Microscopic Visualization of Indole Signaling in *Escherichia coli* Cells

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Introduction:

Bacteria populations under antibiotic stress possess a variety of mechanisms to overcome and survive the toxic effects of antibiotic treatments. One such mechanism involves the production of bacterial persister cells, which are not antibiotic-resistant but antibiotic-tolerant instead. While antibiotic-resistant bacteria may live and reproduce during the course of antibiotic treatments, persister cells shut down cellular functions and remain dormant during antibiotic stress instead. This mechanism allows persister cells to survive antibiotic treatments and results in chronic or recurrent infections in their hosts including humans, making them a growing concern in the field of medicine [15]. Persister formation has been found to occur in antibiotic-susceptible bacteria populations predominantly during the transition between the growth phase and stationary phase of overall bacterial community growth. While several research studies have been conducted to identify key genes, proteins and stress responses associated with the development of persister cells, complete knowledge of the mechanism responsible for the formation of persister cells has yet to be obtained [9, 15].

It is well known and understood among the scientific community, however, that many bacterial species are able exist together as dynamic communities. They have achieved this ability to survive in adverse environmental conditions such as nutrient deficiencies, competition for resources and antibiotic stress through the development of intercellular communication strategies. One of these strategies, in which bacteria secrete and transmit small diffusible signal molecules, is known as intracellular signaling. Signal molecules allow bacteria to sense environmental conditions and to communicate with other bacterial cells in order to coordinate and execute multicellular responses to the environment. One such signal molecule is indole [10]. Indole is known to be produced by over eighty-five bacterial species and can perform diverse

signaling roles in different bacterial strains. These roles include the modulation of biofilm formation, virulence, stress responses and manipulation of gene expression and membrane potentials [5]. Additionally, multiple non-indole producing species have been observed to develop mechanisms to utilize indole. Due to the versatility of actions that it can perform, indole is gaining widespread attention in the scientific community.

Escherichia coli is one of the many species that produces and utilizes indole for intracellular communication. In *E. coli*, the enzyme tryptophanase hydrolyzes the amino acid tryptophan into pyruvate, ammonia, and indole in a catabolic reaction pathway illustrated below in Figure 1 [10, 13]. Tryptophanase is encoded by the *tnaA* gene located in the *tnaCAB* operon which is induced by high concentrations of tryptophan. Extracellular tryptophan enters *E. coli* cells through the *tnaB* transporter. Induction of tryptophanase also helps to reduce high tryptophan levels via amino acid metabolism [13].

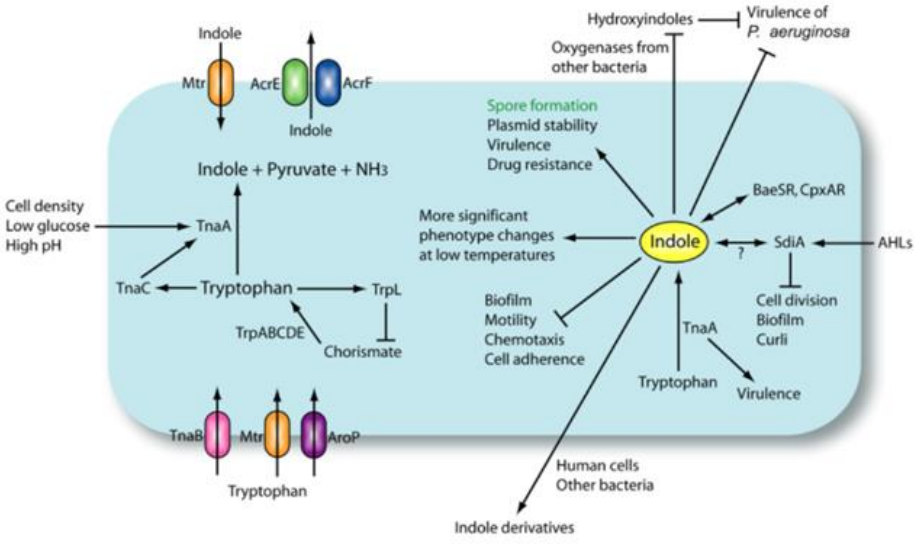


Figure 1. A diagram of the production of indole and its effects. On the left, the degradation of tryptophan into indole via the enzyme tryptophanase is shown. On the right, the various mechanisms that indole has been observed to play a role in are shown. In *E. coli*, indole plays an important role in the survival of bacteria cultures under antibiotic treatment [10].

In *E. coli* strains, the metabolite indole serves as a chemical messenger between isolates in a population of cells during antibiotic stress. Indole helps *E. coli* bacteria overcome antibiotic stress by turning on drug efflux pumps and oxidative-stress protective mechanisms [9]. Indole also serves as a proton ionophore and inhibits *E. coli* cell division. As an ionophore, it reduces the electrochemical potential across the cytoplasmic membrane of bacterial cells and deactivates the Min site selection system. This action prevents the localization of the tubulin homologue FtsZ and the formation of the divisome complex, effectively preventing cell division [2]. These mechanisms performed by indole allow E. coli cells to become more dormant under antibiotic stress in order to survive and proliferate again after antibiotic treatment has ceased. Interestingly, research studies have shown that in a culture of *E. coli*, most of the individual bacterial cells themselves are less resistant to antibiotic stress than the overall population in the culture. The few isolates that are highly resistant to antibiotic stress have been observed to optimize the survival of the other less resistant cells by producing and exuding indole to them at a fitness cost [9]. This altruistic mechanism ensures the survival of the overall population as a whole. This is a subject of long term interest in the Wilson lab and the focus of my thesis.

