Race to Evolution: A Study of The Role of Progressive Bottlenecks in the Evolutionary Development of Antibiotic Resistance

[redacted] **Final Report (Spring 2019)**

Abstract

Antibiotic resistance is a growing concern in society, as misuse and overuse of in medical and agricultural industries have led to rapid development of antibiotic resistant bacteria. The mechanism for this bacterial resistance have been characterized by Luria and Delbruck to not be induced the presence of selective pressures (like antibiotics), but rather mutations arise spontaneously that subsequently confer a selective advantage to mutants. This phenomena can also be visually observed on antibiotic landscapes by a recent experiment by Michael Baym at Harvard Medical School, however questions still remain on how the progression of increasing antibiotics relative to stark antibiotic bottlenecks affect the growth of antibiotic resistant bacteria. To that end, we extended Baym's experiment and observed the growth of *E. coli* in Petri dishes of step-wise antibiotic concentrations. Our results were inconclusive to whether the progression of increasing antibiotic concentrations supports more rapid bacterial growth into higher concentrated regions.

Introduction

Since the discovery and use of antibiotics against bacterial infection, bacteria have progressively gained resistance to antibiotic treatment (1). The development of resistance against selection pressures can be expected through natural selection and evolution of species, however the rapidity of bacterial resistance has been exacerbated due to human overuse and misuse of antibiotics clinically, agriculturally, and otherwise (2). This issue is especially apparent in the medical field due to the misuse and lack of adherence to prescribed antibiotic medication, in addition to the lack of novel drug development in the pharmaceutical industry (3).

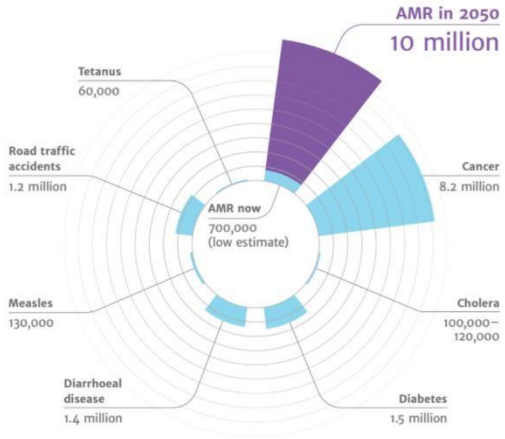

Figure 1. *Radial graph of current sources of death in blue relative to the predicted amount of deaths in 2050 due to antimicrobial resistance (AMR) in purple (5).*

This is such a pressing global concern that in 2014, the UK Prime Minister David Cameron commissioned the "Review on Antimicrobial Resistance (AMR)" to assess the emergence of drug resistance and to propose concrete actions and solutions (4). The review estimated that a total of about 700,000 people already die every year from drug resistant strains of common bacterial infections, HIV, TB and malaria, and that based on the United Nations report on World Population Prospects, the burden of deaths from bacterial resistance could increase to 10 million lives each year by 2050, outpacing the present day deaths from cancer (5) (***Figure 1***). This demonstrates that the morbidity and mortality due to bacterial infection will become a public concern if improved awareness and elucidation of bacterial antibiotic resistance mechanisms in relation to alternative treatment solutions are not explored.

On the topic of mechanisms of antibiotic resistance, a well-known foundational experiment was conducted by Max Delbruck and Salvador Luria in 1934 (6). Luria and Delbruck were curious if genetic mutations are induced in response to selective pressure from the environment or if they arise spontaneously through random mutation that is then selected for by the environment. They proposed that if mutations arose from the former and were induced, then bacterial cultures run in parallel would have roughly the same number of mutants. However if the later were true and mutations arose spontaneously, then there would be a high variability in mutant formation, as either no mutation would arise or a mutation would arise that proliferated in lineage to it's descendants and would have a selective advantage to outcompete and largely outnumber nonmutant bacteria (***Figure 2***). Upon finding high variability in number of mutants, they concluded mutations are not induced by environmental selection, but rather spontaneously arise regardless of selection. The environment can then confer a selective survival advantage to mutants, allowing for increased proliferation of mutant bacteria, but this pressure is not inducing the original mutation.

