PSEUDOMONAS AERUGINOSA GROWTH AND PRODUCTION OF EXOTOXIN A IN STATIC AND MODELED MICROGRAVITY ENVIRONMENTS.

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Pseudomonas aeruginosa is a ubiquitous, water-borne bacterium and opportunistic pathogen, which may thrive in water and other environments. It may form a biofilm of cells in an extracellular polymeric matrix, on the mucous membranes of the lungs in cystic fibrosis patients and on many other surfaces. An important virulence protein of *P. aeruginosa* is Exotoxin A (ETA) (Campa et al., 1993). The microgravity environment of spaceflight (10^{-4} to 10^{-6} x g) can provide important information as to how the physiology of terrestrial organisms is affected by gravity. Modeled Microgravity (MMG) systems are used to simulate the gravitational effects of spaceflight on cells. Previous studies have shown changes in protein expression in *P. aeruginosa* PA103 (ATCC 29260) bacteria (Pulcini et al., 2004) and up-regulation of virulence factors in *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (Nickerson et al., 2004) in response to MMG. Variations in the protein expression of *P. aeruginosa* in MMG reflect alterations in metabolic and physiological functions, and also in putative pathways responsible for the production of *P. aeruginosa* virulence factors (Pulcini et al., 2004). The virulence of *P. aeruginosa* is suspected to be enhanced in MMG. However, the growth and physiology of *P. aeruginosa* has not been well studied in MMG conditions. The goal of this study was to establish the growth of this microorganism in MMG systems, and to determine how Exotoxin A production is related to different stages of growth. It is anticipated that similar experiments will be performed in spaceflight. Fundamental questions concerning the effects of spaceflight on microorganisms in relation to crew health risks remain unanswered.

The MMG system used to study the influence of gravity on the growth cycle was the clinostat. Samples in a clinostat still experience unit gravity (g), however, the constant rotation of the samples results in the g-vector being time-averaged to near-zero (Klaus et al., 2001). MMG is the term used to describe the resultant state of clinorotation. Vertical rotation (VERT) was utilized as a rotational control to the clinostat. In the vertical system, the resultant force gravity vector is parallel to the gravity vector of the Earth. Static control (STAT) conditions were achieved inside clinostat tubes in a stationary position.

To assess the effects of MMG, the lag, log and stationary phases of growth of the ETA producing strain, PA103, were studied. The experiment inoculum was prepared in two different ways. One was suspended in glycerol (20 % final concentration) and frozen at -80°C (FZN) while the other was suspended in water and refrigerated at 4°C (REF). Plate count results were used to adjust the inoculum suspension to ca. $5x10^8$ CFU/ml before freezing or refrigeration. Both inocula were added to MSDM2 (Modified Simple Defined Media 2) at a ratio of 1:10 and used to inoculate 5 ml syringes each with 2 ml of medium to simulate flight growth chamber specifications. FZN inoculum growth cycle sampling times were time zero, 30 min, 3 h, 6 h, 9 h, 12 h, 15 h, 18 h, 21 h, and 24 h. For the REF inoculum, growth cycle sampling times were time zero, 6 h, 12 h, 15 h, 18 h, 21 h, and 24 h. For both inocula and every time point, syringes were placed in a STAT control and in a MMG environment. VERT controls were included only for the REF inoculum. Time points differ between the FZN and REF inocula due to space constraints in the VERT rotator and relevance to ETA production. MMG and VERT samples were rotated at 15 RPM and all samples incubated at 37°C. Samples were drop plated on R2A agar and incubated for 24 h at 30°C. Optical density (OD) readings were taken at 540 nm. The remaining culture was fixed with formalin (2.0 % final concentration). ETA was quantitated by an ELISA assay developed for spaceflight experiment BACTER on STS-107. Anti-*Pseudomonas* Exotoxin A (Sigma) was used as the primary antibody, Anti-Rabbit-HRP (Sigma) as secondary antibody and orthophenylenediamine (OPD) as the HRP indicator substrate. A dilution series of standard ETA (CalBioChem) was included. Optical density was read at 490 nm and sample concentrations were determined from the standard curve in ng/ml.

