added to the DALT tank, which was programmed at 10mAmp per gel, for 1 hour and, at 35mAmp per gel, for a maximum of 24 hours. Gels could be monitored by observing the bromophenol blue line across the gel (Fig. 3). When this line reached the bottom of the gel the second dimension was assumed to be finished. A number of 2-D gel electrophoresis runs had to be discarded because of streaking of proteins across the gels which did not allow for proper separation based on molecular weight. Acrylamide De-Gas procedures were done to minimize bubbling prior to gel casting.

Table 10. Iso-Electric Focusing Voltage Currents.

Step 1 – Ramp	500 V	1000 V/hours
Step 2- Focus	500 V	2000 V/hours
Step 3 – Ramp	3500 V	10 KV/hours
Step 4 – Focus	3500 V	48 KV/hours

Table 11. Second Dimension Electrophoresis Buffers.

Equilibration Buffer (EB)		
	0.5 M Tris/HCl pH 6.8	25 ml
	Urea	90 g
	99% Glycerol	75 ml
	SDS	10 g
	MQ H_2O	ad 250 ml
Strip Buffers		
	DTT	3.5mg/ml in EB
	Iodoacetamide	45 mg/ml in EB
SDS Running Buffer		
	Tris	60.5 g
	Glycine	288.0 g
	SDS	20.0 g
	MQ H_2O	20.0 liters

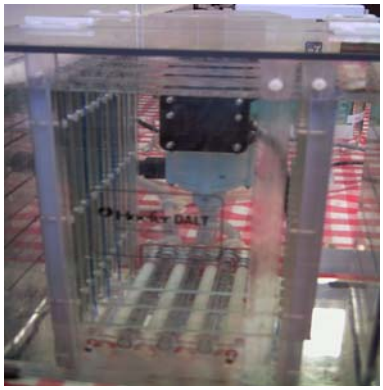


Figure 3. The DALT Tank. This tank houses the second dimension electrophoresis that is, molecular weight separation of proteins.

Table 12. Sodium-Dodecyl-Sulfate-Poly-Acrylamide Gel Components.

Final 12 % T (1.5 mm width)		
(Volume in ml)	12 Gels	23 Gels
Acrylamide 40% 29:1	339	555
1.5 m Tris-HCl pH 8.8	275	450
MQ Water	462	756
10 % SDS (Sodium Dodecyl Sulfate)	11	18
10% APS (Ammonium Persulfate)	11	18
10 % TEMED	1.6	2.6
Total volume	1100	1800

Gel Staining.

The Silver Nitrate and SYPRO Ruby Protein Gel Stain methods were used to stain gels**.** Table 13 describes the preparation of staining solutions. Silver staining is the most sensitive, non-radioactive staining method (Sonawane et al., 2003). It requires a high purity reagents and precise timing for reproducible, high-quality results. All staining and developer solutions were prepared freshly. After the run, the proteins were fixed by incubating the gels in fixation solution with gentle agitation on a shaking platform for at least 1 h at room temperature. The gel slabs were rinsed with 50% ethanol (3 changes, 20 min per change) with gentle agitation. The gels were sensitized with a sensitization solution for 1 min. The solution was discarded and the gel slabs were rinsed with 3 changes of double distilled water (20 seconds each). Staining solution was added to the gel and gently agitated for 20 min at room temperature. The silver nitrate solution was discarded, the gels were quickly rinsed with distilled water (2 changes, 20s per change) and transferred to the developing solution and gently agitated with a rocking motion until

Table 13. Preparation of Staining Solutions.

Silver Nitrate Staining:

Fixation solution:		
Ingredient(s)	Volume	Final Concentration
Ethanol	500 ml	50 %
Acetic acid	120 ml	12 %
37% formaldehyde	0.5 ml	0.05 %
dH_2O		380 ml
Sensitization:		
Ingredient(s)	Volume	Final Concentration
$Na_2S_2O_3.5H_2O$ (Sodium Thiosulfate)	0.2 g	0.02 %
DdH_2O		1000 ml
Staining solution:		
Ingredient(s)	Volume	Final Concentration
Silver nitrate	2 g	0.2 % (w/v)
37% formaldehyde	0.75 ml	0.075 %
dH_2O		1000 ml
Development solution:		
Ingredient(s)	Volume	Final Concentration
Na_2CO_3 (Sodium Carbonate)	60 g	6 %
$Na_2S_2O_3.5H_2O$	4 mg or 20 ml of solution	0.0004 %
37% formaldehyde	0.5 ml	0.05 %
dH_2O		1000 ml
Stop solution:		
Ingredient(s)	Volume	Final Concentration
Acetic Acid	50	5 %
dH_2O		950 ml

SYPRO Ruby Protein Gel Staining:

Step	Reagent	Basic Protocol
Fixation	50% Methanol, 7% Acetic Acid	100 ml for 30 minutes
		100 ml for 30 minutes
Stain	SYPRO Ruby gel stain	60 ml, overnight
Wash/destain	10% Methanol, 7% Acetic Acid	100 ml for 30 min

spots reached the desired intensity (~1 min.). To terminate the developing reaction, the gels were immediately transferred to the stop- solution. On the other hand, the SYPRO Ruby protein gel staining method was used for a number of gels instead of the silver nitrate method, in order to obtain enhanced-quality gel images. After the second dimension electrophoresis, the gel was placed into a clean container with 100 ml of fix solution and agitated on an orbital shaker for thirty minutes. This procedure was done twice with fresh fixative each time. For the second step, 60 ml of SYPRO Ruby gel stain. (Molecular Probes) were added after the fixative had been drained and recycled. The gel with stain was agitated on an orbital shaker overnight. The gel was then transferred to a clean container and washed in 100 ml of destain solution for 30 minutes. This transfer step aids to minimize background staining irregularities and stain speckles on the gel. Prior to imaging, the gel was rinsed in MQ water twice for 5 minutes each to prevent possible corrosive damage to the imager.

Gel Imaging.

The Typhoon Imager (Amersham Typhoon 8600 Variable Mode Imager) was used to detect chemiluminescence/fluorescence from the ruthenium in the SYPRO Ruby stained gels. The imager works with an excited laser, and the pixel-by-pixel fluorescence measurement eliminates fluorescent blooming and provides better resolution than conventional systems. Proteins expressed as a result of HARV modeled microgravity (H-MMG) were compared visually against the static (H-STAT) and rotational (H-HZN) controls.

RESULTS

Clinostat Experiments.

These experiments were performed to determine the effects of MMG on subsequent growth and physiology of *P. aeruginosa* PA103 (Guadarrama et al., 2005a & b). An additional objective was to determine the effects of inoculum storage conditions on the results. Results show that freezing the inoculum generates lower cell numbers at inoculation, slower growth, and lower cell numbers in stationary phase than subsequent experiments done with freshly refrigerated inoculum. Samples were rotated at 15 RPM with static controls, and incubated at 37^{o}C for 3, 6, 9, 12, 15, 18, 21 and 24 hours.

