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| Tips and Tools

Use of a microgravity analog to explore the effects of simulated microgravity on the development of *Escherichia coli* K12 biofilms

Janelle Hicks,[1] Collin Topolski,[2] Alba A. Chavez,[1] Hugo A. Castillo[1]

AUTHOR AFFILIATIONS See affiliation list on p. 4.

ABSTRACT The rapid development of space technologies and the increase of human presence in space has brought the discussion of the effects of microgravity on cells into the undergraduate classroom. This paper proposes an idea to simulate microgravity on a bacterial culture, suitable for an introductory microbiology laboratory. For this purpose, we show the use of a 2D clinostat designed for microbial studies, along with traditional microbiology techniques such as optical density, plate counts, and biofilm biomass measurement to test the effect of simulated microgravity on the growth of *Escherichia coli* K12. This exercise aims to facilitate further discussions on the effects of microgravity on bacteria growth and communication, as well as the use of technology to simulate space and predict physiological changes in cells.

KEYWORDS clinostat, EagleStat, microgravity, gravity, biofilm

Who has not wondered what it must feel like to experience microgravity, that free-floating condition that astronauts and objects experience when they escape the pull of gravity on Earth and fly into space? This fascination has grown in the last decade with the increasing presence of images or videos of experiments at the International Space Station on television and social media and it has become a topic of interest for research and education laboratories around the globe (1). Granted, experimentation with real microgravity is available almost exclusively for research purposes and so its simulation on different biological models has become a valuable alternative.

To explore the phenomenon of microgravity, analogs (such as 2D clinostats) are well-studied systems that simulate microgravity effects on cells and bacteria (2–4) and are an opportunity for hands-on experimentation in undergraduate microbiology laboratories. The objective of this paper is to facilitate the understanding of the biological effects of microgravity on bacteria by measuring the differential development of biofilms on *Escherichia coli* K12 cultures grown under simulated microgravity conditions for 24 h. For this purpose, we propose a set of experiments that use a 2D clinostat (5) along with widely used techniques to count bacteria and measure biofilm biomass (6, 7). Moreover, this experiment relates undergraduate students in space-related majors to the palpable effects of reduced gravity conditions on microorganisms and supports the development of microbiology skills and scientific analysis by engaging scientific curricula with modern and appealing concepts of space science in traditional microbiology laboratories.

PROCEDURE

Activity overview and timeline requirements

The activities presented in this paper require the intervention of the instructor and students over a period of 3 days, two of which will be dedicated to student work in a

Editor Megan K. Barker, Simon Fraser University, Burnaby, British Columbia, Canada

Address correspondence to Hugo A. Castillo, castilh2@erau.edu.

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typical microbiology lab of 2-h sessions each and 1 day of instructor work for 1 h, for a total of 5 h combined 10 (Fig. 1A).

Equipment and bacterial growth under gravity and simulated microgravity

This experiment uses the EagleStat (5), a 2D clinostat designed for bacterial cultures that runs both treatment (microgravity) and control (gravity) samples simultaneously using regular sterile 2-mL screw-cap plastic tubes as growth vessels (Fig. 2A and B). Laboratories interested in using this instrument for educational purposes can contact the corresponding author to obtain a unit on loan or for instructions to construct the device, previous agreement to intellectual property rights. Alternatively, a commercially available analog known as Rotary Cell Culture System or RCCS (https://synthecon.com/pages/rotary_cell_culture_systems_13.asp) equipped with disposable rotating growth vessels (https://synthecon.com/pages/disposable_vessels_rotating_wall_vessel_synthecon_29.asp) can be used and alternative protocols are provided in the supplementary material.

As shown in Fig. 1A, an overnight culture (OC) is started by inoculating 2 mL of nutrient broth (NB) in a culture tube with an *E. coli* K12 (Carolina Biologicals cat #155067) colony, incubated at 30°C under constant agitation (250 rpm) for at least than 15 h (day 1). On the day of the experiment (day 2), a 1:100 (OC:NB) solution is prepared at a volume enough to fill at least four tubes, enough to measure cell growth (CFU and optical density) and to measure biofilm development from both treatment and control conditions. Make sure that the tubes do not show any visible bubbles when they are

