Research Article

Effects of Microgravity and Clinorotation on the Virulence of *Klebsiella, Streptococcus, Proteus*, and *Pseudomonas*

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ABSTRACT

To evaluate effects of microgravity on virulence, we studied the ability of four common clinical pathogens—*Klebsiella*, *Streptococcus*, *Proteus*, and *Pseudomonas*—to kill wild type *Caenorhabditis elegans* (*C. elegans)* nematodes at the larval and adult stages. Simultaneous studies were performed utilizing spaceflight, rotation in a 2D clinorotation device, and static ground controls. Nematodes, microbes, and growth media were separated until exposed to true or modeled microgravity, then mixed and grown for 48 hours. Experiments were terminated by paraformaldehyde fixation, and optical density measurements were used to assay residual microorganisms. Spaceflight was associated with reduced virulence for *Klebsiella* and *Streptococcus*, but had negligible effect on *Enterococcus* and *Pseudomonas*. Clinorotation generated very different results with all four organisms showing significantly reduced virulence. We conclude that clinorotation is not a consistent model of the changes that actually occur under microgravity conditions. Further, bacteria virulence is unchanged or reduced, not increased during spaceflight.

Key words: Virulence; Spaceflight; Nematodes; Clinorotation; Microgravity; Microorganism

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ACRONYMS

FPA	Fluid Processing Apparatus
GAP	Group Activation Pack
ISS	International Space Station
MRSA	Methicillin-resistant *Staphylococcus aureus*

INTRODUCTION

The possibility that microorganisms would become more virulent in spaceflight and pose a health risk to astronauts has been a recurring concern. However, experiments to evaluate this possibility have generated complex, and sometimes confounding results. Bacteria, particularly motile organisms, tend to divide more rapidly in space, most likely due to increased access to nutrients while suspended in liquid medium (Benoit and Klaus, 2007; Kacena et al., 1999). The structure of biofilms differs in microgravity and this may also impact virulence (Altenburg et al., 2008; Kim et al., 2013; Searles et al., 2011). However, virulence is affected by both the characteristics of the microbe and the ability of the infected host to control and eradicate the pathogen. *Salmonella* grown in space and returned as live cultures at ambient temperature appeared to be more virulent when subsequently injected into mice on Earth (Wilson et al., 2007). However, these results are not necessarily predictive about the interactions of hosts and pathogens in space.

The worm *C. elegans* is a powerful model system to study host-pathogen interactions (Hammond et al., 2009; Harvill and Miller, 2000; Sifri et al., 2005). *C. elegans* will consume the microorganisms in their medium unless the organisms are so virulent as to kill nematodes first (Hammond et al., 2009; Smith et al., 2002). We have adapted this model to measure virulence in spaceflight by incubating *C. elegans* with the test microbe for 48 hours while in space, fixing the cultures with paraformaldehyde, and measuring the numbers of *C. elegans* surviving to the end of the co-culture by optical density (Hammond et al., 2009). The nematodes are hatched in space and by adjusting the time period and food sources necessary for them to progress through their life cycle, one can selectively measure the virulence of organisms towards larval or adult *C. elegans*.

Using this assay, we previously found that *Listeria*, *Enterococcus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), and *Candida* were less virulent for both adult and larval nematodes in space (Hammond et al., 2013b). By contrast, spaceflight had negligible effect on the virulence of *Salmonella* (Hammond et al., 2013c). Clinorotation is a favored ground-based emulation of many of the features of microgravity. Clinorotation randomizes gravity's vector, allowing biologics of differing size and density to co-localize under minimal shear stress (Hammond and Hammond, 2001). We previously found that clinorotation reproduced the effects of spaceflight for some, but not all, microbes tested. In clinorotation, *Candida* and *Enterococcus* were less virulent for larval worms but not adult worms, whereas virulence of MRSA, *Listeria*, and *Salmonella* were unaffected in tests with both adult and larval worms (Hammond et al., 2013b; Hammond et al., 2013c).

The purpose of the present study was to use our spaceflight-adapted nematode assay to evaluate the effect of microgravity versus clinorotation on the virulence of four additional common clinical pathogens—*Klebsiella*, *Streptococcus*, *Proteus*, and *Pseudomonas*.