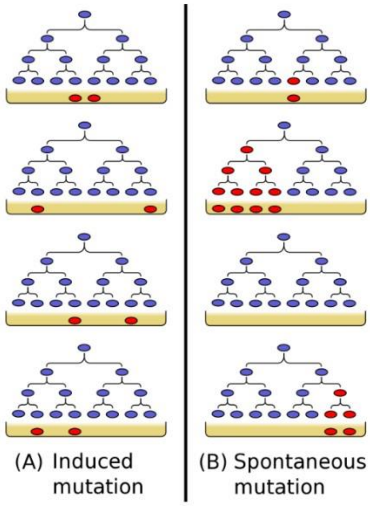

Figure 2. *Depiction of possible outcomes of the Luria-Delbruck experiment. Red are mutants and blue are non-mutant bacteria.*

This discovery is relevant to antibacterial resistance as intuitively it may seem that bacteria become resistant in response to the selective pressure conferred by antibiotics, but in reality random mutations arise independently from antibiotic pressure, and then when then arise, mutant bacteria with antibiotic resistance can survive antibiotic treatment and outcompete and nonmutant bacteria. This phenomenon is eloquently depicted in an experiment in a Science publication by Michael Baym and Roy Kishony at Harvard Medical School (7). In the paper, E. Coli bacteria are introduced into a large petri dish in which there are bands of increasing doses of antibiotics. As the bacteria grow perpendicularly towards these bands, their growth is abruptly stopped at the border of each new band of higher antibiotics, until a single bacteria develops a mutation that confers antibiotic resistance to itself and it's descendants, allowing them to grow into the new band of antibacterial resistance (***Figure 3 A-C***). These increasing bands of antibiotic resistance nicely exemplify Luria's and Delbruck's findings, as well as demonstrate selective advantage that population bottlenecks confer to mutants with antibiotic resistant mutations. However, the study does not address how

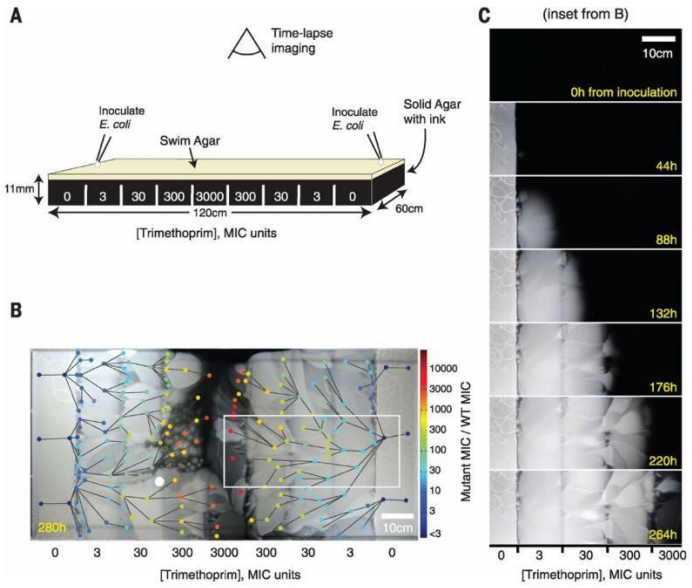

Figure 3. *Figure from Baym, M., et. al. (2016). Nature. Depicts (**A**) agar cross section of base agar and swim agar, (**B**) observed bacterial lineages and (**C**) observed progression of E. Coli growth over time.*

varying the magnitude of the selective bottleneck, or the jump in antibiotic concentration between bands, would affect the "speed of evolution" in bacteria, meaning, how quickly a population can evolve to withstand the highest concentration of antibiotics . While through the work of Luria and Delbruck we know that the magnitudes of environmental selective pressures does

not affect or induce mutations of resistance, we are curious how the population traits conferring greater antibiotic resistance arise under progressive, lower magnitude selection pressures, relative to a single, high magnitude pressure. Do mutations allowing for lower levels of antibiotic resistance enable a population bacteria to more easily develop traits for high levels of antibiotic resistance, or does the population's need to independently develop many more mutations delay this "speed of evolution".

To that end, we propose to evaluate the speed of growth of E. Coli to regions of high antibiotic from a

region without any antibiotic, both through progressive steps in increasing antibiotic concentration, and through a single sudden increase in antibiotic concentration. In this "race to evolution", we hypothesize that that progressively selecting for antibiotic resistant mutants allows for bacteria to more rapidly migrate into high antibiotic concentrated regions in comparison to the migration of bacteria from no antibiotic regions to high antibiotic concentrated regions. The results for this experiment have interesting parallels to bacterial resistance in healthcare, as many experts cite poor medication adherence and prolonged low-dose antibiotics treatments as a contributor to bacteria developing antibiotic resistance (I learned this in class, need to find source).