Experimental Background and Methods:

In previous research conducted in the Wilson lab, *Escherichia coli* was used as a model organism to study the mechanisms of persister cell formation and survival [3, 4]. Such mechanisms have been a long-term interest of the lab. In past experiments, it was found that tryptophanase was linked to the formation of persister physiology in this bacteria. Mass spectrometry of true persister cells in *E. coli* had revealed that tryptophanse was far more abundant (by 2,000 fold) in these cells compared to other cells that had survived antibiotic stress *in vitro*. This suggested that over-expression of this protein may be an exclusive feature of true

persister cells. Fluorescent microscopy of lysed *E. coli* cells stained with FM 5-95 (a membrane-staining dye) revealed that cells with over-expression of tryptophanase survived prolonged, lethal antibiotic treatment, some of more than ten hours duration. The use of flow-cytometry methods had demonstrated that tryptophanase seemed to play an essential role in persister physiology since 91% of cells collected after antibiotic treatment were shown to contain tryptophanase [7]. These discoveries pointed to a correlation between tryptophanase and antibiotic tolerance in *E. coli*, leading the lab to hypothesize that tryptophanase and its production of indole play an pivotal role in the regulation and survival of persister cells and that further study of the precise mechanisms involving tryptophanase and indole signaling might contribute to effective drug treatment against persister cells.

Since *E. coli* populations have been observed to have special interactions between isolates with different degrees of resistance to antibiotic treatments, an experiment was proposed in which microscopy would be used to visualize *E. coli* isolates and their interactions with each other under antibiotic treatment "in real time." The basic design of this experiment is as follows: A sample of live *E. coli* cells from an overnight culture is treated with an antibiotic that would cause antibiotic stress and potentially lysis of the cells, and immediately transferred to a microscope slide so that the behaviors of individual isolates and effects of the antibiotic treatment could be observed under microscopy. This design took into consideration several experimental factors and conditions, such as the types of *E. coli* strains used, the concentration of the antibiotic used, and the conditions in which the cells would be observed under the microscope. No previous method has been created previously in the Wilson Lab for the visualization of live bacterial cells (as opposed to lysed cells) undergoing antibiotic treatment. Therefore, this is a novel experiment in the Wilson lab.

Strains:

Initially, three strains of *E. coli* were selected for this experiment and are listed below in Table 1. *E. coli* strains MG 1655, Gl40, and GL607 were all obtained from Dr. Kevin D. Young of the University of Arkansas for Medical Sciences.

Strain	Features	Reference
MG1655	Wild-type *E. coli*, Sex: F-, Chromosomal Markers: λ^{-1}, ilvG^{-}, *rfb*-5, *rph*-1	[12]
GL40	*E. coli* containing a Super-Folding Green Fluorescent Protein (SF-GFP) that is fused to TnaA tryptophanase Parent Strain: MG1655	[12]
GL607 (*ΔtnaA)*	A strain of *E. coli* that involves deletion of the genes *tnaA* and *tnaB* in order to remove tryptophanase	[11]

Table 1. Strains used in the experiment and their features

These three strains were selected for this experiment due to special features that they either naturally process or were genetically modified to possess. The strain MG1655, a wild-type *E. coli* strain containing tryptophanase and therefore able to produce indole [12], was therefore was selected to serve as an experimental control. The GL40 strain contained a Super-Folding Green Fluorescent protein (SF-GFP), which is fused to tryptophanase hereby tagging it for visualization under fluorescent microscopy [12]. Since tryptophanase often localizes at the poles of *E. coli* cells, we hypothesized that, under fluorescence microscopy, we would see fluorescent green regions at the poles of bacterial cells. Unfortunately, an issue transpired in which the mercury bulb needed for fluorescent microscopy burned out. Therefore, the GL40 strain was omitted from the experiment. Finally, the GL607 strain (referred to in the lab as the *ΔtnaA* strain) was a strain that was constructed by the replacement of the *tanA* and *tnaB* genes with a *kan* cassette. In effect, this strain lacked tryptophanase and therefore the ability to produce indole [11]. We hypothesized that, under antibiotic treatment, this strain would potentially lyse faster than the other two strains due to the lack of indole signaling activity.

Growth Conditions of *E. coli* Strains

Materials:

- LB (Luria Bertani) broth powder (Fisher BioReagents, Cat #BP1426-2)
- LB agar powder (Fisher BioReagents Cat ##BP1423-500)
- Sterile water

Instruments:

- Autoclave (ADV-PB, 250°F heat, 15 psi pressure, liquid cycle = 30 minutes)
- Incubator (New Brunswick Scientific, US patent #3002895)
- Spectrophotometer (HP, diode array, 845A)

Method:

Tubes containing five milliliters of LB broth each were sterilized via autoclaving and used as a growth medium for overnight *E. coli* cultures. For the experiment, three 5 ml LB tubes were used. One tube was aseptically inoculated with MG1655 and one tube was aseptically inoculated with GL607. The third tube contained only sterile LB broth and was used as a control to ensure that the tubes were inoculated aseptically. It also served as a blank for optical density readings. After inoculation, the strains were allowed to grow at 37°C for twenty-four hours with shaking (250 rpm) for twenty-four hours until the cultures reached an OD_{600} of approximately 2.0 (1.943 for the GL607 strain and 1.881 for the MG1655) strain. These cultures were then used for visualization of cell isolates via microscopy.

