EVALUATION OF THE EFFECTS OF INDOLE ON STRESS TOLERANCE AND PERSISTER FORMATION IN ESCHERICHIA COLI

By

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Abstract: Bacteria can adapt to many unfavorable conditions that can arise in their environment. Persister cells are a specific phenotype that can occur within a small fraction of a genetically identical population of cells. This phenotype is characterized by a state of temporary metabolic quiescence that enables the individual cell to survive stresses such as antibiotics that might kill the majority of the population. This presents a significant challenge to the treatment of chronic bacterial infections because persister cells enable the continuation of an infection and can enable the emergence of antibiotic resistance.

The compound indole, produced by the enzyme tryptophanase, is one of many proposed signals that promotes persister cell formation. Tryptophanase mainly produces indole during the stationary phase of bacterial growth, which occurs after the logarithmic stage of growth when persister cells are typically assayed.

Using *Escherichia coli* as a model, we evaluated the effect indole on stationary phase bacteria as it pertains to several factors of persister cells and indole signaling. Utilizing a qualitative dye, the ability for persister cells to resume growth following antibiotic treatment was examined based on differences in the presence of indole. The ability of indole to function as a signaling molecule that can induce the persistence phenotype in other cells was assayed by comparing the survival of indole non-producing cells when grown in a culture with indole-producing cells. Finally, we sought to explore the model for indole signaling by evaluating the effect on survival that could be introduced by removing the expression of proteins that are believed to be involved in indole signaling. We saw that low concentrations of indole induces a physiological change that influences cells to leave the persister phenotype. This may provide a competitive advantage over cells that are not influenced by indole and would be beneficial to bacterial in an infection while clinical treatment is occurring. A disadvantage may also occur in these cells over long-term exposure to antibiotic; the persister cells that are influenced to regrow become susceptible to antibiotic treatment. This research provides an important understanding of how indole impacts persistence by studying persistence when indole is most physiologically relevant.

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CHAPTER I

INTRODUCTION

Persistence is a phenomenon that enables cells to survive exposure to antibiotics or other stressors within their environment. Persistence is a heterogeneous phenotype that can occur even within a genotypically homogeneous population of bacterial or eukaryotic cells (1,2). These "persister" cells survive exposure to an antibiotic without possessing a gene that supplies resistance to the drug in question. The core of the persistence phenotype is the adoption of a dormant state that cells can leave (1) This allows the population regrow to continue an infection (1). This phenomenon enables chronic infections to occur when the persister cells regrow inside of a host, human or animal, after treatment for the infection has ceased (4). These chronic infections can include the bacteria species *Escherichia coli, Mycobacterium tuberculosis, Staphylococcus aureus* and many others (4). Persister cells also play an important role in establishing antibiotic resistance because a few cells in the population can survive the initial exposure (5,6). These surviving cells can mutate to acquire antibiotic resistance traits (5,6).

Persistence is fundamentally different from resistance. Antibiotic resistance is defined as the ability of cells to survive and replicate while an antibiotic is present (3). Persisters survive but will only continue to replicate if the antibiotic is removed from the environment. (3). There are a wide variety of ways that persistence is triggered. Many of the persister forming methods are stochastic in nature. The most well studied of these are toxin-antitoxin systems (7). External factors (e.g., environment or from other cells in the population) can also trigger the formation of persister cells. These signals can be present alongside indole and can alter the amount of persister cells in a culture. Antibiotics can signal increases of persisters present in a population of bacteria (3, 8). The amount of a particular antibiotic that is used will always be constant in these experiments. Other factors can be harder to control or observe such as the cell density and cellular starvation levels (3).

The classical method used to identify persisters in a culture is by measuring the population's death once exposed to antibiotics. The presence of persister cells then can be identified by a biphasic killing curve where the rate of cell death caused by an antibiotic will change over time (3,5). This occurs because the core characteristic of a persister cell is metabolic dormancy that enables it to survive exposure to antibiotics (3). After the antibiotic is removed, those persisters regrow (3). The antibiotic being removed before recovery separates persisters from antibiotic resistant cells which can repopulate while the antibiotic is still present.

Many factors induce the persistence phenotype in bacteria, one of which is the compound indole (9). This compound is expected to induce persistence by activating stress response pathways in bacterial species that produce it. Indole is produced by the

tryptophanase enzyme in *Escherichia coli*. It is associated with a number of cellular pathways and was implicated in influencing persister formation relatively recently (10). Introduction of physiologically relevant quantities of indole has been shown to increase the number of persister cells in a bacterial population (10). Indole also increases expression of stress responses such as the phage-shock response and the oxidative stress response in these cultures (10). This implies that such pathways are involved in the mechanism of indole-induced persistence (10).

There is controversy as to the effect of indole on persistence (7, 9, 10, 11, 12). Some researchers believe that indole provides a beneficial effect to persister cells (9,10). Their data suggests that the mechanism of indole signaling promotes the transcription and activation of a variety of proteins that causes the cell to adopt the persister phenotype (10). Other researchers have shown that indole production is reduced by the activity of other systems that lead to persister cell formation and use this as evidence that indole itself does not induce persistence (7). The controversy likely stems from the wide array of pathways that indole is involved in as a signaling molecule. Such pathways can react in different ways from each other and may help or hinder persister formation (10). Amongst these pathways are those that typically respond to phage infection, hydroxyl radicals, and other stressors that are not inherently related to the activity of antibiotics (10).

Inducing persistence is not the only role that indole performs in a cell. It influences a wide variety of cellular functions leading to a variety of phenotypes. Most notable is that indole can influence colony density. High extracellular concentrations of indole, above 1mM, will reduce colony density (12, 13). Negative effects like this may influence persistence.

The choice of antibiotic is important when studying persister cells. Firstly, antibiotics are classified into one of two categories. Bacteriostatic antibiotics stop bacterial growth but do not actually kill the organisms (14). This prevents an infection from worsening and does lead to the end of the infection (14). The second group of antibiotics are bactericidal. These antibiotics are directly lethal to bacteria (14). Utilizing bactericidal antibiotics is necessary to study persister cells.

Research Objectives

We predict that indole will increase persister cell formation in the stationary phase of growth. We believe that the increase in indole upon entry to the stationary phase will upregulate the pathways that indole influences (10, 13). Working in the stationary phase specifically allows us to evaluate indole signaling within the full context of cellular activity that it is typically produced in. We believe that this increase in indole concentration during the stationary phase is likely one of the factors that enables indole to induce the persister cell phenotype (13). The current model of indole signaling states that it occurs when extracellular indole diffuses back into a cell (10). It does not necessarily diffuse back into the cell that produced it. We attempted to address several questions about indole signaling and persister formation in the stationary phase:

1. Will indole promote an increase in persistence within the stationary phase?
2. Does indole influence the ability of persister cells to regrow after antibiotic exposure?
3. Does indole influence cells that do not produce it to the same degree that it influences cells that do produce it?

4. Are genes that are upregulated when indole is added to a culture part of the pathway through which indole induces persistence (10)?

Thesis organization:

Chapter two is a **review of the literature**. This includes publications that provided data or values such as reagent concentrations that were used in experiments within as well as publications that outline clinical relevance of the phenomena that is studies in this thesis.

Chapter three provides an explanation of the experimental methods performed in this research

Chapter four describes the results of the experiments and provides context as to the meaning of the results. It also explains modifications to previous methods when an additional variation or modification of an experiment deviated from the established methods for a specific, noteworthy, and worthwhile reason.

Chapter five provides the conclusions of the research as well as a discussion of future research questions that are sparked by the results from this research. This chapter will include some repetition of the results from chapter four.

CHAPTER II

REVIEW OF LITERATURE

Persister cells are growing in interest with the increasing global concern of antibiotic resistance. Recently several researchers in the field worked collectively to establish proper methods for the study of persisters (3). This begins with the necessary step of consistently defining persistence to avoid confusion between resistance and tolerance. Antibiotic persistence is defined as "the ability of a subset of the population to survive exposure to a bactericidal drug concentration" (3). One key factor to determine true persistence is the presence of a biphasic killing curve upon exposure to an antibiotic in its bactericidal concentration (3, 5). This is identified by the rapid killing of most cells in a population followed by a slower killing of the remaining cells (3, 5). The second, reduced rate of killing corresponds to persister cells (3, 5). Persister cells retain their vulnerability to antibiotics if/when they regrow (3). Despite this, the survival from multiple rounds of exposure can produce antibiotic resistant mutants (6). This potential mutation to resistance makes persisters clinically relevant, and the wide range of cellular mechanisms and methodologies of study has historically created controversies within the field.

A second clinical relevance occurs when persisters regrow in an infection once a patient stops taking antibiotics.

This work builds on previous data from our laboratory that found that the enzyme tryptophanase was 100-fold overexpressed in persister cells (15). Previous work in this laboratory utilized mass spectrometry to compare the proteomes of persister cells to those of non-persister cells that were harvested utilizing fluorescence-activated cell sorting (15). Two fluorescent proteins with different inducers were used, and media was changed to alter which inducer was present (15). This sorting strategy meant that persisters retained the original fluorescent protein, which was diluted in live cells that were replicating but no longer producing it while they were transcribing a different fluorescent protein. Tryptophanase was shown to have a 100-fold increase in abundance within the persister cells (15). The addition of indole was also shown to induce tryptophanase expression, likely because the reaction catalyzed by the tryptophanase enzyme is reversible (15).

Other effects of indole have been described as well (10, 9). These include biofilm formation, which is itself a method for cells to survivor antibiotics and other stresses (25). Other phenomena that it influences include cellular motility, virulence and tolerance to acid across species and kingdoms (17, 18, 19, 20, 21).

Indole has been studied for its effects on persister formation in *Escherichia coli* and other species of bacteria. Indole has been classified as a signal that encourages the formation of the persister cell phenotype, but its effect on persistence is controversial (7,

9, 10). Some laboratories have shown that indole can directly trigger persister formation under exposure to antibiotics (10).

Previous research has shown that indole functions as a signaling molecule that influences bacterial populations to form persisters. Indole is produced by all wild type cells within the culture, but not every indole producing cell will become a persister. Previous studies with indole compared the survival of a wild type strain of *E coli* and strains with a deletion for either the tryptophanase enzyme or the Mtr tryptophan transporter after antibiotic killing (10). All three strains were grown under a combination of conditions. The first was growth in a rich or minimal medium (10). The second condition was growth with or without added indole (10). The tryptophanase knockout mutation displayed similar survival to the wild type in the minimal medium, but survival without indole in the rich medium was around an order of magnitude worse than the wild type (10). Some of the difference in the survival might be attributed to the nutrient limitation in the minimal medium (10). The improvement of the wild type though, is likely also due to the ability of the wild type to produce its own indole in the rich medium, with the potential for the total indole in the culture to remain within the beneficial range of concentrations (13). Effects of Mtr deletions are weaker. Deletion of Mtr may reduce the cell's ability to produce indole but indole does not require the protein to cross the plasma membrane (10). When evaluating indole, there is limited reason to remove Mtr or any other transport protein.

The arguments against indole induced persistence are based on improvements in persistence when indole is removed. This can be evaluated directly by deleting the tryptophanase enzyme. It can also be studied indirectly when another change reduces

tryptophanase expression. Studies that have found this are not consistent with our model or with the research used to establish our model.

In the primary example of this argument, a toxin type protein called YafQ causes a reduction in tryptophanase levels (7). Increasing YafQ was shown to increase persister formation (7). This presents a system where persister formation decreased in spite of a reduction in indole production. It is important to note that toxin-antitoxin systems, of which YafQ is a part of, are themselves a common method of persister formation (7). While this may suggest that the loss of indole is responsible for the increase in persister cell formation, other effects of YafQ could also be responsible for the results. Such effects of YafQ may cause a net increase in persister cells.

Indole functions as an intercellular signal. This functionality allows the indole produced by a few cells to impact neighboring cells. Within a population of bacteria there are a few "highly resistant isolates (HRIs)" which are less likely to be harmed by exposure to an antibiotic than the typical cells of the population (9). These HRIs are of extremely low abundance (9). In those ways, HRIs seem like persister cells. The primary value of these individuals to us is that they are believed to improve survival of the whole population in a charitable fashion (9). This factor is an important difference from persister cells. Like our lab's previous results with persister cells, tryptophanase is overexpressed within the HRI cells (9). Indole produced by the HRIs is believed to protect the less resistant isolates (LRIs) that constitute the majority of any given population of bacteria (9). Tryptophanase overexpressing HRIs can be as rare as 1% of the population while still improving survival of the population (9). The role of indole in both phenomena, as well as the rarity of both, does however imply that the two may be

related. This phenomenon may not be mutually exclusive with the persister phenotype. A cell does not need to be active for indole to diffuse out of it.

Indole concentration differs between the interior of cells and the growth media (or supernatant). Supernatant concentrations will typically reach between 0.5 and 1.0 millimolar during the stationary phase of growth (13). The intracellular concentrations of indole will be much greater: during logarithmic growth the concentration will typically be constant at around 20 millimolar (13). The transition to the stationary phase is accompanied by a significant but transient spike of indole production that produces an intracellular concentration of 40 to 60 millimolar, more than double the log growth concentration, before falling back down to the logarithmic phase concentration (13). Extracellular indole increases to its stationary phase concentration of 0.5 to 1.0 millimolar during this period (13). This data provides a novel hypothesis towards indole functioning to arrest logarithmic growth; a function that could be relevant to the formation of persister cells (13). We think that the so called "indole pulse" is related to persister formation. The variance in indole's concentration may be relevant to its effect on persistence. There is currently room to further evaluate the relationship between indole concentration and persistence.

Tryptophanase expression and indole production are altered by factors other than growth phase. One factor that alters tryptophanase expression is believed to be temperature (22). Lower temperature appears to increase tryptophanase expression and 30^{o}C has been used by some laboratories in an attempt to highlight the effects that it has on a culture (10, 22).

