Paul W. Baker, Michelle L. Meyer and Laura G. Leff*

Escherichia coli growth under modeled reduced gravity

Bacteria exhibit varying responses to modeled reduced gravity that can be simulated by clino-rotation. When Escherichia coli was subjected to different rotation speeds during clino-rotation, significant differences between modeled reduced gravity and normal gravity controls were observed only at higher speeds (30-50 rpm). There was no apparent affect of removing samples on the results obtained. When E. coli was grown in minimal medium (at 40 rpm), cell size was not affected by modeled reduced gravity and there were few differences in cell numbers. However, in higher nutrient conditions (i.e., dilute nutrient broth), total cell numbers were higher and cells were smaller under reduced gravity compared to normal gravity controls. Overall, the responses to modeled reduced gravity varied with nutrient conditions; larger surface to volume ratios may help compensate for the zone of nutrient depletion around the cells under modeled reduced gravity.

Mail address:

*corresponding author. Dept. of Biological Sciences, Kent State University, Kent OH 44242, USA. lleff@kent.edu

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1. Introduction

Several studies have shown that biological systems, including bacteria, can be affected by reduced gravity [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12]. For *E. coli* (ATCC 4157), actual microgravity results in a shortened lag phase, an increase in exponential growth rate and higher final cell count during the stationary phase in comparison to normal gravity controls [4, 5, 6, 8]. This is because in a normal gravity environment the bacteria slowly sediment but in a microgravity environment the bacteria remain in a geostationary position resulting in a zone of reduced nutrients and increased waste products around the cells [4].

In many of the previous studies, experiments were performed under actual microgravity conditions. However, ground-based experiments can be performed using a device called a rotary cell culture system (RCCS; Synthecon) that models reduced gravity. Vessels attached to the RCCS rotate around the horizontal axis at a slow speed so that any object within the vessel will perpetually freefall but as the vessel is continuously rotating, the object spirals around itself and remains in a relatively geostationary position [13]. This system appears to model microgravity affectively because similar results were obtained when *E. coli* was grown under actual microgravity compared to cells grown in the RCCS [5, 6, 8]. Although there are theoretical studies that predict the minimum speed necessary for modeled microgravity conditions [14, 15, 16], no studies have examined the optimal conditions required to model reduced gravity for bacteria.

In this study, a non-motile strain of *E. coli* (ATCC 26) was grown under different nutrient conditions in modeled reduced gravity. Growth was assessed based on cell number, using the Live/Dead *Bac*Light kit, DAPI (4',6-diamidino-2-phenylindole), and fluorescent in situ hybridization (FISH), and cell size. In addition, the affect of speed of clino-rotation and removal of samples from the vessels during an experiment was addressed.

2. Materials and Methods

2.1. Experimental design

Escherichia coli ATCC 26 was grown in 0.2% (w/v) nutrient broth (NB) or minimal medium (M9) for 24 h at 30°C. After growth, cultures were centrifuged at 6000 g for 20 min, resuspended in filtered sterile distilled water and 1 ml was inoculated into each vessel to give a final density of 10^6 cells per ml. A similar method of inoculation was used in a previous study [17]. The standard experimental set-up consisted of three slow turning lateral vessels (STLV – Synthecon, Houston, TX, 240 ml volume, 9.2 cm internal diameter) rotating on a RCCS around the horizontal axis (modeled reduced gravity). Simultaneously, negative controls were established by placing another RCCS so that the three STLV rotated around the vertical axis (normal gravity). Air enters into the vessels at a constant rate and permeates into the medium through a diffusible membrane in the center core of the vessel.

The effect of rotation speed was investigated using a range of speeds from 2 to 50 rpm (the maximum setting of the RCCS). A sample was removed from each STLV after 24 h of growth in 0.2% NB. One portion of each sample was used directly for enumeration of live and dead bacteria. The remaining portion of each sample was preserved (PBS buffer: 8% paraformaldehyde) and stored at 4°C until analysis using DAPI and fluorescent in situ hybridization (FISH). Bacteria were enumerated as described below.

