Research Article

Differing Responses in Growth and Spontaneous Mutation to Antibiotic Resistance in *Bacillus subtilis* and *Staphylococcus epidermidis* Cells Exposed to Simulated Microgravity

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ABSTRACT

Bacteria of the genera *Bacillus* and *Staphylococcus* are frequent inhabitants of the International Space Station (ISS) and represent possible opportunistic pathogens. The effect of simulated microgravity on growth and the frequency of mutation to antibiotic resistance in the model surrogate organisms *Bacillus subtilis* (*B. subtilis*) and *Staphylococcus epidermidis* (*S. epidermidis*) were investigated. The test organisms were cultivated for six days in Rotating Wall Vessel (RWV) clinostats either in the vertical (simulated microgravity) or horizontal (1 *g* control) orientation. Parameters measured were: optical densities (ODs); viable counts; frequencies of resistance to rifampicin (RFM); and frequencies of double resistance to RFM and trimethoprim (TMP). The results indicated that the response to simulated microgravity differed in the two microorganisms. Both *B. subtilis* and *S. epidermidis* grew to higher ODs and cell numbers in simulated microgravity. However, the frequencies of mutation, both to RFM resistance and double resistance to RFM and TMP, were observed to increase significantly in simulated microgravity-grown *B. subtilis* but not in *S. epidermidis*.

Key words: Antibiotic Resistance; *Bacillus subtilis*; Clinorotation; International Space Station; Microgravity; Microorganisms; Mutation; Rifampicin; *Staphylococcus epidermidis*; Trimethoprim

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INTRODUCTION

Preparations are underway for long-duration missions through interplanetary space to destinations such as the Moon, near-Earth asteroids, or Mars (International Space Exploration Coordination Group, 2013). For more than five decades, human spaceflight missions into Low Earth Orbit (LEO) have rendered a wealth of information about the challenges of space travel and their effects on human health. Future missions beyond LEO are more likely to expose astronauts to higher risks to their health and performance. Of particular importance is the documented dysregulation of astronauts' immune systems during long-term missions (Crucian et al., 2009; Guéguinou et al., 2009), combined with the reported enhanced virulence of some microorganisms exposed to the stresses of the spaceflight environment, especially microgravity (Klaus and Howard, 2006; Ott et al., 2012). These two phenomena

acting together could lead to an increase in astronaut infections by opportunistic pathogens during extended missions (Klaus and Howard, 2006). Committees representing both the National Research Council (NRC) and the International Space Exploration Coordination Group (ISECG) have identified the need to better understand health risks during space exploration, and both organizations have recognized the ISS as the best available platform to conduct research activities to address these challenges (ISECG, 2013; National Research Council, 2014).

Although space stations start out as essentially sterile environments, microbes rapidly colonize numerous ecological niches, to which they adapt and evolve in response to selective pressures unique to the spaceflight environment (Novikova, 2004; van Tongeren et al., 2007). Although preflight protocols minimize the risk of astronaut infection by true pathogens, a number of opportunistic pathogens have been isolated from space station crew quarters and from their human inhabitants, including species of the genera *Bacillus, Citrobacter, Enterobacter, Enterococcus, Escherichia, Flavobacterium, Haemophilus, Klebsiella, Morganella, Proteus, Pseudomonas, Ralstonia, Serratia, Staphylococcus, Stentrophomonas, Streptococcus,* and *Yersinia* (Ilyin, 2005; Klaus and Howard, 2006). In fact, fungal infections, viral diseases, styes, and infections of the urinary tract, upper respiratory tract, and subcutaneous tissue all have been documented from STS-1 to STS-108 missions (Sams, 2009). In particular, *Bacillus* and *Staphylococcus* spp. are the most ubiquitous organisms from the ISS, especially from crew quarters, debris, and lint (Venkateswaran et al., 2014). Not surprisingly, *S. epidermidis* was the most frequently encountered organism in the ISS microbiome, due to its close association as a skin commensal of humans (Venkateswaran et al., 2014).

Knowledge about the efficacy of antimicrobial interventions to treat infections during spaceflight is limited given the difficulty to assess the interplay between pharmacokinetics and physiologies of host and microbiome, both of which are altered in the spaceflight environment (Wotring, 2012). Decreasing microbial antibiotic susceptibility in the microgravity environment has been demonstrated from experiments performed on *Salyut 7* (Tixador et al., 1985) and the Space Shuttles *Challenger* (Lapchine et al., 1986) and *Discovery* (Tixador et al., 1994). In addition, experiments performed on space station *Mir* showed that the frequency of mutations to streptomycin resistance in the *E. coli rpsL* gene was increased by 2- to 3-fold in space compared to ground controls, and the spectrum of mutations observed was clearly altered (Fukuda et al., 2000; Yatagai et al., 2000). Experiments onboard the ISS have demonstrated that horizontal transfer of antibiotic resistance plasmids can occur among both Gram-positive and Gram-negative bacteria (De Boever et al., 2007). The above observations indicate that the possibility must be considered of antibiotic-resistant strains emerging, becoming dominant types in the microbiomes of crew members, and causing health problems.

