Combined Antibiotic Effects on Bacterial Persistence in *Escherichia coli*

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Abstract

Persistence is a phenomenon in which a genetically uniform population of bacteria, when exposed to an antibiotic to which they are sensitive, show a biphasic death curve. A portion of the population dies at a certain rate, whereas the rest of the population, the persisters, die at a slower rate. This is different than genetic resistance, because when a population of only persisters is regrown and then given the same antibiotic, the same biphasic death curve is produced. The persistence is not genetically conferred to the next generation, but appears in the same fraction of the population as in the previous test. I have characterized persister curves in *Escherichia coli* in response to combinations of antibiotics, including ampicillin, tetracycline, and streptomycin. When the individual antibiotics were used, the death curves showed persistence in progressively steeper curves as drug concentration increased. However, when two different antibiotics were used together, the death curve resembled the curve of one of the cultures with a single antibiotic. The combined antibiotic curve was similar to the steeper of the two single drug curves. I also observed persister curves in high concentrations of drugs that are clinically described as bacteriostatic. In addition, I explore the potential for increasing the efficiency of bacterial research using the Envision multilabel plate reader and a strain of *Escherichia coli* containing a luminescent plasmid, and by using MATLAB to organize and plot the data.
Introduction

Bacteria are single-celled organisms that are classified as prokaryotic, meaning they do not have membrane-bound nuclei or organelles. *Escherichia coli* is rod-shaped and propelled by long flagella. It is a gram-negative bacterium, so called because it does not retain color when died with the Gram stain, implying the presence of an outer membrane ("*Escherichia coli*", *The Columbia Encyclopedia*).

In recent years, the popular media has been flooded with stories of *E. coli* outbreaks, illness, and death related to consumption of common food items. In 2006, an outbreak of *E. coli* O157:H7 caused 199 infections and 3 deaths in the United States, all resulting from consumption of fresh spinach ("Update on Multi-State Outbreak of E. coli O157:H7 Infections From Fresh Spinach", Centers for Disease Control). Normally, though, *E. coli* harmlessly inhabits the intestinal tract of humans and other animals and provides protection from other kinds of harmful bacteria, in addition to aiding digestion ("*Escherichia coli*", *The Columbia Encyclopedia*).

Many different types of antibiotics are used in clinical and theoretical biology, with varying mechanisms of action. Ampicillin is an antibiotic often used to treat and study *E. coli*. Ampicillin is part of the penicillin family of antibiotics, which are defined by a β-lactam structure. β-lactams, which include both penicillins and cephalosporins, are often used to treat infection in humans because they are effective against many kinds of bacteria, but are not very toxic to humans (Blumberg and Strominger 1974). Even low concentrations of penicillins, compared with effective concentrations of other antibiotics, interfere with the formation of outer cell wall material, so that an actively growing cell will eventually undergo lysis, or cell death (Blumberg and Strominger 1974).
Tetracycline is a broad-spectrum antibiotic that is commonly used clinically because it is cheap and has relatively few side effects. It inhibits bacterial growth by binding to bacterial ribosomes and preventing protein synthesis (Speer et al. 1992). Streptomycin is also an antibiotic that kills bacteria by inhibiting protein synthesis at the ribosome (Luzzatto et al. 1968). Indolmycin is currently being studied by Professor Jason Sello at Brown University, and works by inhibiting transcription. It has potential for the treatment of notorious drug-resistant infections such as MRSA.

The growth cycle of bacteria can be described by six phases (Figure 1): the lag phase, in which the growth rate is zero; acceleration phase, in which the rate of growth is increasing; exponential phase, in which the bacteria are rapidly dividing and their numbers are growing exponentially; retardation phase, in which the growth rate decreases as the carrying capacity of the environment is approached; stationary phase, in which there is no more growth; and phase of decline, in which the bacteria are dying (Monod 1949).