Indole signaling influences several pathways. The most relevant pathways to persister cell research appear to be the oxidative stress response and phage shock response pathways (10). There is some evidence that the two pathways are interconnected because attempts to activate one may activate the other (10). This may also just mean that both pathways are not very specific and easily activated by similar signals. Important proteins in both pathways are associated near the plasma membrane (10). This localizes them in an ideal location to detect indole as it diffuses into the cell.

The correlation between indole induced persistence and upregulation of the phage shock proteins suggests that indole disrupts the membrane potential of a cell when it is transported into or out of it (10). The phage-shock response is a system found within cells that protects against extracytoplasmic stress such as filamentous phage infection (24). It also protects against temperature extremes and the effects of proton ionophores (24, 25). A common trait of the signals that upregulate the phage-shock response is the disruption of the proton motive force (24). Amongst the specific genes of the phage shock response, PspB and PspC are cytoplasmic membrane proteins that are responsible for sensing the condition that induces the phage shock response. They then interact with PspA which is responsible for inducing the response (24, 26). This role makes the three proteins relevant targets for study in relation to the activity of indole.

The oxidative stress response is also linked to indole and persistence. It is a second method that cells use to survive environmental stresses. The oxidative stress response primarily protects cells from the damaging effects of peroxide, oxygen radicals, and other threats containing reactive oxygen species (27). One of the key proteins for induction of the response is the transcription regulator OxyR (27). OxyR is commonly

found in the cytoplasm near cell membranes (10,28). This similarity to phage shock proteins might be key in understanding why both responses are upregulated by indole.

The oxidative stress response is also of interest because of a model that claims that all bactericidal antibiotics perform their killing activity by creating oxygen radicals (28). This claim that reactive oxygen species are the primary function of bactericidal antibiotics has been directly countered by two papers that showed that the same antibiotics will function without the formation of reactive oxygen species (28, 29, 30). The first used a comparison of the effect of ampicillin, norfloxacin, and kanamycin on cultures grown in either the presence or absence of oxygen, and therefore with or without the ability for reactive oxygen species to form (29). It found that ampicillin and norfloxacin had comparable activity regardless of the ability of reactive oxygen species to form (29). Kanamycin was less effective without oxygen, but the effectiveness could be restored with nitrate addition (29). That addition provided an alternate pathway for respiration within the cells (29). The second laboratory showed that thiourea would increase the minimum inhibitory concentration (MIC) of various antibiotics, but the effect would disappear at higher concentrations of the antibiotic (30). The significance of that result is that thiourea's protective effect disappeared within clinically achievable antibiotic concentrations (30). This laboratory showed that hydroxyl radical formation decreased with that antibiotic addition. Radical formation could be reduced to the point that cells that were treated with thiourea were not different from the untreated cells (30). Thus, both reports present evidence that hydroxyl radicals are not required for bactericidal antibiotic activity. However, the results do not prove that there is no effect of hydroxyl radicals on bactericidal killing.

The tryptophanase protein is associated with other roles than persister formation. Biofilm formation is one of the most important effects influenced by tryptophanase (17). Biofilms are a population wide strategy that some bacteria use to survive environmental stressors. With biofilms, indole interacts with proteins that trigger a reduction in biofilm formation (32). This is the inverse of the effect that we expect indole to have on persister formation. Recognizing how indole and tryptophanase influence biofilm formation is important because trying to influence one could influence the other. Tryptophanase's involvement in a second survival strategy besides persister formation shows that it is a multifaceted tool for cell survival. Like persister cells, biofilms also display a quiescent phenotype from some of the cells involved.

Indole is associated with bacterial biofilm formation. Biofilms, like the persister phenotype, are a method of surviving environmental stressors and there are a variety of effects of indole pertaining to biofilm formation. Indole is not the exclusive factor in biofilm formation but simply one of many factors. As a molecule used for quorum sensing, indole interacts with the SdiA protein in a manner that reduces biofilm mass by about one third (32).

A significant factor that links persister cells and biofilms is that both are a method of protecting a population from stressors. Different responses to stress can be responsible for increasing either of the two conditions. Indole interacts with a variety of proteins relevant to stress responses:

- Indole's interactions with *SdiA* will reduce biofilm mass (32).

- Indole's interactions with YmgB (also called AriR) will reduce biofilm formation under non-stressed conditions (32).
    - Deletions of *ymgB* followed with addition of indole reduces acid resistance, and presumably resistance to other stressors (32).
- Additional stress response genes that are associated with indole signaling include *ycfR*, *ibpAB*, and a variety of efflux pumps (32).
    - *YcfR* protects cells from stressors such as heat, peroxide, and acidic conditions in addition to triggering indole production (32).
    - It is likely that *YcfR* regulates biofilm formation through mediation of this stress response (32). Indole signaling is likely a part of that pathway (32).
    - The *IbpAB* operon is induced as part of the cellular response to oxidative stress (32).
        - *IbpAB* increases tryptophanase expression as a part of this response (32).

Through the many proteins that influence indole production, the expression of persister and biofilm phenotypes is modified to further influence cell survival.

The mechanism to induce biofilm or persister phenotypes may also involve the potential for indole to disrupt cellular division (31). This occurs when the concentration of indole reaches a point significantly higher than the expected extracellular concentration (13, 31). The mechanism by which this affects cells is uncertain (31). Cell division and tryptophanase localization appear to be related (30). Tryptophanase localizes

near the poles of cells near cellular replication machinery (23, 30). This localization may be significant to the functions of tryptophanase.

Persistence is a route towards the emergence of antibiotic resistance in populations of bacteria. Previous studies have shown that persisters can acquire resistance after multiple rounds of exposure to the same antibiotic (5). Researchers were also able to associate increasing persister frequency with increasing mutation rates, primarily by evaluating the emergence of resistance across different bacterial strains (5). Persisters may experience damage from antibiotics that can further increase mutation rates.

Persistence likely enables antibiotic resistance to occur without being directly causal to resistance mutation. Antibiotic resistance has been shown to appear in persister cells and rates of resistance correlate with rates of persistence (5, 6). DNA-damaging fluoroquinolone antibiotics can cause mutations to become more frequent in cells that survive exposure to them (6). DNA may not be perfectly repaired after a persister survives exposure to a fluoroquinolone (6). In rare situations, this may lead to beneficial mutations such as antibiotic resistance. This kind of change would require recovery from the antibiotic, which requires that persister cells be present at the time of treatment (6). While fascinating, this phenomenon is rare outside of fluoroquinolone antibiotics.

Persister cells are clinically relevant as a common route for the emergence of chronic infections. The organism studied in this work is *Escherichia coli*; persister cells of this species are a common cause of chronic urinary tract infections and other medical issues (4). Persisters may survive a typical clinical treatment of antibiotics well enough that a patient's immune system is not capable of eliminating the threat entirely. Persister

formation makes complete treatment of an infection difficult (4). Even without antibiotic resistance occurring as a result, typical clinical treatments may not be sufficient to eliminate an infection with a significant population of persister cells.

Toxin-antitoxin (TA) systems are the best studied method of persister formation. TA systems are comprised of two proteins. The toxin protein hinders cellular performance while the antitoxin inhibits the toxin's activity (7). A disruption in the balance of toxin and antitoxin molecules that allows toxins to accumulate is believed to induce a dormant state in bacteria, which has been established as the core trait of persister cells (7). Like indole, TA systems will only induce persistence in a fraction of a population (7).There are multiple TA systems, a mutant of one such system, HipA7, was used as a positive control in some early tests outlined in this thesis due to its known nature as an overproducer of persister cells (33). Another toxin molecule known as YafQ has been shown to reduce indole signaling while increasing persister formation, thereby establishing some controversy as to indole's importance in persister formation (7).

The stringent response also induces persistence. This response utilizes guanosine tetraphosphate and guanosine pentaphosphate, collectively referred to as (p)ppGpp (7). The stringent response is of particular interest because it primarily functions to respond to nutrient starvation. This makes it a relevant factor when studying cells in the stationary phase (7). While it can be triggered by nutrient starvation, the stringent response is also stochastically activatable in an exceedingly small number of exponentially growing cells and it can also be triggered by environmental conditions that induce production of the (p)ppGpp signal (7).

The SOS response for DNA repair also induces persistence (7). This response functions both as a complementary pathway to stress signals and as a method to repair DNA when a persister cell returns to normal metabolic activity (7). The wide variety of relevant factors can complicate a study of persister cells; the design experiments must be carefully considered so that only the factor of interest is expected to change.

CHAPTER III

MATERIALS AND METHODOLOGY

Bacterial Strains

Escherichia coli strains MG1655 and MG1655-hipA7 were obtained from Professor Thomas Hill of the University of North Dakota (33). *Escherichia coli* strainsBW25113, JW1297-1, JW1298-1, JW1299-1, JW3686-7, JW3933-3, and JW5437-1 were obtained from the *E. coli* Genetic Stock Center at Yale University (35). The MG1655 and GL607 strains were both transformed with the pBAD plasmids containing the mCherry and MEGFP genes to create four new strains, which will be referred to as MG1655-mCherry, MG1655-GFP, GL607-mCherry, and GL607-GFP.

***Escherichia coli* Strains**

Table 3.1 Strains and Plasmids

A list and description of the strains (A) and plasmids (B) that were used within this thesis.

A

Strains	**Description**	**Reference**	**Purpose**
MG1655	Wild type strain	Blattner et al. 1997 (36)	Wild type strain
GL607	MG1655ΔTnaAB	Li et al. 2013 (31)	Indole negative strain derived from MG1655 wild type.
MG1655-hipA7	MG1655 based strain with mutations at G22S and D291A locations within the hipA7 gene.	Korch et al.2003 (33)	Mutant with increased persister production.
BW25113	Wild type strain	Grenier et al. 2014 (37)	Wild type background utilized in other mutants.
JW1297-1	BW25113ΔPspA	CGSC (35)	Mutant with deletion for a gene associated with indole induced persistence.
JW1298-1	BW25113ΔPspB	CGSC (35)	Mutant with deletion for a gene associated with indole induced persistence.
JW1299-1	BW25113ΔPspC	CGSC (35)	Mutant with deletion for a gene associated with indole induced persistence.

JW3686-7	BW25113ΔTnaA	CGSC (35)	Indole negative strain in new wild type background.
JW3933-3	BW25113ΔOxyR	CGSC (35)	Mutant with deletion for a gene associated with indole induced persistence.
JW5437-1	BW25113ΔRpoS	CGSC (35)	Mutant with deletion downstream of other genes of interest.

Plasmids

B

Plasmid	**Description**	**Reference**	**Purpose**
mCherry-pBAD	pBAD plasmid with arabinose induced gene insert for mCherry fluorescent protein	Addgene.org (38)	Plasmid with ampicillin resistance and mCherry fluorescent protein to identify cells containing plasmid.
mEGFP-pBAD	pBAD plasmid with arabinose induced gene insert for mEGFP fluorescent protein	Addgene.org (39)	Plasmid with ampicillin resistance and mEGFP fluorescent protein to identify cells containing plasmid.

Strain construction

Strain construction was performed using electroporation to introduce the pBAD plasmid variants into the MG1655 and GL607 strains of *E. coli*. To do this, cells were cultured overnight on LB agar at 37ºC of the parent strains. Individual colonies were selected for inoculation into 5 mL of LB broth. These selected colonies were regrown

overnight with 250 RPM shaking and 2.5 mL of those cultures were reinoculated into 500mL of LB broth and grown until their optical densities at 600 nM (OD600) reached a point between 0.5 and 0.7. The cultures were pelleted by centrifugation for 20 minutes at 4200 RPM at 5^{o}C. The pellets were subject to a process of resuspension in water, chilling on ice, and centrifugation two times. The final pellet was suspended in 0.5mL iced water and moved to microcentrifuge tubes. Two replicates of each strain were produced this way, and one sample of each strain was introduced to 0.5 ug of plasmid. Each replicate of a strain received a different plasmid variant. Cell and plasmid mixtures were mixed by tapping and moved to electroporation cuvettes. Electroporation was performed with the parameters of 2.5 kV and 25 uF with a pulse control of 200 ohms. The resulting strains were replated onto LB supplemented with 100ug/mL ampicillin. The resulting cell growth showed that the transformation was successful because the plasmids' ampicillin resistance gene was necessary for growth on this media.

Persister Assay

To assay persister cell counts, two different methods were used. In either variation, overnight cultures were grown at 37ºC while shaking at 250 RPM in liquid Luria Broth (LB). Both tests would also include plating onto LB agar before other procedures were performed in order to obtain initial counts of colony forming units (CFUs). Cells were plated at dilutions ranging from 1/100 to 1/1000000.

To evaluate changes in persister formation when the cells are exposed to antibiotic on agar. These tests utilized the MG1655 wild type and related mutants. Antibiotic exposure occurred in a novel way wherein cells from overnight cultures were plated onto LB agar and incubated at 37ºC for an hour before 100ug/mL ampicillin was added to the plate via misting. From a stock concentration of 25mg/mL, 5 sprays of the mister delivered 0.1mL of the ampicillin stock onto the 25mL of agar on the plates. Another hour of incubation was followed with the application of penicillinase in the same manner. The plates were then incubated overnight to allow colonies to grow.

For traditional persister assays, overnight cultures of cells were exposed to antibiotics and then plated to obtain CFU counts. This test utilized either 100 ug/mL ampicillin or 0.5 ug/mL ciprofloxacin. Antibiotic exposure occurred in either 5mL LB agar in test tubes or 200mL LB agar in 500mL Erlenmeyer flasks. Cells were plated immediately before antibiotic addition and at various time points afterwards at dilutions ranging from 1/100 to 1/10000000. Plates were incubated at 37ºC overnight before counting. This method was also modified to perform many of the other tests performed in this thesis.