Opportunistic infections are often treated with combinations of two antibiotics that differ in their mechanisms of action. Two antibiotics that were prescribed together to combat acute and recurrent infections are rifampicin (RFM) and trimethoprim (TMP) (Stein et al., 1988). Although the RFM-TMP combination regimen is no longer recommended (Zander et al., 2010), they are still indicated as components of multidrug therapies (Centers for Disease Control and Prevention, 2011; Cosgrove and Avdic, 2013). In addition to its clinical relevance, RFM resistance studies have been invaluable in understanding cellular processes and global responses of bacteria to different environments (Maughan et al., 2006; Maughan et al., 2004; Nicholson and Maughan, 2002). RFM is a broad-spectrum antibiotic that inhibits bacterial transcription initiation (Wehrli et al., 1968) by binding to the β-subunit of RNA polymerase in the mRNA exit channel, 2-3 nucleotides downstream from the active site (Campbell et al., 2001). Bacterial resistance to RFM (RFM^R) results from mutations in the *rpoB* gene encoding the β subunit of RNA polymerase, particularly in a small area called Cluster I corresponding to the RFM binding site (Jin and Gross, 1988). By comparison, TMP is a folate analogue that exerts its antimicrobial activity by competitively inhibiting dihydrofolate reductase (DHFR), an enzyme that catalyzes the reduction of dihydrofolate to tetrahydrofolate. Resistance to TMP most commonly stems from a chromosomal mutation in the *dfrA* gene that results in the

production of a DHFR that binds less tightly to TMP (Gleckman et al., 1981). In *S. aureus,* TMP^R is caused by mutations that change DHFR conserved amino acids L41F, F99Y/S, or H150R (Vickers et al., 2009). TMP^R can also result from elevated expression or activity of DHFR or dihydropteridine synthase (DHPS). In addition, it has been reported that mutations in *rpoB* causing RFM^R can up-regulate expression of the DHFR gene *dfrA*, also resulting in TMP^R (Kane et al., 1979). Thus, simultaneous resistance to both RFM and TMP can be linked mechanistically.

To explore the development of multiple antibiotic resistance in opportunistic pathogens during long-term human habitation in space, an experiment involving the Biological Research in Canisters (BRIC) hardware on the ISS was devised. The mission, designated BRIC-18, is to be described elsewhere in detail. In preparation for the BRIC-18 mission, the present communication describes ground-based studies using spaceflight analogues: (i) a rotating wall vessel (RWV) clinostat served as a generator of simulated microgravity; (ii) two Gram-positive model organisms, *Bacillus subtilis* and *Staphylococcus epidermidis*, served as surrogates of opportunistic species found in human space habitats, and; (iii) RFM and TMP were selected as test antibiotics.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions

Strains used were *B. subtilis* WN1532 (*trpC2*) from the authors' strain collection and *S. epidermidis* strain ATCC12228 obtained from the American Type Culture Collection, Manassas, VA. Medium for general cultivation was Trypticase Soy Yeast Extract (TSY) medium containing (g/L): Tryptone, 15; Soytone, 5; NaCl, 5; Yeast Extract, 3; K_2HPO_4, 2.5; glucose, 2.5; final pH 7. For semisolid plates, agar was added to TSY at 15.0 g/L. As appropriate, the antibiotics RFM and TMP (Sigma-Aldrich) were added to TSY at final concentrations of 5 μg/mL and 5 μg/mL, respectively.

Simulated Microgravity Experiments

Simulated microgravity was provided using two 4-place Rotary Cell Culture Systems (RCCS-4, Synthecon Inc., Houston, TX), each fitted with four 10-mL High Aspect Ratio Vessels (HARVs) (for details, refer to: http://www.synthecon.com/; accessed 11/6/14). In each experiment the two RCCS-4 units were operated simultaneously at 14 rpm, one unit in the vertical orientation producing simulated microgravity and the other in the horizontal orientation serving as the 1 *g* control. In accordance with the planned BRIC-18 flight experiment on ISS, cultures were incubated for 6 days at laboratory-ambient temperature (~23˚C).

Sample Analyses

Cultures were transferred from HARV chambers into sterile 50-mL conical centrifuge tubes. Optical densities at 660 nm (OD_{660}) were determined in a spectrophotometer. For viable counts, cultures were diluted serially tenfold in Phosphate Buffered Saline (PBS), dilutions plated on TSY, and colonies counted after incubation at 37˚C for 24 hours. To select for RFM^R mutants, cultures were concentrated by centrifugation, plated without dilution onto TSY+RFM plates, and colonies counted after incubation at 37˚C for 24 hours. The frequency of mutation to RFM^R was calculated by dividing the total number of RFM^R mutants by the total number of viable cells in each culture. To determine the frequency of mutation to both RFM and TMP, single RFM^R colonies were picked onto TSY plates containing TMP and scored for growth after incubation at 37˚C for 24 hours.

Statistical Analyses

Basic statistical parameters and One-Way Analysis of Variance (ANOVA) were computed using either Kaleidagraph version 3.6.2 (Synergy Software, Reading, PA), or an online statistical calculator (http://vassarstats.net/anova1u.html; accessed 11/6/14). Meta-analysis of data from multiple experiments was conducted using an online meta-analysis calculator (Health Decision Strategies, http://www.healthstrategy.com/meta/metainput.htm; accessed 11/6/14).