Though most commonly known for causing disease, *E. coli* is also widely used in research because it relatively easy to grow and manipulate. Not only are studies done relating specifically to treating *E. coli* infection in humans, but also to investigate other biological occurrences, because *E. coli* can be genetically manipulated and used as a basic genetic model for more complex organisms.

I have been particularly interested by the phenomenon of bacterial persistence, which is best described by Balaban et al. (2004): “A fraction of a genetically
homogeneous microbial population may survive exposure to stress such as antibiotic treatment. Unlike resistant mutants, cells regrown from such persistent bacteria remain sensitive to the antibiotic.” In other words, when a genetically uniform population of bacteria, such as *E. coli*, is exposed to antibiotics to which they are sensitive, not all cells die at the same rate. Most of the cells die at a certain exponential rate, while a fraction of the cells dies at a slower exponential rate, such that when the death curve of the entire population is graphed on a logarithmic scale, the curve looks similar to the one in Figure 2. In *E. coli*-C, I found that 1 in $10^3$ cells dies at the slower death rate, and is classified as a persister. The first part of the curve represents the death rate of the non-persisters, and the second part of the curve represents the death rate of the persisters. Once the number of non-persisters drops below the number of persisters, the slope of the curve is more affected by the death rate of the persisters than that of the non-persisters.

![Figure 2](image.jpg)  
*Figure 2.* Death curve of *E. coli*-C treated with 100 µg/mL ampicillin at Time 0. The two phases of the curve represent the sensitive cells and the persister cells. The best fit lines were created by splitting the data into two sets at the point where the death rate appeared to change phases.
Persistence is distinct from antibiotic resistance because persistence is not heritable (Keren et al. 2004). That is, if a culture of cells treated with antibiotics until only persisters remain is then washed and inoculated in a fresh medium, the same death curve results. Keren et al. (2004) observed this several times with the same culture and found a nearly identical death curve each time. While there are genes associated with variation in frequency of persisters, there is no genetic difference between normal cells and persisters (Lewis 2007).

Persisters are genetically normal cells, which, according to an expression profiling of RNA in isolated persister cells, show significantly less transcription of genes used in energy production and some non-essential functions (Lewis 2007). Balaban et al. (2004) showed that persisters are cells that are not actively dividing. *E. coli* that express a fluorescent protein were grown on plates with narrow grooves such that the cells could only grow and divide along the groove, which was one cell thick. This left fluorescent strings of cells that had grown from individual cells. The cells were exposed to

![Figure 3](image-url)  
*Figure 3.* (Balaban et al. 2004) Evidence that persisters are characterized by a lack of cell growth.
ampicillin for several hours, and then the ampicillin was removed and the medium was washed. Figure 3 shows images taken from this experiment. The rapidly growing cells form fluorescent lines. The red arrow identifies a persister cell, which does not form a string because it is not actively dividing. When ampicillin is added, the strings of dividing cells die, leaving the persisters, which then begin to divide once the antibiotic is removed.

The exact mechanism of persistence and the reasons for its evolution are not known for certain, although many explanations have been suggested. Kussel et al. (2005) suggest that persistence evolved as a defense mechanism against variable environmental conditions, and created a model that showed the relative benefits and tradeoffs of the proportion of persisters in a population in environments of differing variability. On the other hand, Klapper et al. (2007) suggest that persister behaviors can be explained by cell senescence; that is, growth rate and substrate usage decline as a cell ages, but the cell becomes more tolerant to antibiotics.

Another aspect of persistence that is not well studied is the persistence response to different types of antibiotics. In clinical pharmacology, antibiotics are often classified as bactericidal or bacteriostatic. Bactericidal drugs kill bacteria, whereas bacteriostatic drugs merely inhibit growth. Persistence would not necessarily occur in the presence of antibiotics that are bacteriostatic by this definition, because the bacteria are not being killed and have nothing against which to persist. It is suggested, however, that antibiotics should not be broadly classified as bactericidal or bacteriostatic based on their activity against certain bacteria, but rather which concentrations of the antibiotics have certain effects (Rahal and Simberkoff 1979). That is, low concentrations of antibiotics that are
considered bactericidal may have bacteriostatic effects, whereas high concentrations of bacteriostatic antibiotics may be bactericidal. Of the antibiotics I used, ampicillin and streptomycin are considered bactericidal, and tetracycline and indolmycin are considered bacteriostatic.