MATERIALS AND METHODS

Test Organisms

The following microorganisms were obtained from the American Tissue Type Collection (Manassas, VA): *Klebsiella pneumoniae* ATCC 8052; *Streptococcus pneumoniae* ATCC 51915; *Proteus mirabilis* ATCC4630; and *Pseudomonas aeruginosa* ATCC BAA-47. Wild type N2 Bristol nematodes were purchased from the *Caenorhabditis* Genetics Center at the University of Minnesota, Twin Cities, MN. Worms were expanded on nematode growth medium plates seeded with *E. coli* and eggs were prepared by standard techniques (Hammond et al., 2009; Smith et al., 2002).

Chemicals and Reagents

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Spaceflight Hardware

To conduct experiments in spaceflight, we made use of the fluid processing apparatus (FPA) (Hammond et al., 2009; Hoehn et al., 2004). The FPA is a glass tube that is configured to isolate four separate volumes between moveable rubber septa. By advancing the plunger, the contents of each chamber can be serially mixed via a bypass channel, allowing experiments to be activated and terminated in sequential steps (Hoehn et al., 2004)

(Figure 1). To conduct the assay, *C. elegans* or buffer, growth medium for the bacteria, and microorganisms are mixed approximately 40 hours into spaceflight, once the hardware reaches the International Space Station (ISS). The microorganisms and worms were allowed to interact for 48 hours at ambient temperature and the experiment was then terminated by introducing paraformaldehyde as a fixative. The now stabilized FPAs are stored at ambient temperature on the ISS until returned to ground for measurements of OD_{620} measured as a quantification of microorganisms. Figure 2 summarizes the experimental design.

Gas exchange during the assay is enabled through the use of hydrophobic PTFE Teflon™ membranes (pore size 0.02 μm>700 kPa (100 psi) water entry pressure) (Hammond et al., 2009) in the septum located at the far end of the FPA most distal to the plunger (Hammond et al., 2009; Hoehn et al., 2004). All the samples use zero head space, meaning that the membrane is always wet, as there is no air bubble. Gore-tex membranes were selected since they preserve gas exchange when wet.

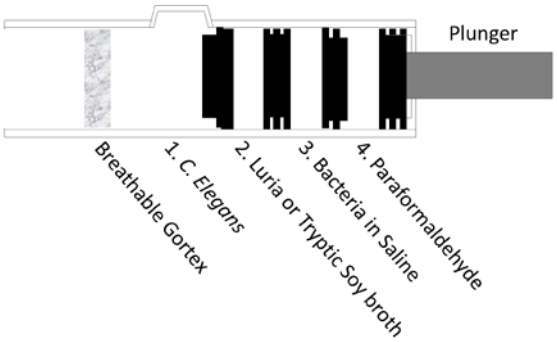

Figure 1. Flight Processing Apparatus (FPA). The left end of the FPA is plugged with a breathable Gortex membrane. To activate the experiment, the plunger is advanced to mix *C. elegans* in chamber 1 with Luria or Tryptic Soy broth in chamber 2, and microorganisms in saline in chamber 3. At the end of 48 hours, the plunger is further advanced to empty the paraformaldehyde from chamber 4 into the mixture and terminate the experiment.

The next chamber contains 2 ml of S-basal medium with 5 mg/liter cholesterol plus 5000 N2 *C. elegans* eggs. The eggs hatch in flight as the shuttle flies to the station and are ready to interact with the test microorganisms on ISS. In ground controls >80% of the worms hatched. In experiments designed to measure virulence towards larval worms, no additional food was provided in the first chamber, which causes the hatched eggs to arrest at the L2 life cycle larval stage. In experiments designed to measure virulence towards adult worms, the first chamber also included 1×10^9 heat-killed *E. coli*. This food source allows the eggs to progress to the L3/L4 stage of their life cycle before they were mixed with the test microorganism (Corsi, 2006). The quantity of *E. coli* was adjusted so that this food source was depleted approximately one-half day before the test microorganisms were introduced to the worms. The *C. elegans* eggs are extracted in the lab at the Space Life Sciences Building at Kennedy Space Center and are loaded into the flight and ground control hardware; the flight samples undergo late load on the space shuttle. At 22°C, the worms develop into L4 larvae by the time of co-culture with the target bacteria on the ISS, which is 40 hours post-launch and 48 hours post egg extraction (Corsi, 2006; Hammond et al., 2009; Hoehn et al., 2004). By culturing the worms at ambient temperature, as opposed to the more customary 30°C used in many *C. elegans* studies, we were able to slow their growth, thereby minimizing egg-laying and completion of the life cycle within the interval of the virulence assay. This lower temperature also slows the growth rate of the target microorganisms, as we have previously noted (Hammond et al., 2009).

