**Abstract**

Most of what we know today about the evolution of antibiotic-resistance is focused on the selections of lethal antibiotic concentrations, which allows the detection of rare mutant strains that show strong phenotypic traits. However, this is just the tip of the iceberg when it comes to the evolution of antibiotic-resistance genes. In fact, high-resolution competition assays show that resistant bacteria are selected at relatively low concentrations of antibiotics. Sub lethal concentrations of antibiotics are found mostly in non-medical conditions, such as wastewater treatment plants and food and water used in agriculture and farming. We will show that there are many evolutionary pathways within the resistance gene *blaTEM-50* in *E. coli.* These pathways change depending on the type and concentration of antibiotics they are exposed to. Focusing on the β-lactam treatments of cephalosporins, and a combination of penicillins with inhibitors, we see how the multiple concentrations of antibiotics accelerate evolution.

**Introduction**

In natural and clinical settings bacterial pathogens are exposed to a wide range of antibiotic concentrations, this is often associated with non-medical use of antibiotics. We see sub lethal concentrations of antibiotics in wastewater all around the world, ranging from 1μg/L to 64 μg/L in waste water treatment plants and hospital effluent water supplies (*Watkinson 2009*). This water cannot be completely filtered out before being used for agriculture, delivering these low concentrations of antibiotics to agriculture and animals that have access to it for drinking. Also, there have been some ranchers and farmers who use antibiotics to promote lean muscle production in animals and counts for up 13% of antibiotic use, which have been eliminated in accordance with the FDA Guidance. (*Wegener 2003).* Based off of the excessive use of antibiotics, both in medical and non-medical settings, it is critical to focus on various concentrations of antibiotics and their effects on the evolutionary pathways within bacterial cultures that are exposed to them.

Beta-lactam antibiotics were first introduced around 1940, with Penicillin being the first clinically used antibiotic. Since then, the world has been flooded with beta-lactam antibiotics because of their high efficiency and low toxicity to the human body. This includes cephalosporins, which have the same mode of action as penicillin, but are less susceptible to penicillinases. All β-lactam antibiotics disrupt the synthesis of the peptidoglycan layer of the bacterial cell wall. Because of the overuse of these β-lactam antibiotics, bacteria have evolved to produce an enzyme, called a β-lactamase, which has the ability to deactivate the β-lactam ring of the β-lactam antibiotics. One of the genes that encode for a β-lactamase is TEM-1.

In 1963, the TEM beta-lactamase emerged among gram-negative bacteria, and it rapidly increased in frequency to become the most frequent beta-lactamase in most pathogenic gram-negative populations. TEM stands for the name of the patient from whom this enzyme was isolated, Temoneira. TEM beta-lactamases have been found in Escherichia coli, and other gram-negative bacteria. TEM-1 is considered the wild-type, denoted here as (0000). Over 170 TEM variants have been found clinically, where forty-one are single mutants, i.e., they have exactly one amino acid substitution, and the majority have at most four amino acid substitutions. In this study, we focus on TEM-50, which is one of the clinically found isolates with four substitutions. We have created all 16 possible variations of those mutations using site directed mutagenesis.

Because of the widespread use of beta-lactam antibiotics, there have been additional approaches that utilize a combination of mechanisms based on activators for beta-lactamases such as clavulanic acid, sulbactam and tazobactam (5). To avoid β-lactamase activity, some antibiotics are given with these β-lactamase inhibitors, resulting in a more effective treatment. These inactivators destroy the β-lactamase activity, therefore enhancing the ability of the drug to destroy the cell wall. An Inhibitor-resistant TEM (IRT) is a bacterial strain that produces an inhibitor-resistant enzyme that breaks down these beta-lactamase inhibitors. Within the TEM-50 gene, there are two substitutions that contribute to the IRT phenotype, M69L (IRT-5) and N276D (not yet isolated) (*Chaibi 1999)*.

We asked whether the evolutionary trajectory within the *blaTEM-50* gene would change if treated with various antibiotic treatments, including inhibitors, and if adding inhibitors to the treatment would have an effect on which variant allele is selected for. We will show that there are many evolutionary pathways within the resistance gene *blaTEM-50* depending on the type and concentrations of antibiotic treatment used. We will be focusing on the treatments that combine the β-lactam antibiotic, specifically penicillins, plus one inhibitor*.*

**Materials and Methods**

*Strains and Cultures*

The E. coli strain DH5-αE in which the alleles expressed were mutant constructs from the blaTEM-1 gene in the pBR322 plasmid (1). We obtained cultures from phosphate-buffer stocks and incubated in 5 mL of Luria Broth with Tetracycline (5mL tetracycline/ 1 Liter of LB). In order to get the optimum number of bacterial cells in a culture (1.9 X 105 cells per mL of broth), we used the equation:

V1 = , (1)

where

1.9×108 is the expected number of cells per mL, V1 is the volume of culture we are solving for in milliliters, V2 is the volume of broth we are using for the experiment in milliliters, we will use 5 mL here, and O.D. is the apparent absorption of the culture.

The O.D of 200μL samples of each strain was taken in the Eon Microplate Spectrophotometer, 96 well plates. With these, the volume of culture needed to transfer to the fresh 5mL Mueller Hinton broth was calculated to run the experiment.

For example, O.D. for a TEM-1 strain was 0.218. Substituting this into equation (1) and solving for V1 yields:

V1=0.012 mL or V1 = 12μL.

So, 12μL of culture is transferred to 5mL Mueller Hinton broth to run the experiment.