Preparation, Mounting, and Visualization of Microscopy Slides:

Materials:

- 1% sterile LB-agarose solution
- Clean glass slides (75mm x 25 mm, Fisher Scientific, Cat #12-544-4)
- Clean coverslips (25mm x 25 mm, Fisher Scientific, Cat #12-540C)
- 25 μg/μl ampicillin stock solution (in sterile water)
- 250 μg/μl ampicillin stock solution (in sterile water)
- 1000 μg/μl ampicillin stock solution (in sterile water)
- Scotch tape

Instruments:

- Digital Heatblock (VWR Dry Block Heater)
- Vortex-Mixer (Fisher Scientific)
- Epi-fluorescence microscope (XYL-146Y, Amscope)
- Digital camera (MU 1000-CK, Amscope)

Method:

The antibiotic ampicillin was selected for used in the experiment. A stock solution of 1% sterile LB-agarose solution was prepared and stored at 4°C. A uniform mixture of LB-agarose and ampicillin was utilized to ensure that cells would be exposed to a consistent concentration of ampicillin on the microscope slides. The stock of LB-agarose was microwaved for approximately fifteen seconds until liquefied. Fifteen microliters of molten LB-agarose was pipetted into a 1.5 ml Eppendorf tube and transferred to a heat block at 47.5 °C. The molten LB-

agarose was allowed to cool down to this temperature to ensure that the heat-sensitive ampicillin would not degrade. After the LB-agarose has adjusted to the temperature of the heat block, 2.1 µl of ampicillin stock was added to the LB-agarose solution and vortex-mixed in order to create an uniform solution. The mixture of LB-agarose and ampicillin was then stored in the heat block at 47.5°C so that the mixture remained in liquid form. For the experiment, three different stock concentrations of ampicillin were used for two trials each (one for each strain). A reference table is provided below.

Stock Concentration of Ampicillin	Amt. of Stock Ampicillin	Amt. of LB-Agarose Solution	Amt. of Cell Sample Added to Pad	Final Concentration of Ampicillin
25 µg/µl	2.1 µl	15 µl	4 µl	2.5 µg/µl
250 µg/µl	2.1 µl	15 µl	4 µl	25 µg/µl
1000 µg/µl	2.1 µl	15 µl	4 µl	100 µg/µl

Table 2. The concentrations of stock solutions of ampicillin, the amounts of stock ampicillin and LB-agarose that were mixed together to produce pads, and the amount of cells that were added to the pads in order to create the final concentrations of ampicillin. Each stock concentration were used twice; one for each strain.

For the preparation of each LB-agarose/ampicillin pad, a layer of scotch tape covered the ends of a clean glass microscope slide. Then 17.1 microliters (2.1 µl of stock ampicillin and 15 µl of LB-agarose) of the LB-agarose/ampicillin mixture was pipetted onto the middle of the glass slide, and a second glass slide was immediately placed on the top of it. The slides were then pressed together in order to trap the LB-agarose/ampicillin solution and create a thin "pad." They were then transferred to a refrigerator at 4°C for three minutes in order to allow the pad to solidify. After three minutes, the slides were taken out. The top slide was carefully separated from the bottom slide by "sliding" the slides apart rather than pulling the two slides apart. The result was a glass slide with a thin pad of solidified agarose. The slide was then allowed to sit and dry in order to ensure further solidification of the pad. Next, the wet mounting of cells took

place. Four microliters of *E. coli* cell culture were then pipetted on top of the pad on the slide and gently (but firmly) covered with a cover slip. The tapes were then removed from the slide.

The slide was then transferred to the microscope stage and visualized with a magnification of 100X. A digital camera attached to the microscope was programmed to take time-lapse pictures of the cells every five seconds for at ten to twenty minutes per slide.

Results:

Experimental Conditions:

Initially, fluorescence microscopy was planned to be utilized in order to visualize the presence of SF-GFP tagged tryptophanase in *E. coli* cells from the GL40 strain. However, the mercury bulb and related fluorescence microscopy equipment were not in working order, so the GL40 strain and fluorescence microscopy were omitted from the experiment. Cultures of GL607 and MG1655 were grown overnight until they reached an OD_{600} of 1.943 and 1.881 respectively. A total of six slides were used for the experiment; three slides each contained four microliters of GL607 cells, and the other three slides each contained four microliters of MG1655 cells. Three different doses of ampicillin were used: 2.5 μg/μl, 25 μg/μl, and 100 μg/μl. Each of the three doses were administered twice via the LB-agarose pads, once to a MG1655 cell sample and once to a GL607 cell sample. After the microscope slides were mounted with cells, each slide was observed under a magnification of 100X for at least ten minutes. Pictures of isolate interactions are provided below for each slide.