Based on results of the rotation speed experiment, other experiments were conducted under modeled reduced gravity conditions using a rotation speed of 40 rpm (centrifugal-force equivalent to 80.7 cm s^{-2}). In these experiments, *E. coli* was grown in either 0.2% NB or minimal medium (M9) under modeled reduced gravity and compared to normal gravity conditions. Samples were recovered after 24 h and 48 h and processed as above to examine the potential impact of removal on the results. This part of the study was repeated except samples were recovered after 48 h only to examine the potential impact of sample removal. Biovolumes of 200 cells per sample were measured from the samples collected at 48 h.

2.2. Live/Dead staining

Dead and live bacteria were stained using the Live/Dead BacLight™ kit as previously described [18]. Samples were diluted in filtered sterile deionized water and stained for 20 min using the Live/Dead kit (Molecular Probes, Eugene, Oregon, USA). Bacteria were filtered through a 0.2 µm black polycarbonate filter (Osmonics, Inc., Minnetonka, MN, USA), overlaid onto 0.45 µm type HA membrane filter (Millipore, Danvers, MA, USA). The cells that stained red with propidium iodide (PI, dead) and the cells that were stained green with SYTO 9 (live) were enumerated using epifluorescent microscopy.

2.3. DAPI

Total bacterial counts were enumerated using DAPI (4',6-diamidino-2-phenylindole) staining [19]. Samples were filtered through 0.2 µm black polycarbonate filter overlaid onto a 0.45 µm HA membrane filter. The bacteria were stained with 2 ml of DAPI solution (1.5 mg ml^{-1}) for 3 min. The cells were counted via epifluorescent microscopy.

2.4. Fluorescent in situ hybridization (FISH)

Bacteria were enumerated using FISH using the method developed by Lemke *et. al*. [20]. Bacteria were concentrated onto a 0.2 µm Anodisc filter (Whatman, Clifton, N.J., USA) overlaid onto a 0.45 µm HA membrane filter. The Anodisc was washed with 1 ml 0.2 µm filtered sterile deionized water, and then washed with 0.1% (w/v) Nonidet P40. After 40 µl hybridization solution (6x SSC, 0.02 M Tris [pH 7], 0.1% SDS and 0.01% Poly A) containing 5 ng µl-1 Texas red-labeled probe EUB338 [5'-JGCTGCCTCCCGTAGGAGT-3'] [21] (Sigma-Genosys, The Woodlands, TX, USA) was added to each Anodisc, they were incubated for 4 hours at 48°C in the dark. After the incubation, the Anodiscs were washed twice with 400 µl washing buffer (0.9 M NaCl, 0.02 M Tris [pH 7.2] and 0.1% (w/v) SDS). To each, 80 µl washing buffer was added and they were incubated at 48°C for another 10 min and washed twice with 400 µl washing buffer and then twice with 400 µl filtered sterilized deionized water before enumeration via epifluorescent microscopy.

2.5. Biovolumes

DAPI stained cells were viewed using a Zeiss Axioskop fluorescent microscope equipped with a digital image capture system (RT Slider, Diagnostic Instruments, Inc.). The fiber length and fiber breadth of >200 cells were measured using MetaMorph software (Universal Imaging Corp.). During this study, the biovolumes of only individual cells (not clumped) were determined and dividing cells that had not yet separated were excluded from the analysis. Based on these measurements, the biovolume was calculated assuming the bacteria were elliptical using the following equation:

$$\text{Biovolume} = \left[(\text{L-W}) \times \pi \times \left(\frac{\text{W}}{2} \right)^2 \right] + \left[4 \times \pi \times \left(\frac{\text{W}}{2} \right)^3 \right]$$

Where L is length and W is width.

2.6. Statistics

Data were analyzed to using one-way ANOVA using SPSS statistical package for Windows (SPPS Inc., Chicago, USA). Post hoc tests were performed using Tukey's test. Significant differences were defined as cases where $P<0.05$.