To measure the growth of test microorganisms in the absence of worms, control FPAs were set up with just 2 ml of S-basal medium plus cholesterol, adjacent to the gas exchange membrane.

The second chamber contained 1 ml of growth medium for the microorganisms: Luria Broth for *Streptococcus*, and Tryptic Soy Broth for *Proteus*, *Klebsiella*, and *Pseudomonas*. We have previously validated this mixture of media as optimizing the growth of both microorganisms and the *C. elegans* (Hammond et al., 2009; Hammond et al., 2013a).

The third chamber contained 0.75 ml of phosphate buffered saline with 1×10^7 live microorganisms in static phase. The microorganisms remain in stasis until mixed with growth medium at the time of experimental

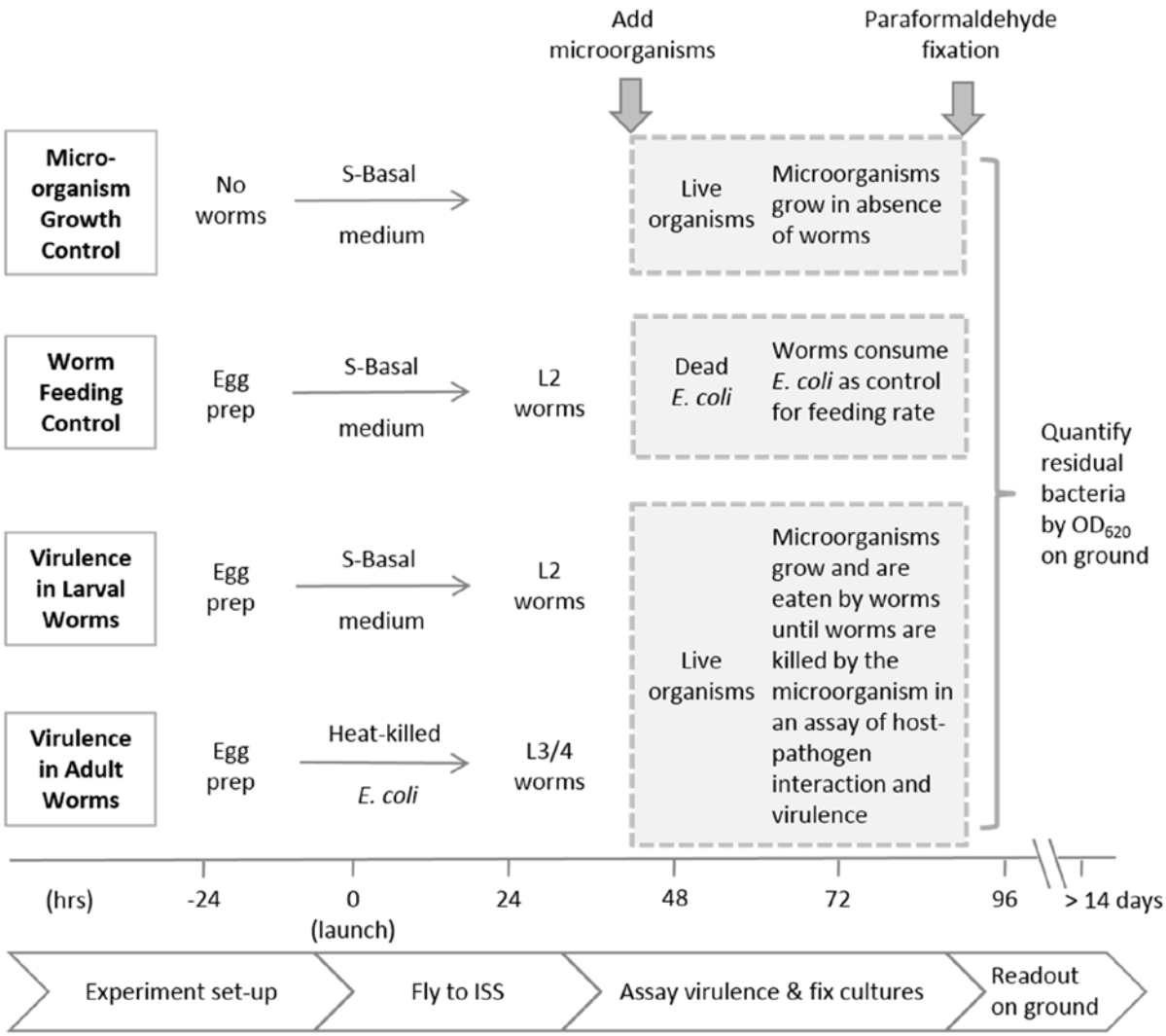

Figure 2. Design and timeline of the experiments. Growth controls were set up with live microorganisms grown in the absence of worms. Worm feeding controls were conducted with L2 larvae and heat-killed *E. coli*. Virulence in adult and larval worms was evaluated by preparing *C. elegans* eggs just before launch. All Group Activation Packs (GAPs) were loaded one day before launch and passed off. Eggs hatching in basal medium are growth-arrested at the L2 stage, whereas larvae hatching in the presence of heat-killed *E. coli* as a food source can mature into L3/4 larvae. Virulence assays were activated approximately 46 hours after launch and terminated by fixation after an additional 48 hours. The timeline reflects hours prior to and after launch, which is defined as zero.

activation. In feeding control studies, heat-killed *E. coli* replaced live microbes (Hammond et al., 2013b; Hammond et al., 2013c).

To activate the experiment, the first three chambers are mixed together to bring the hatched *C. elegans* in contact with the microorganisms that are now fed with the Luria or Tryptic Soy broth. After 48 hours of co-culture, the experiment is stopped by mixing in 0.5 ml of 12% paraformaldehyde fixative from the fourth chamber.

The FPAs are assembled in groups of eight around the perimeter of a cylindrical container known as a Group Activation Pack (GAP) and plunged simultaneously with a crank pressure plate mechanism and crank handle (Hammond et

al., 2009; Hoehn et al., 2004). Each experiment was set up in triplicate. One set of GAPs was flown in space on STS 125 to the ISS.

Ground Controls

Ground controls included a second set of GAPs rotated on a clinorotation device to maintain cells in suspension, and a third set of GAPs were maintained under static conditions on ground. Spaceflight and ground controls were performed in identical hardware and with identical timing, except that the ground controls procedures were offset by 30 minutes to allow for any delays in astronaut communications from the ISS. All experiments were matched to shuttle middeck ambient temperature, which averages between 21°C and 23°C (Thirsk et al., 2009).