Determination of Bactericidal and Inhibitory Concentrations

To determine the minimum inhibitory concentration (MIB) and the minimum bactericidal concentration (MBC) of ciprofloxacin and cefoxitin, we performed a test to find the concentrations of the antibiotic that stopped growth and that kills 99.9% of cells in the exponentially growing culture (40). To determine these concentrations, overnight cultures of *E. coli* MG1655-hipA7 were grown overnight at 37ºC and 250 RPM shaking in LB media and diluted 1:100 into fresh LB. Cultures were grown back into the late exponential phase. (O.D. 600 equal to 0.8). They were then plated onto LB agar plates containing various concentrations of the antibiotics. If colonies did not grow on the plates, it meant that the antibiotic concentration was at or above the MIC. The MBC was determined by exposing many exponential growing cultures to different concentrations of the antibiotic and plating the cultures onto agar to evaluate decreases in colonies. Plating for the MBC was performed at dilutions of 1/10 to 1/1000.

Killing Curves

Traditional killing curve tests were performed to confirm the presence of persister cells in our strains after growth in our conditions. Cultures were grown overnight in LB media at 37ºC and 250 RPM shaking. After ciprofloxacin was added to a culture, cells were plated at multiple time points over the curse of several hours. Plates were made using dilutions of cultures ranging from 1/100 to 1/10000000. Countable plates (typically 2 or 3) were averaged to determine the final count at a given time point.

Variations of the killing curve diluted overnight culture at 1/200 and regrew them to reach the early stationary phase (O.D. 600 of 1.2 or greater).

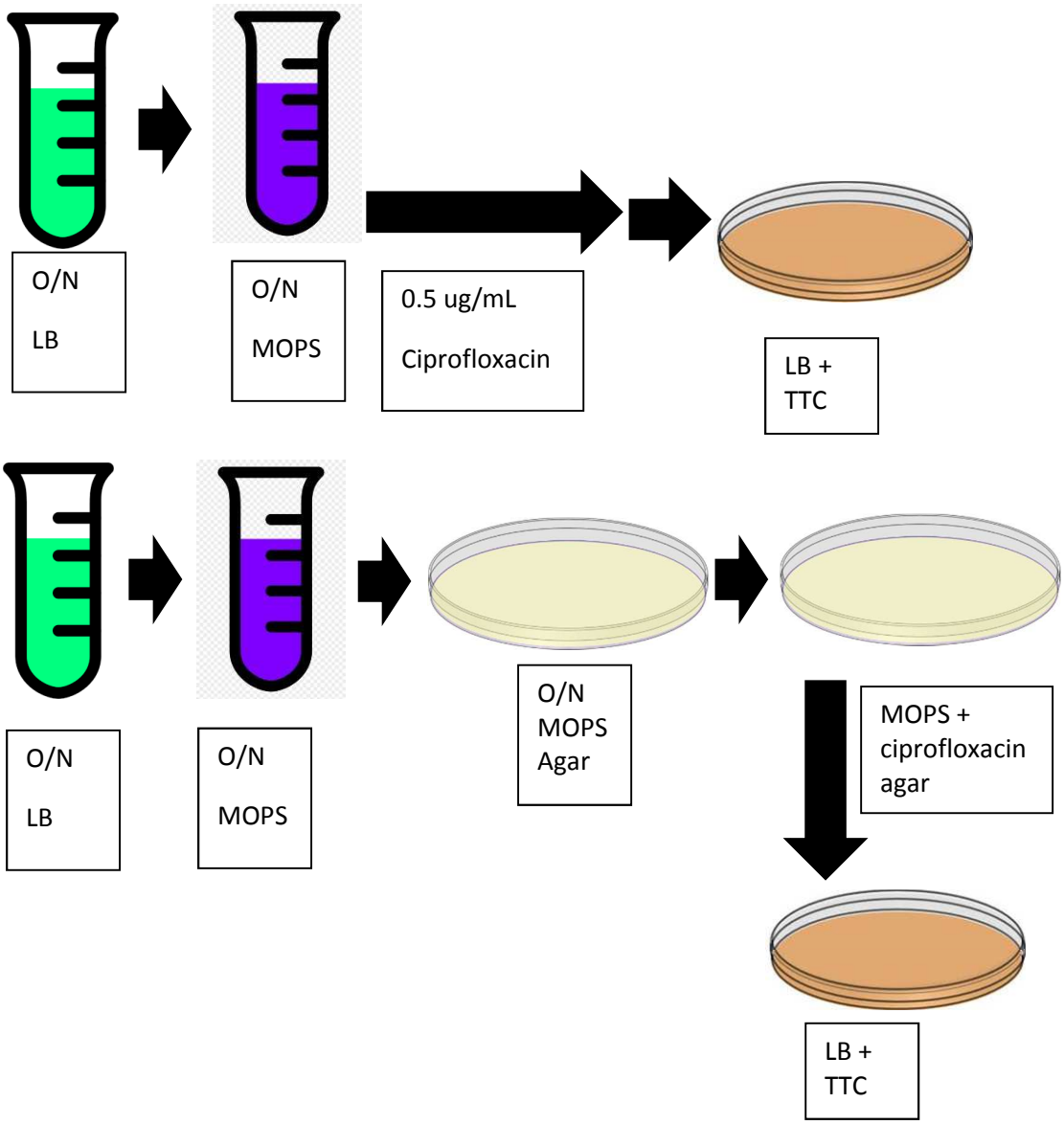
TTC Assay

This assay detects living, active cells. The reagent 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) is a redox sensitive reduced into 1,3,5-triphenylformazan (TPF) in cells that are actively respiring (16). Overnight cultures were grown in LB at 37^{o}C and 250 RPM shaking and then inoculated at 1/200 dilutions into 5mL of either MOPS minimal media or MOPS minimal media supplemented with 0.25 mM tryptophan. Cultures were regrown to the stationary phase under the same conditions.

The first variation of this assay was based in liquid media. One sample of each strain from each condition was placed on a nitrocellulose filter on LB agar containing TTC. Each liquid culture was then exposed to 0.5 ug/mL ciprofloxacin and incubated for one hour at 30^{o}C. Samples from each were then moved onto separate nitrocellulose filters on LB agar containing TTC. Both the controls and experimental samples were checked hourly for color development.

The second variation of this assay was based on solid media. Samples from each culture were taken at the same time as the controls and placed on nitrocellulose filters on MOPS agar media containing the same reagents as the liquid medias they came from. These cultures were grown overnight to accumulate biomass and moved onto new MOPS agar plates that contained 0.5 ug ciprofloxacin. They incubated for one hour at 30^{o}C and were moved to LB agar plates with TTC where color development was observed.

Figure 3.1: An outline of the TTC procedures. O/N means overnight culture.



MOPS Minimal Media

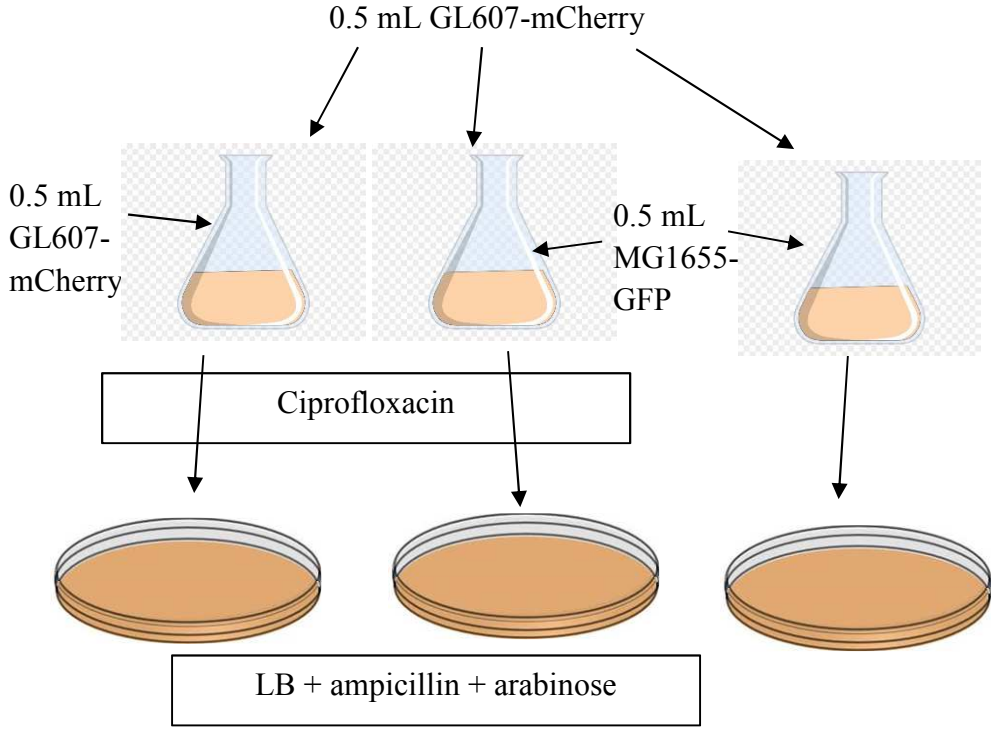
Table 3.2: The recipe for the MOPS buffer based minimal media that was used in some experiments.

Ingredient	**Volume per Liter**
MOPS buffer, ≥99.5%	100mL
Water	880mL
Potassium Phosphate, dibasic	10mL
Casamino Acid, 10%	10mL

Mixed Culture Experiment

MG1655-GFP and GL607-mCherry overnight cultures were grown at 37°C and 250 RPM shaking in the presence of 100 ug/mL ampicillin. We inoculated 600 mL of fresh LB containing 100 ug/mL ampicillin with 1.5 mL of GL607-mCherry. This culture was separated equally into two other flasks to create three flasks with 200 mL LB each to ensure equal inoculation of the strain. One flask was inoculated with an additional 0.5 mL of GL607-mCherry. The other two flasks received 0.5 mL of MG1655-GFP. One of them was set aside to monitor for changes that occurred for reasons other than antibiotic addition. These cultures were grown to stationary phase and 0.5 ug/mL ciprofloxacin was added. Cultures were plated at various times on LB agar containing 100 ug/mL ampicillin and 0.2% arabinose to induce fluorescence. Plates were counted using a transilluminator to fluoresce colonies long enough to take a photograph.

Figure 3.2: A visible guide to how the mixed culture experiment is performed. Incubation periods are excluded.



Peroxide Priming Experiments

Overnight cultures were diluted 1:100 into fresh 5mL LB test tubes and grown for around four hours into early stationary phase (O.D.600 = 1.2) (13). These cultures were then exposed to 0.3 millimolar hydrogen peroxide and incubated for one hour. They were then exposed to 0.5 ug/mL ciprofloxacin. Survival was recorded by plating to determine the concentration of CFUs in the cultures. Cells were plated at 1/10 to 1/1000 dilutions onto LB agar.

Kovac's Assay

To compare indole production in cells, we modified the Kovac's test for use with UV-vis spectroscopy. Overnight cultures of various *E. coli* strains were centrifuged at 4200 RPM for 10 minutes in an ultracentrifuge for 10 minutes and the supernatants were decanted into microcentrifuge tubes. For measurement, 200 uL of sample supernatants were mixed with 300 uL Kovac's reagent (Sigma Aldrich 60983), and 125 uL of ethanol in microcentrifuge tubes. These tubes were then mixed by inversion. The Kovac's reagent visibly changes color from yellow to purple upon exposure to indole. Absorbance was then measured in a UV-Vis spectrophotometer at the wavelength 530 nm (A530) using a mixture of fresh LB, Kovac's reagent, and ethanol in the same ratio as a blank. This test was later repeated using a microcentrifuge rotating at 13500 RPM.

Microscopy

Slides were prepared with 8uL of low melting temperature agar placed on a slide cover glass and covered with a second cover glass to spread the agar thin. After sitting for one minute to cool, the two glasses were slid apart so that the agar remained on one. Then 3uL of a culture sample were placed on the agar and the cover glass was set on a

microscope slide. The slide was left sitting to allow the agar to further solidify. Slides were then subjected to light microscopy under a 100x objective lens with immersion oil.

CHAPTER IV

RESULTS

Ciprofloxacin and Cefoxitin MBC and MIC

To determine the proper concentrations of ciprofloxacin and cefoxitin to introduce to cultures, we performed MBC and MIC testing. Cultures were first plated onto agar plates containing various concentrations of the antibiotic incubated overnight. Colonies were then counted. Results showed that growth ceased between a concentration of 0.01 ug/mL and 0.1 ug/mL for ciprofloxacin and at 10 ug/mL for cefoxitin.

To better identify the MBC of ciprofloxacin, fresh cultures were exposed to various concentrations of the antibiotic for one hour and then plated onto LB agar. This second test was designed to find a decrease in colony number as antibiotic concentration increased. We found that the minimum number of colonies occurred at 0.05 ug/mL. We recorded this as the MIC. A 10-fold increase of the MIC was selected as the MBC following standard practices within the laboratory. This is a slightly low MBC, but

it is comparable to values used by other labs (3, 7). The final values are recorded in Table 4.1.

Antibiotic	MIC	MBC
Ciprofloxacin	0.05 ug/mL	0.5 ug/mL
Cefoxitin	10 ug/mL	100 ug/mL
Ampicillin		100 ug/mL (24)

Table 4.1: MIC and MBC values for all antibiotics used in this thesis (15). Ampicillin's MBC was taken from recent work in the lab.