Methods

Experimental Procedures

To test our alternative hypothesis, we extended Baym's experiment by creating circular petri dishes section into regions of different antibiotic concentrations. Each plate was separated into three sections of varying antibiotic concentration, the top region having no antibiotic, the bottom right having a low concentration of antibiotics, and the bottom left having a high concentration of antibiotics (***Figure 4A***). This allows E.Coli inoculated in the top antibiotic free region to migrate into the bottom left high antibiotic concentrated region in two ways: 1) Clockwise through the bottom right low concentration region and 3) Counterclockwise directly into the bottom left high concentration region. This allowed us observe the speed of migration through progressively increasing antibiotic concentration (i.e. clockwise) and the speed of migration directly into high antibiotic concentrations (i.e. counterclockwise). Our null hypothesis was that migration of bacteria is independent of antibiotic concentration and bottom left high antibiotic concentrated region first as migration would be largely dependent on proximity to plate region.

Plate Preparation

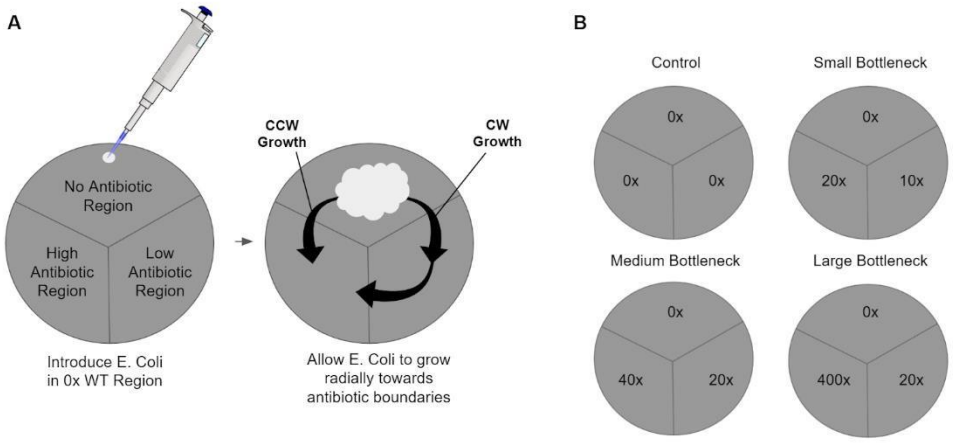

Figure 4. ***(A)*** *Depiction of bacterial inoculations and growth towards the bottom left region of high antibiotic concentrations in the counter-clockwise (CCW) and clockwise (CW) directions.* ***(B)*** *Depiction of plate setup with antibiotic concentrations in each region listed.*

Plates were prepared using similar methods and materials to those used in Baym, M. et. al. (2017) Nature publication (7). Base agar was composed of 2% BD Bacto Agar mixed with 2 percent black ink to allow improved contrast between bacterial growth and the agar. Antibiotic concentrations were introduced into the base agar, with sections divided by autoclaved popsicle stick dividers to keep sections of antibiotics separate. After hardening, a swim agar that was composed of 1% BD Bacto Agar was poured on top. This allowed for a mobile phase for bacterial migration. Antibiotics would vertically diffuse from the base agar into the swim agar (7). Sterile plates were used for all experiments.

Four plates of varying antibiotic concentrations were prepared (***Figure 4B***). Plate 1 was the control with no antibiotics. Plate 2 had small antibiotic concentration steps, with one section being free from antibiotics, one section having 10 times the dosage a wild type E. coli would be able to survive (hereby referred to as 10x), and one section being 20x. Plate 3 had medium antibiotic concentration steps, with one section being 0x, one section being 20x, and one section being 40x. Plate 4 had medium antibiotic concentration steps, with one section being 0x, one section being 20x, and one section being 40x.

Antibiotic Treatment

The antibiotic used ciprofloxacin was used for all experiments. Antibiotic concentrations were determined relative to the concentration that wild-type E. Coli can withstand. Therefore 0x is no antibiotic concentration, and 1x is the minimum inhibitory concentration (MIC) for WT E. Coli growth which is 3*10^3 microgram per milliliter (7). Greater concentrations were scaled from 1x concentrations.

Incubator Set Up

Incubator system was reused from previous experiments in which we observed the radial growth of E. Coli (Module 5). The Arduino code was modified to include both a lower and upper control limit, in which we set the lower temperature limit to be 27 degrees Celsius and the upper limit to be 29 degrees Celsius. The light bulb used to warm the incubator would turn on if the temperature was less than 27 degrees, and would stay and only turn off when the temperature was greater than 29 degrees. This allowed for more consistency in lighting as well as controlled incubator temperatures.