Persistence exists in the presence of stresses other than single antibiotics. It has been suggested that persistence is used as a bacterial defense mechanism against other types of stresses, such as heat shock and genomic damage. There is very little information in the literature regarding this. It is also unknown how persisters respond to combinations of antibiotics. For example, do the cells that persist against one antibiotic also persist against another antibiotic? In this case, the effectiveness of two antibiotics on bacterial persisters would not be additive. It is also unknown whether the same cells persist against combinations of different types of stresses, such as heat shock and antibiotics together.

An understanding of persistence is important to clinical bacteriology. Bacteria that suppress some cellular functions in a portion of the population become less sensitive to antibiotics and exhibit this tolerance in the presence of different types of antibiotics (Keren et al. 2004), which makes it much more challenging to combat an infection. Persistence has been observed across a wide range of bacteria and antibiotics (Dhar and McKinney 2007), and nearly all clinically used antibiotics (Wiuff and Andersson 2006). Many bacteria that have shown evidence of persisters are bacteria known to be harmful to humans in addition to E. coli, such as Mycobacterium tuberculosis, pneumococci, and Pseudomonas aeruginosa (McCune et al. 1956, Spoering and Lewis 2001, Tuomanen et al. 1986). Other antibiotics that result in persister behaviors in addition to ampicillin
include other \( \beta \)-lactams, and ofloxacin, a fluoroquinolone antibiotic (Cozens et al. 1986, Spoering and Lewis 2001, Tuomanen 1986). However, current pharmacodynamic models tend not to take into account persistence (Wiuff and Andersson 2006), which could be dangerous if used for ineffective treatment of bacterial infection.

A clinical description of bacterial persistence, as given by McDermott (1958), is as follows: A series of patients with a drug-susceptible infection were treated with antibiotics, and all appeared healthy. Most of these patients remained free of infection, but a small portion showed signs of illness again some time later. The bacteria isolated from these patients were not drug resistant, and the patients responded to another course of the same antibiotic treatment, showing evidence of persistence. If the bacteria had developed resistance to the antibiotic, they would not have responded to the second treatment. Instead, McDermott (1958) described this persistence as the ability of a bacterium to “play dead”.

It is suggested, though, that infection with a bacterium that shows persistence may not be inherently more dangerous than that with a bacterium that does not show persistence, because persisters may only cause what is known as a latent infection, which is the presence of bacteria that does not cause fatal disease (McKay et al. 1957). Also, some “unusual” \( \beta \)-lactams are more effective at killing slow-growing bacteria (persisters) than others, such as MT 141 and CGP 17520, although the reason why this is the case is not known (Cozens et al. 1986, Tuomanen 1986).

Although there is thorough research on the presence of persistence in many species of bacteria in response to many antibiotics, many questions remain open in the body of knowledge. First I wanted to explore the possibility of using the PerkinElmer
Envision, a 96-well plate reader, to measure the density of luminescent cell colonies in order to expand and streamline persister experiments. A similar device has previously been shown to accurately reflect the number of colony-forming units (CFU’s) in an actively growing culture of bacteria (Kishony and Leibler 2003). I determined that the measurements were also accurate in a stress environment, such as one containing antibiotics. This is necessary for creating a persister curve.

Following this, I looked at the bactericidal/bacteriostatic clinical dichotomy as it relates to persistence. I found that bacterial cultures inoculated with antibiotics that are clinically used bacteriostatically, such as tetracycline, show evidence of persistence when used at concentrations above which they act bactericidally.