Effect of Indole on Persisters

We compared survival of the MG1655 wild type strain of *E. coli* to the survival of an indole-negative strain (GL607). The MG1655-hipA7 strain was included as well as a positive control due to the overabundance of persister cells that it produces (14). We expect that increasing the amount of indole that cells are grown with will increase the amount of persisters. In this experiment, stationary phase cells were plated onto agar, incubated for one hour, then misted with ampicillin, incubated for one hour, and misted with penicillinase. We saw that around the indole negative mutant that was grown with 1.0mM indole displayed 66% of the persisters that it displayed when grown with 0.5mM indole. (Figure 4.1). The wild type had a 37% increase in persisters compared to the mutant grown with 0.5 mM indole. The difference between both mutant cultures and the wild type lacks statistical significance, likely due to the high variance in survival within the wild type strain, but the difference between the two variations of the mutant is significant enough to say that the increase in indole does reduce survival under the conditions of this experiment. We suspect that the difference may be caused by potential toxic effects of indole on the mutant grown with 1.0mM, but they may be occurring in both strains. Because the samples are beginning to grow again once they are moved to the plate, it is possible that they are experiencing a much higher amount of indole than is appropriate for this new growth.

This data is inconsistent with our model because increased added indole negatively influenced mutant survival. We speculate that the decreased survival experienced by the mutant likely stems from the known toxic effect of indole while beneficial effects were not successfully induced (13).

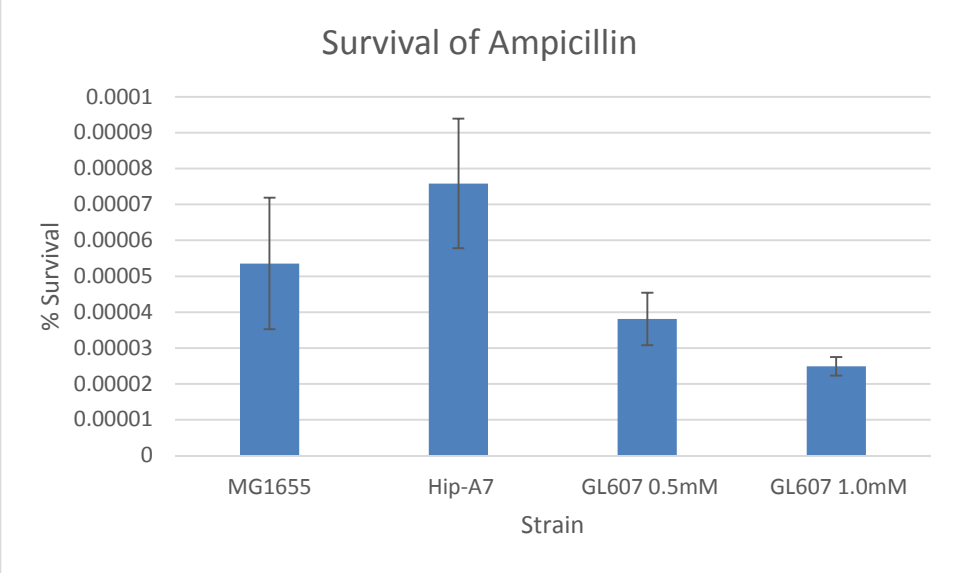


Figure 4.1: Percent survival of *E. coli* strains. Surviving colonies represent persister cells. Percent survival is calculated based on comparison of plates that underwent treatment with ampicillin and penicillinase to untreated plates that were plated at the same time from the same overnight culture. Error bars represent differences between biological replicants. Technical replicants from the same biological replicant were averaged together.

N_b=3, N_t=2

P values (two tailed) are as follows:

MG1655 vs Hip-A7: 0.207

MG1655 vs GL607 with 0.5mM indole: 0.245

MG1655 vs GL607 with 1.0mM indole: 0.0547

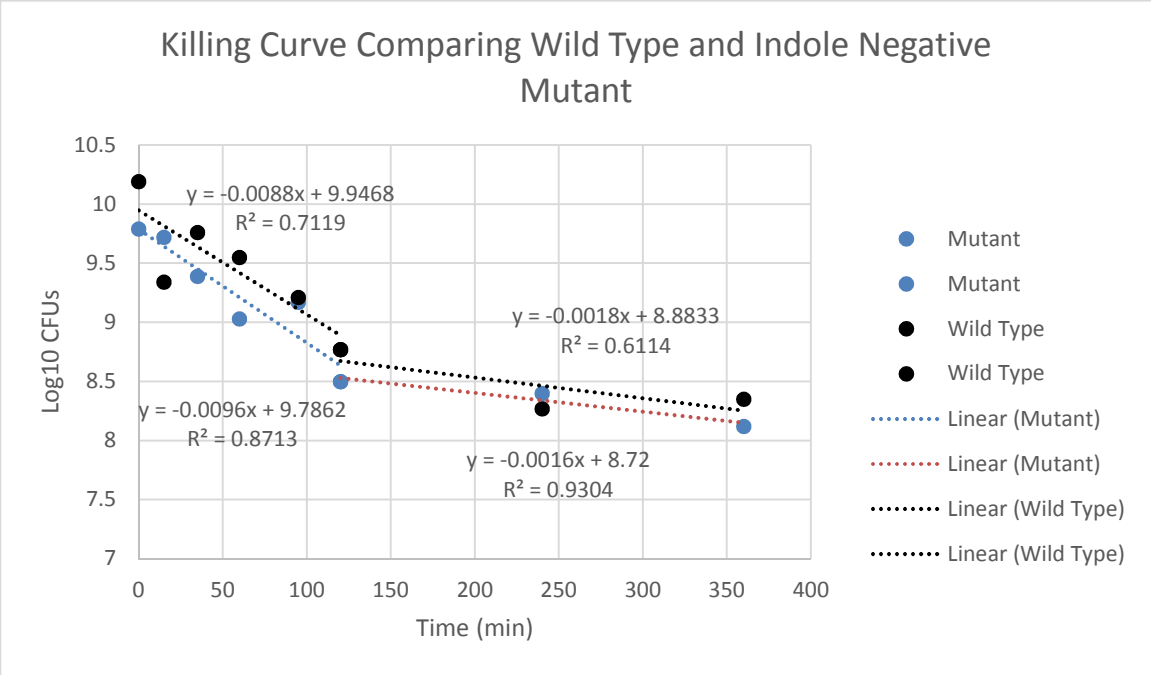
GL607 0.5mM vs GL607 1.0mM: 0.0420

Killing Curve to Confirm Persisters Within Wild Type and Indole-Negative Strain

To test that persister cells were occurring in cultures, a killing curve test was performed on wild type MG1655 and the indole negative mutant GL607. Liquid cultures were grown to stationary phase, exposed to Ciprofloxacin, and plated to count colony forming units (CFUs) over the course of 360 minutes (6 hours). Between 120 to 180 minutes into the experiment, the bactericidal activity of the antibiotic decreased (Figure 4.2). This decrease in death rate represents the much slower death of persister cells that occurs once the normal stationary phase cells are largely dead (3). While not as extreme as would be expected from cells grown in the exponential phase of growth, the changes in rate are still apparent for these stationary phase cells (3).

There was little if any difference between the two strains other than the initial number of cells (Figure 4.2). This suggests that the production of indole did not improve survival of the wild type. This falsifies the model that indole directly improves persistence. The results may also be caused by a variety of other processes in the stationary phase that support persister formation, such as starvation (3). This is likely because only around 10^2 to 10^3 cells are being killed. This suggests a need to redesign experiments to better evaluate stationary phase cultures. Changing the test to reduce other stationary phase causes of persistence could potentially occur by either performing the experiment early in the stationary phase or by adding stationary phase concentrations of indole to exponentially growing cultures. Both methods carry risks of changing other potential factors, such as what proteins are translated, that could be relevant to the stationary phase effects of indole.

Figure 4.2: The killing curve comparing the wild type and the indole negative mutant.



Killing Curve to Evaluate Exogenous Indole on Indole-Negative Mutant Cells

To evaluate the ability for indole to influence the indole-negative mutant, a second killing curve was performed that compared two cultures of the mutant. One culture was grown in LB while the other culture was grown in LB supplemented with 1.0 mM indole dissolved in methanol. This version of the killing curve was performed upon logarithmic phase cultures, but the supplemented indole is akin to stationary phase extracellular indole concentrations (13). From this test, we first observed the appearance of the biphasic killing curve, confirming that persisters were still produced under both conditions (Figure 4.3). There is little difference in the early, fast killing period (Figure 4.3). The second, slower portion of the biphasic curve provides an observable difference between the two. The culture grown with indole displayed a much greater decrease in CFUs during the later period where persister cells are killed by the antibiotic (Figure 4.3). This seems counter to our expectations; the added indole seems to affect the survival of persister cells negatively rather than influencing the formation of persister cells. This is not an expected result; differences were expected to appear in the earlier curves with fast antibiotic killing rather than in the second slopes. This may indicate that the persisters that appear in the culture with added indole are either more vulnerable or are leaving the persister state earlier and becoming vulnerable to the ciprofloxacin. A similar trend appears in later experiments.

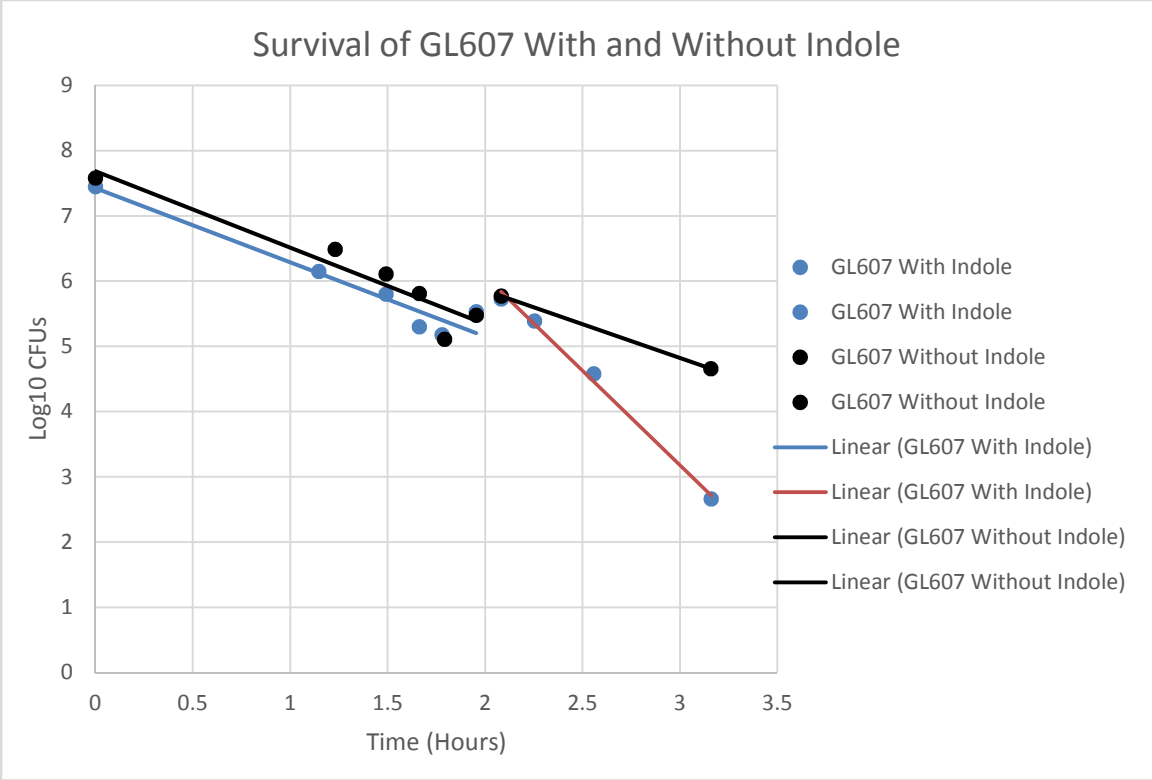


Figure 4.3: Change in CFUs over time for the culture grown with indole and the culture grown without indole.

Formulas:

GL607 with indole, early: $y = -2.895x + 11.865$ $R^2 = 0.9947$

Later: $y = -1.1374x + 7.4243$ $R^2 = 0.9375$

GL607 without indole, early: $y = -1.1714x + 7.6842$ $R^2 = 0.9104$

Later: $y = -1.032x + 7.9194$ R^2 excluded due to error in one time point

TTC Test to Evaluate Cellular Recovery from Antibiotics

To determine if indole influences a culture's ability to recover from antibiotic stress, we employed the redox sensitive dye TTC. We designed this test to evaluate the recovery of cells after exposure to ciprofloxacin in liquid and on agar. The wild type and indole-negative mutant were grown to stationary phase in MOPs minimal media with or without supplemented tryptophan. Performance was evaluated based on the observable appearance of color on sample. The appearance of color represents metabolic activity of a sample, which means that cells have left the persister state.

We first set up control samples to find differences between the growth of the strains before antibiotic was added. The quickest to grow were the indole-negative mutant that was grown with tryptophan and the wild type that was grown without tryptophan. The wild type grown with tryptophan was slower and the mutant grown without tryptophan was the slowest. These controls show that the presence of tryptophan alone does not directly influence colony growth of the mutant and wild type in the same way. This means that indole does influence the rate at which the cells will regrow, but these controls also include non-persister cells. We also learned from the controls that the cultures do not all regrow at the same rate when an antibiotic is not present.

After antibiotic exposure, we could observe how the different cultures regrew. For both the cultures exposed to antibiotic in liquid media and on solid media, metabolic activity took longer to appear than in the control samples. This is indicative that cells were not active immediately. We first tested cells exposed to antibiotic while in liquid media. Under this method, the wild type grown with tryptophan displayed metabolic

activity first. This shows that the production of indole provided a beneficial effect. When compared to the previous experiment, the results of both suggest that indole has some effect on cells leaving the persister state.

The cells that were exposed to antibiotic on agar were more difficult to comprehend. The mutant with added tryptophan displayed activity the earliest, while the mutant without tryptophan displayed activity at the latest point. The wild type cultures displayed activity at rates that were indistinguishable from each other. Based on observations of the samples, this appears to be directly related to the visible biomass of the samples; the larger (and taller) that the colony appears to be, the quicker activity returned. This is most likely due to the antibiotic being unable to reach many of the cells than persistence. It could potentially be related to another defense mechanism such as biofilm formation that can occur in high populations of cells. This portion of the experiment would require modification for understandable results to be obtained.