RESULTS

Time Course of *Bacillus subtilis* and *Staphylococcus epidermidis* Growth in Simulated Microgravity

As part of ground-based preparation for the BRIC-18 experiment to the ISS, *B. subtilis* and *S. epidermidis* cells were cultivated in the vertical (simulated microgravity) or horizontal (1 *g*) orientation in clinostats. To match conditions under which cells were to be subjected on the ISS, cells were grown for 6 days at ISS ambient temperature (~23˚C). To track the progress of growth, viable counts were determined from samples removed at daily intervals (Figure 1). *B. subtilis* cells initiated growth without a lag period and grew exponentially for the first 2 days at essentially the same rate, regardless of clinostat orientation (Figure 1A). In contrast, *S. epidermidis* cultures lagged for 1 day before entering exponential growth phase, and simulated microgravity-grown cultures appeared to grow at a slightly faster rate (Figure 1B). In both organisms, simulated microgravity-grown cultures grew to a slightly higher exponential cell number than did the 1 *g* controls (Figure 1). Once cultures reached the stationary phase, cell viability of both organisms was observed to decline; however, viability of cells cultured in simulated microgravity declined at a slower rate than in those grown at 1 *g* (Figure 1).

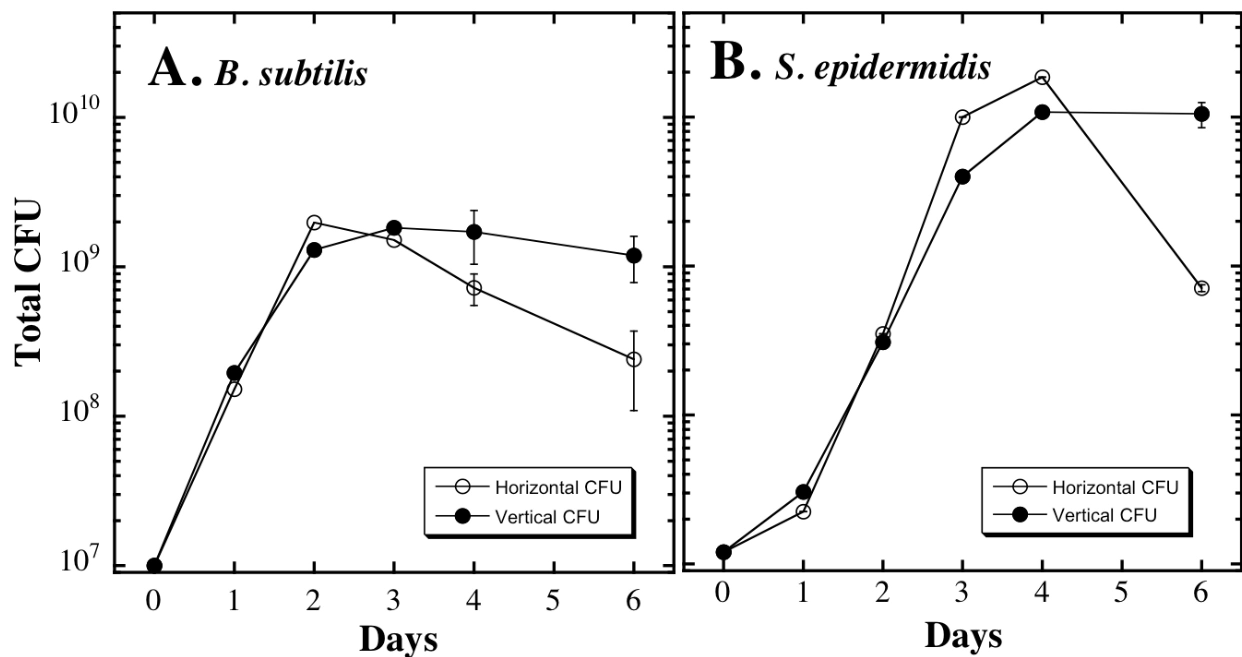

Figure 1. Time course of growth measured as viable counts of *B. subtilis* (A) and *S. epidermidis* (B) cells incubated in 10-mL HARVs either in simulated microgravity (vertical; filled circles) or 1 *g* (horizontal; open circles) orientation. Values are averages ± standard deviations (*n* = 4).

Six-Day Culture Experiments in Clinostats

It was observed that both test organisms exhibited a higher final cell density after cultivation for 6 days in simulated microgravity compared to the 1 *g* controls (Figure 1). To more rigorously test this observation, both *B. subtilis* (Figure 2) and *S. epidermidis* (Figure 3) cells were cultivated for 6 days in 10-mL HARVs in simulated microgravity vs. 1 *g* (*n* = 4). To achieve greater statistical power, multiple Trials (either 3 or 4) of each experiment were performed.

Growth

Six-day cultures of *B. subtilis* were harvested and growth was measured by optical density (OD) (Figure 2A) and viable counts (Figure 2B). In all three trials, *B. subtilis* cultures exposed to simulated microgravity exhibited greater cell mass, as measured by OD (Figure 2A), and

greater numbers, as measured by viable counts (Figure 2B), than did cultures cultivated in parallel at 1 *g*. Statistical analysis of the data by ANOVA revealed that in all 3 Trials the OD values were statistically significant (Figure 2A), and the viable count data was statistically significant in Trails 1 and 3, but not Trial 2 (Figure 2B).