I also explored the effects of combinations of antibiotics on persistence. Sufya et al. (2003) reported exposing a culture of E. coli to an antibiotic until only persisters remained in the population, and then the surviving cells were immediately exposed to another antibiotic. They found that persisters maintained their shallow death curve when exposed to the second antibiotic. However, I wanted to look into the effects of combinations of antibiotics. According to Yeh et al. (2006), “two drugs may have no interaction, or they may interact synergistically or antagonistically to increase or suppress their individual effects”. This uncertainty also applies to the potential effects of antibiotic combinations on persistence in a bacterial culture.

I did experiments to investigate these effects, using pairwise combinations of ampicillin, tetracycline, and streptomycin. Though these drugs are not all classified as bactericidal, I used bactericidal concentrations of each in order to observe persistence. I expected one of two results: either the cells that persist against one antibiotic are also
persisting against the other antibiotic, or the cells persisting against either antibiotic are mutually exclusive of each other (Figure 4 (a) and (c)). In each situation, I would expect the part of the death curve that corresponds to only persisters remaining in the culture would differ between these two hypotheses. In the first hypothesis, in which persisters are persisting against both antibiotics, the second part of the death curve for the combination of drugs would be the same as the curves for the individual antibiotics. If the curves of the individual antibiotics are not the same, then the curve of the combination would take shape of the steeper of the two curves. If the other case is correct, where persisters against one antibiotic are not persistent against the other antibiotic, then I would expect the death curve of the combination of drugs to be steeper than both of the individual drug curves, perhaps as steep as the first part of the curve when mostly sensitive cells are in culture (Figure 4 (b) and (d)).

Figure 4. Two hypotheses of persistence against multiple drugs. In a) and c), the same cells persist against both antibiotics. In b) and d), the cells that persist against each antibiotic are mutually exclusive.
I found that the persister curve of *E. coli* in response to a combination of antibiotics very much resembles the steeper curve of the two single antibiotic curves of the same concentration. For example, persistence is seen with 500 µg/mL ampicillin and with 500 µg/mL tetracycline, although the tetracycline curve is steeper, showing a faster death rate. When the drugs are combined at those concentrations, so that one culture contains both 500 µg/mL ampicillin and 500 µg/mL tetracycline, the death curve resembles that of the culture containing only 500 µg/mL tetracycline. This implies that the cells that persist against ampicillin are the same cells persisting against tetracycline. The same results were seen with other two-drug combinations, which suggests that persisters may persist regardless of interactions between combinations of antibiotics and that the mechanism of persistence is independent of the antibiotics which are present.

**Materials and Methods**

The strain *E. coli-C + PCS-λ* was used for all experiments. *E. coli-C* was chosen because it had the highest density of cells in an overnight culture of several strains tested, which made it easier to observe persistence. The *E. coli* was transformed with the PCS-λ plasmid, which causes a bacterium to produce luciferase, a bioluminescent protein. A single colony of *E. coli-C* was grown overnight (12-24 h) in 3 mL LB broth, Lennox (Difco) broth shaking at 37°C. The culture was then diluted 1:10 by adding 1 mL of the overnight to 10 mL LB broth and put it back in the 37°C incubator for two hours. By this time, the culture had reached an optical density at 600 nm between .600 and .800, indicating that the cells were in exponential growth phase. Preliminary experiments had shown that cells need to be in exponential growth phase to see evidence of persistence,
because some types of antibiotics, such as ampicillin, are considerably more effective when cells are actively growing than when they are in stationary phase (Keren et al. 2004).

**Petri Plate Assays**

At this point, Time 0, an appropriate amount of antibiotic was added to each flask. The appropriate concentrations were determined in preliminary experiments, which would kill bacteria quickly enough that persistence is observable, but not so quickly that enough samples could not be plated to observe the phenomenon.

The cells were incubated, shaking, at 37°C for the duration of the experiment. Samples were taken every half hour for the first two hours. The flask was then kept shaking in a 37°C incubator for the rest of the experiment, and samples were taken every hour until hour 7.