The exact configuration and design of the clinorotation device employed has been described in detail previously (Hammond et al., 2009; Hoehn et al., 2004). In order to match the conditions for spaceflight and ground controls as much as possible, the FPAs were loaded into the same GAPs used in spaceflight. The GAP was mounted in a clinorotation device such that it rotated around its central longitudinal axis. The FPA thus moved in a circle around the GAP's axis of rotation, as opposed to each FPA rotating on its own axis. The GAP had a diameter of 10.2 cm and FPAs rotated around the center axis at a radius of 3.5 cm.

The axis of rotation was perpendicular to Earth's gravitational field, resulting in a complete randomization of the gravity vector. Given the rotation axis and a rotation speed of 5 rpm, cells in the FPA would experience a centripetal acceleration of approximately 9.7×10^{-4} *g* (Klaus et al., 1998). Centripetal motion of the nematodes or bacterial cells would be negligible under such forces when compared with non-rotating, unit gravity controls. This clinorotation model approximates one aspect of microgravity in terms of preventing net sedimentation without stirring. Constant reorientation of the culture effectively nullifies cumulative sedimentation of the bacteria, but does not necessarily result in uniform distribution of the suspended organisms (Klaus, 2001; Klaus et al., 1998). Furthermore, when there are rotating particles of differing sizes or densities, such as bacteria with nematodes, the rotation rate cannot be set so as to maintain both components in a motionless state; there will be varying degrees of relative motion between the different components and the fluid (Horneck et al., 2010).

We used conditions that have previously been shown to optimally maintain bacteria in suspension in this specific hardware (Klaus et al., 1998). The rotation rate was determined by using the equations linking the density of the particle and the medium, the viscosity of the medium, the effective Stokes radius of a bacterial cell, and the radius of the container (Klaus et al., 1998). Although the nematodes are far larger than the bacteria, our model does not rely on the forces of the clinorotation apparatus to keep the two co-localized. The nematodes are highly motile, which keeps them in suspension and allows them to swim freely toward the bacteria in response to chemotactic signals.