Growth Cycle.

Refrigerated inoculum samples produced higher cell numbers than the frozen inoculum. The growth decline by time point 21 hours in the frozen inoculum reflected instability during the stationary phase (Fig. 4). The cause has not been determined. However, the decline was consistent in all replicates and occurred in both MMG and HZN conditions. The MMG culture numbers declined steadily from 12 to 21 h, while the HZN numbers declined rapidly from 18 to 21 h.

P. aeruginosa PA103 growth was not affected by the MMG of the rotating Clinostat compared with horizontal rotation or static conditions. There were no significant differences between the means for the MMG vs. the controls (Fig. 4).

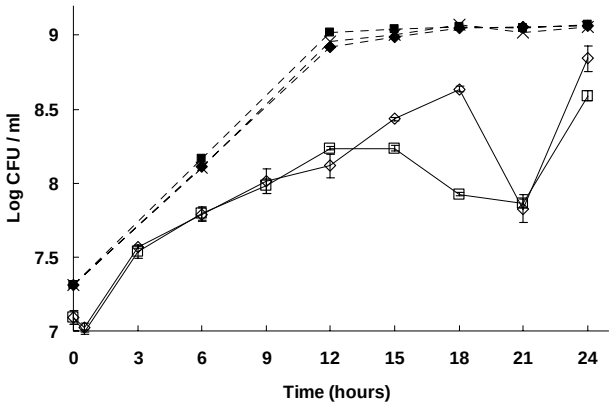


Figure 4. Growth cycle of *Pseudomonas aeruginosa* PA103. Frozen inoculum (n=3), ◊ Static, □ MMG. Refrigerated inoculum (n=3), ♦ Static, ■ MMG, X HZN. Bars indicate standard deviation of the mean.
determined.

Exotoxin A Production.

Samples from time points 0, 6, 9, 12, 18, 21 and 24 hours were assessed for ETA production. ELISA results showed Exotoxin A production to increase during the log phase of growth for both the frozen and refrigerated inocula (Fig. 5). The same growth cycle pattern was observed for the ETA production portion of the experiments. The 24 hour stationary phase result for both sets of inocula reflects a statistically comparable amount of toxin production.

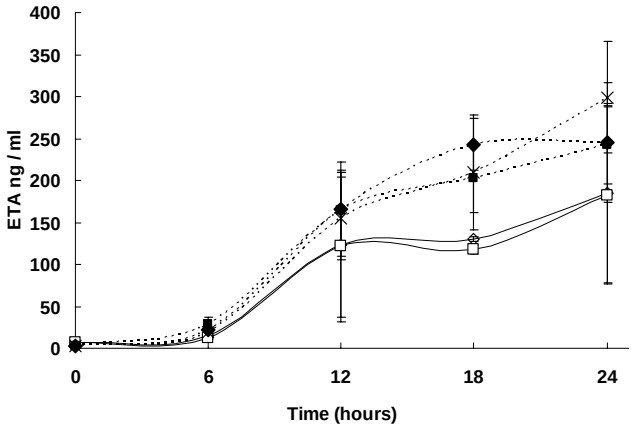


Figure 5. ETA Production of *Pseudomonas aeruginosa* PA103 in the Clinostat. Frozen inoculum (n=3), ◊ Static, □ MMG. Refrigerated inoculum (n=3), ♦ Static, ■ MMG, X HZN. Bars indicate the standard deviation of the mean.

Proteomics.

Two samples from the refrigerated inoculum set, Modeled Microgravity (MMG) and Horizontal rotation control (HZN) from the 24h time point, were assessed for protein expression. Imaging of these gels suggests that MMG may inhibit the expression of proteins that otherwise were present when the gravity vector was functional in the HZN treatment, (Fig. 6). Color differences in gels may be due to intensity of staining.

(A) (B)

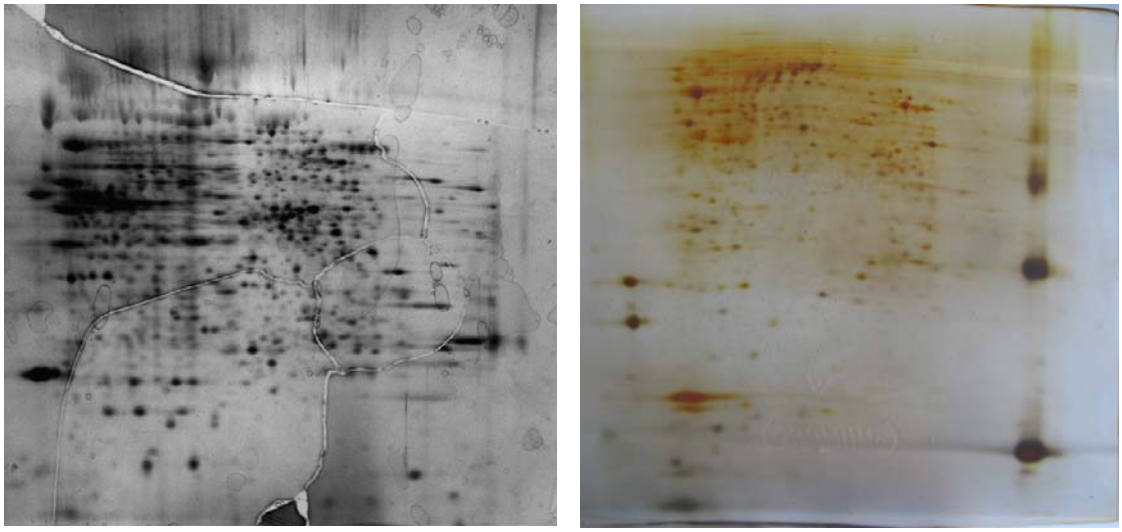


Figure 6. Two-Dimensional Gels from Clinostat Experiments. (A) 24h Horizontal rotated control sample (HZN). (B) 24h Modeled Microgravity Clinostat rotated sample (MMG).

HARV Experiments.

Growth Observations.

P. aeruginosa PA103 was also exposed to the modeled microgravity environment of the High-Aspect-Ratio-Vessel (HARV). As previously described in the materials and methods section, three different conditions or treatments were established: H-STAT, H-HZN and H-MMG, in which aeration was allowed and nitrate added in the medium in some of them (Fig. 7 panels A, B, & D, refer also to Table 6 and Appendix A). A yellow/green pigmentation was exhibited by aerated and non-aerated samples containing nitrate in the horizontal and static controls (Fig. 7 panels A, B & D); while horizontal rotation controls (Fig. 7A&D) displayed more pigmentation in the non-aerated samples.