We also used 4 cameras simultaneously. Two computers were used, each connected to two cameras, and a computer video software called SkyStudio Pro was used to capture images from each plate every 30

seconds. By using two computers, we avoided issues with programs crashing. We recorded for 180 hours total with no issues.

Observed Measurements and Video Analysis

After inoculation, plates were stored in the incubator and were observed using video cameras that captured images from each plate every 30 seconds. These images were then combined into time lapse videos for high-level observation. MATLAB was also used to extract normalized grayscale pixel brightness (ranging from 0 to 1) at 27 points in three separate regions per bacterial concentration slice along the plate. This allowed for quantitative image analysis of the bacterial growth on the plates as bacteria grow white along the black agar surface. The three separate regions for each slice also allowed for analysis into the bacterial migration as you could deduce the direction bacteria were growing (***Figure 5***).

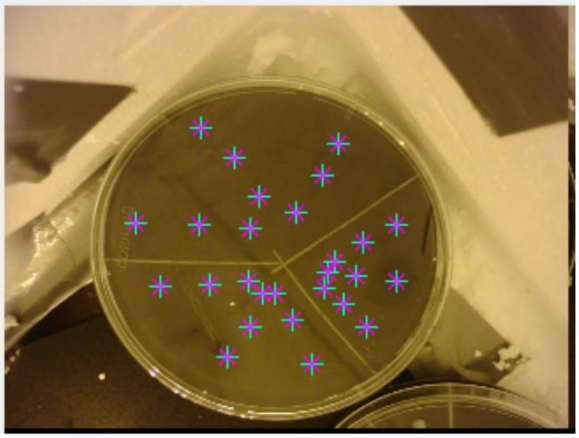

Figure 5. *Image of plate during MATLAB data analysis. Final brightness values for a given region or border were calculated by averaging all data points in each region or border along selected radial coordinates.*

Results

In the two cases where bacterial growth penetrated into the region with the highest concentration of antibiotic, it did so growing from the region without any antibiotic (***Figure 6 & 7***). This is exceptionally true in the case of the plate with a 10x and a 20x region, where the bacteria grew into the 20x region before it did the 10x. This can be observed in ***Figure 8*** where a clear sharp increase in brightness in the yellow curve

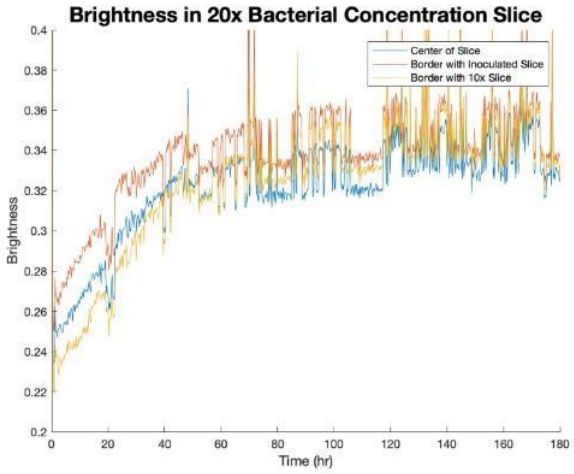

Figure 6. *Graph of bacterial growth measured by brightness in different regions of the 20x slice of the 0x/10x/20x Plate. Outlying spikes can be attributed to the incubator light being on for a given picture. Immediate growth is observed at the 0x/20x border (red plot).*

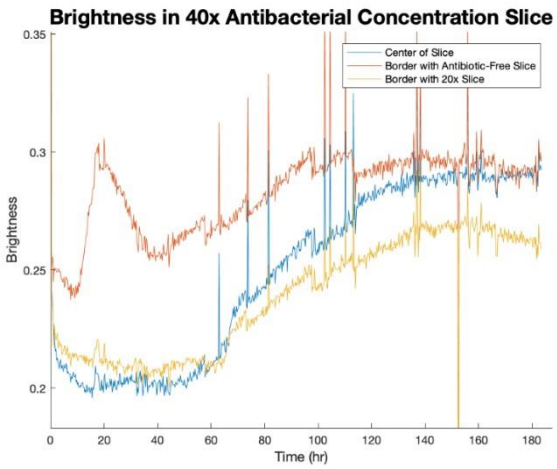

Figure 7. *Graph of bacterial growth measured by brightness in different regions of the 40x slice of the 0x/20x/40x Plate. Outlying spikes can be attributed to the incubator light being on for a given picture. Immediate growth is observed at the 0x/40x border (red plot).*