3. Results

When *E. coli* was grown under a range of different rotation speeds, there were significantly higher numbers of live cells (stained with SYTO 9) at 30-50 rpm under modeled reduced

gravity in comparison to normal gravity; while dead cells (stained with PI) were only significantly higher at 50 rpm (Figure 1). Based on DAPI staining, total cell counts were significantly higher at rotation speeds between 30-50 rpm under modeled reduced gravity compared to normal gravity (the modeled reduced gravity and the normal gravity controls for each speed were run simultaneously). Differences among rotation speeds were not statistically examined because each speed was performed in a separate experiment, even though reproducibility was quite high.

When *E. coli* was grown in minimal medium (at 40 rpm), there were no significant differences between normal and modeled reduced gravity on day 1 using any of the fluorescent stains (Figure 2a). However, on day 2, the number of live cells

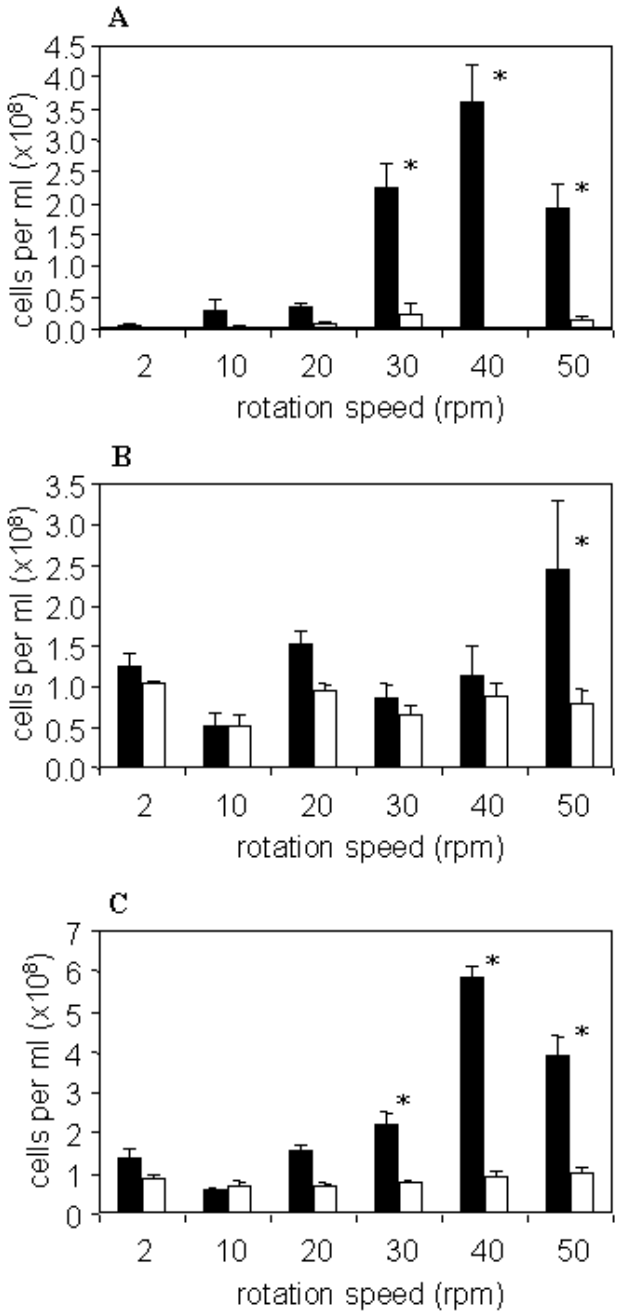

*Fig. 1.: Abundance of E. coli ATCC 26 grown in 0.2% (w/v) nutrient broth at rotation speeds ranging from 2-50 rpm based on staining with SYTO 9 (A, Live), PI (B, Dead) or DAPI (C). The speeds 2, 10, 20, 30, 40 and 50 rpm correspond to centrifugal-forces of 0.2, 5.0, 20.9, 45.4, 80.7 and 126.1 cm s-2, respectively (diameter of STLVs are 9.2 cm). The black bars represent modeled reduced gravity while the white bars represent normal gravity. This * denotes significant differences (P< 0.05) between modeled reduced gravity and normal gravity.*