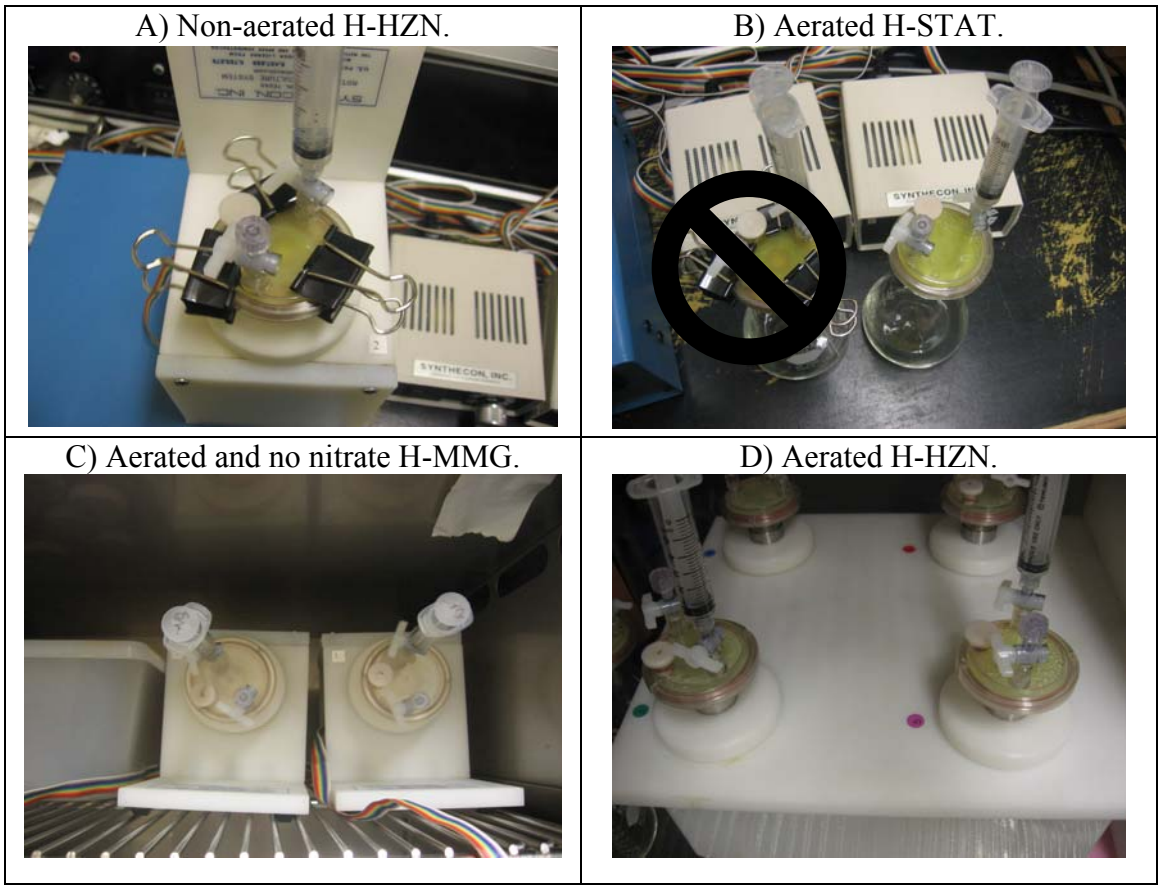


Figure 7. HARV Growth Observations. Pictures show samples in vessels after a 12 hour incubation at 37°C. The sample medium was supplemented with nitrate unless stated otherwise. (A) Non-aerated H-HZN. (B) Aerated H-STAT. (C) Aerated with no nitrate H-MMG. D) Aerated H-HZN. Also, every picture reflects the aeration/medium condition corresponding to panels in the Growth Cycle and ETA Production graphs.

Pigmentation did not appear in the aerated/no nitrate MMG vessels (Fig. 7C), or in the aerated/nitrate or non-aerated/nitrate vessels (pictures not shown). The yellow/green pigmentation suggests the presence of pyochelin and possibly pyocyanin, which may be affected by the nitrate in the medium and by MMG rotation. Both of these pigments are virulence factors (Table 4). Furthermore, for all rotated and static samples containing nitrate, (aerated or non-aerated), nitrogen bubbles were observed continuously after 12 hours of incubation (Fig. 7 A, C & D). A syringe was attached to every vessel to

allow for removal of these nitrogen bubbles in order to prevent interference with MMG and possibly low-shear conditions.

Growth Cycle.

P. aeruginosa PA103 growth was not affected by H-MMG under the HARV (RCCS) conditions of these set of experiments. Samples were rotated at 15 RPM, except for static controls, and incubated at 37^{o}C for 12 and 24 hours. There were no differences between the means for the H-MMG vs. the controls. The growth cycle for H-HZN and H-MMG vs. H-STAT control varied according to medium components rather than because of rotational treatment. It appears that the lack of nitrate in the medium in the aerated HARV caused a lower cell density than the control (Fig. 8 C vs. D). Also, provided nitrate was not present in the medium, the non-aerated HARV seemed to exert a positive effect on the growth cycle for all three treatments (H-MMG, H-HZN & H-STAT) (Fig. 8 A vs. B). Panels B & D in figures 8 and 9 do not show samples exposed to H-HZN or H-MMG conditions, respectively, because of the limited number of rotators available. Only H-STAT samples were duplicated, and both plate count and ETA results for these two replicates were highly constant. This indicates the reliability of the HARV system.