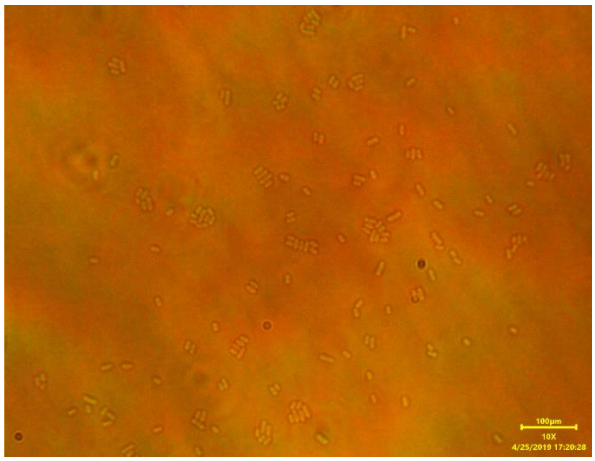
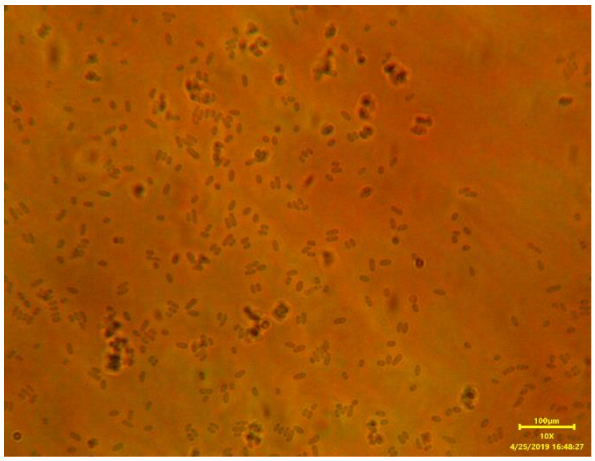
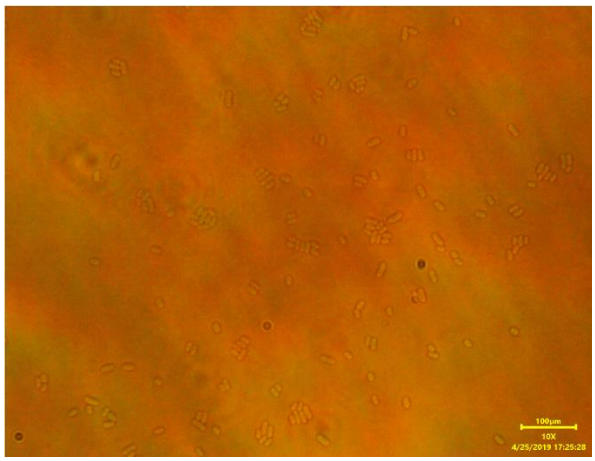
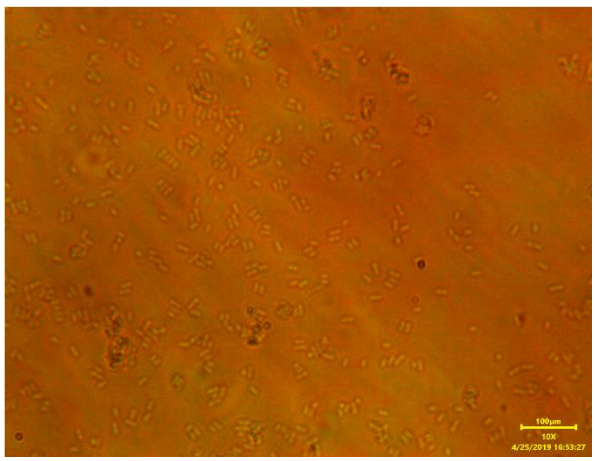
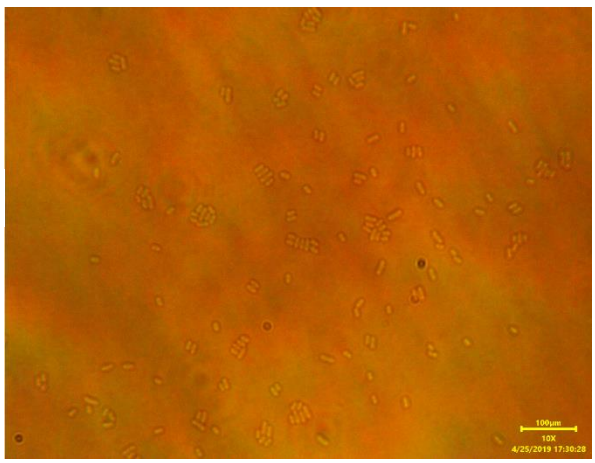
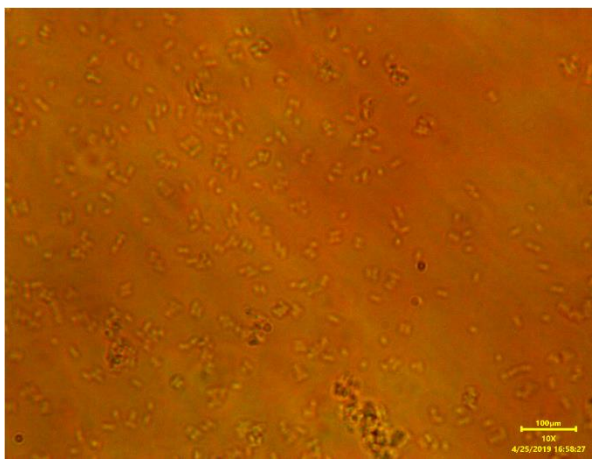
Time Elapsed:	GL607 Cells	MG1655 Cells
Zero Minutes		
Five Minutes		
Ten Minutes		

Figure 2: Visualization of GL607 cells and MG1655 cells exposed to 2.5 µg/µl of Ampicillin under microscope.