When a sample was taken from one of the flasks, it was then at a dilution at which the colony-forming units that appeared on the plates the following day could be accurately and consistently counted, as determined by previous experiments. Ten-fold serial dilutions were made by adding a 100 µL sample to 1 mL LB broth and vortexing it for 2 seconds, and then repeating until the desired dilution was reached. Once diluted, each sample was spread on an LB plate. For example, a $10^{-5}$ dilution was made by taking 10 µL from the flask and adding it to 1 mL LB broth. After mixing, 100 µL was added from the dilution to another 1 mL LB broth and mixed. 10 µL from this second dilution tube was spread on the plate, such that the number of colonies that grew represented $10^{-5}$ times the number of colony forming units (CFUs) in one milliliter.
The plates were kept in the 37°C incubator overnight (12-24 h) and then counted the number of colonies on each plate. The data were graphed using Microsoft Excel. Logarithmic best-fit lines were added to the graphs by splitting each data set into two sets at the point where I visually determined the slope to change.

96-well Plate Assays

The 96-well plate assays were done with gradients of antibiotics along the rows and columns of the plate, such that there was a gradient of one antibiotic along the rows and another along the columns. Three antibiotics were used: ampicillin, tetracycline, and streptomycin (Fisher Biotech). The antibiotics were diluted from stock concentration with LB broth. Using a multi-channel pipettor, 20 µL of five times the desired concentration of antibiotic was put in each well, so that each well had 40 µL of antibiotics. If one or zero antibiotics were required in a particular well, the equivalent volume of LB broth was used in order to keep the volume of liquid in the wells consistent. 60 µL of the *E. coli* culture was then put in each well for a total of 100 µL of liquid culture plus antibiotics in each well.

The 96-well plate were put into the PerkinElmer Envision, an electronic plate-reader, and a program was used that measured the amount of luminescence in each well every fifteen minutes for approximately twenty-four hours. For this entire time, the 96-well plate remained at 37°C and not shaking. The data were graphed using MATLAB.
Results

Multilabel Plate Reader

Although Kishony and Leibler (2003) showed that luminescence directly and linearly corresponded to the number of CFUs in a well during bacterial growth, I wanted to test whether this holds true for a culture under stress, such as in the presence of antibiotics, and whether it was possible to observe persisters in this way. I performed an experiment using 100 ug/mL ampicillin both on the Envision and using Petri plates, and compared the data. I used three replicates of each plate during the plate assay, and five replicates of each well on the Envision. I fit a linear regression to the curve and the intercept was such that 220 units of luminescence corresponding to one CFU (Figure 5). The R-squared value of the line was 0.9455.

Figure 5. Luminescence compared to colony-forming units.
Persistence Against Bacteriostatic Antibiotics

Next, I explored the effects of bactericidal concentrations of drugs that are used clinically at bacteriostatic concentrations (Luzzatto et al. 1968, Speer et al. 1992). I found that *E. coli* shows persistence in the presence of high enough concentrations of tetracycline, using data from both the Envision (Figure 6(a)) and Petri assays (Figure 6(b)). I also found evidence of persistence in response to indolmycin (Figure 6(c), Figure 6(d)).

**Figure 6.** Death curves of *E. coli* for two drugs that are traditionally classified as bacteriostatic. (a) Tetracycline on the Envision. (b) Tetracycline Petri plate assay. (c) Indolmycin on the Envision. (d) Indolmycin Petri plate assay.
Antibiotic Combinations

In order to determine the effects on persistence of combining antibiotics, I ran 96-well plate assays using gradients of two antibiotics. By combining ampicillin and tetracycline (Figure 7(a)), I found that the same number of cells was persisting with the combination of the two drugs as persisted with only one drug, implying that the same cells persist against both drugs. I then tried similar experiments combining ampicillin and streptomycin, and tetracycline and streptomycin (Figure 7 (b) and (c)).
I overlaid the average curve of certain concentration combinations to get a clearer idea of the nature of how the different curves correspond (Figure 8(a)). This makes it easier to see how the bacteria behave, on average, in response to each antibiotic environment. I also confirmed this data by repeating part of the experiment on Petri plates (Figure 8(b)).
Figure 8. Average persister death curves with 500 µg/mL ampicillin, 500 µg/mL tetracycline, and both (a) on the Envision and (b) in a Petri plate assay.
Discussion