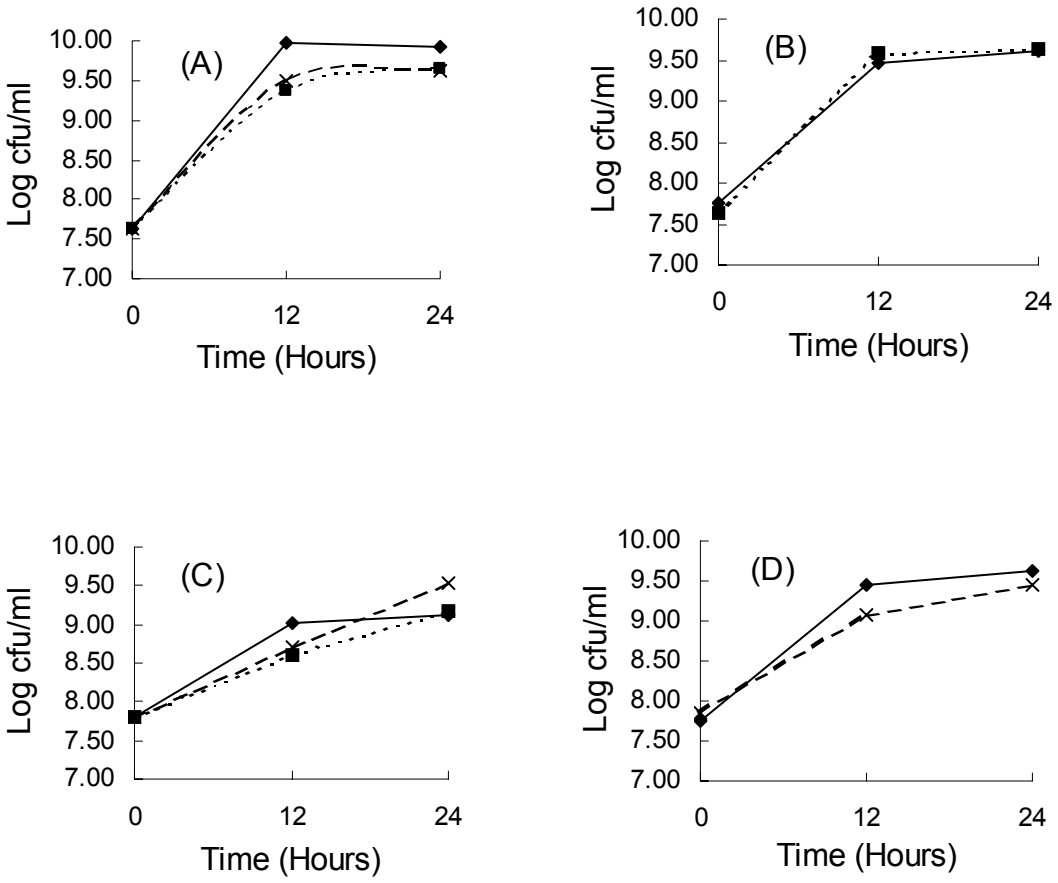


Figure 8. Growth curve of PA103 in the HARV. Experimental treatments/conditions: ♦ H-STAT, X H-HZN, and ■ H-MMG. The sample medium was supplemented with nitrate unless stated otherwise. (A) Exp.1, non-aerated; (B) Exp. 1, aerated; (C) Exp. 2, aerated with no nitrate; (D) Exp. 2, aerated.

Exotoxin A Production.

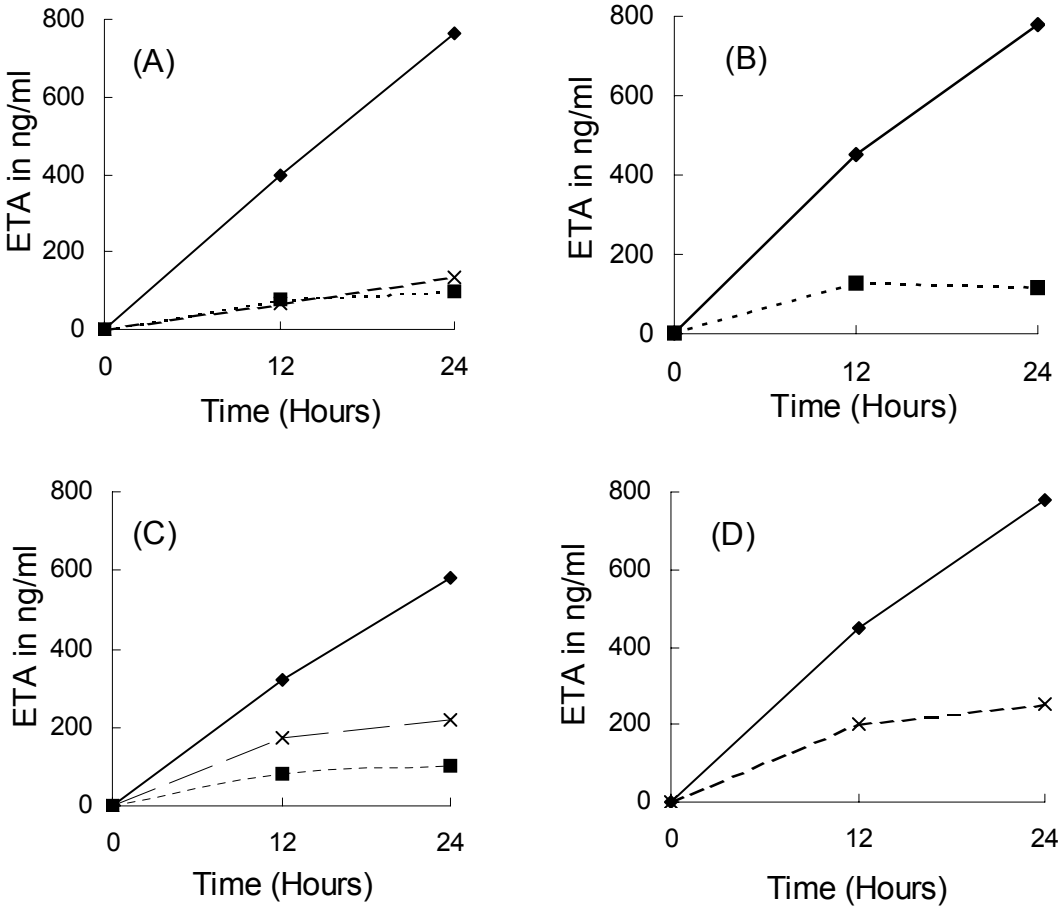


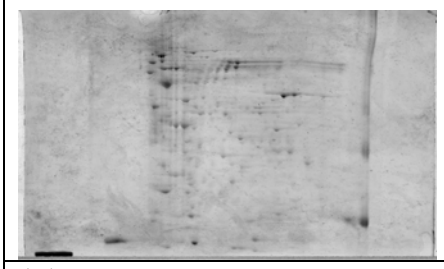
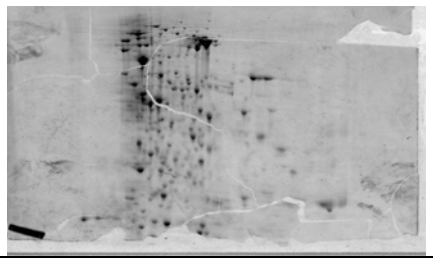
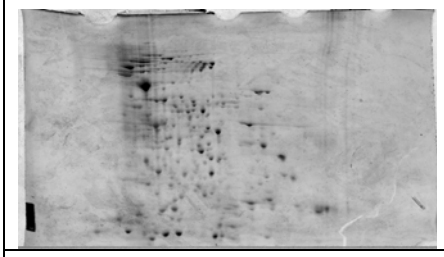
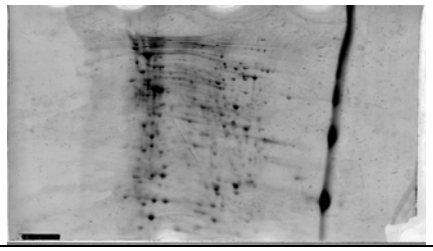
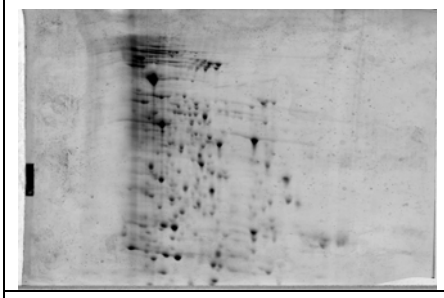
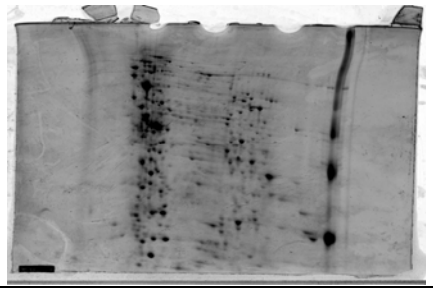
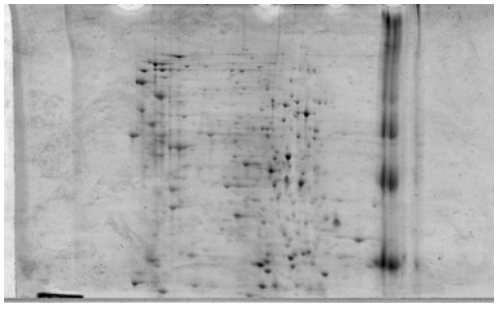
Figure 9. ETA Production in PA103 in the HARV. Experimental conditions: ♦ H-STAT, X H-HZN, and ■ H-MMG. The sample medium was supplemented with nitrate unless stated otherwise. (A) Exp. 1, non-aerated; (B) Exp. 1, aerated; (C) Exp. 2, aerated and no nitrate; (D) Exp. 2, aerated.