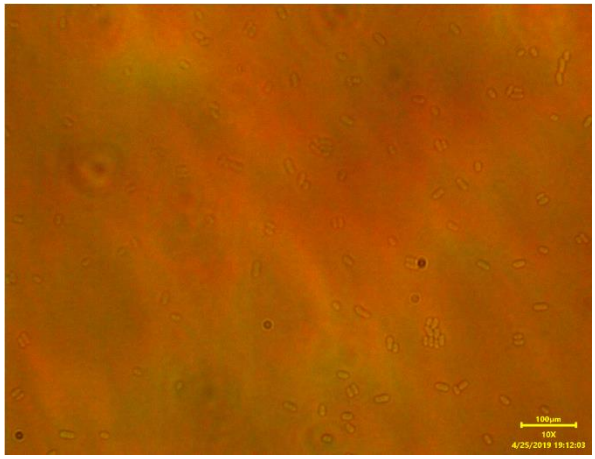
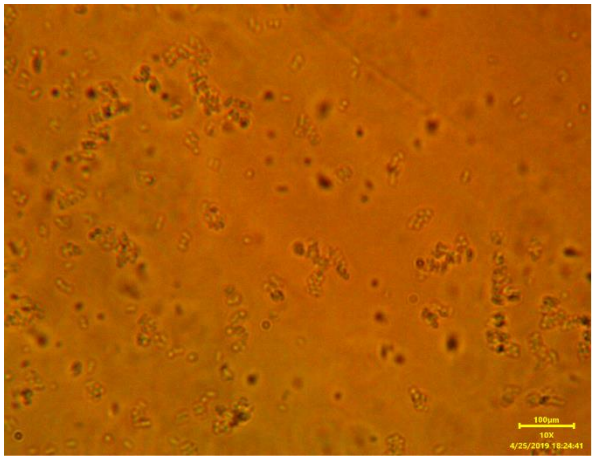
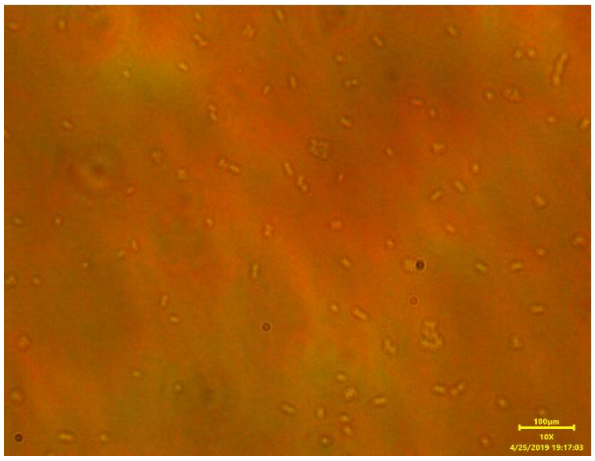
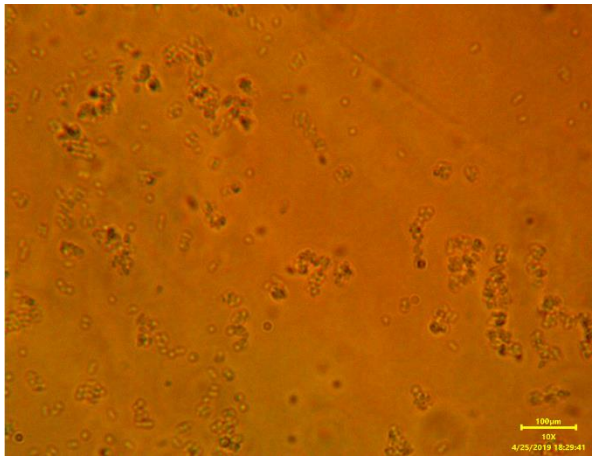
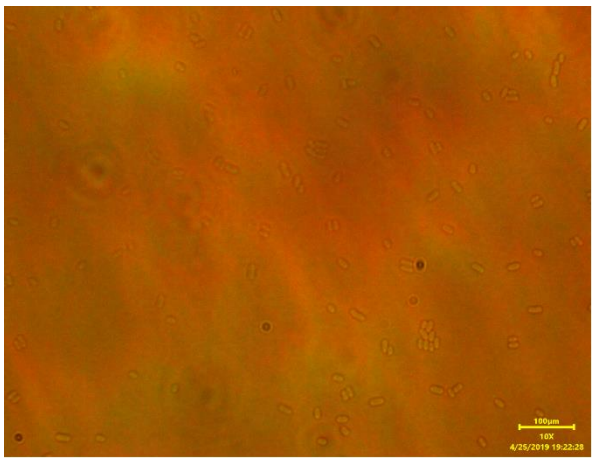
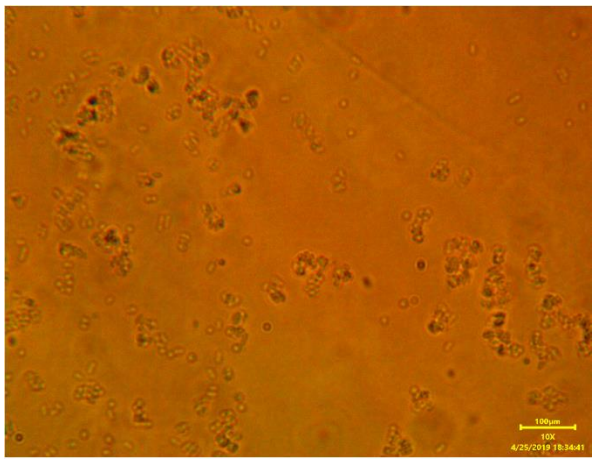
Time Elapsed:	GL607 Cells	MG1655 Cells
Zero Minutes		
Five Minutes		
Ten Minutes		

Figure 3: Visualization of GL607 cells and MG1655 cells exposed to 25 µg/µl of Ampicillin under microscope