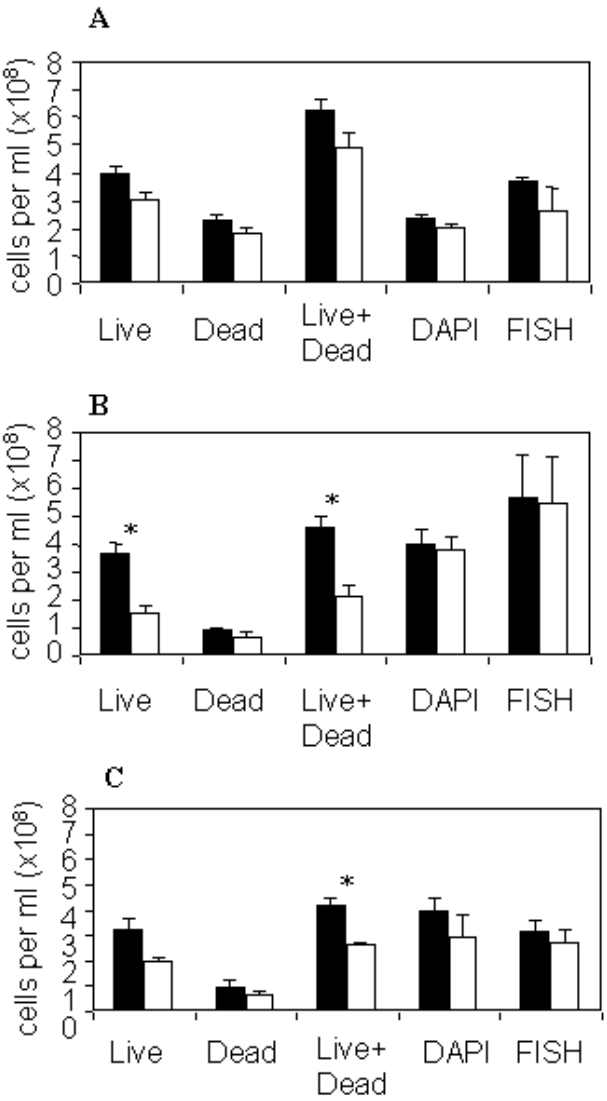

*Fig. 2.: Abundance of E. coli ATCC 26 grown in minimal medium at 40 rpm and first sampled after 24 h (A) and then sampled again after 48 h (B). In the second experiment (C), STLVs were sampled only once after 48 hours. The black bars represent modeled reduced gravity while the white bars represent normal gravity. This * denotes significant difference (P< 0.05) between modeled reduced gravity and normal gravity.*

(stained with SYTO 9) and the live+dead cells (stained with SYTO 9 or PI) were significantly higher under modeled reduced gravity compared to normal gravity (Figure 2b). Likewise, when the STLVs were sampled on day 2 only, the live+dead cells were significantly higher under modeled reduced gravity compared to normal gravity (Figure 2c). Biovolumes of *E. coli* grown in minimal medium and sampled on day 2 revealed no significant differences between modeled reduced gravity and normal gravity (Figure 3).

When *E. coli* was grown in 0.2% nutrient broth (at 40 rpm), counts on day 1 were significantly higher using SYTO 9 and DAPI but not PI and FISH under modeled reduced gravity compared to normal gravity (Figure 4a). On day 2, only DAPI counts were significantly higher under modeled reduced gravity relative to normal gravity (Figure 4b). When the STLVs were sampled on day 2 only, there were no statistically significant differences between modeled reduced gravity and normal gravity using three replicates (data not shown). However, when the experiment was repeated and the data was pooled together (six replicates in total, the numbers of bacteria and trends were similar in both experiments.), the number of live, live+dead and DAPI stained cells were significantly higher under modeled reduced gravity compared to normal gravity (Figure 4c). Bacterial cells grown in 0.2% nutrient broth were significantly smaller during modeled reduced gravity in comparison to normal gravity on day 2 (Figure 3). Bacteria grown in nutrient broth in modeled reduced gravity were usually separate from each other and smaller than under normal gravity. In contrast, under normal gravity many of the cells were longer and in the process of division.