In all cases (Fig. 9), ETA production in static controls (H-STAT) continued to increase from 12 to 24 hour incubation, while concentrations in the rotated vessels (H-MMG, H-HZN) tended to increase more slowly between 12 and 24 hours. Elimination of

nitrate in the aerated vessels had a negative effect on the toxin production in the static control (Fig. 9 C vs. D).

Proteomics.

Two-dimension gels were obtained from samples at different stages of PA103 growth cycle for H-MMG, and H-HZN at 15 RPM and H-STAT controls, incubated at 37^{o}C for 12 and 24 hours. Basic visual comparisons were done among all Typhoon^{RT} (Amersham) imaged gels from every different HARV bioreactor treatment and time point (Zero hour Control, H-STAT, H-MMG and H-HZN). No clear differences in protein expression profiles were observed (Fig. 10). The 24 h samples from every condition showed a lower number in protein spots than their 12 h counterparts. We suspect this maybe due to time dependent degradation by indigenous PA proteases. However, the ETA ELISA concentrations in these samples increased between 12 and 24 h incubation, which suggests that protease digestion was negligible. It is possible that these differences are related to staining or other 2-D gel factors. Because these gels were SYPRO Ruby stained, there were small black specs in the background, which would normally be removed prior to digital image comparison by the software.

24 hour	12 hour
(A) STAT 	(B) STAT 
(C) HZN 	(D) HZN 
(E) MMG 	(F) MMG 
(G) FAMQ. Zero hours. 	Figure 10. Two Dimension gels from HARV experiments. All samples were non-aerated and supplemented with nitrate. H-STAT (A) 24h, (B) 12h; H-HZN (C) 24h, (D) 12h; H-MMG (E) 24h, (F) 12h; Control* (G) at 0h. *(FAMQ=Filtered-Autoclave-Milli-Q) bacterial suspension control.

DISCUSSION AND CONCLUSIONS

Bacteria possess the unique ability to withstand hostile environments and to readily adapt to frequent changes in the environment. They are more adaptable than mammalian cells, which, in contrast, benefit from regulated and rather constant conditions. As a result, the mechanisms that govern bacterial growth, virulence and gene/protein expression in response to environmental signals are of major interest in basic and applied research. Bacterial infections on Earth could be better understood by targeting key virulence factors in investigations applying a series of environmental stresses. As we have seen, modeled microgravity experiments have been useful in this respect. In the present work, we have studied the effects of modeled microgravity on the virulence of *P. aeruginosa* 103 by using growth, toxin detection and protein expression (proteomic) assays.

Clinostat Experiments.

The growth cycle of *P. aeruginosa* was apparently not affected by the MMG conditions of the Clinostat, relative to the static and horizontal controls. For all samples, ETA production increased most rapidly during the log phase of growth with both the frozen and refrigerated inocula. The refrigerated inoculum showed mean ETA production greater in the horizontal rotation system compared to the Clinostat samples and, a mean ETA production lower in the Clinostat than in the static control, however, these differences were not statistically significant. ETA production from the frozen inoculum was slightly lower in the Clinostat than in the static controls. The difference

between the initial bacterial populations of the frozen vs. the refrigerated inocula did not affect the overall ETA production throughout the growth cycle, neither was there a major difference in net production when one type of inoculum was compared against the other. In Clinostat syringes, the rotational velocity of the MMG system (15 RPM) had a minor effect on growth and ETA production. The variability in ETA concentrations may be due to inconsistency of the ETA standards, although this was accounted for in the statistical analyses.

Frozen and refrigerated inocula showed overall differences probably due to selection of cells during storage. Possibly, a particular population within the same strain may have been selected in the frozen inoculum. The Clinostat experiments underlined the need to use inocula prepared in the same way throughout a series of trials. It also highlighted the need to ensure that reliable standards are used for analyses such as ELISA, taking into account potential loss of potency. Standardization of procedures and materials is critical for spaceflight experiments, to avoid the interference of variables such as microbial selection in stored inocula.

There was less ETA produced in the MMG system than in the static and horizontal controls at 18 h and after 24 h incubation with the refrigerated inoculum. Statistical analysis of the ETA results showed that the ETA standards were stable for these experiments. Results for refrigerated samples in their time–treatment combinations using analysis of variance (ANOVA) indicated a strong interaction between incubation times and treatment (MMG vs. HZN vs. STAT) after more than 6 h incubation. From 12 h incubation, ETA varied significantly with increasing incubation time ($p<0.0001$) and

the time: treatment interaction was strong (p=0.0100). This indicates that the effects of one variable (ETA production) cannot be considered without taking into account the other variable (incubation time). However, it would be expected that ETA production would increase as growth progressed.

Previous proteomic analyses (Pulcini et al., 2004) indicated that clinorotation at 8 or 80 RPM resulted in differences in expression of over 20 proteins, 6 of which are associated with virulence factors in *P. aeruginosa*. Visual gel analysis comparisons between HZN and MMG samples after 24 hours of incubation revealed differences in staining intensity but did not provide enough data to draw any further conclusions (Fig. 9).

HARV Experiments.