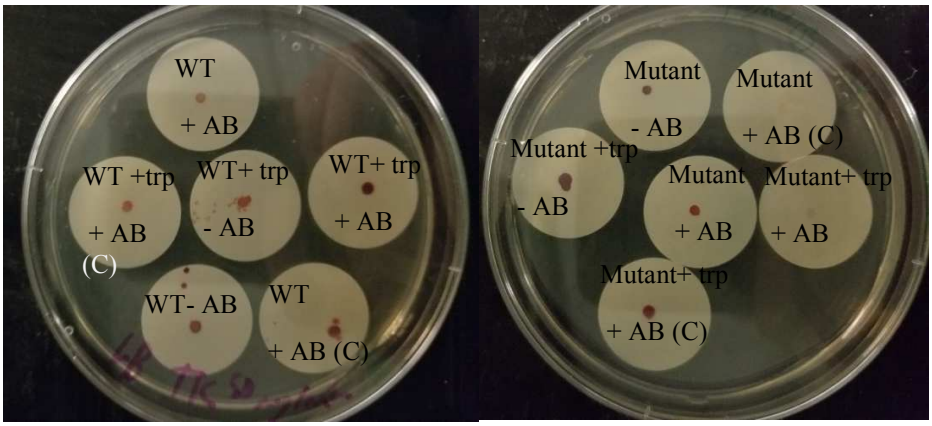


Figure 4.4: TTC plates containing one of each sample. Each sample shown is from the conclusion of the experiment. The left plate contains the wild type samples. The right plate contains the GL607 indole-negative mutant samples. In the labels, AB means antibiotic (ciprofloxacin). Cells that were exposed to ciprofloxacin on agar are denoted by "(C)."

Mixed Culture Test

Indole is expected to function as an intracellular signal (10). Under our hypothesis, a cell that produces indole would increase the number of persister cells of our indole-negative mutant strain. Since previous results have disagreed with our original hypothesis, we expected that any beneficial or harmful effect of indole on persistence would be equal between a strain that produces indole a strain that does not produce indole but is exposed to it. To test this, we established a mixed culture comprised of the wild type and indole negative mutant. Cells within this culture were also distinguished with red and green fluorescent proteins. Cultures in this test were grown to stationary phase before ciprofloxacin was introduced. Along with the other data, table 4.3, which covers one iteration of the test, was included for later discussion.

We first compared persister cell counts between the mixed culture and a culture of the indole negative mutant. We observed that the mixed culture displays greater, but varied, survival of the antibiotics than the culture containing only the indole-negative mutant (Figure 4.5). The cells that are surviving at and cultured at 1 and 3 hours are persister cells. This result may have some biological significance, and further testing will be performed to further evaluate it.

We also observed that the ratio of wild type (green) cells to indole-negative mutant (red) cells within the mixed culture were consistent over time as the antibiotic killing occurred (figure 4.6). We observed that the mutant was slightly higher represented throughout the entire course of the experiment. This most likely occurred because indole-negative cells reach a higher cell density in the stationary phase (13). That would cause

the inoculum of mutant cells to be higher when the culture was initially established. This result suggests that the indole being produced is influencing both strains within the mixed culture.

Time	Culture	Mutant CFUs/mL ($x10^9$)	Wild Type CFUs/mL ($x10^9$)	Percent of Wild Type in population	Culture Survival %
0 Hours	GL607 mCherry	3.51	0	0	100
0 Hours	Mixed + Control	0.89	0.85	47	100
0 Hours	Mixed Test	1.29	0.90	41	100
1 Hour	GL607 mCherry	0.698	0	0	20
1 Hour	Mixed Test	0.347	0.230	40	26
3 Hours	GL607 mCherry	0.604	0	0	17
3 Hours	Mixed Test	0.261	0.250	49	23
24 Hours	GL607 mCherry	0.635	n/a	n/a	18
24 Hours	Mixed Test	0.140	n/a	n/a	6.3
24 Hours	Mixed + Control	0.90	n/a	n/a	52

Table 4.2: Data from a single iteration of the test was performed over a total of twenty-four hours. The mixed positive control refers to a mixed culture that was not exposed to ciprofloxacin.

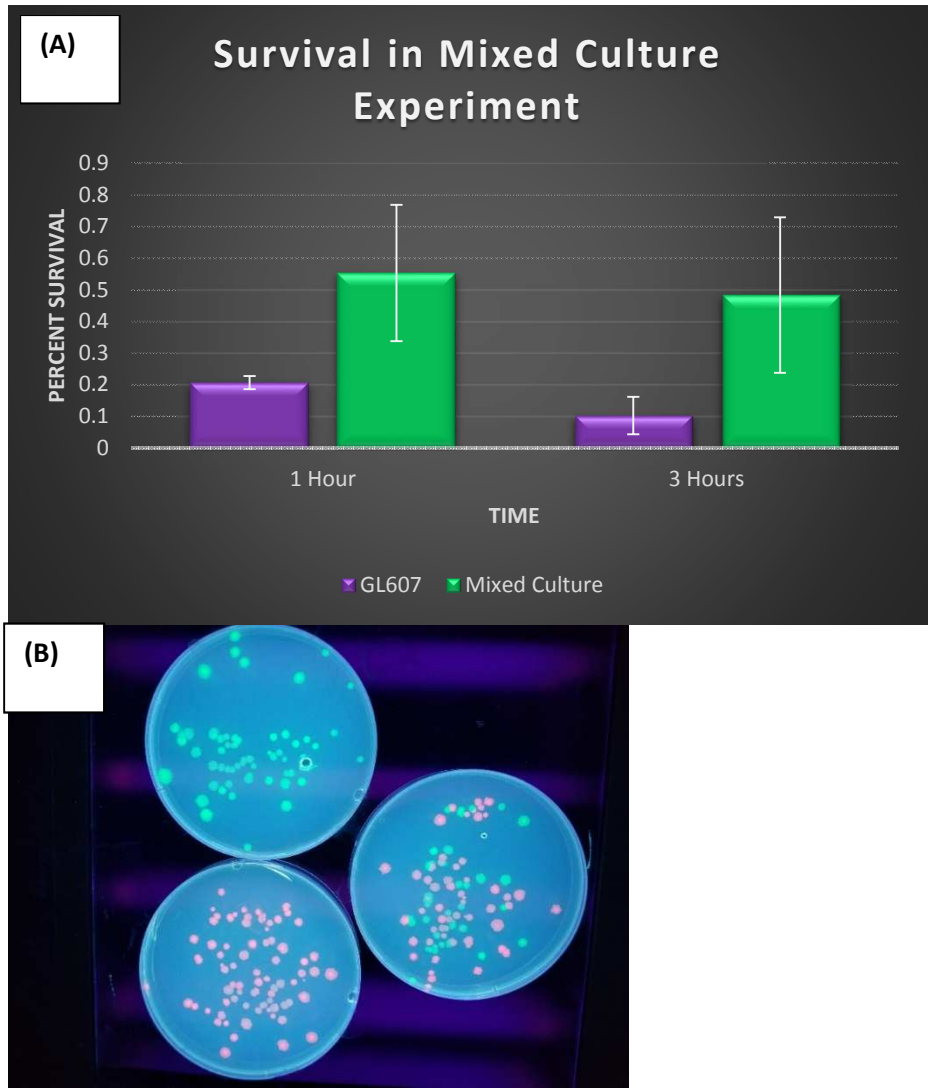


Figure 4.5: Persister formation improves in the mixed culture. (**A**) Survival comparison of the mixed culture and GL607 culture. Survival is based on CFUs at the time of plating compared to CFUs before ciprofloxacin addition. (**B**) A sample image of plates fluorescing containing two mixed culture plates and one mutant only plate.

N_t=3

P values (2 tailed):

Percent survival at 1 hour, mixed culture vs GL607 only: 0.109

Percent survival at 3 hours, mixed culture vs GL607 only: 0.121

Change in percent survival from 1 hour to 3 hours for GL607: 0.03

Change in percent survival from 1 to 3 hours for the mixed culture: 0.76

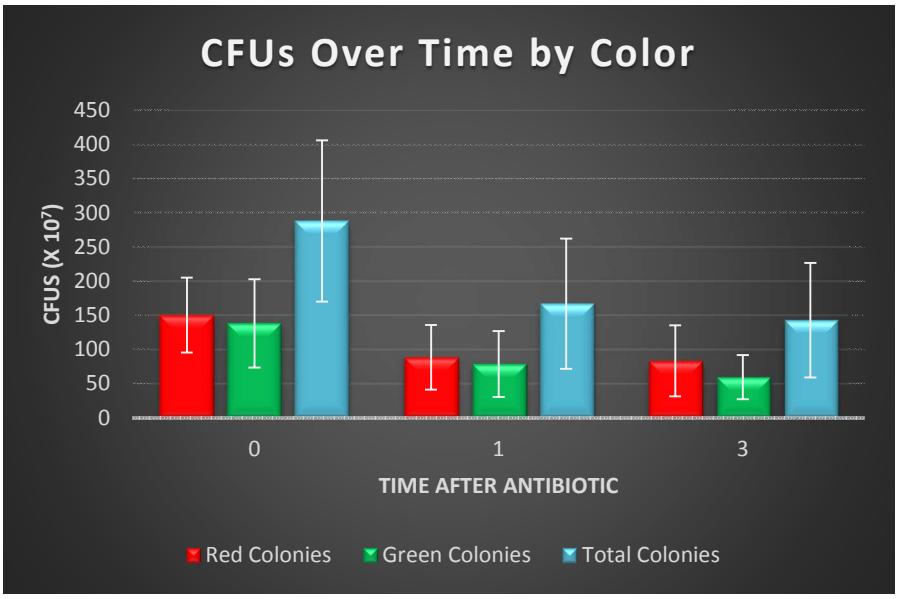


Figure 4.6: Percentage cells by color at the points in the mixed culture.

N_t=3

P values (2 tailed):

Total colonies, time 0 to time 1: .240

Total colonies: time 1 to time 3: 0.757

Peroxide Priming

We sought to evaluate if known indole regulated genes were involved in indole's influence on persister cell formation. Because genes in the oxidative stress pathway are upregulated by indole, we attempted to induce activity of the pathway before introducing ciprofloxacin to early stationary phase cultures. This was performed by adding peroxide in a concentration below its MBC to cultures of our wild type and indole-negative mutant.

We first tested how the wild type responded to different concentrations of peroxide, to determine what concentration to use for future testing. We found that 0.1mM peroxide worked well for this test. Data from several additional trials are included as well (table 4.3). These trials include incubation with 0.3mM peroxide and a wild type culture that was exposed to ciprofloxacin when other strains were exposed to peroxide. Survival is recorded as the ratio of the concentration of CFUs present one hour after ciprofloxacin to the concentration of CFU's before ciprofloxacin. This means that death caused by peroxide addition does not impact the survival statistic, and the experiment is designed to minimize that factor (Table 4.3). We observed a 20 to 30 percent increase in survival when the wild type was treated with peroxide, suggesting an increase in persister formation (Table 4.3). Peroxide caused very little cell death in most cultures that were exposed to it (Table 4.3).

The indole-negative mutant's survival only increased by 10 percent when peroxide was added. This suggests that indole is relevant to the results seen in the wild

type (Table 4.3). This test supports the hypothesis that activating the oxidative stress response can improve persister formation, especially when indole is present as well.

Culture	CFUs Before Peroxide ($x10^9$)	CFUs Before Ciprofloxacin ($x10^9$)	CFUs After Ciprofloxacin($x10^9$)	Percent Survival
GL + peroxide	2.79	2.74	0.98	35%
GL - peroxide	2.81	2.70	0.79	29%
MG-1	1.73	1.75	0.45	26%
MG-2	1.70	1.66	0.23	14%
MG 0.3mM Peroxide	1.41	1.01	0.51	50%
MG 0.1mM Peroxide	2.29	2.33	0.98	44%

Table 4.3: CFU counts for the peroxide priming experiment. Percent survival refers to CFUs after ciprofloxacin compared to the CFUs before ciprofloxacin.

Legend

GL+: GL607 strain with 0.3mM peroxide

GL-: GL607 strain without added peroxide

MG-1: MG1655 strain without peroxide that was exposed to antibiotic when samples were exposed to peroxide

MG-2: MG1655 strain without peroxide that was exposed to antibiotic at the same time as other samples

MG 0.3: MG1655 strain with 0.3mM peroxide

MG 0.1: MG1655 strain with 0.1mM peroxide

Comparison of Extracellular Indole in *E. coli*

Different *E. coli* mutants may produce different amounts of indole, especially if the mutation is in a gene related to indole activity. To test this, we utilized a modification of the Kovac's test for indole to be used with UV-vis spectroscopy (13). This test first required the development of a standard curve over a range of indole concentrations. Achieving readable results in the standard curve required using indole concentrations far below those that we expect to occur in *E. coli* cultures (Figure 4.7). For this reason, the supernatant samples were diluted to 1:10 and to 1:100.

From the indole-negative mutant, we saw no absorbance (Table 4.4). This was expected from the strain and confirmed the validity of the test. The MG1655 wild type and the MG1655-*hipA7* mutant displayed comparable results to each other. We would expect this because while the *hipA7* mutant does overproduce persister cells, it is not known to change indole production. Compared to our standard curve, the values we received from various dilutions of the samples did not correlate with expected extracellular concentrations in the indole-positive cells. It is possible that our wild type and our hipA7 mutant produce significantly less indole than the expected range of 0.5mM to 1.0mM. We suspect that our methods do not enable complete separation of extracellular indole from the cell pellet upon centrifugation. Indole's nonpolar nature may limit its ability to separate from the cell pellet.