4. Discussion

Clino-rotation is an effective method for modeling microgravity [13] and many previous studies have used a variety of different rotational speeds and specially designed vessels or high aspect rotating vessels [8, 11]. In this study, only at higher rotation speeds were significant differences observed between modeled reduced gravity and normal gravity. This result was surprising because based on theoretical calculations [14] the minimum speed for bacterial cells to begin to experience a centrifugal force is 0.25 rpm. It is assumed that at this point the bacteria are in a relatively geostationary position. One potential cause of the discrepancy between the theoretically and empirically determined rotation speeds is that any excess insoluble carbon dioxide may remain as small bubbles in suspension providing rotation speeds were sufficient to counter buoyancy (note: air was always entering the STLVs through a diffusible membrane, which should maintain aerobic conditions). A previous study suggested that the increase in carbon dioxide could result in the

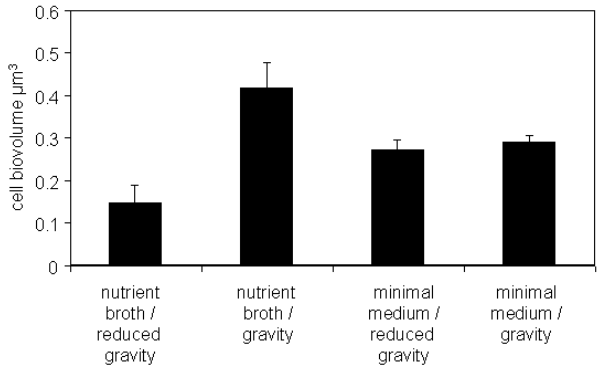

Fig. 3.: Biovolume of E. coli ATCC 26 cells grown in 0.2% (w/v) nutrient broth and minimal medium on day 2 of incubation.

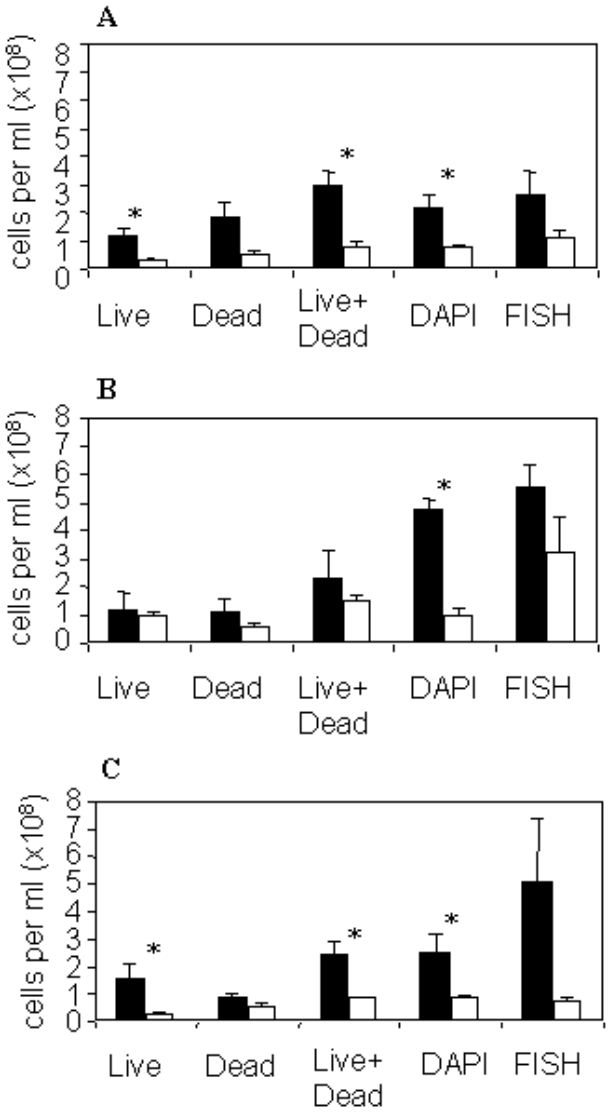

*Fig. 4.: Abundance of E. coli ATCC 26 grown in 0.2% (w/v) nutrient broth at 40 rpm and first sampled after 24 h (A) and then sampled again after 48 h (B). In the second experiment (C), STLVs (six replicates) were sampled only once after 48 hours. The black bars represent modeled reduced gravity while the white bars represent normal gravity. This * denotes significant difference (P< 0.05) between modeled reduced gravity and normal gravity.*

reduction of the lag phase [12]. Another possibility is that gravitational effects on cells of different sizes and shapes are different making it impossible to predict how they will sediment under normal gravity conditions [22].