Samples grown in the HARV appeared to show no overall effect of MMG from rotational conditions as compared to static controls. This is consistent with previous findings where MMG did not exert a significant effect on the growth cycle of PA (England et al., 2003) or *E. coli* (Lynch et al, 2004). In contrast, samples from cultures grown under rotational conditions (both MMG and 1 G) accumulated a lower amount of ETA. Rotated cultures from all conditions appeared to produce ETA more slowly in late logarithmic and early stationary phase (12h vs 24h). The absence of nitrate and lack of aeration negatively affected the growth cycle and ETA production of PA103 for all treatments. This suggests that the lack of nitrate and aeration might have affected cell metabolism and molecular regulation impairing the capability to produce or accumulate ETA. This may be related to PA103 ETA being secreted by the type II system. Earlier

studies provide genetic evidence linking respiration-linked terminal reductases to type II protein secretion (DiChristina et al., 2002). These reductases, which are part of the respiratory electron transfer chain, depend on soluble terminal electron acceptors such as dissolved oxygen, nitrate, sulfate, or carbon dioxide, which diffuse passively into the cell or are taken up by transmembrane transport systems (DiChristina et al., 2002).

Cultures may also be influenced by MMG treatment in addition to being affected by the presence of nitrate in the medium. The absence of pyochelin, and possibly pyocyanin, in a number of HARV samples (Fig. 10) may partially explain why MMG treatments produced less ETA. For example, pyocyanin is reduced to leukopyocyanine which in turn can reduce Fe (III) and release Fe from transferrin, which led to the postulation that pyocyanin may help *P. aeruginosa* obtain the iron needed for persistent and expanding infections (Sorensen & Joseph, 1993). Furthermore, rotated and static samples that contained nitrate and were aerated or non-aerated, contained bubbles continuously after 12 hours of incubation (Fig. 10 A, B & D). Because nitrate was present in the medium, PA was able to switch to a nitrate reduction mode to acquire oxygen and produce nitrogen gas inside the vessel.

Preliminary proteomic analyses were done at different stages of the PA103 growth cycle in H-MMG at 15 RPM and in controls. Hammond and Hammond (2001) as previously mentioned, recommended an average HARV rotational speed that ranged between 10 and 60 RPM, where 25 RPM was the optimal, to allow for minimal shear stress on the cells. These analyses were done on samples that were non-aerated and contained nitrate in their medium. Basic visual comparison of gels containing samples

from all HARV bioreactor treatments (H-STAT, H-MMG and H-HZN) revealed no visible differences in protein spot profiles (Fig. 13). Moreover, the 24 h samples from every condition showed a lower number in protein spots than their 12 h counterparts. We suspect this is due to time dependent degradation by indigenous PA proteases. However, the protein ETA, as determined by ELISA, was apparently not affected. Further proteomic and genomic work needs to be done in order to understand if MMG and/or low shear may influence gene expression. Past investigations showed that the absence of particular genes may contribute to increased virulence (Nickerson et al., 2004). Because MMG by definition is an analogous system to the weightlessness of space flight, it may help to understand if the interaction of space crew with pathogenic bacteria in a self-contained environment and subsequent infection risk with the addition of proposed regenerative life support systems, including waste remediation (Nickerson et al., 2000).

Modeled Microgravity as a Tool for Space Flight Experiments.

We investigated the short-term effects of MMG on bacterial virulence and found that our results differed from those of previous studies, for example, the observation that MMG in itself did not affect virulence in terms of ETA production. Possible reasons for these differences include the nutritional, metabolic and environmental versatility of *P. aeruginosa*. The strain PA103, which was used in these experiments, is motile and this motility may have tended to counteract MMG effects (Bouloc and D'Ari, 1991; Klaus, 2000). While the rotation speed of 15 RPM used for these experiments is lower than the 25 RPM typically used for HARV reactors (Nickerson et al., 2000), speeds from 8 to 80 RPM (Pulcini et al., 2004) or even 7 to 150 RPM (England et al., 2003) have also been

used. Also, other investigations have reported that bacterial cells grown during space flight reached a higher density than those grown under normal gravity conditions (Kacena et al., 1999 b) or MMG systems (England et al., 2003, and Lynch et al., 2004). One explanation for this is that due to the lack of sedimentation in a *true* weightless environment there is more opportunity for more contact between bacterial cells and nutrients present in the medium, as well as for waste removal from the surrounding fluid environment immediate to the cells (Klaus et al., 1997). Earlier work showed that bacteria, including PA (England et al., 2003), *E. coli* (Kacena et al., 1999 b & Lynch et al., 2004) and *S. enterica* serovar Typhimurium (Nickerson et al., 2004 and 2000 & Wilson et al., 2002a and b), grown in the HARV were shown to have increased virulence but with no overall growth differences. In the present work we show that rotational conditions do not seem to stimulate the production of the virulence factor Exotoxin A. Production of virulence pigments, however, (pyochelin and possibly pyocyanin) were affected. Consequently, because of the extended duration of space shuttle missions (about 14 days on average), International Space Station (about 3 to 6 months) and future missions to the Moon and Mars (6+ months), longer-term investigations are required to determine the effect of MMG in comparison with spaceflight studies on bacteria to validate its actual purpose: simulate reduced gravity. It might be possible that bacteria develop adaptations or reflect variations (i.e., genotypic or phenotypic) in response to long duration MMG similar to those displayed during actual weightlessness. Another reason why our results differ from those of earlier studies, apart from using an opportunistic pathogen, is because of assessment protocols. Nickerson et al., (2000)

tested bacterial virulence by measuring lethal dosage levels in mice, whereas, we tested virulence by quantifying virulence ETA levels under different static and rotational conditions. This suggests that more refined analyses need to be performed to assess the influence of MMG on bacterial systems.

We conclude that *P. aeruginosa* virulence in terms of ETA production does not appear to be enhanced by MMG. In addition, both the Clinostat and HARV produce consistent results in the analysis of virulence in relation to ETA production by PA103. As far as equipment operation is concerned, we found that the HARV could be adapted to experiment needs (e.g. aerated vs. non-aerated), allowed for more sample volume to be tested and facilitated the handling of treatments and controls. Changes in gravity and/or shear remain to be further analyzed to confirm if virulence is affected only in certain pathogenic bacteria and/or if it is a result of aeration and/or medium components.

Future research could include studying the effects of different speeds in the Clinostat and the HARV High-Aspect Ratio Vessel (Synthecon) MMG systems on the growth cycle and protein and gene expression of PA103, including shear stress effects on ETA production. In addition, it needs to be examined if absence of particular genes may contribute to increased virulence (Nickerson et al., 2004). Time-effect relationships need to be assessed. Long-term duration space missions could be modeled by the assembly of continuous bioreactors using the HARV principle and adding a higher number of sampling time points. Phenotypic and genotypic changes should be analyzed using plate counts, cytotoxicity and toxin quantification assays in addition to proteomic and genomic methods.

True pathogenic and opportunistic bacteria like *P. aeruginosa* possess the ability and versatility to survive in a variety of environments including that of space flight. As a result, there is a growing interest to develop means to control the infection process of these microbes by the use of therapeutic treatments and countermeasures that will decrease the chances and consequences of these infections occurring during space flight and on Earth (Nickerson et al., 2000).