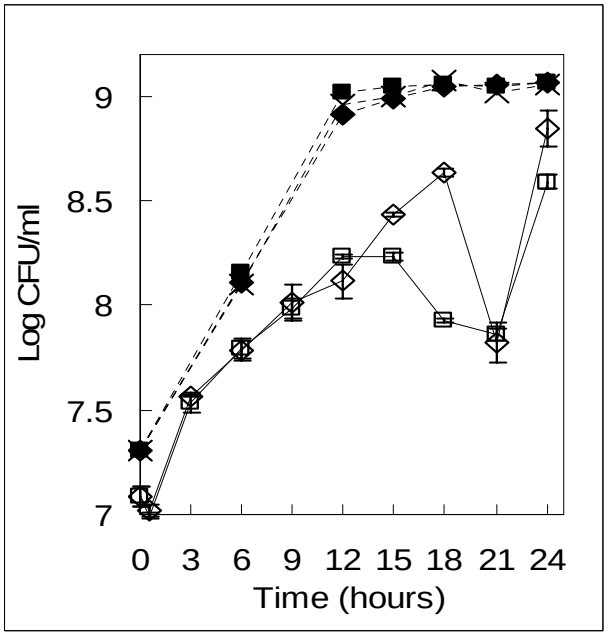

Figure 1. *Growth cycle for frozen inoculum (n=3), ◊ Static, □ MMG, and refrigerated inoculum (n=3), ♦ Static, ■ MMG, X Vertical.*

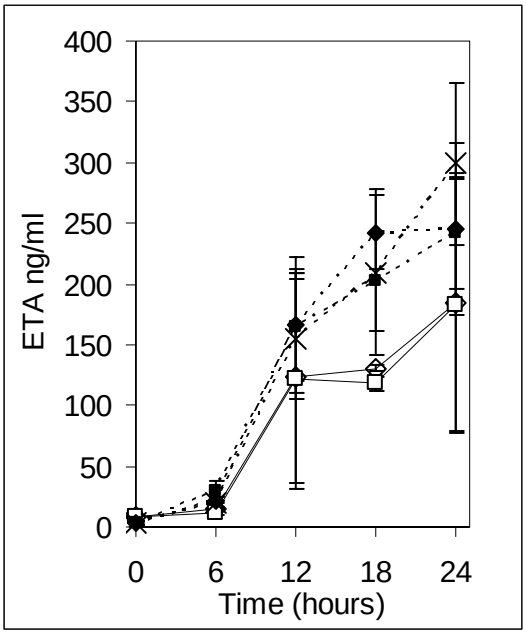

Figure 2. *ETA production for frozen inoculum (n=3), ◊ Static, □ MMG, and refrigerated inoculum (n=3), ♦ Static, ■ MMG, X Vertical.*

The growth cycle of *Pseudomonas aeruginosa* was apparently not affected by the MMG conditions of the clinostat (Figure 1 and 2). The initial cell numbers in samples from the FZN inoculum diluted in MSDM2 were consistently lower than those from the REF inoculum. The FZN inoculum cultures also reached lower numbers in the stationary phase, and there was a decrease in CFU/ml between 18 and 21 h that recovered by 24 h (Figure 1). This decline and recovery did not occur with the REF inoculum (Figure 2). The overall ETA production rate was fairly constant during the log phase with both inocula. For the FZN inoculum, the ETA concentration in the MMG was essentially equivalent to the STAT control (Figure 3). With the REF inoculum, the ETA concentration was somewhat higher in the vertical rotation system when compared to the MMG and STAT samples (Figure 4). The difference between the bacterial populations of the FZN vs. the REF inocula apparently affected the overall ETA production throughout the growth cycle (Figures 1, 2, 3 and 4). Mean ETA concentrations in samples from the REF inoculum were generally higher than from the FZN inoculum. Differences between the FZN and REF inocula may have been the result of selection of a particular population during freezing.

As demonstrated here, due to the differences that can be induced in the experimental sample by pre-experiment storage conditions, it is crucial importance that all experiments, especially for spaceflight, are done with the inoculum prepared in the same way to obtain consistent and comparable results. Future work will focus on studying the effects of different speeds in the clinostat and another MMG system such as the Synthecon HARV (High-Aspect Ratio Vessel), on the growth cycle of PA103, including shear stress consequences on growth and virulence. In addition, proteomic analyses will be performed at intervals over the growth cycle to determine variations in protein expression which may be related to virulence.

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