Assay Validation

We have previously validated our assay (Hammond et al., 2009; Hammond et al., 2013b; Hammond et al., 2013c). Microgravity appears to have negligible effect on *C. elegans'* ability to feed on microorganisms. Although launch is associated with brief intense vibration, multiple astronauts have confirmed that vibration on ISS is too gentle to see by eye or feel on a vibration profile instrument (personal communications—David Wolf, Rich Linnehan, and Larry DeLucas). We reproduced launch gravitational stresses in a centrifuge at Bioserve Space Technologies, University of Colorado Boulder, and found no effect on *C. elegans'* consumption of dead *E. coli* over 24 hours after increased *g* exposure or swimming pattern under a microscope.

C. elegans presented with a nonpathogenic food source during spaceflight consume virtually all of the available killed *E. coli*, just as they do on the ground (Hammond et al., 2009; Hammond et al., 2013b; Hammond et al., 2013c). However, when *C. elegans* were cultured with pathogenic *Salmonella typhimurium*, nearly all the *Salmonella* survived and the *C. elegans* died. This suggests that *C. elegans* in spaceflight do not become more resistant to virulent microorganisms (Hammond et al., 2009; Hammond et al., 2013c).

Post-spaceflight examination of worms grown with microorganisms revealed a mixture of *C. elegans* with curved shapes and needle shapes

(data not shown). The needle shape is characteristic of dead worms, whereas a curved shape is characteristic of live worms. We verified that paraformaldehyde fixes live *C. elegans* very quickly and preserves the curved shape characteristic of live worms. This result verifies that some worms survived the 48 hour co-culture with microorganisms and were alive at the time that paraformaldehyde was added. Thus, our virulence assay was conducted within the dynamic range with both dead and live worms present at the end of the co-culture with microorganisms.

Statistics

Statistics were performed using Student's 2-tailed unpaired t-test. Data are presented as the mean ± 1 SEM of quadruplicates.

RESULTS

Figure 3 illustrates the virulence of the four microorganisms towards nematodes in static/ground condition, clinorotation on ground, and microgravity of spaceflight. When cultured alone as growth controls, the four microorganisms grew as well as or better in spaceflight as they did in matched ground-based cultures, whereas clinorotation induced a striking increase in growth (Table 1). A comparison of worm/microorganism co-cultures with the growth controls revealed that the raw OD_{620} of microorganisms incubated with worms was higher than the raw OD_{620} of microorganisms cultured alone (Table 1). This does not reflect light absorption by the worms, as their light absorption at this wavelength is negligible (Hammond et al., 2009). Instead, it appears the increased OD_{620} in the microorganism/worm mixtures reflects debris from worms that have been killed by the microorganism. We therefore calculated for each condition the difference in OD_{620} for microorganism mixed with worms minus the OD_{620} for microorganisms cultured alone. We used the delta (Δ) OD_{620} as an index of virulence to compare the different culture conditions. Decreased virulence is indicated by a lower ΔOD_{620} due to consumption of more microorganisms by the *C. elegans*. Table 1 provides the raw data used to calculate the Δ OD_{620} and Figure 3 is based on those ΔOD_{620}.