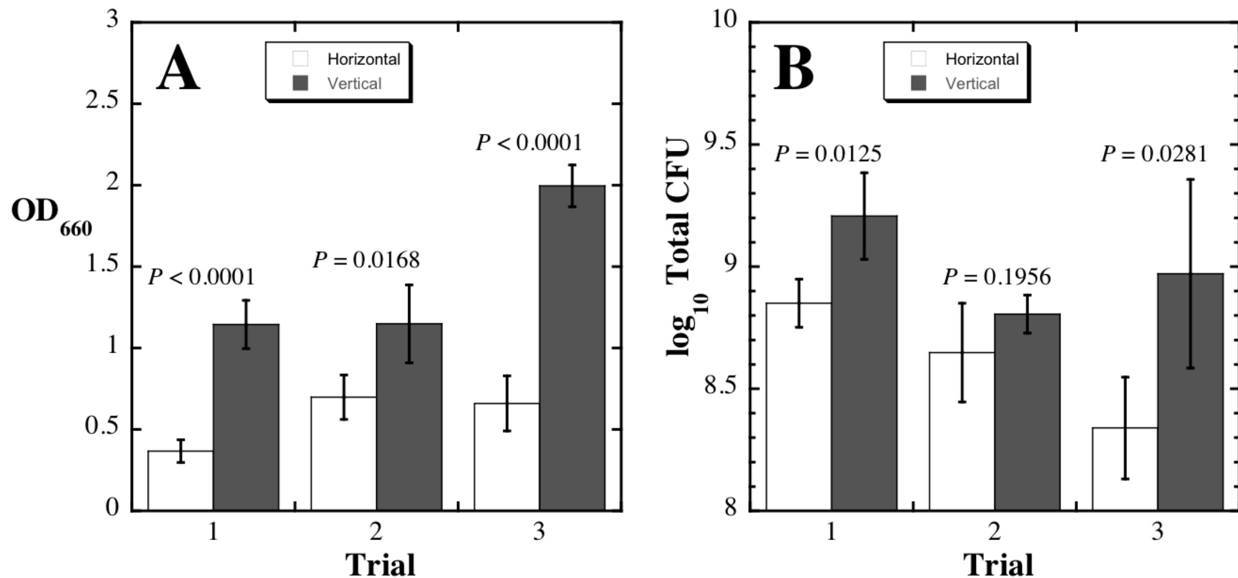

Figure 2. Optical density (A) and total cell number (B) of horizontal (open bars) and vertical (filled bars) cultures of *B. subtilis* cells after 6 days of incubation. Data are depicted as averages and standard deviations (*n* = 4) of three separate trials. Above each pair of bars is displayed the *P* value derived from ANOVA. $P < 0.05$ was considered statistically significant.

The same experiment was performed with cultures of *S. epidermidis* (Figure 3). Six-day cultures of *S. epidermidis* were harvested and growth was measured by OD (Figure 3A) and viable counts (Figure 3B). In all 3 Trials, simulated microgravity-grown cells exhibited significantly greater cells mass as measured by OD (Figure 3A). When viable counts were measured, it was observed that cell numbers were greater in simulated microgravity-grown cells in all 4 Trials, and the differences were statistically significant in Trials 2, 3, and 4, but not Trial 1 (Figure 3B).

Frequency of mutation to RFM^R

To determine the frequency of mutation to RFM^R, cells from the same cultures as described in Figures 2 and 3 were harvested, concentrated by centrifugation, and plated onto TSY plates containing RFM (Figure 4). When *B. subtilis* cultures were analyzed, a higher frequency of mutation to RFM^R was observed in the simulated microgravity-grown cultures in all 3 Trials, and the differences were statistically significant by ANOVA in Trials 2 and 3 (Figure 4A). In sharp contrast, when *S. epidermidis* cultures were examined, the differences in mutation frequency in all 4 Trials were found to be not statistically significant, and indeed in Trials 2, 3, and 4 a lower frequency of mutation to RFM was noted in the simulated microgravity-grown cultures (Figure 4B). Thus it appeared mutation to RFM^R was affected by simulated microgravity differently in *B. subtilis* vs. *S. epidermidis.*

Frequency of mutation to RFM^R and TMP^R

RFM^R resistant colony isolates were picked onto TSY containing TMP to score for TMP^R

(Figure 5). Data from *B. subtilis* showed that in all 3 Trials the simulated microgravity cultures exhibited a higher proportion of mutants resistant to both, RFM and TMP, than did the cultures from the 1 *g* controls; however, these differences were not significant at the $P < 0.05$ level by ANOVA (Figure 5). In the case of *S. epidermidis* cultures, growth on TSY+TMP plates was slow and quite variable, leading to ambiguous results which are not reported here.