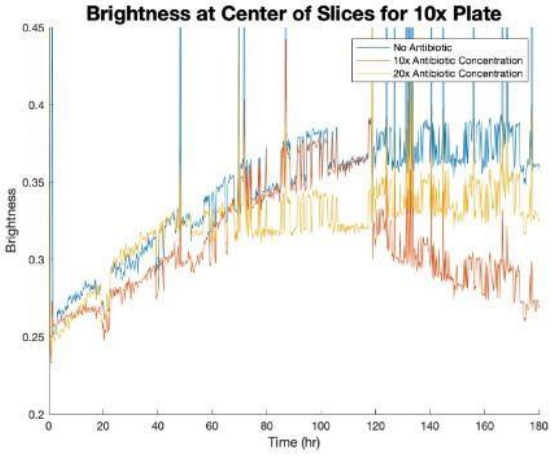

Figure 8. *Graph of bacterial growth measured by brightness in the 0x, 10x, and 20x regions of the 0x/10x/20x Plate. Outlying spikes can be attributed to the incubator light being on for a given picture. Also note that at t = 118 hours, the plate was moved, reflected by the rapid decrease in brightness of the 10x plot. Bacterial growth was observed early in the 20x region, suggesting growth into the 20 x region before the 10 x region.*

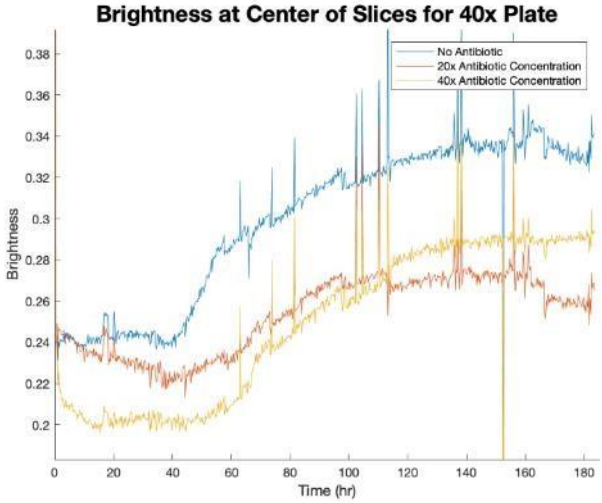

Figure 9. *Graph of bacterial growth measured by brightness in the 0x, 20x, and 40x regions of the 0x/20x/40x Plate. Outlying spikes can be attributed to the incubator light being on for a given picture. Bacterial growth was observed early in the 0x region, then spread to the 20x region and finally the 40x region.*

denoting the bacterial growth in the 20x region coincides with the increase in the inoculated blue curve, but not in the yellow curve. This can also be observed in the timelapse provided in the supplemental. This can be contrasted to the plates with 20x and 40x concentration slices, where the bacterial growth began in the 20x region before the 40x (***Figure 9***). Bacterial growth however was not able to penetrate into the 400x region in the one sample which included such a high concentration, inhibiting out ability to see the bacterial behavior when encountered with an exceptionally high increase in antibiotic concentration when provided with a smaller "stepping stone" evolutionary step. This is shown in **Figure *10***, where the yellow curve denoting the brightness in the 400x slice stays constant the entire time. The control sample was unable to grow throughout the entire plate, likely due to effects outside the scope of the experiment. All plates have timelapses provided in the supplemental (***Supplemental Videos 1-4***).

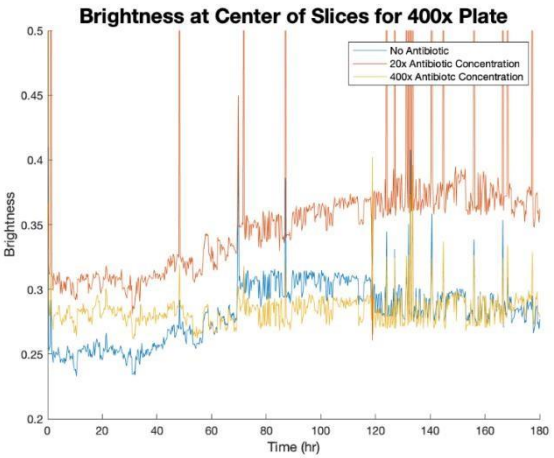

Figure 10*. Graph of bacterial growth measured by brightness in the 0x, 20x, and 400x regions of the 0x/20x/400x Plate. Outlying spikes can be attributed to the incubator light being on for a given picture. Bacterial growth was observed in the 0x and 20x regions but not the 400x region.*