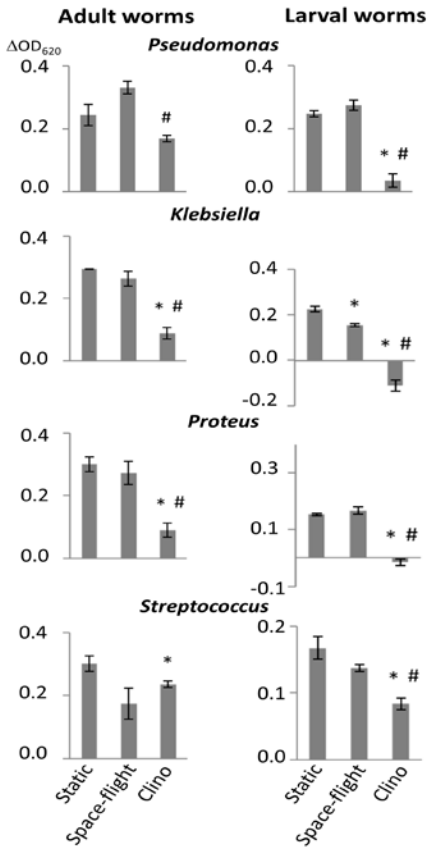

Figure 3. Virulence of four microorganisms towards adult and larval worms in ground/static, spaceflight, and clinorotation. Microorganisms were cultured with nematodes in identical hardware under static conditions, clinorotation, and space-flight and fixed with formaldehyde after 48 hours of co-incubation. Delta (Δ) OD_{620} values are the OD_{620} for microorganism in the presence of larval or adult worms minus the OD_{620} for microorganism cultured alone. Error bars indicate ± 1 SEM of quadruplicates. Two-tailed unpaired t-tests were used to estimate the significance between the ΔOD_{620} for spaceflight or clinorotation, versus static conditions and clinorotation versus spaceflight. * indicates $p<0.05$ relative to static controls, # indicates $p<0.05$ for clinorotation versus flight. The positive ΔOD_{620} with the static control suggests that there is some debris generated when microorganisms are incubated with larvae or adult worms (or that the microorganisms grew better in the presence of *C. elegans*). Smaller ΔOD_{620} indicates more consumption of microbes and/or less generation of debris under spaceflight. Either explanation is consistent with decreased virulence. Assays of virulence were done concurrently in spaceflight, static ground, and clinorotation.

Table 1. OD_{620} of Microorganisms after Growth and Virulence Assays. OD_{620} of microorganisms grown in medium alone (growth) or after addition into cultures of larval or adult worms. Cultures in identical hardware were maintained under static/ground, spaceflight, or clinorotation conditions. After 48 hours of growth, samples were fixed with paraformaldehyde. Values are the mean + 1 SEM of quadruplicates.

	Pseudomonas	*Klebsiella*	*Proteus*	*Streptococcus*
Growth Static/Ground	0.263 ± 0.106	0.307 ± 0.022	0.386 ± 0.055	0.256 ± 0.013
Larval Worms Static/Ground	0.511 ± 0.0103	0.532 ± 0.012	0.539 ± 0.004	0.423 ± 0.017
Adult Worms Static/Ground	0.508 ± 0.034	0.601 ± 0.001	0.686 ± 0.023	0.557 ± 0.024
Growth Spaceflight	0.227 ± 0.017	0.302 ± 0.013	0.307 ± 0.003	0.207 ± 0.050
Larval Worms Spaceflight	0.501 ± 0.016	0.458 ± 0.006	0.475 ± 0.013	0.344 ± 0.005
Adult Worms Spaceflight	0.558 ± 0.020	0.565 ± 0.024	0.580 ± 0.038	0.381 ± 0.050
Growth Clinorotation	0.553 ± 0.012	0.597 + 0.010	0.617 ± 0.011	0.440 ± 0.080
Larval Worms Clinorotation	0.589 ± 0.021	0.485 + 0.025	0.602 ± 0.012	0.523 ± 0.008
Adult Worms Clinorotation	0.722 ± 0.010	0.685 + 0.018	0.708 ± 0.022	0.676 ± 0.011

Pseudomonas was slightly numerically more virulent in spaceflight, but not statistically significantly when tested with adult worms ($p=0.07$). There was no difference in virulence when assayed with larval worms (Figure 3). *Streptococcu*s was slightly less virulent in spaceflight when tested with adult worms ($p=0.06$), but showed no difference with larval worms. Virulence of *Klebsiella* was reduced by spaceflight when assayed with larval worms ($p<0.01$), but not adult worms. Virulence of *Proteus* was not different in spaceflight.

When assayed under clinorotation conditions with adult or larval worms, all four organisms were significantly less virulent compared to static controls. The one exception was *Pseudomonas* with adult worms where the clinorotated samples were significantly less virulent than samples from spaceflight, but did not reach statistical significance when compared to static controls.

DISCUSSION

The microbe, the host, and the interplay between them must be considered to understand whether virulence changes pathogenicity (Harvill and Miller, 2000; Sifri et al., 2005). Ours is the first system to use a direct in vivo assay to evaluate a microbial virulence system in space (Hammond et al., 2009). By employing the *C. elegans* host-pathogen model, we can assay virulence in spaceflight and terminate the assay with fixative for subsequent ground-based analysis. This eliminates any confounding variables associated with re-entry and delays. We have verified that neither spaceflight nor clinorotation changed the feeding rate of nematodes, allowing changes in bacterial consumption to accurately reflect virulence (Hammond et al., 2013c).

C. elegans display an innate, or immediate, immune response and share many cellular and molecular structures and control pathways with higher organisms (Harvill and Miller, 2000; Sifri et al., 2005). For example, they are able to produce antimicrobial peptides and enzymes in response to microbes (Ewbank, 2006). A brief exposure to bacteria "immunizes" the worms and allows them to survive a subsequent exposure that would otherwise prove lethal—a phenomenon referred to as "conditioning" (Anyanful et al., 2009). *C. elegans* do not have a true adaptive or secondary immune response that higher order organisms generate upon repeated exposure to the same microbe. Nonetheless, several studies have shown good concordance between the virulence of *Salmonella* assayed in *C. elegans* and virulence in the mouse systemic infection assay (Jelsbak et al., 2012; Paulander et al., 2007).