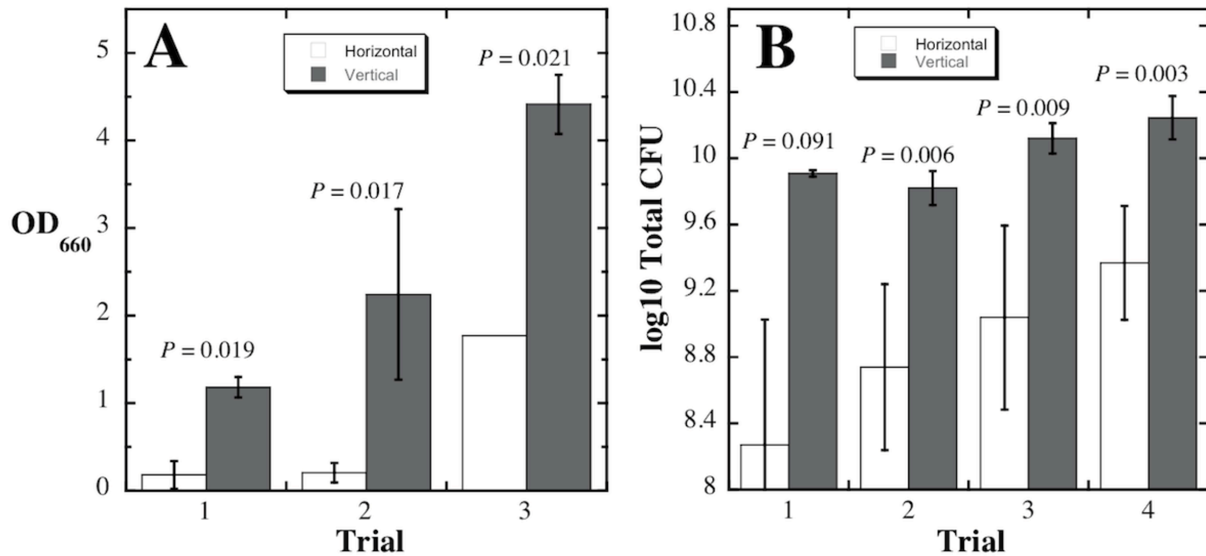

Figure 3. Optical density (A) and total cell number (B) of horizontal (open bars) and vertical (filled bars) cultures of *S. epidermidis* cells after 6 days of incubation. Data are depicted as averages and standard deviations (n = 4) of three (OD) or four (CFU) separate trials. Above each pair of bars is displayed the *P* value derived from ANOVA. $P < 0.05$ was considered statistically significant.

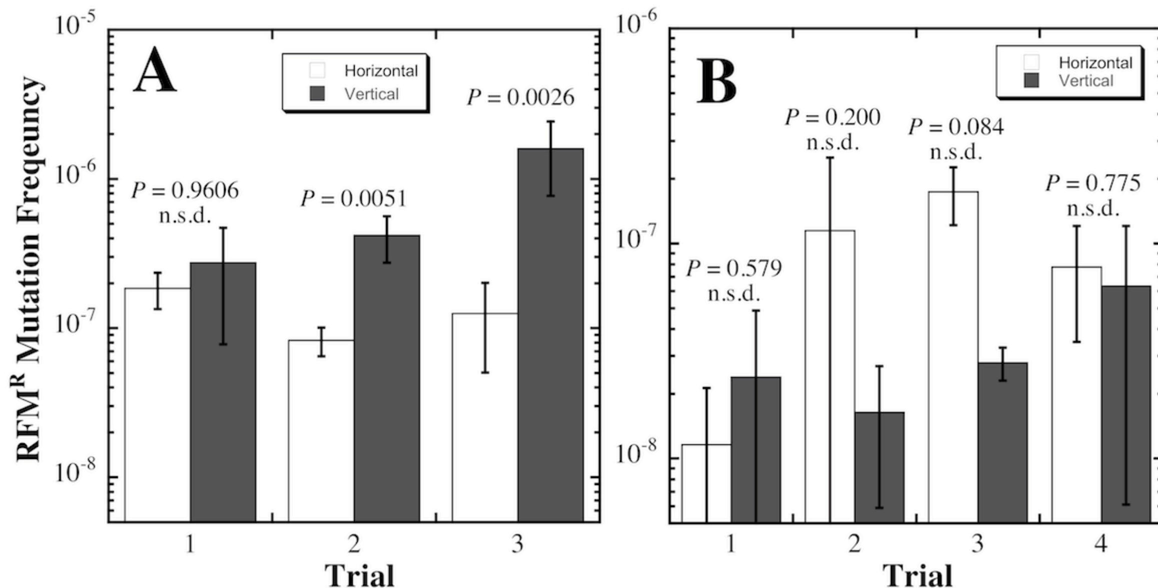

Figure 4. Mutation frequency to RFM^R by *B. subtilis* 168 (A) and *S. epidermidis* (B) after 6 days of clinorotation in either the horizontal (open bars) or vertical (filled bars) orientation. Data are depicted as averages and standard deviations (n = 4) of three (A) or four (B) separate trials. Above each pair of bars is displayed the *P* value derived from ANOVA. $P < 0.05$ was considered statistically significant.