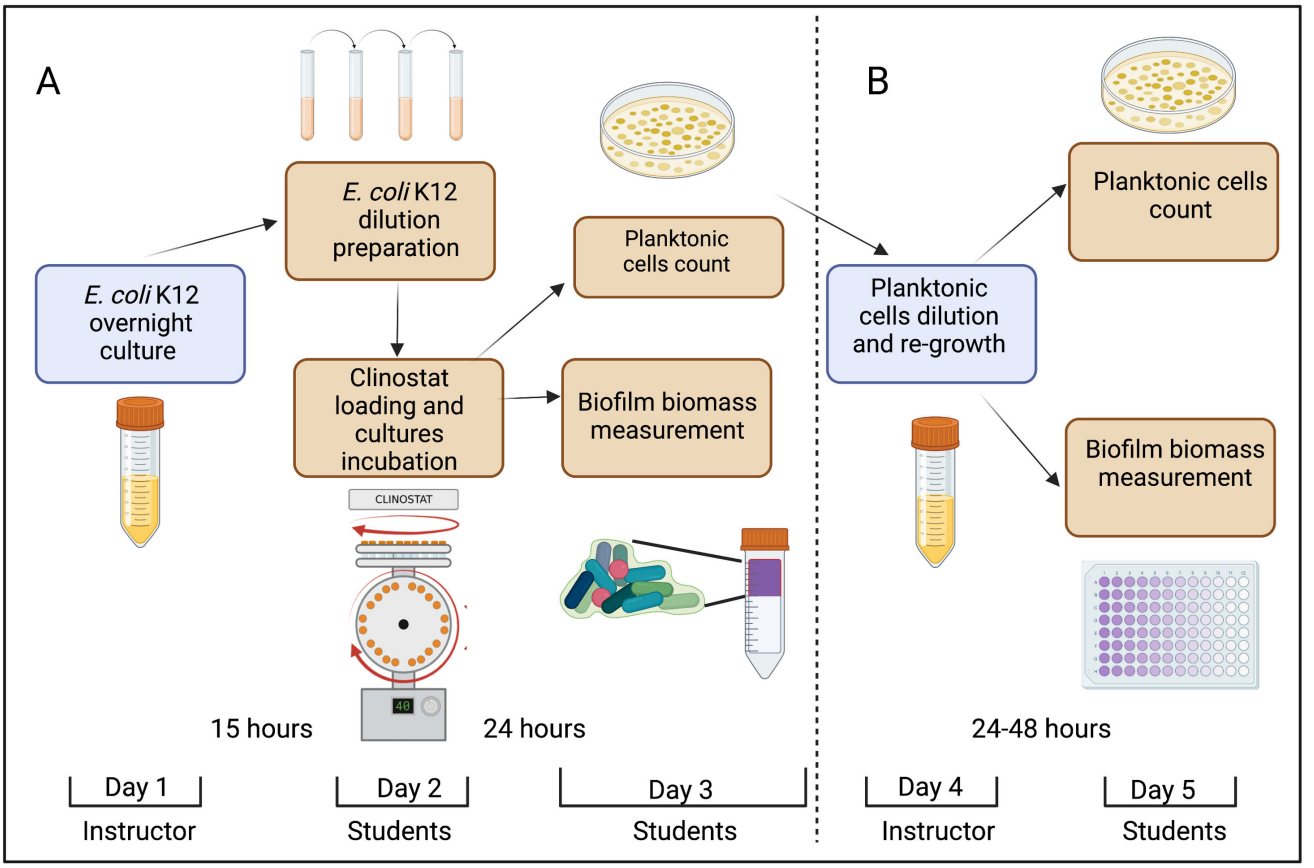

FIG 1 Schematic of the activities required, by students and instructor, to study the effect of simulated microgravity on the growth and biofilm development in *E. coli* K12. This experiment is intended for undergraduate (A) and graduate students (B).