The validity and reliability of MMG in relation to spaceflight experiments remains to be elucidated. Results to date, including the present study, have been inconsistent. The data reported here suggest that MMG minimally affects the growth, physiology or virulence of PA103. Factors other than gravitational variation, such as aeration, medium formulation, shear, and mixing may be the basis of some reported affects. In addition, different species or even strains of bacteria may respond differently. Nevertheless, MMG is a readily accessible tool in an era of limited spaceflight opportunity.

Results could potentially serve as a basis for novel methods to control bacterial pathogenesis on Earth and space missions. If these results are accompanied by further proteomic research and complemented by sensitive genomic analyses, they could also provide detailed information on the responses of bacterial cells to environmental factors including those of modeled microgravity and spaceflight.

LITERATURE CITED

Blumentals, II, MM Kelly, M Gorziglia, JB Kaufman and J Shiloach. 1987. Development of a defined medium and two-step culturing method for improved Exotoxin A yields from *Pseudomonas aeruginosa*. Appl. Env. Micro. 53:2013-2020.

Bouloc, P and D'Ari, R. 1991. *Escherichia coli* metabolism in space. J. Gen. Microbiol. 137: 2839-2843.

Bouma, JE and DL Pierson. 1997. Effect of simulated microgravity on populations and functional activity of a gnotobiotic microbial community. Abstr. Gen. Meet. Am. Soc. Microbiol. 97: 413.

Brock, TD, MT Madigan, JM Martinko and J Parker. 1994. The Biology of Microorganisms. Prentice Hall. Pp. 64.

Brown, RP. 1999. Effects of space flight, clinorotation, and centrifugation on the growth and metabolism of *Escherichia coli*. A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy. University of Colorado.

Chapman, DK. 1989. Do the design concepts used for the space flight hardware directly affect cell structure and/or cell function? Ground based simulations. Moffet Field, CA, Ames Research Center.

Cioletti, LA, DL Pierson and SK Mishra. 1989. Microbial growth and physiology in space: a review. Visited on Jan 2006. http://205.149.4.69/spacebio/modules/mb resource/space_microbio.doc.

DiChristina, TJ and JR Haas. 2002. Effects of Fe(III) chemical speciation on dissimilatory Fe(III) reduction by *Shewanella putrefaciens.* Environ. Sci. Technol. 36: 373-380.

England, LS, M Gorzelak and JT Trevors. 2003. Growth and membrane polarization in Pseudomonas aeruginosa UG2 grown in randomized microgravity in a high aspect ratio vessel. Biochim. Biophys. Acta. 1624:76-80.

Ferguson, JK, GR Taylor and JB Mieszkuc. 1975. Biomedical results of Apollo. Microbiological Investigations. Chapter 2. 83-103.

Fang, A, DL Pierson, DW Koenig, SK Mishra and L Domain. 1997. Effect of simulated microgravity and shear stress on Microcin B17 production by *Escherichia coli* and on its excretion into the medium. Appl. Environ. Microbiol. 63(10) 4090-4092.

Fang, A, DL Pierson, SK Mishra and L Demian. 2000. Growth of *Streptomyces hygroscopicus* in a rotating-wall bioreactor under simulated microgravity inhibits rapamycin production. Appl. Microbiol. Biotechnol. 54(1)33-36.

Ferguson, JK, GR Taylor, RC Graves, RM. Brockett and BJ Mieszuck. 1975. Biomedical Results of Apollo. NASA Scientific and Technical Information Offices. Chapter 2. National Aeronautics and Space Administration, Washington, D.C.

Frank, D. 1997. The exoenzyme S regulon of *Pseudomonas aeruginosa*. Mol. Microbiol. 26:621–629.

Galloway, DR. 1993. Role of exotoxins in the pathogenesis of *P. aeruginosa* infections, p. 107-127. *In* Campa, M, M Bendinelli and H Friedman (ed.), *Pseudomonas aeruginosa* as an opportunistic pathogen. Plenum Press. New York and London.

Gao, H, PS Ayyaswamy and P Ducheyne. 1997. Dynamics of a microcarrier particle in the simulated microgravity environment of a rotating-wall vessel. Microgravity Sci. Technol. X(3)154-165.

Gasset, G, R Tixador, B Eche, L Lapchine, N Moatti, P Toorop, and C Woldringh. 1994. Growth and Division of *Escherichia coli* under microgravity conditions. Res. Microbiol. 145, 111-120.

Gmunder, FK and A Cogoli. 1988. Cultivation of Single Cells in Space. Appl. Microgravity Technol.1:115-122.

Guadarrama, S, E deL Pulcini, SC Broadaway and BH Pyle. 2005a. Analysis of *Pseudomonas aeruginosa* growth and virulence in modeled microgravity. J. Grav. Physiol. 12(1) 249-250.

Guadarrama, S, E deL Pulcini, SC Broadaway, and BH Pyle. 2005b. *Pseudomonas aeruginosa* growth and Exotoxin A production in static and modeled microgravity environments. J. Grav. Space Biol. 18(2) 85-86.

Hammond, TG and JM Hammond. 2001. Optimized suspension culture: the rotating wall vessel. Am. J. Physiol. Renal Physiol. 281: F12–F25.

Ingber, D. 1999. How cells (might) sense microgravity. FASEB. 13: 3-15.

Juergensmeyer, MA, EA Juergensmeyer and JA Guikema. 1999. Long-term exposure to spaceflight conditions affects bacterial response to antibiotics. Microgravity Sci. Technol. 12(1) 41-47.

Kacena, MA, PE Leonard, P Todd, MW Luttges.1997. Low gravity and inertial effects on the growth of *E. coli* and *B. subtilis* in semi-solid media. Aviation Space Environ. Med. 68(12) 1104-1108.

Kacena, M.A., CA Merrel, B Manfredi, EE Smith, SM Klaus, and P Todd. 1999 a. Autolysis of *Escherichia coli* and *Bacillus subtilis* cells in low gravity. Appl. Microbiol. Biotechnol. 52(3) 437-439.

Kacena, MA, CA Merrel, B Manfredi, EE Smith, SM Klaus, and P Todd. 1999 b. Bacterial growth in space flight: logistic growth curve parameters for *Escherichia coli* and *Bacillus subtilis*. Appl. Environ. Microbiol. 51: 33-36.

Klaus, DM, S Simske, P Todd, L Stodieck. 1997. Investigation of space flight on *Escherichia coli* and a proposed model of underlying physical mechanisms. Microbiol. 143: 449-455.

Klaus, DM, P Todd, A Schatz. 1998. Functional weightlessness during clinorotation of cell suspensions. Adv. Space Res. 21(8/9) 1315-1318.

Klaus, DM. 2001. Clinostats and Bioreactors. Gravi. Space Biol. Bull. 14:2.