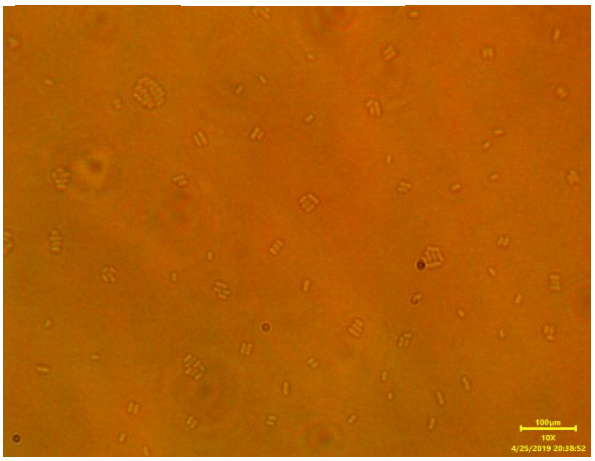
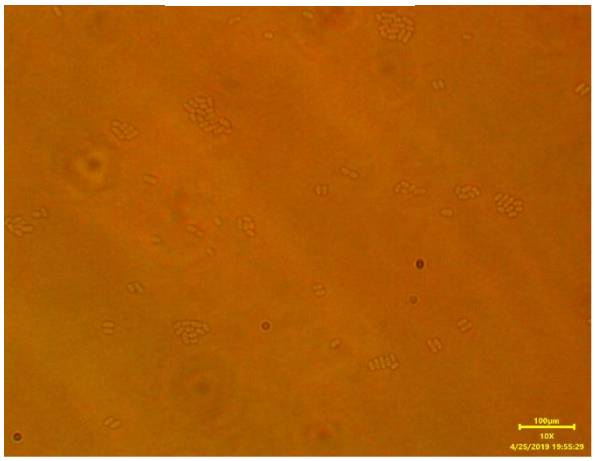
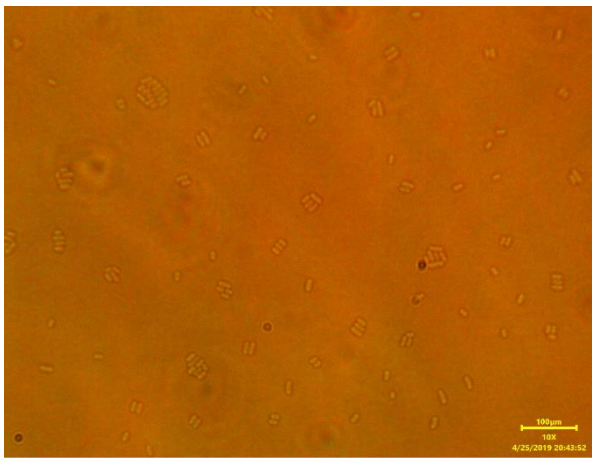
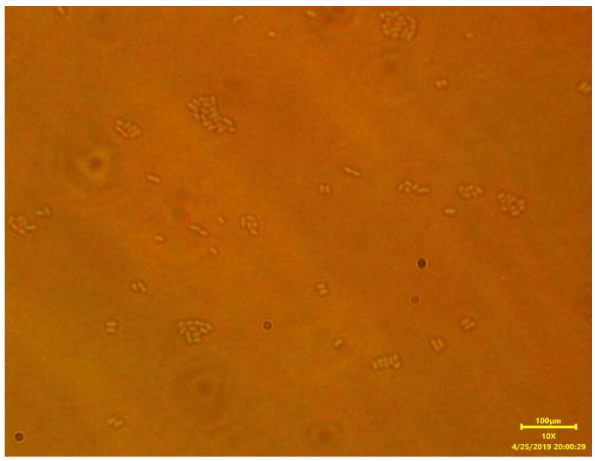
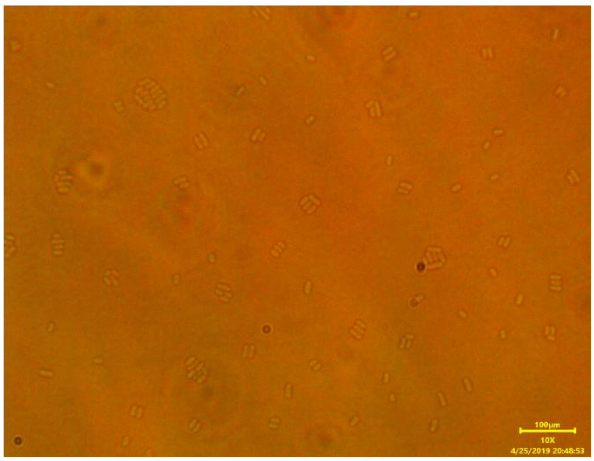
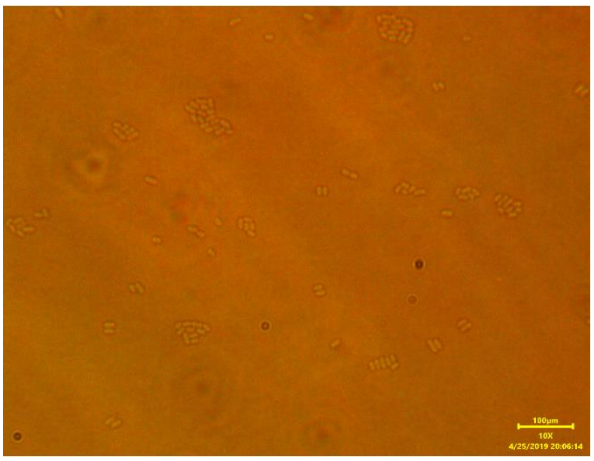
Time Elapsed:	GL607 Cells	MG1655 Cells
Zero Minutes		
Five Minutes		
Ten Minutes		

Figure 4: Visualization of GL607 cells and MG1655 cells exposed to 100 µg/µl of Ampicillin under microscope.