Implications of the Multilabel Plate Reader

The first experiment that I did showed very clearly that luminescence as measured by the Envision directly relates to the number of colony-forming units per milliliter of culture. A challenge that might have arisen while using the Envision is that cells under stress, such as those trying to survive in the presence of antibiotics, may not express the luciferase gene, and therefore not create luminescence in the machine. If this were the case, however, I would expect that persistence would not be observable; rather, the luminescence curve would decrease steeply and steadily. Instead, luminescence showed a biphasic curve, corresponding to that of a persistent culture of living cells.

This was important in order to be confident about the later data I used from the Envision. For the other 96-well plate assays, I was able to assume that the luminescence in a well directly corresponded to the number of CFUs in that well at that time. I determined this by graphing the number of CFUs I determined by counting colonies on plates against the units of luminescence on the Envision at the same time points. The MATLAB linear regression had a y-intercept at 220, suggesting that 220 units of luminescence correspond to one CFU. The R-squared value of the line was 0.9455, which implies a very high degree of correlation. Therefore, I assumed that this relationship held true in all experiments, but chose to confirm results on Petri plates in some cases in order to assure accuracy.

The Envision could to help improve research on persistence and bacterial growth in general. By measuring luminescence, the machine can accurately estimate the number of living cells in a culture. It can take more samples at a greater frequency than can be
done by hand. I was easily able to take 96 or 192 samples every 15 minutes for 24 hours, with only the amount of time it takes to prepare the 96-well plates. This allows for the possibility of a much larger number of experiments to be run, as well as experiments covering a wider range of variables with more replicates. For example, tests can be done with a range of antibiotic concentrations and many replicates of each, at the same time. This has the potential to streamline bacterial research.

Classification of Antibiotics

In clinical medicine, the line between bactericidal and bacteriostatic antibiotics holds strong; bactericidal antibiotics kill greater than 99.9% of bacteria, and bacteriostatic antibiotics only inhibit growth (Kohanski et al. 2007). In theoretical bacteriology, however, it appears more that bactericidal and bacteriostatic designations are the ends of a continuous spectrum. That is, low concentrations of antibiotics that are considered bactericidal may have bacteriostatic effects, whereas high concentrations of bacteriostatic antibiotics may be bactericidal. I found that this relationship held true for the different categories of antibiotics in terms of persister behavior, as observed along the top row and left column of the assays in Figure 7.

I looked for persistence in the presence of a wide range of concentrations of a few different antibiotics. I was able to see clearly that in a bactericidal drug, ampicillin, very low concentrations only inhibited bacterial growth, moderate concentrations created a clear persister curve, and high concentrations killed nearly all the bacteria almost immediately (in the first couple of hours). This is shown clearly along the first column of Figure 7 (a). In a bacteriostatic drug, tetracycline, low and moderate concentrations of
the drug inhibited growth, whereas high concentrations created a persister curve or killed the entire population very quickly, as in the first row of Figure 7(a).

From these large death curve assays done on 96-well plates, I was able to quickly determine which concentration would yield a persister curve when I repeated the experiment on Petri plates. This is important, as a bacteriostatic concentration of an antibiotic would not kill bacteria quickly enough to yield a persister curve. In my undergraduate research, I characterized the persister curve of ampicillin in detail. Ampicillin is widely considered bactericidal. I found that a clear persister curve is created when the culture was inoculated with 100-1000 µg/mL ampicillin, or a ten-fold range of concentrations. Lower concentrations did not do more than inhibit bacterial growth, and higher concentrations killed the bacteria too quickly to plate enough time points to create a persister curve.