In minimal medium, total bacterial counts showed no differences between modeled reduced gravity and normal gravity, but numbers of live cells were higher under reduced gravity. The results differed to some degree from previous studies where *E. coli* was grown in minimal medium [3, 4, 5, 6, 7, 8]. In prior studies on a different strain of *E. coli* (that exhibits low motility), total bacterial counts (based on direct counts using a haemocytometer) were significantly higher under actual (not modeled) reduced gravity compared to normal gravity. In our study, total counts based on DAPI staining were not altered by modeled reduced gravity, in contrast to the number of live cells, in minimal medium. However, under high nutrient conditions, cell numbers were higher under reduced gravity as has been reported in studies using other strains of *E. coli* in minimal medium [4, 6]. Differences between this study and earlier work may be attributable to the observation the responses to reduced gravity were greater under actual microgravity conditions compared to clino-rotation [5] and differences in enumeration methods and strains used. Overall, higher numbers of cells under reduced gravity may result from the more uniform distribution of cells compared to normal gravity controls where bacteria are subjected to sedimentation [4].

Few studies have examined the effects of reduced gravity on bacterial cell sizes and only one study, using *Streptomyces hygroscopicus*, showed that biomass was reduced when grown under model reduced gravity compared to normal gravity [23]. In a study on *E. coli*, certain results suggest that cells were larger under reduced gravity but the majority of results obtained revealed no alteration of cell size [2]. This finding is consistent with our study, as we also found that cell size was not affected in minimal medium. However, cell size was reduced, in our study, under higher nutrient conditions. However, caution in measuring biovolume should be noted because of problems in resolution and halo effects [24]. However, this method has been extensively used for indirectly determining the biomass of indigenous bacteria in aquatic environments [24, 25].

The effects of stopping the STLVs and removing samples during an experiment were also examined. The outcome of removing a sample during the course of an experiment includes the introduction of an air bubble, mixing within the STLV, and returning the samples to a normal gravity environment. Previous studies suggest that shear forces may occur if the STLVs are not completely filled, but due to the small size of bacteria, shear forces may play a less significant role in comparison to larger organisms [14, 16]. In both minimal medium and nutrient broth, there was no apparent affect of sample removal.

The total numbers of bacteria based on DAPI staining and based on summing the cells stained with SYTO 9 (live) and PI (dead) were not always consistent with each other. The discrepancy between the results from the Live/Dead *Bac*Light kit and DAPI may be because some of the bacteria growing under normal gravity did not stain with SYTO 9. Prior studies have shown that sometimes the Live/Dead *Bac*Light kit underestimates the number of bacteria compared to total bacteria staining using either DAPI or acridine orange (P.W Baker and L.G. Leff, unpublished). In addition, DAPI sometimes underestimates total bacterial number when cells undergo a starvation/stress response [26]. Other studies have also shown this discrepancy and suggest that bacteria that are more metabolically active may stain more brightly with SYTO 9 making them easier to enumerate [27, 28].

Overall, the effects of modeled reduced gravity on the bacteria examined depended on the nutrient conditions as well as the method of enumeration. Under minimal nutrient conditions, cell size was not affected by modeled reduced gravity and there were few differences in cell numbers. However, in high nutrient conditions, cell numbers and sizes were affected by modeled reduced gravity. The smaller cells found under modeled reduced gravity conditions have a greater surface to volume ratio that may have facilitated their acquisition of resources from the zone of nutrient depletion surrounding the cells. Whereas, under normal gravity conditions sedimentation of the bacteria does not confine them to a particular location

5. Acknowledgements

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6. References

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