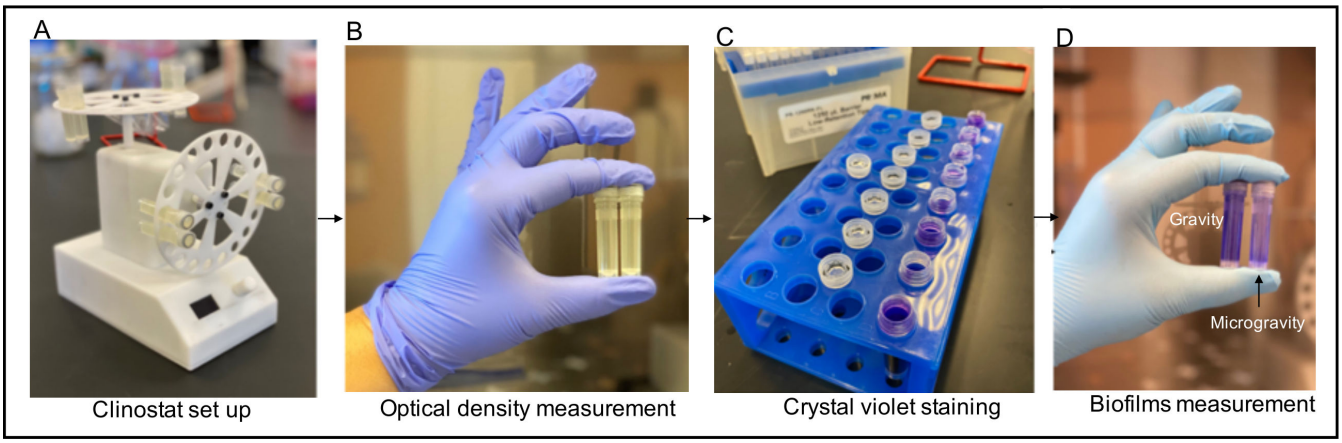

FIG 2 Overview of *Escherichia coli* cultures growth under simulated microgravity showing the placement of growth vessels (tubes) on the EagleStat (A), a side-by-side comparison of cell density in a treatment and control tubes after a 24-h incubation at 30°C (B), the crystal violet staining step to measure biofilm development on the tubes (C), and the differential accumulation of biofilm biomass as a result of the treatment (D).

in horizontal position. The presence of bubbles would disrupt the low-shear conditions required for the simulation of microgravity. Once the tubes are filled, equal numbers are placed on the microgravity and gravity positions on the clinostat set at 8 rpm within a microbiological incubator at 30°C for 24 h.

Measuring the effect of simulated microgravity

The total cell number and the formation of biofilms will measure the effect of simulated microgravity on cell growth. The rationale behind microgravity analogs is that cells growing under simulated microgravity experience a constant state of free-falling; therefore, decreasing their probability to attach to the solid surface of the growth vessel to form biofilms. After incubating the cells for 24 h (day 3), the clinostat is stopped and taken out of the incubator for cell sampling. To estimate the number of bacteria grown under both treatment and control, 100 µL aliquots are taken from half of the tubes on each treatment to start a 10-fold serial dilution up to 10^{-7}. Prior to sampling, the tubes are gently mixed inverting the tubes two times with the purpose of resuspending the cells without disrupting the biofilms. The last four dilutions of the dilution series are then plated on nutrient agar to estimate CFU/mL. Here, we recommend using the drop-plate technique as it is faster and more efficient in the use of media than the traditional spread-plate method (supplemental material). Biofilm biomass is measured on the other half of the tubes using the crystal violet (CV) assay (Fig. 2C and D). In brief, tubes are gently rinsed with 1× phosphate-buffered saline three times to remove planktonic (free-floating) bacteria and the excess liquid by taping the tubes on paper towel. To stain the biofilm cells, crystal violet (0.1% aqueous solution) is added to the tubes, incubated for 30 minutes, washed, and solubilized using 91% ethanol to measure optical density. The higher concentration of CV or absorbance measured is directly proportional to the amount of biomass in the tubes. For the statistical analysis of the differences in total cell number and biofilms between treatments, we recommend a two-sample *t* test using any available statistics software or online calculator.

Materials, intended audience, and future perspectives

The materials needed for the bacteria culture preparation, clinostat setup, colony count, and biofilm measurement procedures are listed in the supplementary materials section. The microbiological media can be prepared from its components or can be purchased ready to use to save instructor time and to make it more suitable for microbiology laboratories with smaller audiences or limited space. This exercise is intended for undergraduate students but can be used for graduate courses by adding a second

procedure that shows the selection of cells with an increased biofilm-forming phenotype by the treatment (Fig. 1B). This study can be introduced into the curriculum for biology majors that want to include a test that highlights the importance of space-related conditions on cell growth and phenotype differentiation.

CONCLUSION

The experiment described relates students to microgravity simulation and to some of its effects on cells. It is relatively inexpensive, and it applies techniques frequently found on basic microbiology syllabi. The biofilm assay proposed here is a clear indication of phenotype changes in bacterial cultures after exposure to an environmental factor as novel as microgravity. It is advised that an aliquot of the cells obtained after incubation are stored frozen as glycerol stocks or preserved chemically for RNA extraction in order to continue with more detailed studies of phenotype changes or differential gene expression for more advanced microbiology courses.

AUTHOR AFFILIATIONS

[1]Department of Human Factors and Behavioral Neurobiology, Embry-Riddle Aeronautical University, Daytona Beach, Florida, USA
[2]Department of Mechanical Engineering, Embry-Riddle Aeronautical University, Daytona Beach, Florida, USA

AUTHOR ORCIDs

Hugo A. Castillo http://orcid.org/0000-0001-5122-0992

AUTHOR CONTRIBUTIONS

Hugo A. Castillo, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental materials (jmbe00062-23-s0001.docx). Protocols for the simulation of microgravity on bacterial cultures.

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