As illustrated in Figures 2, 3, and 4, neither strain of cells appeared to lyse under any of the ampicillin concentrations used. Therefore, no quantitative data from this experiment was obtained. However, the images do present some interesting differences between the two strains. Time-lapse photos of MG1655 cells in conditions of the first two ampicillin concentrations revealed that they were more motile and clustered in appearance than the other strains. Additionally, there appeared to be more cells present in the MG1655 microscopic data under the first two ampicillin concentrations than for the other strain. Proposed reasons for the observed phenomena and lack of quantitative data are discussed in the following section.

Discussion:

For this experiment, the wild-type MG655 *E. coli* strain and the genetically mutated GL607 *E. coli* strains were used. Since the GL607 *E. coli* strain lacked tryptophanase and therefore the ability to synthesize and transmit indole signal messengers, we hypothesized that this strain would lyse faster under ampicillin treatment than the wild-type strain. When a cell lyses, the membrane and cell wall are broken down. This would make the cell appear to "pop" and somewhat disappear under microscopic view. However, this phenomenon was not observed in any of the six slides, so the microscopic data did not appear to support this hypothesis. The lack of lysis may be attributed to a number of different factors such as the conditions in which the cells were viewed under the microscope, the concentration of the antibiotic used, and the growth conditions of the cells themselves.

The production of indole is not a constant process that occurs throughout the growth of *E. coli*. Instead, indole production typically occurs primarily during the transition from exponential phase to stationary phase of cell population growth. During this period of transition, *E. coli* cells produce a substantial amount of indole. Research studies have demonstrated that supernatant

indole concentration in LB medium can reach a maximum of 0.5 to 1mM which allows most of its processes that induce the inhibition of cell division and modulation of bacterial biofilms to occur [6]. Additionally, the concentrations of cell-associated indole can transiently reach as much as 60 mM during entry into stationary phase of cell growth. This significantly high concentration of indole has been proposed to be part of a phenomenon known as the "indole pulse", in which indole is produced at a more rapid rate in *E. coli* cells than is exuded from the cells. This pulse is said to be responsible for the inhibition of cell growth and division, allowing the culture to enter a stationary phase before the exhaustion of nutrient resources in the growth medium occurs. The pulse therefore can help maintain long-term survival of the cells [6]. In the experiment conducted, the cell cultures were grown to an OD_{600} of about 2.0. *E. coli* cells typically transition to stationary phase at an OD_{600} between 0.6 and 1.0 in LB broth, although some studies suggest that this transition may occur at an OD_{600} of 0.3 [14]. Since the OD_{600} of these cultures were well past this range, it is possible that the cultures were too overgrown for the ampicillin to have any noticeable effect on them. It may be possible that ampicillin would have a more noticeable effect on cells at an earlier phase of growth.

Ampicillin was selected as the antibiotic for this experiment. As a β-lactam antibiotic, ampicillin inhibits the synthesis and maintenance of the cell membrane in *E. coli*, leading to disruption and lysis of the membrane [1]. Initially, the ampicillin stocks available in the lab were either degraded or expired. Therefore, it was necessary to produce a new, working ampicillin stock before the experiment could take place. In order to ensure that the newly created ampicillin stock was working, an experimental test was performed. LB broth cultures (5 milliliters each) of MG1655 and GL40 were created and allowed to grow to OD_{600} levels of 0.646 and 0.689, respectively. Fifty microliters of 25 mg/ml ampicillin were added to each culture and the OD_{600}

readings were taken about thirty minutes later. The OD_{600} levels fell to 0.151 and 0.149 for GL40 and MG1655 respectively. Therefore, the new ampicillin stock was clearly functional and the live *E. coli* cells were not totally resistant to the ampicillin. It is possible that the final concentrations of ampicillin used in the experiment were not adequate, or that the mixture of ampicillin and LB-agarose negatively affected the effectiveness of ampicillin. In the design of the experiment, a mixture of LB-agarose and ampicillin for the microscope pads was used rather than simply using LB-agarose pads, pipetting the cells onto the pads, and then pipetting ampicillin onto the cells. It was believed that the latter method would not be highly effective for two reasons. First, it could not be ensured that all *E. coli* cells would be exposed to the same amount of ampicillin throughout the pad. Secondly, this method was more time intensive; rather than simply pipetting cells onto the pad, both the cells and ampicillin had to be pipetted onto the pad before transferring the slide to the microscope. It was feared that the cells would lyse faster during this transition and therefore loss of qualitative data could occur. Unfortunately, lack of quantitative data rather than loss of qualitative data occurred instead, so the precise method used for the administration of ampicillin should be reevaluated and modified in future experiments. However, the experiment did result in a method that successfully allows one to visualize live bacteria cells under the microscope.

The qualitative data observed suggested that the MG1655 cells were generally more mobile and appeared in higher quantities than the other strain. While an interesting observation, there is likely no compelling reason for this occurrence. The higher amounts of cells could be attributed to simply having obtained cells from a region of the tube that had more cells present in it than in the rest of the tube. While the MG1655 strain had relatively more mobile cells, they still remained in the same positions on the slide. The clustering of MG1655 cells could be simply

have been due to more overcrowding of cells on the slide. Overall, the cells from both strains appeared to be quite stationary and almost dormant in appearance. *E. coli* cells grow more actively and effectively at temperatures of 37°C rather than at room temperature. Thus, the transfer of live *E. coli* cells from environments at 37°C to environments of room temperature (such as the microscope stage) could provide another potential reason for the lack of lysis and action in the cells. The cells could have become more stationary due to an unfavorable drop in temperature during transfer from the live culture to the microscope stage. In future experiments, this should be corrected for by the utilization of a stage warmer apparatus and tests for viability of the cell cultures.