Conclusion and Discussion

From these results, we fail demonstrate if progressively selecting for antibiotic resistant mutants improves migration rate into high antibiotic concentrated regions in comparison to the migration of bacteria from no antibiotic regions to high antibiotic concentrated regions. This is because in the 0x/10x/20x plate, the E. coli migrated to the higher 20x concentrated region before the 10x. In our two other plates (0x/20x/40x and 0x/20x/400x) the E. Coli migrated to the lower concentrated areas first. These results hint that there may be an antibiotic concentration "threshold" in which our alternative hypothesis can be confirmed. This would be supported by our lower concentrated plates having not following quicker direct migration from the 0x region to the 20x region compared to the progressive growth observed in higher concentrated plates. However this can not be definitively concluded e without further experiments and more standardized methods to reduce possible experimental error. One source of error that could have confounded our results was our inexpertise with bacterial culturing. This inexperience may have resulted in contamination or errors in agarose preparation and antibiotic application. Furthermore, the creation of both a base and swim agar may have allowed for antibiotic horizontal diffusion that would have made our defined antibiotic regions not entirely accurate. In addition, as Luria-Delbruck proved, in an experiment on the factors which lead to evolution many separate trials need to be taken, and our experiment was only able to twice get conclusive data. Regardless of this, our experiment was great exposure to bacterial culturing techniques, as well as demonstrated the overall ability of E. Coli to mutate and grow in the presence of antibiotics. Upon simplification of the agarose layering, this would be an interesting experiment for future POLS groups to undertake, as it involves a variety of lab techniques used in previous

modules in addition to introducing students to concepts of antibiotic resistance. Perhaps it could even be a DIY project for other groups to help increase both the awareness of bacterial resistance and it's potential to drastically influence the future of healthcare.

Sources

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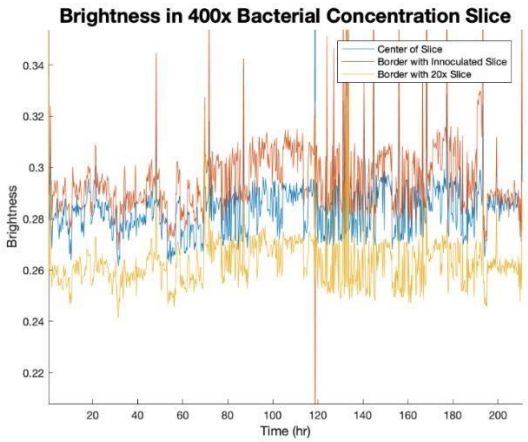
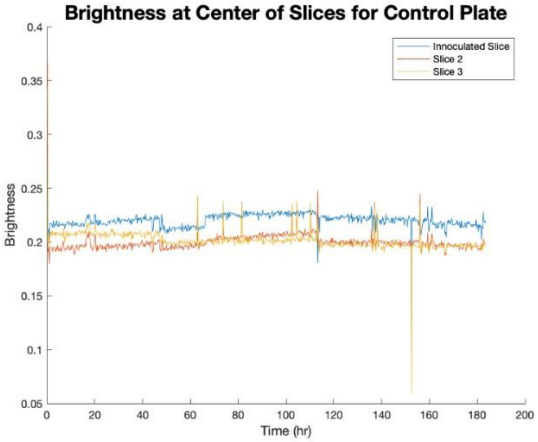
Supplemental Videos and Figures

Supplemental Videos 1-4. Time-lapse videos from each plate over a period of 200 hours. Videos are located in the DropBox folder containing this report.



Supplemental Figure 1*. Graph of bacterial growth measured by brightness in different regions of the 400x slice of the 0x/20x/400x Plate. Outlying spikes can be attributed to the incubator light being on for a given picture. Bacteria was not observed to grow into the 400x region.*



Supplemental Figure 2*. Graph of bacterial growth measured by brightness in different regions of the control plate. Bacteria was observed to grow along the edges of the plate but not in regions observe above.*

Supplemental Videos Information

Supplemental Video 1. Top slice: 20x, bottom right slice: 0x, bottom left slice: 10x.

Supplemental Video 2. Top slice: 40x, bottom right slice: 20x, bottom left slice: 0x *Supplemental*

Video 3. Top slice: 400x, bottom right slice: 20x, bottom left slice: 0x

Supplemental Video 4. Control Video, no antibiotics.