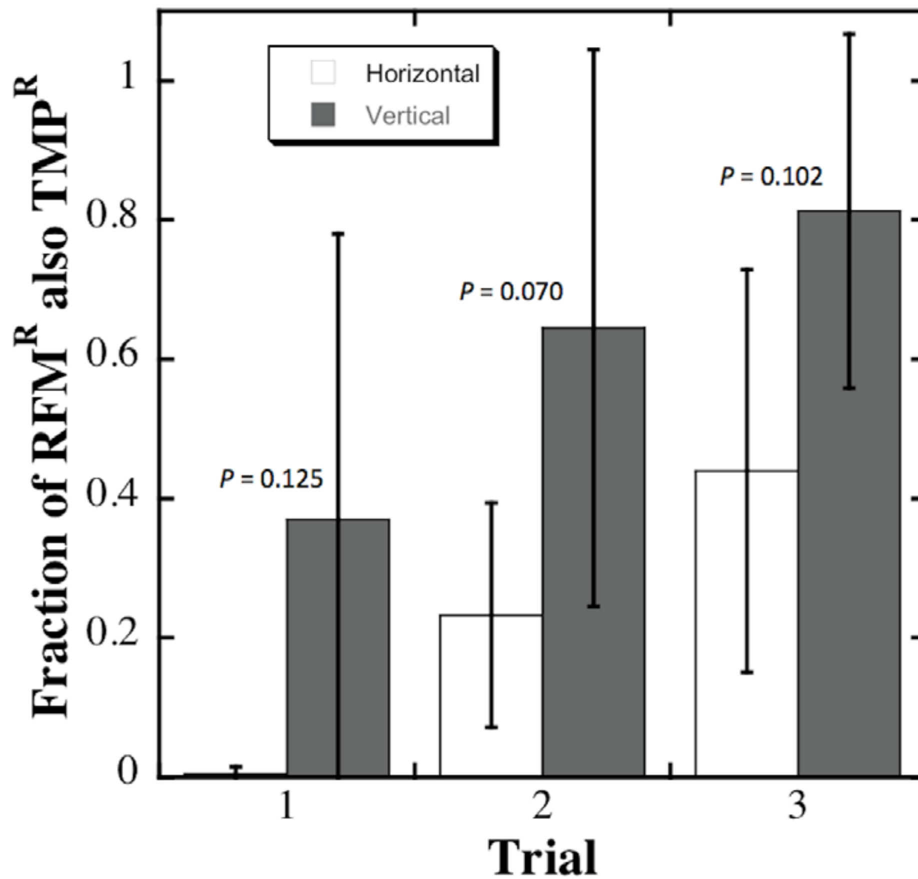

Figure 5. Frequency of simultaneous mutation to RFM^R and TMP^R in *B. subtilis*. Data are averages and standard deviations (n = 4) from 3 independent trials.

Meta-Analysis of the Data

Meta-analysis is often used to increase statistical power for detection of small effects by analyzing and comparing data from multiple trials (Cohn and Becker, 2003). Therefore, meta-analysis was performed on the data collected in the present study. In the case of *B. subtilis*, meta-analysis revealed that all parameters measured (OD, viable counts, frequency of RFM^R mutants, and frequency of RFM^R, TMP^R double mutants), were significantly higher in cells cultivated in simulated microgravity (Figure 6). In contrast, meta-analysis revealed that in *S. epidermidis*, OD, and viable counts were significantly higher for cells grown in simulated microgravity, but that the frequency of mutation to RFM^R was not (Figure 7).

DISCUSSION

Despite being a subject of intense research [reviewed in (Horneck et al., 2010; Klaus and Howard, 2006; Nickerson et al., 2004)], to date no coherent model has emerged adequately describing how microgravity affects bacterial growth and metabolism. This situation results from (i) infrequent opportunities to access spaceflight habitats, and (ii) limitations on the ability to perform sophisticated, well controlled on-board experiments in the spaceflight environment. Although it is impossible to replicate the microgravity environment on the surface of Earth, a number of ground-based systems have been designed to simulate the effects of microgravity (Anken, 2013). Among these, the RWV clinostat system has become