The current experiments found only modest changes in the virulence of *Pseudomonas*, *Klebsiella*, and *Streptococcus*, and no changes in the virulence of *Proteus* in spaceflight. This contrasts with our previous studies of *Listeria*, MRSA, *Salmonella*, and *Candida albicans* (*C. albicans)* that all showed significantly reduced virulence in spaceflight when tested with both larval and adult *C. elegans* (Hammond et al., 2013b; Hammond et al., 2013c).

When tested under conditions of clinorotation, the current report showed significantly reduced virulence of *Pseudomonas*, *Klebsiella*, *Proteus, and Streptococcus.* We have also previously found that *Candida* and *Enterococcus* were less virulent for larval worms, but not adult worms, when tested under clinorotation; whereas virulence of *Salmonella*, MRSA, and *Listeria* were unaffected in clinorotated tests with both adult and larval worms (Hammond et al., 2013b; Hammond et al., 2013c). Thus, using shear force to offset gravity did not consistently produce the same effect on virulence as did true spaceflight microgravity. This discordance may reflect variability in how different microorganisms are affected by the shear forces that are required to offset gravity in the clinorotation model.

Many bacteria proliferate better in clinorotation compared with static conditions, including the four microorganisms evaluated in the present study. If the numbers of microorganisms overwhelm the feeding capacity of the *C. elegans*, the microorganisms may appear to be more virulent than static controls with lower bacterial numbers. This may have been at play in some of our assays. On the other hand, our previous experiments with *Listeria* and *Enterococcus*, using the same experimental set-up, did not find enhanced growth in clinorotated samples compared to static ground controls or flight samples. Yet the virulence of *Listeria* and *Enterococcus* was reduced in flight, but unchanged in clinorotation (Hammond et al., 2013b).

In orbit, net forces on an object (gravity and centrifugal acceleration) are effectively nulled out, resulting in a perpetual free-fall condition. In low-Earth orbit, only a small residual force (generally referred to as microgravity) remains. In contrast, clinorotation randomizes the influence of gravity so there can be no net directional acceleration or force acting on an object. Residual accelerations for the clinorotation device used in this study are less than 10^{-3} *g* which, according to Stoke's Law, means that non-motile bacteria will move through the medium at a rate 1,000 fold less than bacteria in a static system. This is important, as the magnitude of the applied *g* force determines biological outcome in some systems (Brown et al., 1976). Both culture modalities, space-based and clinorotation, minimize motion in a suspension culture and prevent the microorganisms from sedimenting (Klaus et al., 2004). *C. elegans*, by contrast, are highly-motile organisms and can remain in suspension in any of these conditions.

When Benoit and Klaus reviewed the literature looking for an explanation as to why microgravity is associated with increased bacterial growth for many but not all bacteria (Benoit and Klaus, 2007), they hypothesized that spaceflight indirectly affects growth by reducing the tendency of bacteria to settle out of liquid medium and reducing the potential for buoyant convection in the vicinity of actively metabolizing bacterium. Would the impact on microgravity be less evident on motile bacteria that can remain dispersed throughout the liquid culture and actively stir the medium in their microenvironment, whether on ground or in spaceflight? Nine of nine studies with non-motile strains (including *Salmonella typhimurium*, *E. coli*, and *B. subtilis*) showed increased growth in microgravity, whereas three of three studies with motile *E. coli* showed no

difference in growth under microgravity (Benoit and Klaus, 2007). Motility also explained the variability in concurrence between results in spaceflight and results from clinorotation used to maintain microorganisms in suspension. As evidence, six of six non-motile bacteria showed increased growth in clinorotation, whereas a motile strain showed no difference (Benoit and Klaus, 2007).

We postulate that motility and shear forces account, at least in part, for the discordance between spaceflight and clinorotation in our studies (Table 2). *Pseudomonas* is a rod-shaped bacteria with a flagellum that provides unipolar motility. Many strains of *Pseudomonas*, including the one used in our studies, form a substantial amount of mucoid exopolysaccharide material. In microgravity, *Pseudomonas* growing on a solid surface forms a column-and-canopy structure not seen on Earth (Kim et al., 2013). However, this would not be relevant in the fluid culture media used in our experiments. *Proteus* are also motile, whereas *Klebsiella* is a non-motile rod. *Streptococci* are non-motile cocci that divide along a single axis to form long chains. Formation of chains would greatly affect the amount of shear force experience by the *Streptococci*. In summary, the effects of clinorotation on bacterial growth and virulence in the *C. elegans* model appear to be influenced by multiple variables, but these likely include motility, and size-dependent shear and Coriolis forces incurred. We find no evidence that microorganisms can become more virulent in spaceflight and pose a health risk to astronauts.