Tetracycline is used clinically as a bacteriostatic antibiotic (Southwick and Durack 1974). However, I found that at certain concentrations, it showed bactericidal behavior and created a good persister curve (Figure 6 (b) and (c)). I did notice, though, that the bacteriostatic drug had a narrower range of bactericidal concentrations than did ampicillin; persister curves were seen only between 200-500 µg/mL, or a 2.5-fold range of concentrations.

I investigated another bacteriostatic drug, indolmycin, for its effects on persistence. It is not a very well studied antibiotic, but I was inclined to study it because it is currently being studied by Professor Jason Sello in Brown University’s chemistry department. Though toxic at high concentrations in the human body, indolmycin shows clinical promise in treating difficult skin infections such as the recently popularized
Methicillin-resistant *Staphylococcus aureus* (MRSA). The data showed evidence of persistence in response to indolmycin, but not enough data was acquired to unquestionably show the presence of persisters because indolmycin is very expensive to produce.

The clinical significance of persister behavior in bacteriostatic drugs is the same as that in bactericidal drugs; if bacteria are not being killed as quickly as they are assumed to be, the infection may be longer lasting and more dangerous. This study, however, does not consider the clinical significance of a patient’s own immune system. Bacteriostatic concentrations of antibiotics are used because bacterial growth only needs to be inhibited for the immune system to kill them on its own (Lewis 2001). Higher concentrations are unnecessary and potentially toxic to the patient’s metabolizing organs. The concentrations of the three antibiotics that I used ranged from 3 to 2,000 times the peak clinical serum concentrations of these drugs after an average dose, which is 3.2 µg/mL for ampicillin and 1.0-2.6 µg/mL for tetracycline (Gordon et al. 1972, Gordon et al. 2005). Only in cases in which a patient is immunocompromised would a bactericidal concentration of an antibiotic be needed.

*Effects of Antibiotic Combination*

The next step in my project was to explore the effects on persisters of antibiotic combinations. I created an array of cultures on a 96-well plate, with a gradient of one antibiotic on each axis. In this way, for each drug, I could see death curves ranging from only inhibited growth to almost immediate death for each drug, and every combination of
the two. The resulting graphs were organized in an array that each combination of drugs was plotted separately, with four replicates for each combination (Figure 7).

I noticed an interesting pattern in the graphs. When two drugs at given concentrations were combined, the resulting persister curve tended to look like the steeper curve of only one drug at the same concentration. That is, if 500 µg/mL ampicillin and 500 µg/mL tetracycline were added to a well, the resulting curve would look very similar to the curve of either 500 µg/mL ampicillin only or 500 µg/mL tetracycline only, whichever curve was steeper. The combined drugs did not kill more bacteria or kill them more quickly than only one drug. This is exemplified in Figure 8(a), which shows a 500 µg/mL ampicillin + 500 µg/mL tetracycline curve which is very similar to the 500 µg/mL tetracycline only curve, whereas the 500 µg/mL ampicillin only curve is less steep than the other two. This is clearer in the ampicillin-streptomycin array, comparing 500 µg/mL ampicillin, 500 µg/mL streptomycin, and both (Figure 7).

In figure 7(b), it is clear that the streptomycin curve is much shallower than the other curves, but all show persistence. The combination of streptomycin and ampicillin created a curve that was almost identical to the curve describing the wells that contained only ampicillin. In figure 7(c), however, the combination of tetracycline and streptomycin results in a curve that looks like the tetracycline curve, and steeper than the streptomycin curve.

In Figure 8(a), it is noticeable that the ampicillin + tetracycline curve increases in population after several hours. This is likely error from the Envision reading that was still seen when the curves were averaged. The curves were a lot cleaner for 100 µg/mL ampicillin and 500 µg/mL tetracycline, which are the same drugs but a different
concentration of ampicillin (Figure 9). These results support the first hypothesis described, as in Figure 4(a), in which the same cells persist against multiple antibiotics. This is significant in that it supports the idea that the mechanism of persistence is uniform in response to many antibiotics, even though the mechanisms of action of these antibiotics differ.