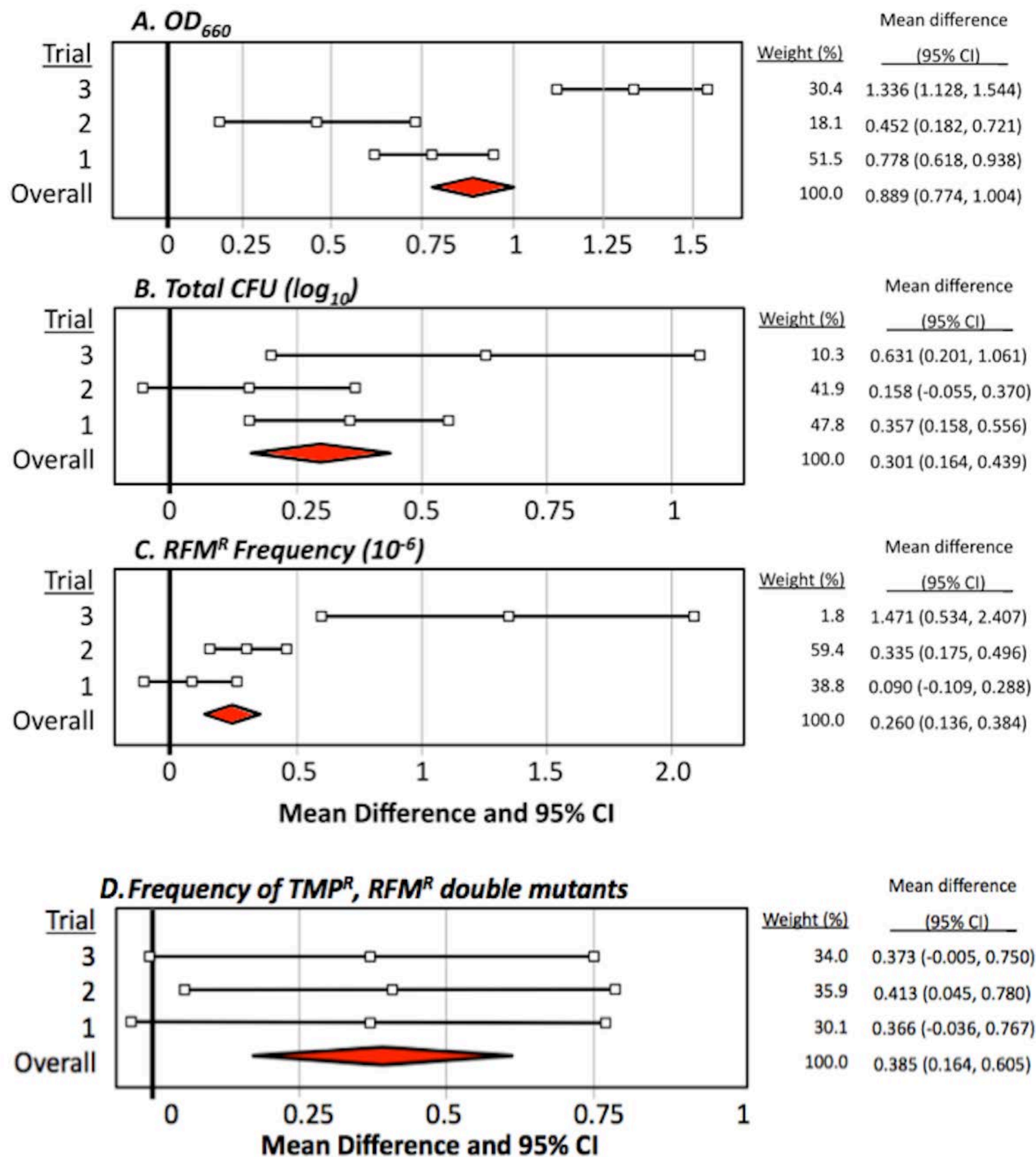

Figure 6. Meta-analysis of *B. subtilis* data for OD (A), Viable counts (B), frequency of mutation to RFM^R (C), and frequency of mutation to RFM^R and TMP^R (D). Relative weights, means, and 95% confidence intervals for each Trial are tabulated to the right and depicted graphically on the left. The red diamonds denote the overall means and 95% confidence intervals for the aggregate data.