Table 2. Effect of Spaceflight and Clinorotation on Virulence of Microorganisms towards Adult and Larval *C. elegans*. The effect of spaceflight and clinorotation on virulence of four microorganisms for adult and larval worms is summarized along with the morphology of the microorganisms. Statistical significance of spaceflight or clinorotation versus static conditions was estimated by two-tailed unpaired Student's t-test.

Microorganism	Shape	Adult Worms Spaceflight	Larval Worms Spaceflight	Adult Worms Clinorotation	Larval Worms Clinorotation
Pseudomonas	Rod Motile	⇔ *p = 0.07*	⇔ *p = 0.2*	⇔ *p = 0.08*	⇓⇓⇓ *p <0.001*
Klebsiella	Rod Non-motile	⇔ *p = 0.2*	⇓⇓ *p <0.01*	⇓⇓⇓ *p <0.001*	⇓⇓⇓ *p <0.001*
Proteus	Rod Motile	⇔ *p = 0.6*	⇔ *p = 0.3*	⇓⇓⇓ *p <0.001*	⇓⇓⇓ *p=0.001*
Streptococcus	Cocci Chains	⇓ *p = 0.06*	⇔ *p =0.14*	⇓⇓ *p = 0.05*	⇓⇓ *p <0.01*

⇓ indicates reduced virulence compared to static/ground controls.

⇔ indicates virulence that is not statistically different from static/ground controls.

CONCLUSIONS

None of the wild type organisms that we have tested to date show increased virulence under either spaceflight or clinorotation (Table 3). Spaceflight decreased the virulence of *Streptococcus* for adult *C. elegans*, which is similar to what we have previously reported with *Candida*, MRSA, *Enterococcus*, and *Listeria* (Table 3). When larval *C. elegans* were the targets, spaceflight decreased the virulence of

Klebsiella, which is what we have previously reported with *Candida, Enterococcus, Listeria*, and MRSA. Spaceflight had minimal effect on the virulence of *Pseudomonas* or *Proteus*, which is similar to what we have seen with *Salmonella* (Table 3). Under clinorotation conditions, *Klebsiella, Proteus, Pseudomonas,* and *Streptococcus* were all less virulent with larval *C. elegans,* as we have reported previously with *Candida* and *Enterococcus* (Table 3). *Pseudomonas* virulence for adult *C. elegans* was unaffected by clinorotation, just as we have previously reported with *Enterococcus, Listeria, MRSA,* and *Salmonella* (Table 3). Overall, *Pseudomonas, Klebsiella, Proteus,* and *Streptococcus* showed far less virulence when tested in clinorotation than was observed in spaceflight (Table 2).

Table 3. Effect of Spaceflight and Clinorotation on the Virulence of Nine Different Microorganisms. Compilation of our laboratory's studies using the *C. elegans* model for assaying virulence in spaceflight and clinorotation. Results for *Listeria monocytogenes, Enterococcus faecalis, Candida albicans,* and Methicillin-resistant *Staphylococcus aureus* (MRSA) are taken from Hammond et al. (Hammond et al., 2013b). Results for *Salmonella* are from Hammond et al. (Hammond et al., 2013c).

	Increased Virulence	Unchanged Virulence	Decreased Virulence
Adult Worms Spaceflight		*Klebsiella* *Proteus* *Pseudomonas* *Salmonella*	*Candida* *Enterococcus* *Listeria* MRSA *Streptococcus*
Larval Worms Spaceflight		*Proteus* *Pseudomonas* *Salmonella* *Streptococcus*	*Klebsiella* *Candida* *Enterococcus* *Listeria* MRSA
Adult Worms Clinorotation		*Enterococcus Listeria* MRSA *Pseudomonas* *Salmonella*	*Klebsiella* *Proteus* *Streptococcus*
Larval Worms Clinorotation		*Listeria* MRSA *Salmonella*	*Candida* *Enterococcus* *Klebsiella* *Proteus* *Pseudomonas* *Streptococcus*

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