Future Directions:

Since the experiments resulted in no strains of cells being lysed under ampicillin treatment, an effective conclusion cannot yet be reached as to whether the presence of tryptophan, and in effect, the production of indole in *E. coli* cells allows the cells to persist longer under antibiotic treatment. Modifications to the experiment design as well as the procurement of specific equipment are in order and would likely lead to more definitive results.

Since the bacterial cells had progressed well into the stationary phase of cell growth, $1/10^{th}$ dilutions of the overnight cultures should be made the following day and at a minimum of three hours prior to the experiments being carried out. During exponential growth, *E.coli* has a doubling time of about twenty minutes resulting in a time requirement of approximately three hours for the cells to reach OD_{600} of about 0.7 [14]. Previous research suggests that the *E. coli* bacteria undergoes the transition between exponential growth phase and stationary phase during this time [5]. Targeting the cells with antibiotics while they are producing large amounts of indole, but are still actively growing, having not yet completely established stationary phase

persister mechanisms, could potentially provide for better visualization of the effects of indole signaling between cells.

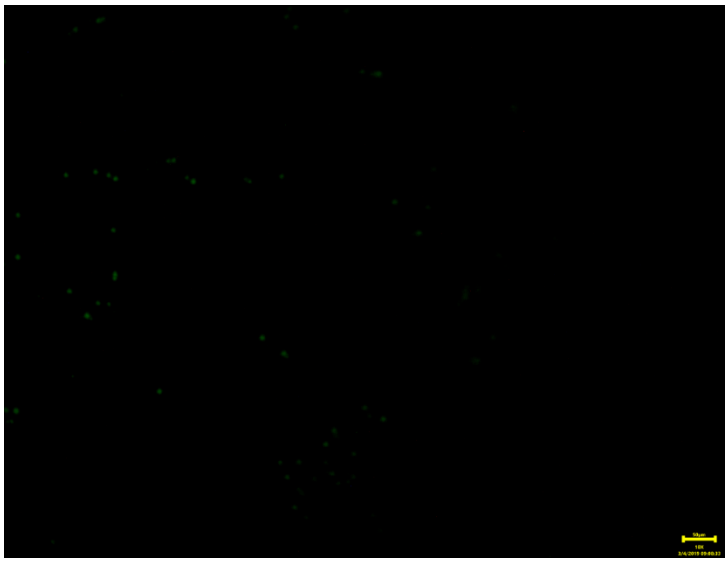


Figure 5. GL40 cell supernatants under fluorescence microscopy. With a protocol now in place to visualize live cells and the repairs of fluorescence microscopy equipment, future experiments can better visualize tryptophanase and its role in persister cell formation and interaction.

The lack of microscopy equipment required for the simulation of *E. coli* growing conditions as well as the disrepair of the fluorescence microscopy equipment needed for adequate visualization of the cells contributed to the difficulties in performing the experiments properly. Fortunately, the Wilson lab is currently in the process of procuring a stage warmer which will allow, in future experiments, the live cells to potentially continue growing and interacting with each other at the temperature of 37°C rather than room temperature. The mercury bulb needed for fluorescence microscopy, as well as other fluorescence microscopy related equipment, are in the process of being repaired. Once such repairs have taken place, live microscopy experiments involving the strain GL40 will be able to be conducted. Therefore, actual visualization of the tryptophanase proteins at the poles of the live *E. coli* cells due to their being fused to SF-GFP tags will be possible. Had fluorescence microscopy been initially available, the experiments would have been conducted under the hypothesis that the few highly resistant cell isolates (indicated by the fluorescent green proteins) would produce indole and

transmit signals to surrounding lowly resistant cell isolates. Such bacterial cells would hypothetically have remained visible for longer periods of time during antibiotic treatments while the other cells lysed. Above is an example of fluorescence microscopy with MG1655 cell supernatants in Figure 5.

Additional modifications to consider in future experiments include the adjustment of the concentration and method of administration of the ampicillin as well as the inclusion other different antibiotics. Future experiments may involve reasonable increases in the dosage of ampicillin until the cell lysis is induced or using a method of ampicillin exposure other than LB-agarose/ampicillin pads. The mixture of ampicillin and LB-agarose currently used presented difficulties in terms of finding a temperature suitable for both substances to function optimally. LB-agarose requires being kept at relatively warmer temperatures in order to remain molten, while ampicillin may degrade at temperatures above 50°C. While the LB-agarose/ampicillin solutions were stored at 47.5°C on heat blocks, it is possible that the ampicillin may have degraded. Future experiments should take into better consideration the heat-sensitive nature of ampicillin. Once an effective concentration and administration of ampicillin is found and experiments using ampicillin are completed, consideration should be given to other types of antibiotics. One study has already suggested that antibiotics that promote oxidative stress by forming reactive oxygen species may inhibit the development of *E. coli* biofilms by increasing the amount of indole signaling in the biofilms [8]. The precise involvement of indole in this process is not yet well understood, so further study of this group of antibiotics may provide grounds for the investigation of other functions of indole signaling and the part it plays in the formation of persister cells.

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