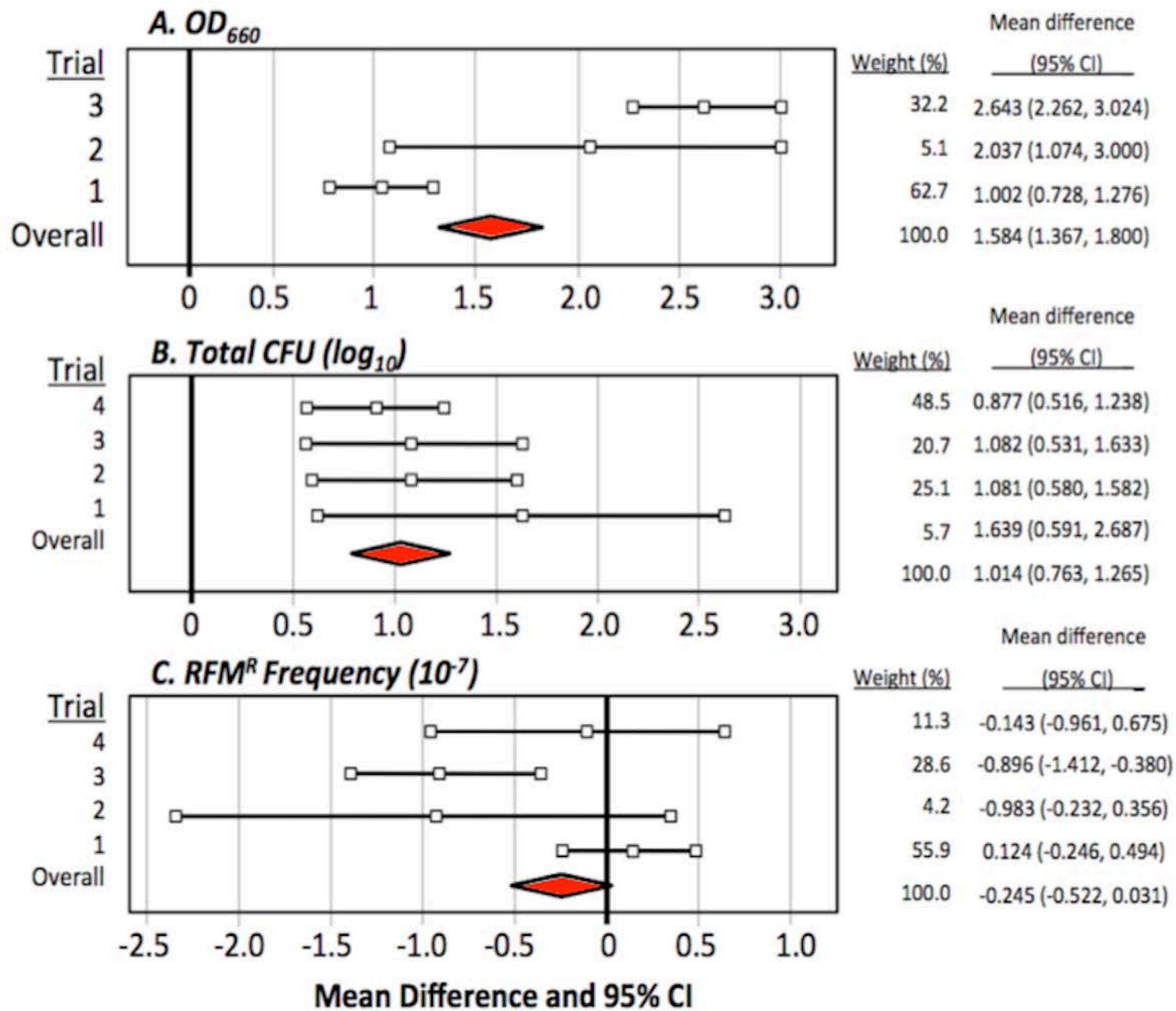

Figure 7. Meta-analysis of *S. epidermidis* data for OD (A), Viable counts (B), and frequency of mutation to RFM^R (C). Relative weights, means, and 95% confidence intervals for each Trial are tabulated to the right and depicted graphically on the left. The red diamonds denote the overall means and 95% confidence intervals for the aggregate data.

widely used as a spaceflight culture analogue (Nickerson et al., 2003). In some cases, alterations in bacterial gene expression and virulence have been found to correlate well between clinostat and actual spaceflight experiments, but not in other cases [reviewed in (Rosenzweig et al., 2014)].

In this communication we report that *B. subtilis* and *S. epidermidis* cultures grew at essentially the same exponential rate and to similar cell densities in simulated microgravity and in 1 *g*, in agreement with recent comparable experiments with clinostat-grown cultures of the Gram-negative bacteria *Enterobacter cloacae, Escherichia coli*, *Citrobacter freudii*, and *Serratia marcsescens* (Soni et al., 2014). It was observed that the number of viable cells declined during the stationary phase in both *B. subtilis* and *S. epidermidis* cultures, but that the rate of decline was markedly slower in simulated microgravity-grown cells of both species. In fact, the

significantly higher OD values and numbers of viable cells in the simulated microgravity-grown cultures were not due to increased exponential growth of cells, but to decreased death of cells in the stationary phase. Because most natural environments are nutrient-limited, microbes in the environment likely spend much of their lives in a stationary phase-like condition (Chubukov and Sauer, 2014; Navarro Llorens et al., 2010). Thus, persistence in the stationary phase is an important mechanism for long-term survival of microorganisms in oligotrophic environments, such as those encountered within the ISS.

It was noted that simulated microgravity-grown *B. subtilis* cells demonstrated a significantly higher frequency of mutation to RFM^R and to RFM^R/TMP^R than did parallel 1 *g*-grown cultures. As a possible reason for this observation, it has been established that both the model bacteria *B. subtilis* and *E. coli* possess well-characterized stationary-phase mutagenesis systems (Gonzalez et al., 2008; Robleto et al., 2012). Furthermore, induction of the stationary-phase mutagenesis system in *E. coli* by starvation has been shown to result in an elevation of clinically-relevant antibiotic resistance mutations (Petrosino et al., 2009). The results from the present experiments suggest that stress resulting from exposure to simulated microgravity in the RWV clinostat may be invoking a similar response in *B. subtilis* cells. In contrast, *S. epidermidis* cells did not exhibit a significant change in the frequency of mutation to RFM^R regardless of orientation in the clinostat. At present, the phenomenon of stationary-phase mutagenesis has not been well studied in *Staphylococcus* spp., but the results presented here suggest that if such a phenomenon exists, it appears to be insensitive to simulated microgravity supplied by clinorotation.

Previous studies of antibiotic resistance in microgravity have focused mainly on transient physiologic changes leading to increased or decreased antibiotic susceptibility (Klaus and Howard, 2006; Lapchine et al., 1986; Tixador et al., 1994; Tixador et al., 1985). In contrast, our results address the emergence of antibiotic resistance in microbes exposed to spaceflight stress resulting from mutations in genes encoding antibiotic targets. Investigation of both aspects is needed for a better understanding of the emergence of antibiotic resistance in the microbiome of the ISS, and the knowledge gained could be applied to similar confined settings on Earth.

The microgravity simulation experiments described here represent preliminary ground studies leading up to the BRIC-18 spaceflight mission to the ISS. Compilation of the ground simulation results reported here with upcoming actual spaceflight data will provide valuable insights into how these two microorganisms respond to the stresses of the integrated spaceflight environment, and possible implications for astronaut health.

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