**Further Research**

There is much room for further research in the field of bacterial persistence. It needs to be better understood in order to effectively treat bacterial infection. There need to be better and more accurate models. If persistence were studied enough that an accurate predictive model of persister behavior existed, clinical treatments could be determined more effectively. These models should be able to take into account all clinically used antibiotics and all possible drug combinations, used on all types of bacteria. More accurate treatment plans for patients could be developed that are more effective at killing infectious bacteria with fewer side effects for the patient. The Envision would be useful in performing such large-scale experiments and testing the accuracy of models.

Persistence has been observed as a bacterial defense mechanism against other types of stresses, such as heat shock and genomic damage. This should be studied in further detail in order to learn more about the nature of persistence as well as the
possibilities of alternative treatment of clinical infections. It would be interesting to see whether the cells that persist against multiple antibiotics are the same cells persisting against other environmental stresses.

There is also a lack of knowledge about the direct clinical effects of persistence. When humans are treated with antibiotics, often the effective dose is enough to slow bacterial growth (i.e. bacteriostatic) so that the patient’s immune system can kill the bacterial infection. There is no data showing that the presence of persisters changes these treatment effects. It would be interesting to see if persisters put extra strain on the human immune system, as they are not killed as quickly as the sensitive cells while they are in the persister state. It is unknown whether bacteria show persistence against the different elements of the human immune system. Observations of infections that persist after antibiotic treatment but have not become resistant to the antibiotic would be useful. It would also be interesting to examine the prevalence of persistence observed in clinical settings such as the one described by McDermott (1958). The fraction of patients who showed signs of illness some time after treatment with antibiotics may or may not correlate to the fraction of persisters in a flask of growth medium and antibiotics.

It has been suggested that biofilms exhibit behavior similar to persisters in the presence of antibiotics (Lewis 2001, Spoering and Lewis 2001). A biofilm is a “population of cells growing on a surface and enclosed in an exopolysaccharide matrix” (Lewis 2001), or a thin layer of partially protected cells on a surface. Biofilms show an increased tolerance to antibiotics, but not heritable drug-resistant mechanisms in the cells. Bacteria in biofilms can tolerate concentrations of antibiotic 10 to 1000 times higher than are necessary to kill planktonic bacteria (Lewis 2001). Spoering and Lewis (2001) suggest
that this tolerance in biofilms is due to the presence of a high number of persisters.

Biofilms exhibit multi-drug tolerance, and Schumacher et al. (2009) state that 60% of infections in the developing world are caused by biofilms.

It would be interesting to compare the death curves of biofilms and persisters, in the hopes of determining whether the antibiotic tolerance of biofilms is due to the presence of persisters and if persisters in a biofilm behave differently than planktonic (growing in liquid) persisters because of other factors unique to a biofilm. There is a challenge in that there is no standardized way to study biofilms (Lewis 2001), but this could be explored as well.

The greatest importance of studying persister behavior is for the clinical applications. Kim Lewis (2007) suggests that persistence may be associated with increased levels of evolved resistance, although there is no data available to support or deny the hypothesis. Biofilms, which show evidence of persistence, are a significant cause of bacterial infection in humans. For the advancement of the field of human medicine, it is necessary to gain as much knowledge as possible regarding the behavior of persisters.

The observations I’ve made confirm these worries. Even drug “cocktails” are not untouched by the effects of persistence. Combining antibiotics is not any more effective at killing persisters than using only one antibiotic, assuming resistance has not been developed. This leaves scientists with the same problems as before in fighting bacterial infection. Research on persisters, especially regarding the effects of types and concentrations of antibiotic treatments, has the potential to be used in the development of
treatment for a variety of bacterial infections that are common causes of serious human illnesses.
Literature Cited


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