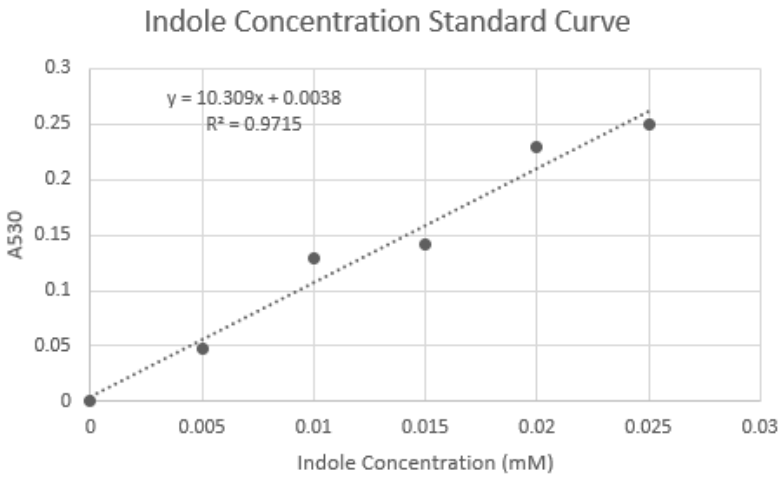


Figure 4.7: Indole concentration standard curve.

Sample	A530	Adjusted Indole Concentration (mM)
MG1655 /10	.133	0.12
MG1655 /100	.02	.16
GL607 /10	0	0
MG1655 hipA7 / 10	.160	.14
MG1655 hipA7 /100	.019	.15

Table 4.4: Samples and absorbance values for the strains as well as the calculated supernatant concentration of indole. The adjusted concentration is equal to the concentration that was found via the standard curve multiplied by the dilution factor.

We performed two other variations of the test. The first was to test indole production in the MOPs media that was used in the TTC test. We suspected that less indole would be produced from the wild type grown in that media. Such a difference may reduce any effect that indole has. The absorbance reading taken from an undiluted sample corresponded to an order of magnitude less indole than the previous samples (Table 4.5). While previous results lead us to conclude that this is not an exact measurement of the indole produced by the sample, it does suggest that less indole is produced when grown in the MOPs minimal media.

Sample	A530	Indole Concentration (mM)
MG1655 MOPs	0.143	.014

Table 4.5: Absorbance values for the Kovac's test performed to evaluate indole production of the wild type strain in minimal media.

The second variation used the wild type and indole-negative strains and changed to a microcentrifuge that was capable of greater RPM. We observed low readings from this method as well (Table 4.6). From these tests, we believe that much of the extracellular indole in the cultures remains within the cell pellet. At best, the Kovac's test may be useful to roughly compare strains but is not sufficient to properly quantify indole concentrations.

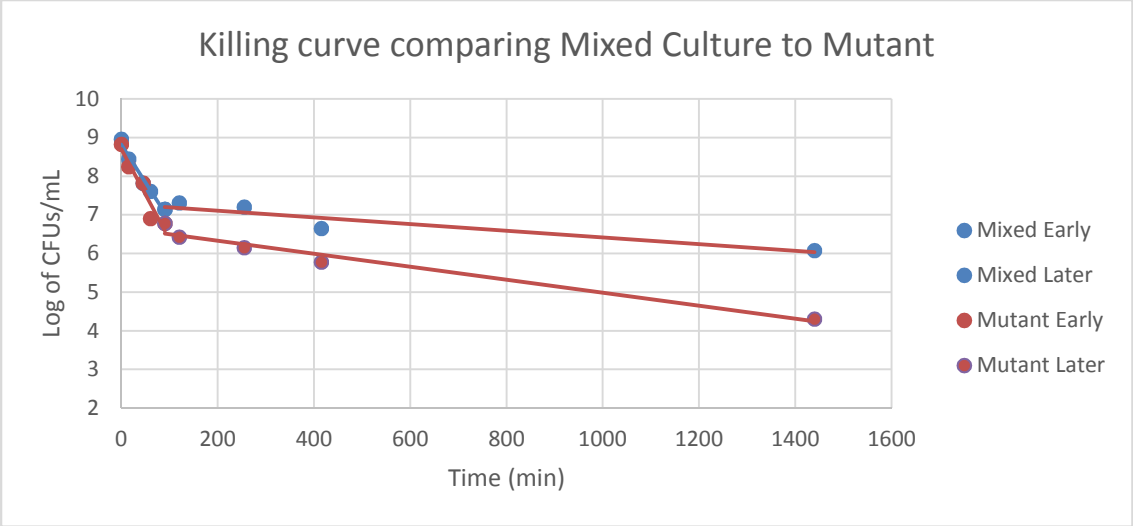
Sample	A530	Adjusted indole concentration (mM)
MG1655 /10	0.194	0.18
MG1655 /100	0.032	0.27
GL607 /10	0	0

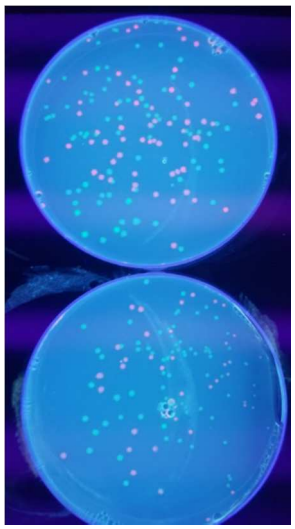
Table 4.6: Values recorded and calculated from the second iteration of the Kovac's test to compare indole production in the MG1655 wild type and GL607 mutant strain.

Early Stationary Phase Mixed Culture Killing Curve

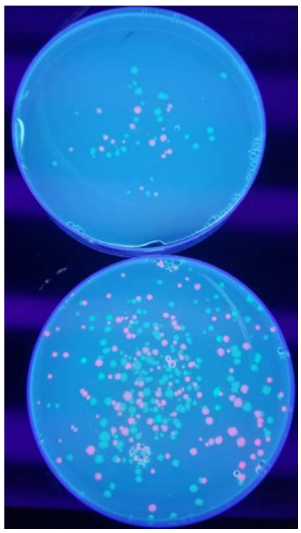
Persisters become more common within the stationary phase of growth (1). We sought to reduce this effect within a previous experiment by repeating it at the early stationary phase. The mixed culture previously displayed a difference from the indole-negative mutant. The indole produced by the wild type in the mixed culture appears to have some influence on the mutant cells that are grown with it. In this iteration of the test, overnight cultures were diluted into fresh LB and grown until O.D.600 reached at least 1.15, a point that is representative of growth slowing upon transition to the stationary phase. This was performed in several segments to make different comparisons.

The first segment of this test was a killing curve to compare the efficacy of ciprofloxacin killing on a mixed culture of the wild type and indole negative mutant versus the indole negative mutant on its own. We predicted that the mixed culture would outperform the mutant like it did in the previous mixed culture test, but that both strains would experience more cell death. This was performed as a curve rather than with a small selection of data points to improve the search for differences. The O.D.600 before antibiotic addition was 1.318 for the mixed culture and 1.260 for the mutant culture. The initial death rate was close between the two cultures, but greater within the mutant. The difference became more pronounced during the second, slower killing period (Figure 4.8). This led to a difference of around $10^{1.5}$ to 10^{2} more cells within the mixed culture. (Figure 4.8). This means that the persisters in the mixed culture are surviving better under longer antibiotic exposure.





Mixed Culture at 0 minutes.



Mixed Culture at 60 minutes.

Mixed Culture at 24 hours.

Figure 4.8: Killing curve comparing the mixed culture (blue) to the mutant culture (red). Both trends are broken into two segments to enable the separation of curves. CFU counts are averaged from countable plates across multiple dilutions from 10^8 to 10^1. This provided at least 2 separate plates at each time point.

The mixed culture within this experiment was also monitored to compare the proportions the two strains that comprised it. We found that the strains were initially nearly equal in proportion, but that the wild type became the majority of cells early under antibiotic exposure (up to 70% wild type) but became closer to equal later (Figure 4.9). The proportion of each strain returns near the starting proportion after 120 minutes, but then becomes more separate again at 255 minutes. At 415 minutes and 1440 minutes, the culture is divided into a ratio of around 45% mutant to 55% wild type. This ratio is what we expected to see throughout the experiment. The greatest difference between strains is at 45 minutes, where the mutant is only about 38% of the culture. It appears that in the early stationary phase, there may be some difference in the rate that each strain in the mixed culture dies at, but that the differences lead to a limited difference by the end of the experiment.

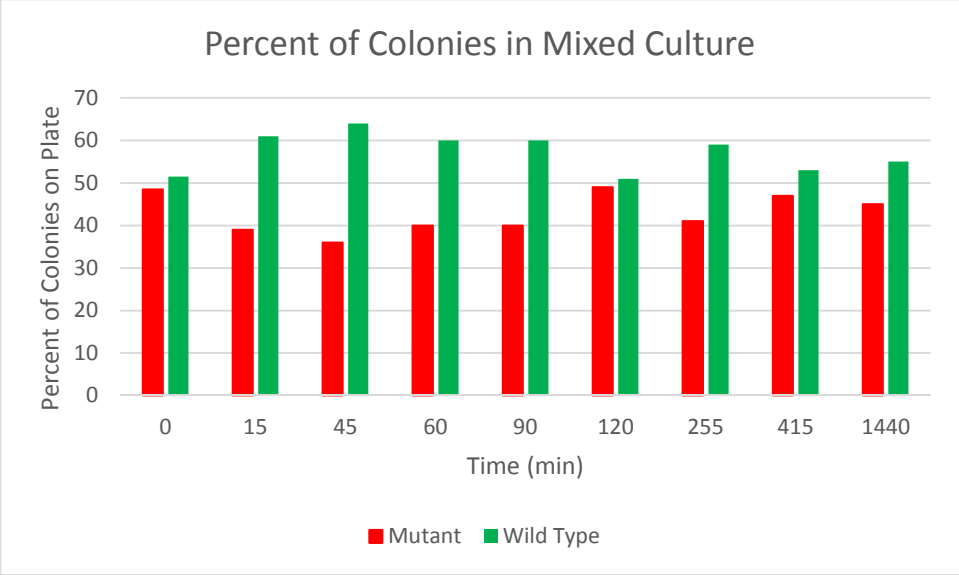
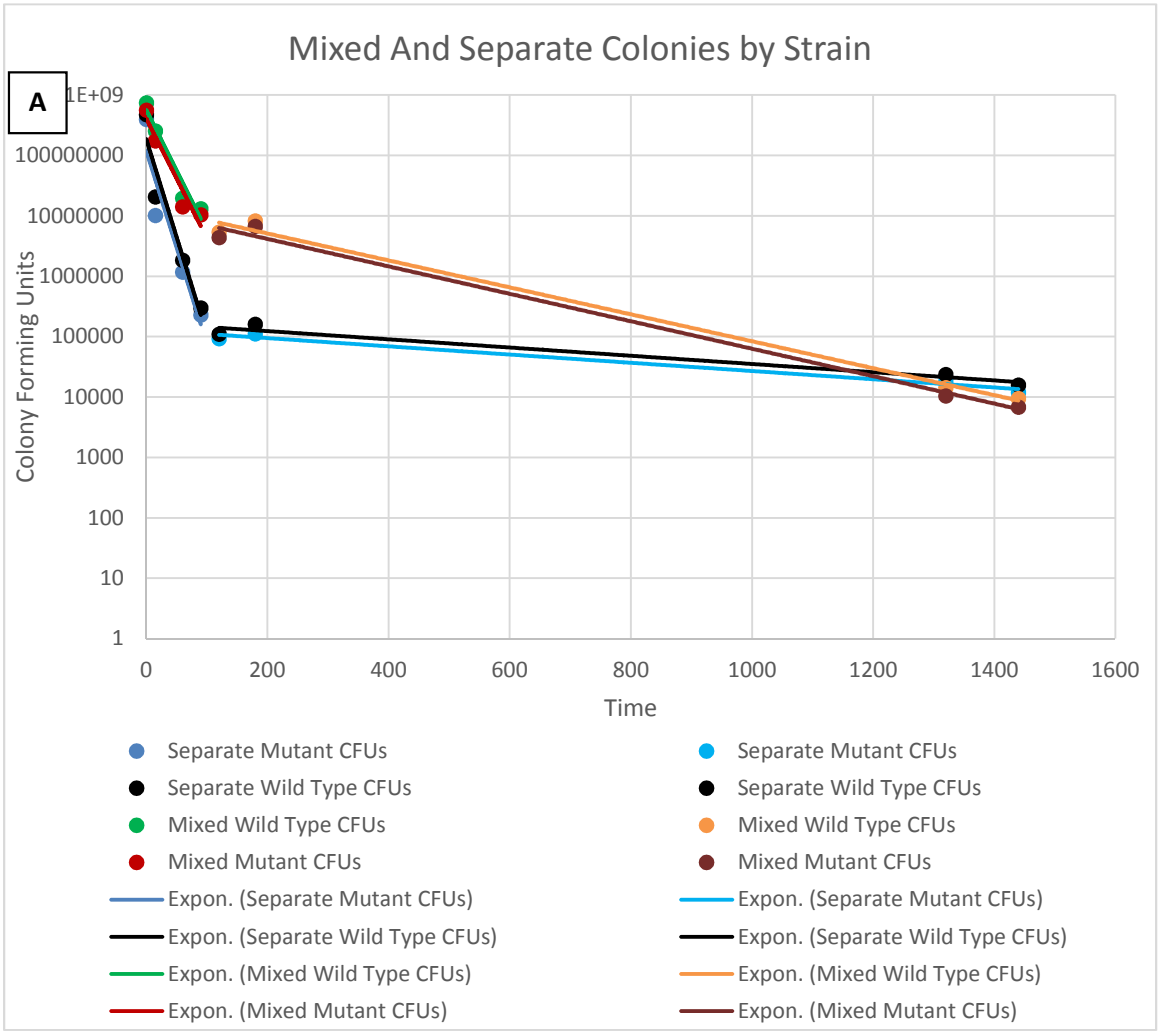


Figure 4.9: The proportion of each strain that the mixed culture is comprised of at the various time points. The ratio was determined from the same plates as Figure 4.8.