Klaus, DM. 2002. Space microbiology: microgravity and microorganisms. *In* Encyclopedia of Env. Microbiology. Pp. 2996-3004. Edited by G. Britton. New York. Wiley.

Konstantinova IV, MP Rykova, AT Lesnyak and EA Antropova. 1993. Immune changes during long-duration missions. J. Leukocyte Biol. 54:189—201.

Lanning, CJ. 1998. Simulated microbial growth in varying inertial conditions. In fulfillment of the 1998, Summer UROP Grant.

Lapchine, L, N Moatti, G Gasset, G Richoilley, J Templier and R Tixador. 1987. Antibacterial activity of antibiotics in space conditions. Nordeney symposium on scientific results of the German spacelab mission D-1. Pp 395-397.

Lewis, ML and M Hughes-Fulford. 1997. Cellular responses to microgravity. Fundamentals of space life sciences, MIT Press.

Leach, CL and PC Rambaut. 1974. Biomedical responses of the Skylab crewmen. Proc. Skylab Life Sci. Symp. 2: 1-4.

Liu, PH, SA Svoronos and B Koopman. 1998. Experimental and modeling study of diauxic lag of *Pseudomonas denitrificans* switching from oxic to anoxic conditions. Biotech. Bioeng. 60(6) 649 – 655.

Lujan, BF. 1994. Human Physiology in Space. NASA Curriculum supplement for secondary schools.

Lynch, SV, EL Brodie and A Matin. 2004. Role and regulation of σ^S in general resistance conferred by low-shear simulated microgravity in *Escherichia coli*. J. Bacteriol. 186(24) 8207-8212.

Menigmann, HD and M Lange. 1986. Growth and differentiation of *Bacillus subtilis* under microgravity. Naturwissenschaften. 73: 415-417.

Nefedov, YG, VM Shilov, IV Konstantinova and SN Zaloguyev. 1971. Microbiological and immunological aspects of extended manned space flight. Life Sci. Space Res. 9:11–16.

Nickerson, CA**,** CM Ott, SJ Mister, BJ Morrow, LB Keliher and DL Pierson. 2000. Microgravity as a novel environmental signal affecting *Salmonella enterica* serovar Typhimurium virulence. Infect. Immun. 68(6):3147-3152

Nickerson, CA, CM Ott, JW Wilson, R Ramamurthy and DL Pierson, 2004. Microbial responses to microgravity and other low-shear environments. Microbiol. Mol. Biol. Rev. 68(2) 345-361.

Pulcini, E deL, SC Broadaway and BH Pyle. 2004. *Pseudomonas aeruginosa* virulence and proteomics in simulated weightlessness. Gen. Meet. Am. Soc. Microbiol, Abstract I-033.

Pyle, BH, GA McFeters, SC Broadaway, CK Johnsrud, RT Storfa and J Borkowski. 1999. Bacterial growth on surfaces and in suspensions. *In* Biorack on Spacehab. Biological Experiments on Shuttle to Mir Missions 03, 05, and 06. European Space Agency SP-1222.

Pyle, BH, SC Broadaway and GA McFeters. 2001. *Burkholderia cepacia* biofilm growth and disinfection in microgravity. 31st International Conference on Environmental Systems, Orlando, FL, July 2001. SAE Technical Paper Series 2001-12-2128. Society of Automotive Engineers, Warrendale, PA.

Pierson, DL. 1983. Medical Microbiology of Crewmembers and Spacecraft during OFT. Shuttle Medical Report. NASA TM 58252. Pp. 49-52.

Pierson, DL, SK Mehta, SK Tyring and DL Lugg. 1997. Reactivation of latent Herpes viruses in astronauts during spaceflight. Immunology/Infection/Air Contamination. Abstract. Gen. Meet. Am. Soc. Microbiol. 97:505.

Ramirez, LES, EA Lim, CFM Coimbra and MH Kobayashi. 2003. On the Dynamics of a Spherical Scaffold in Rotating Bioreactors. Wiley Interscience. http://www.interscience.wiley.com.

Richoilley, G, R Tixador, G Gasset, J Templier and H Planel. 1986. Preliminary results of the "paramecium" experiment. Naturwissenschaften. 73: 404-406.

Sonawane, A, U Klöppner, S Hövel, U Völker and KH Röhm. 2003. Identification of *Pseudomonas* proteins coordinately induced by acidic amino acids and their amides: a two-dimensional electrophoresis study. Microbiol. 149: 2909-18.

Sonnenfield, G. 2005. The immune system in space, including Earth-based benefits of space –based research. Curr. Pharm. Biotechnol. 6: 343-349.

Sorensen, RU and F Jr Joseph. Phenazine pigments in *Pseudomonas aeruginosa* infection, p. 43-58. *In* Campa, M, M Bendinelli and H Friedman (ed.), *Pseudomonas aeruginosa* as an opportunistic pathogen. Plenum Press. New York and London.

Thévenet, DR, R D'Ari and P Bouloc. 1996. The SIGNAL experiment in BIORACK: *Escherichia coli* in microgravity. J. Biotechnol. 47: 89-97.

Virulence Factors in *Pseudomonas aeruginosa.* Website visited on April 2006. *http://textbookofbacteriology.net/pseudomonas.html.*

Wilson, JW,CM Ott, R Ramamurthy, S Porwollik, M McClelland, DL Pierson and CA Nickerson. 2002a. Low-Shear Modeled Microgravity Alters the *Salmonella enterica* serovar Typhimurium Stress Response in an RpoS-Independent Manner. Appl. Environ. Microbiol. 68(11) 5408-5416.

Wilson, JW, R Ramamurthy, S Porwollik, M McClelland, T Hammond, P Allen, CM Ott, DL Pierson and CA Nickerson. 2002b. Microarray analysis identifies *Salmonella* genes belonging to the low shear modeled microgravity regulon. PNAS. 99(21) 13807-13812.

APPENDIX A

HARV EXPERIMENTS TABLE

HARV Experiments Table

Sample No.	Aeration	STAT	HZN	MMG	NO_3	Time Point (hours)	Notes MM=Materials and Methods
1						0	In1-Feb- growth as in MM
2		+			+	12	In1-Feb
3				+	+	12	In1-Feb
4			+		+	12	In1-Feb
5		+			+	24	In1-Feb
6				+	+	24	In1-Feb
7			+		+	24	In1-Feb
8	+	+			+	12	In1-Feb
9	+			+	+	12	In1-Feb
10	+	+			+	24	In1-Feb
11	+			+	+	24	In1-Feb
12						0	In2-Mar-growth as in MM
13	+	+			+	12	In2-Mar
14	+		+		+	12	In2-Mar
15	+	+			+	24	In2-Mar
16	+		+		+	24	In2-Mar
17	+	+				12	In2-Mar
18	+			+		12	In2-Mar
19	+		+			12	In2-Mar
20	+	+				24	In2-Mar
21	+			+		24	In2-Mar
22	+		+			24	In2-Mar