The second segment of this test was performed to check if growing the cells together altered survival or if there was another effect of the mixed growth. For this test, a mixed culture was grown to early stationary phase alongside the wild type and mutant grown separately. The mixed culture reached OD600 1.172. The wild type culture reached OD600 1.164 and the mutant culture reached OD600 1.169. These values are lower than the previous test, meaning that antibiotic exposure occurred earlier in the stationary phase. This may affect survival. Each of the three cultures was treated with ciprofloxacin and plating was performed with the two separate cultures mixed at the time of plating to provide a more direct comparison. The result from this test showed that the mixed culture displayed greater survival during the initial course of antibiotic killing but worse survival during the second, slower period of antibiotic killing (Figure 4.10 A). After 24 hours of antibiotic exposure, the amount of CFUs in the culture was similar between the cultures despite the differences up until that point. The proportion of cells of each strain was similar between the mixed culture and the separate cultures. This data shows that while the mixed culture survived better, indole does not appear to play the role of protecting the cells in the way that was previously thought. There does appear to be some advantage caused by the mixed culture. The difference in both the persister and non-persister death suggests that indole alters the formation and survival of persister cells (Figure 4.10A).



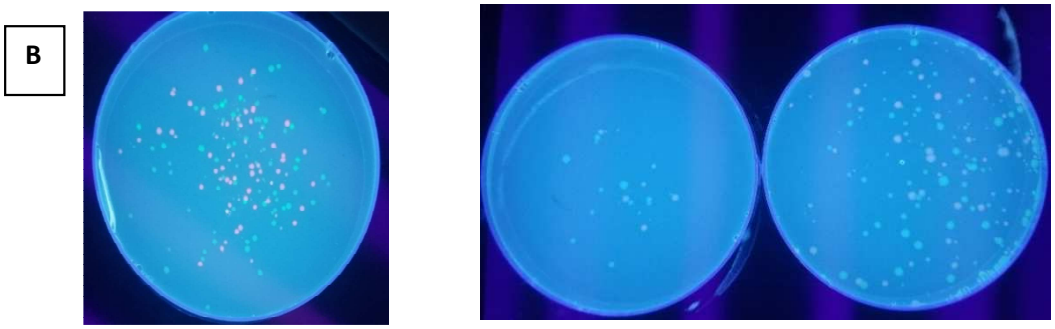
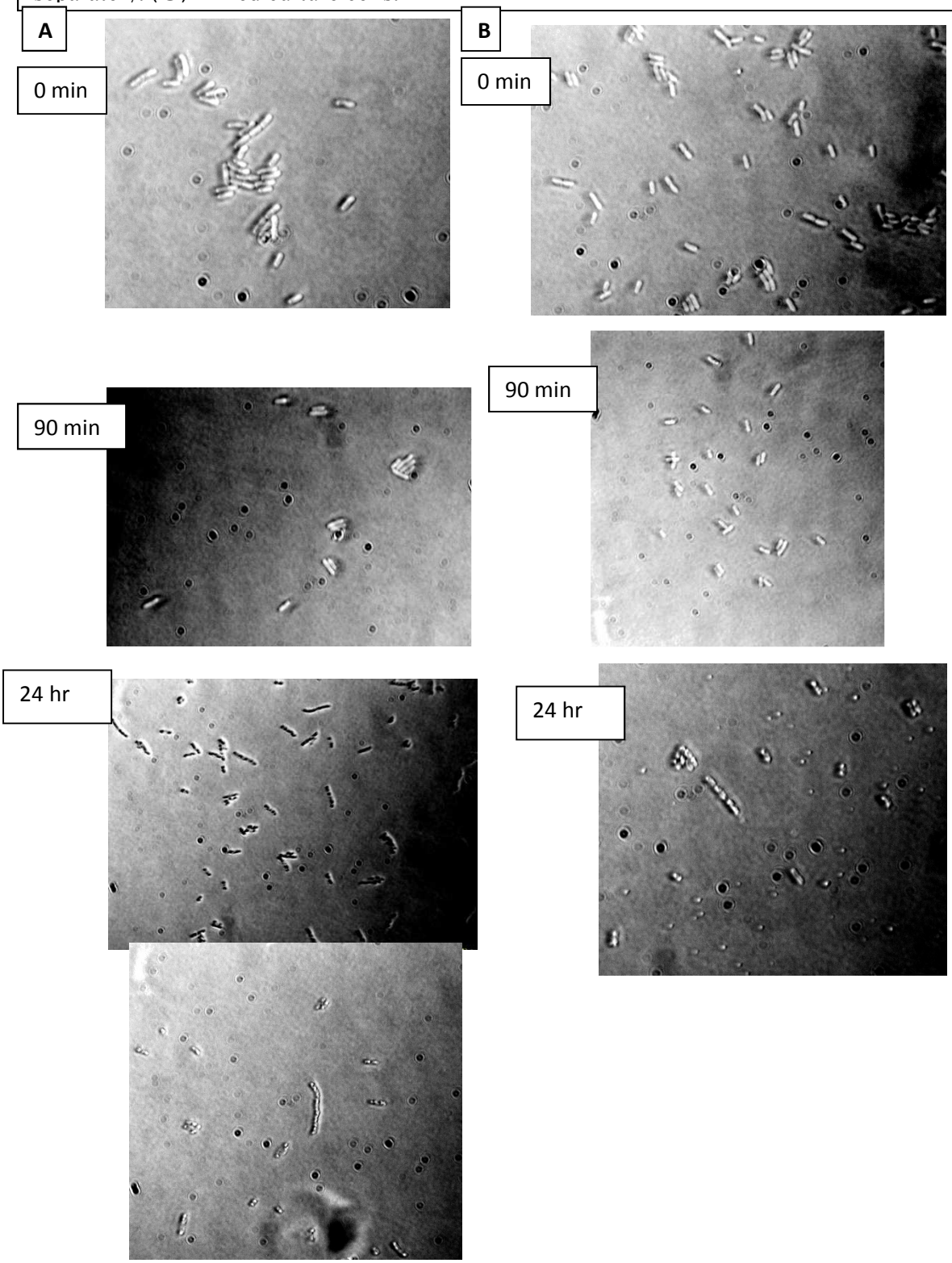


Figure 4.10: (A) Comparison of the amount of CFUs of each strain in each system. **(B)** Sample images from the experiment. The plate on the left is one of the mixed plates at 90 minutes and shows excellent coloration. The plates on the right are both mixed plates from the 24-hour time point and show much weaker coloration but was still sufficient to distinguish cultures.

Microscopy of Mixed and Separate Cultures

To gain understanding of the differences between the cultures from the previous experiment, we evaluated new samples under light microscopy. Samples of cells at various time points were placed on agar pads to fix them to slides. Several photographs were taken at each time point and a few of notable interest are shown. Before antibiotic, each sample displayed a typical rod-shaped morphology (Figure 4.11). After twenty-four hours of exposure to ciprofloxacin, the mutant strain displayed a small number of groups of cells positioned together as a sort of "chain" (Figure 4.11 A). The "chains" are most likely cells that did not separate after replication. We also observed that mutant cells appeared to take on a shorter morphology that was more circular than rod shaped. The wild type also displayed shorter cells, but rod shaped cells were still fairly observable after twenty-four hours of ciprofloxacin exposure (Figure 4.11 B). Cells in the mixed culture were even less likely to display the circular morphology, though they were not as long as the mixed cells were before antibiotic addition (Figure 4.11 C). This may mean that the cells in the mixed culture were beginning to leave the persister phenotype and regrow. Such cells would be subject to the effects of ciprofloxacin. The disruption in DNA replication caused by ciprofloxacin in newly growing cells could cause a loss of cell division. The rounder cells seen in the separate cultures are likely inactive cells that are late stationary phase persister cells.

Figure 4.11: Photographs from microscopy under the 100x objective lens. Selected images are cropped slightly to preserve space. (**A**) Mutant cells grown separately. Two images were included to help illustrate the morphology. (**B**) Wild type cells grown separately. (**C**) Mixed culture cells.



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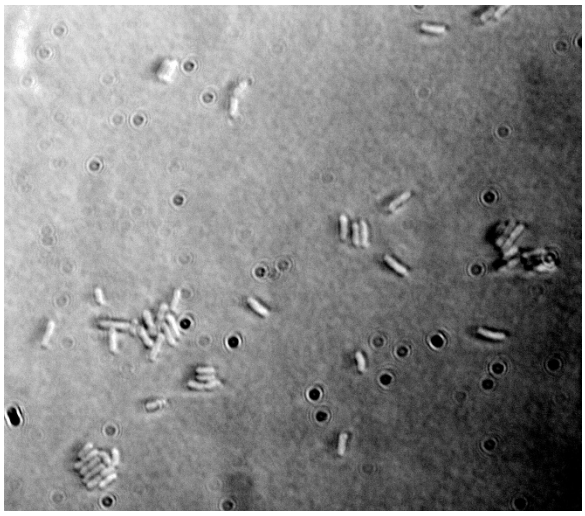

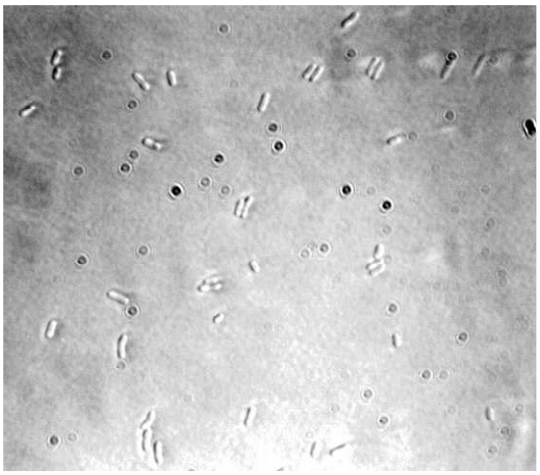

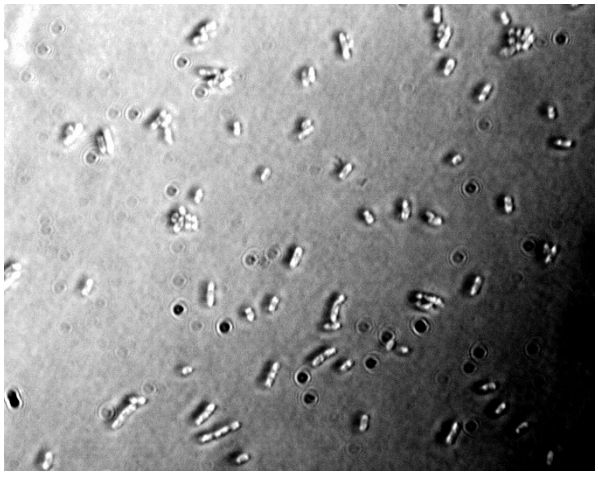

Persister Formation Varies Within Single Gene Deletions

To learn how these various deletions affected persistence, the baseline persister assay was repeated on these strains. The strains were grown overnight, plated, and subjected to 0.5 ug/mL ciprofloxacin and plated after one hour to compare CFU counts before and after the antibiotic was added. We observed wide variance between different cultures of the same strain, placing almost any two strains within one standard deviation of each other. The averages themselves vary respectably, with the strain JW1299-1, the PspC deletion, displaying the lowest average percent survival and JW 1297-1, the PspA deletion, displaying the marginally highest average survival (Figure 4.12). This assay was unable to demonstrate differences between the strains due to the error margins.

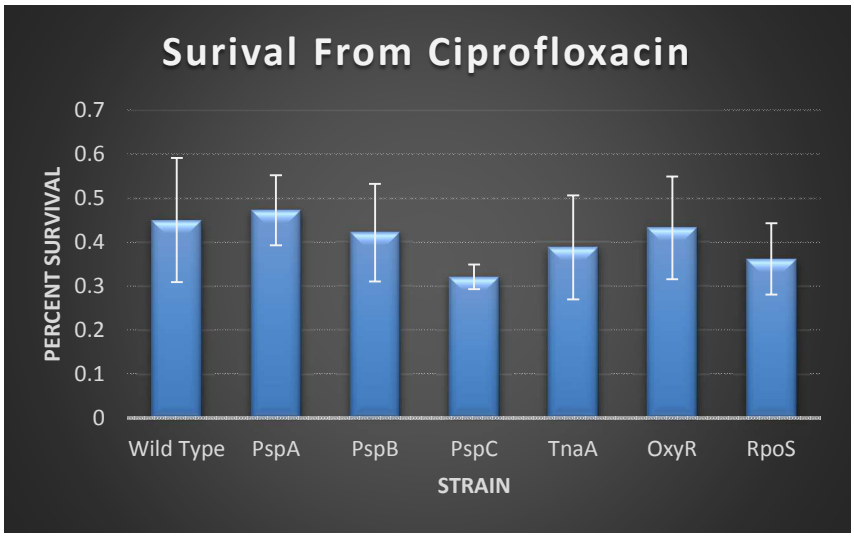

Figure 4.12 Survival percentages of the various new strains based on one hour of exposure to ciprofloxacin. Survival percentage is calculated as CFUs after ciprofloxacin / CFUs before ciprofloxacin. Error is calculated from three technical replicates. Each technical replicate was the average of 2 biological replicates across different plated dilutions.

Peroxide Assay of Single Gene Deletion Strains

We expect genes that are upregulated in the presence of indole to influence persister formation (10). To test this, we applied the peroxide-based assay to a collection of single gene deletion strains that were used in the previous persister assay. These genes are either directly upregulated in the presence of indole or in the same pathway as genes that are upregulated by indole (10, 42). If they are relevant to persister formation, then the deletions should display fewer persisters. We observed a wide variance between multiple cultures of the same strain, like in the persister assay that utilized these strains. Unlike in the trial experiment for this assay, the mutants in this set experienced significant death after the peroxide was added (Figure 4.13). The *oxyR* and *rpoS* deletions displayed incredibly poor survival of the peroxide. Worse performance was expected from them because the deleted genes are directly relevant to surviving stress caused by reactive oxygen species. The degree of death experienced by those strains was greater than expected and prevented meaningful data to be collected about their survival of ciprofloxacin. For this reason, the strains have been excluded from the shown data.

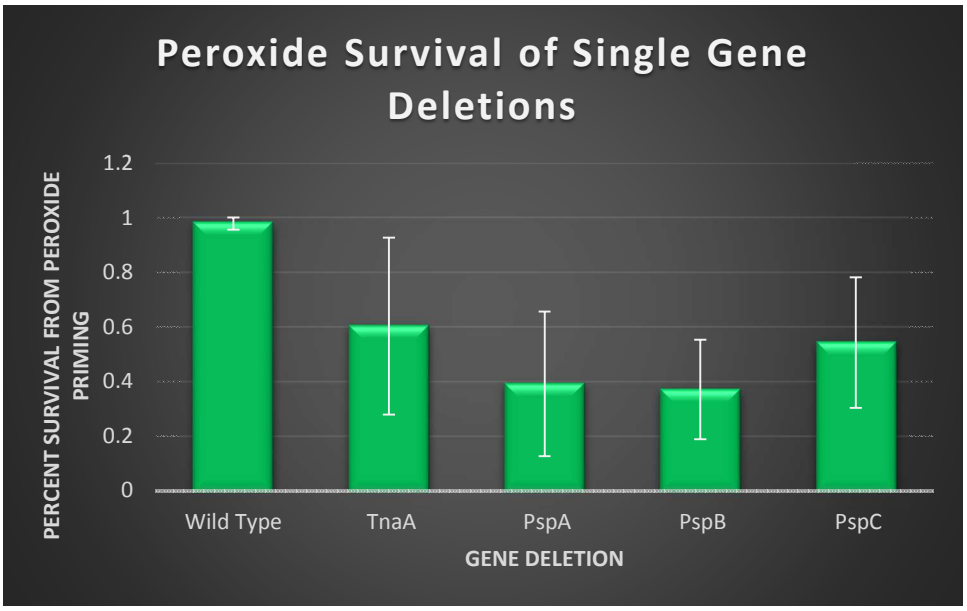

Figure 4.13: Peroxide survival of four of the deletion strains. The RpoS and OxyR strain were excluded from the graph because of how low the percentage of survival was for the two.

Survival of ciprofloxacin followed a trend that appears to be the inverse of the peroxide survival. Strains that experienced limited cell death from the peroxide showed lower survival from the ciprofloxacin (Figure 4.13, 4.14). This means that while different strains were more susceptible to peroxide, it may not have influenced persister formation. This data also implies that the phage shock response overlaps slightly with the oxidative stress response, since deletions of phage shock genes reduced peroxide survival.

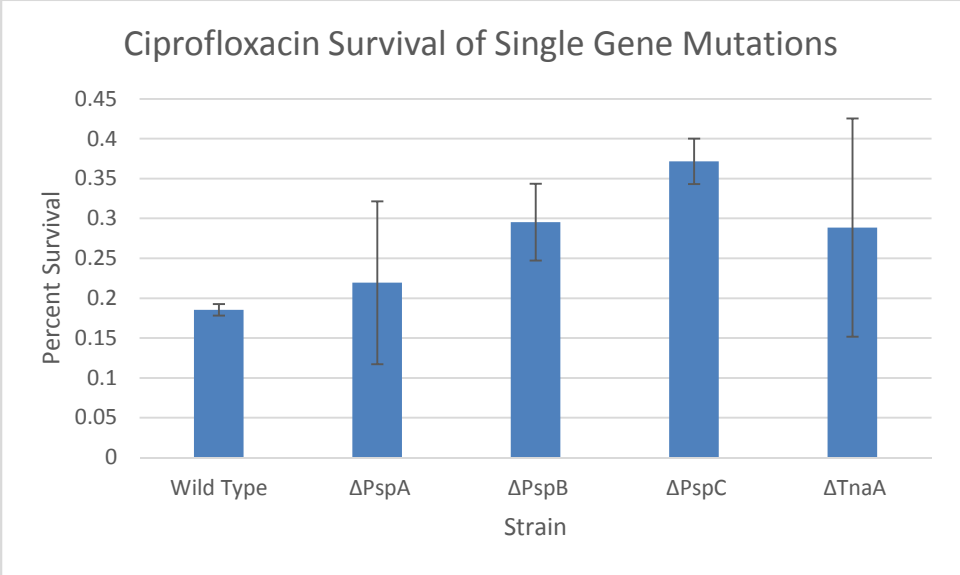

Figure 4.14: Survival of the strains following exposure to the antibiotic ciprofloxacin. The percentage is calculated as the CFUs after antibiotic / CFUs before antibiotic but after peroxide.

P values (2 tailed) are as follows:

Wild Type vs ΔPspA: 0.500

Wild Type vs ΔPspB: 0.031

Wild Type vs ΔPspC: 0.004

Wild Type vs ΔTnaA: 0.207

Kovac's Test of Single Gene Deletions

To learn if the gene deletions caused a difference in indole production, we performed the Kovac's test on them. It has been suggested that indole upregulates the pathways that these genes participate in (10). The reverse may also be true, or there may be a method of feedback occurring within the indole signaling pathway. A change in indole production in the strains would show that such an effect is happening. The BW25113 wild type was used as a baseline of indole production to compare the other strains to, and the indole negative TnaA deletion was included as a negative control to confirm the absence of extraneous indole in the media. For each mutant, we found a reduction in indole concentration in the 1/10 dilution (Table 4.7). The same occurred among the 1/100 dilutions except for the PspC dilution.

Strain	A530	Adjusted Indole Concentration(mM)
Wild Type/10	0.296	0.28
ΔPspA/10	0.183	0.17
ΔPspB/10	0.172	0.16
ΔPspC/10	0.184	0.17
ΔOxyR/10	0.188	0.18
ΔRpoS/10	0.178	0.17
ΔTnaA/10	0.000	0.00
Wild Type /100	0.036	0.31
ΔPspA/100	0.028	0.23
ΔPspB/100	0.027	0.22
ΔPspC/100	0.044	0.39
ΔOxyR/100	0.030	0.25
ΔRpoS/100	0.026	0.21

Table 4.7: Absorbance values for the Kovac's test as performed on the collection of single gene deletion strains. Adjusted indole concentration refers to the indole concentration found from the standard curve multiplied by the dilution factor.

CHAPTER V

CONCLUSION

The core knowledge to take from the data is that the effect of indole is less obvious in the stationary phase of bacterial growth. As cells progress into the stationary phase, persisters become a greater portion of the population. Throughout the physiological changes from exponential to later stationary phase growth, indole influence seems to shift from influencing the speed that persisters regain metabolic activity, to increasing persister formation, to a limited influence that is overshadowed by other mechanisms in the later stationary phase.

There may be a beneficial effect that is reduced compared to the exponential phase of growth. That most likely stems from the increase in triggers of the persistence phenotype that occur by the time that cell cultures have entered the stationary phase. Indole production spikes upon entry to the stationary phase, and both intra- and extracellular concentrations of indole remain higher than the exponential phase

concentration once that spike is resolved (13). Persisters were historically evaluated during the exponential growth phase. Cells are more susceptible to antibiotics during this phase of growth than the stationary phase (3). This provides a clear distinction between the techniques used and the physiological factors surrounding indole; effects that it has on persistence needed to be evaluated at the stationary phase.

When evaluating our data, a few important trends appear. The first is that when attempting to observe survival in the stationary phase, antibiotics are much less effective at cell killing than they are in the exponential phase (9, 10). This limits differences in survival of different cultures and weakens statistical evaluation. A second issue that appears is the variance in survival occurs in many cultures between technical replicates. The original mixed culture test and the tests performed with the single gene deletion set of strains exemplify this. This could be attributed to the stochastic nature of the persistence phenomenon; either the indole induced persistence, or another persistence induce phenomenon such as a toxin-antitoxin system will be inconsistent between cells. We also observed variation in the total stationary phase population of cells. This phenomenon may be related to the variance in persister formation. Statistical analysis becomes very weak because of this, however large differences between averages are still worth discussing.

The experiments outlined within this thesis allow for a few conclusions to be drawn. Firstly, the findings of the TTC test display the ability of cells to recover from antibiotic exposure and the effect that indole has on this process. This is primarily viewed by the comparison of the control samples to the samples that were exposed to ciprofloxacin while in liquid media (Figure 4.4). This experiment showed that the wild

type strain grown with exogenous tryptophan displayed red coloration sooner than other cultures. This represents quicker regrowth of the culture. This result could come from a greater number of surviving or from the cells resuming activity quicker. This test does not provide a way to distinguish between such factors, so the role that the two factors played cannot be precisely determined. This test is much better at evaluating a base level of viability and would better be applied to a wide number of different strains or conditions to look for outliers.

The second conclusion pertains to indole's role as an intercellular signal. This is exemplified by the mixed culture experiment. The first iteration showed a beneficial effect of indole production within the mixed culture (Figures 4.5 and 4.6). The modifications performed later to target cells that were closer to the "indole pulse" seemed to show that the presence or absence of indole was not responsible for improved survival in the mixed culture (Figure 4.10). There appears to be some benefit to the mixed culture that improves survival of cells, but we are not currently able to identify what mechanism is behind the phenomenon. The results from these experiments show the mixed culture experiencing greater cell death after leaving the initial antibiotic killing phase (Table 4.2 and Figure 4.10). This change in fitness suggests that cells in the mixed culture are leaving the persister phenotype. The mixed culture positive control from the original mixed culture test also decreases in CFUs over this time, suggesting that the cultures may enter the death phase (Table 4.2) The specific concentration of indole may be relevant to this behavior, since it does not seem to occur in stationary phase cultures of the wild type.

The third conclusion relates to the peroxide priming test. While the initial trial with MG1655 and GL607 implied that sublethal peroxide could be introduced to the

culture to improve persister formation, the continued testing with the BW25113 based strains were not able to repeat this so successfully (Table 4.3). These strains saw significant but widely varied reductions in CFUs by when the same amount of peroxide was added to them (Table 4.12). This death prevented us from determining if there were changes in persister formation that would allow the strains to survive antibiotics. This death could mean that phage shock proteins have some role in oxidative stress survival. They are associated with the cell membrane and respond to disruptions that occur, which is not dissimilar to the role of OxyR (38). Ultimately, we were not able to confirm that adding peroxide to activate oxidative stress survival mechanisms would improve persister formation.

A fourth conclusion can be drawn from a variety of tests, particularly the early killing curve and the mixed culture tests that involved twenty-four hours of antibiotic exposure (Figure 4.3, Figure 4.10, Table 4.3). These tests showed a novel phenomenon wherein cultures that produced indole or contained added indole displayed greater cell death during the slow killing phase of antibiotic exposure. This suggests that the presence of indole reduces fitness during this period. More work would need to continue to determine the mechanism behind this effect. We believe that this is caused by cells in the persister state returning to normal growth. This creates a decrease in fitness in laboratory cultures, but the effect might create a fitness advantage in real-world infections. Cells that return to growth quicker would have a competitive advantage following the end of an environmental stressor, similar to the effects found by other labs where indole negatively influenced regrowth of species that don't produce it (11).

Our hypothesis that indole improves persister formation in the stationary phase is refuted by this research. There was some increasing number of persister cells when indole was added (Figure 4.8). We saw a greater influence in cells leaving the persister state earlier when indole was included, leading to greater cell death after the initial antibiotic killing phase (Figures 4.3 and 4.10). This is a novel effect of indole that has not been shown in previous experiments that utilized mid exponential phase growth.

5.1 Future Research Directions

There was a phenomenon that occurred within the small number of tests that included overnight incubation with antibiotics. This primarily pertains to the killing curve that compared GL607 death with or without exogenous indole and the early stationary phase mixed culture experiment. In these experiments, a novel trend appeared that differentiates cultures based on the presence of indole. Indole appears to cause cells to leave the persister state over a long period of time in these experiments. This trend appeared outside of the bacterial killing period that we originally targeted, so our data on this effect is limited. This presents interesting questions pertaining to long term stationary phase activity and may imply that indole's concentration has a greater impact that previously believed. A plan of study to evaluate this has begun being formulated within the lab, but there is not yet significant data that can be presented about it.

Another factor to consider would be to evaluate the effect of indole on other species of indole producing bacteria. This route of study would highlight which aspects of indole signaling are the most clinically relevant factors which is an important step in determining how to treat infections when persister cells pose an issue to a patient's

healing. Similarly, the mixed culture test could even be performed by mixing species, and a similar test has actually been done with *E. coli* and *Pseudomonas aeruginosa* which displays how indole can affect cells that aren't meant to produce it (11).

Lastly, one of the early avenues of study was an attempt to expose bacterial cultures to antibiotics while they grew on agar. The purpose of this approach had been to evaluate how indole signaling functions under different conditions, particularly because it would not be expected to diffuse between non-motile cells on agar as easily as it would in liquid. The persister assay utilizing ampicillin and penicillinase as well as the portion of the TTC experiment that exposed cells to ciprofloxacin while on agar represent the efforts to begin that testing process. While this was a novel approach, we were unable to develop a methodology to evaluate persister formation under those conditions. In much the same way that infectious bacteria do not exist in an eternal exponential growth pattern, they also do not exclusively live in a liquid environment with a constant 250 PRM shaking. Evaluation of a cell population's tolerance to antibiotics or other stresses on a solid medium is a more difficult task, and a few modifications of our methods could create a method for such studies.

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