Once the optimal volumes of each culture are obtained, they are then transferred into the new tubes with Mueller Hinton broth (5mL). On a 384-well plate, each well holding a maximum of 100 μL, 80μL of each culture is transferred into the first 12 wells, as the controls, and the last 12 wells as the experiment containing the antibiotic. The antibiotic solution is made by dissolving 10.24 mg of antibiotic per 1 mL of solvent (either pH 6 or pH 8 phosphate-buffer or water depending on the solubility of the drug). The concentration of antibiotic used was based on Minimum Inhibitory Concentrations (MIC’s) taken prior.

Once the samples have all been plated, a membrane is placed over the plate and is placed in the Eon Microplate Spectrophotometer. The temperature was set at 25.1°C for 22 hours. This microplate reader takes O.D. measurements at 600 nanometers every 20 minutes for the entire 22 hours. The O.D. is the measurement of the fraction of light that is absorbed by the solution and depends on the wavelength used (1).

*Growth Rates*

The data obtained from the plate reader is exported and run through the ‘GrowthRates’ program. This program calculates the growth rate based on the growth curve of each sample. This is calculated as the slope of the line at the exponential phase of the growth curve, because bacterial cultures grow exponentially, and the O.D increases as a function of ln(O.D.) (2). This growth rate is the change in number of cells per minute, or can be seen as the change in number of cells per unit of O.D. This can be written as

, (2)

where N is the number of cells at time (t), α is the first order growth rate constant in reciprocal time units (2). (2) can also be written as

(2)

Integrating from t=0 to t=tmax yields

, (3)

where NO equals the initial number of cells present at tO.

When the exponential phase of the growth curve is fit by linear regression, we can see that α is equal to the slope of that line (2).

*Statistical Analysis*

One-Way Analysis of Variance (ANOVA) was used to compare the means of the growth rates we obtained, and to determine if there were significant differences between the mutants. We compared each of the mutants with those that had just one mutation different from each other, going from the wild type, TEM-1, to TEM-50 (Table 1). We were working with a 95% confidence interval so a p-value of less than or equal to 0.05 probability. Not only did we compare the experimental data, we also compared the controls with the experimental data to confirm that the treatments were in fact different than the non-treated samples.

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| Mutants | Binary Allele Code |
| TEM-1 | 0000 |
| M69L (IRT-5) | 1000 |
| E104K | 0100 |
| G238S | 0010 |
| N276D (IRT\*) | 0001 |
| M69L/E104K | 1100 |
| M69L/G238S | 1010 |
| M69L/N276D | 1001 |
| G238S/E104K | 0110 |
| G238S/N276D | 0011 |
| N276D/E104K | 0101 |
| M69L/E104K/G238S | 1110 |
| M69L/E104K/N276D | 1101 |
| G238S/N276D/E104K | 0111 |
| G238S/N276D/M69L | 1011 |
| TEM-50 | 1111 |

**Table 1:** Constructs containing all of the possible mutations in *blaTEM-50.* The left column lists the mutations with the first letter representing the amino acid that was replaced, followed by the position in the protein, and lastly, the new amino acid present. The number ‘1’ represents the mutation present and a ‘0’ represents the no mutation at that specific location. For example, M69L corresponds to Methionine being replaced by Leucine on the 69th position. The two mutations included in this experiment that are inhibitor resistant TEM’s (IRT’s) are denoted.

**Results**

To answer the question of whether selection for new mutations would intensify as the antibiotic concentration either increased or decreased, we measured the growth rates of the 16 variants of the mutations that occur in TEM-50. After measuring the growth rates of the 16 alleles we created adaptive landscapes for each concentration of antibiotic. These adaptive landscapes were created where forward arrows signify selection for new mutations and backward arrows signify selection for reversions, depending on which growth rates are higher. Red arrows represent significance with a p-value ≤ 0.05 and black represent non-significance (p-value ≥ 0.05). After creating these landscapes, we created similarity matrices by calculating the percent similarity of arrow direction among the concentrations of antibiotics. Given the similarity matrices, we can see as the difference in concentration increases, the percent similarity can either decrease (Cefotetan, Cefotaxime, and Ampicillin) or increase (Cefprozil, Ceftazidime, and Amoxicillin) depending on the antibiotic used. To understand the basis for those similarities and differences, we considered each set of landscapes separately.

In each landscape there are different alleles that prove to be the ‘most fit’, referred to as the global optimum. As the concentration of each antibiotic change, the global optimum also changes.

*Cefprozil*

For the antibiotic Cefprozil, (Figure 1 A-C) we see the global optimum change as the concentration of changes. A triple mutant is the optimum at the highest concentration and double mutants are the optima at the two lower concentrations. We also observed the largest number of significant differences in growth rates at the middle concentration of 10 μg/mL, which was also the concentration where we observed the greatest number of significant improvements resulting from reversions. In the similarity matrix for Cefprozil, we notice a slight increase in similarity as the concentration difference increases from 8 μg/mL to 12 μg/mL from 56% to 68%. Also, all three adaptive landscapes have 44% similarity.

*Ceftazidime*

We see similar selective patterns when growth rates were measured in the presence at different concentrations of Ceftazidime. At the highest concentration of 0.125 μg/mL, a triple mutant is selected as the global optimum; however, at lower concentrations double and single mutants become the global optima. We also observed the largest number of significant differences in growth rates at the highest concentration of 0.125 μg/mL. In the similarity matrix for Ceftazidime, we see a relatively high percentage of similarity of 75% from 0.0625 μg/mL to 0.1 μg/mL and 84% when comparing 0.1 μg/mL to 0.125 μg/mL . The closer the concentrations are, the higher the similarity of the adaptive landscapes. All three adaptive landscapes treated with Ceftazidime have 66% similarity.

*Cefotetan*

When looking at the selective patterns of the growth rates measured in the presence of Cefotetan, we noticed that triple mutants are selected as global optima at the two lower concentrations of 0.0312 μg/mL and 0.0625 μg/mL, while a double mutant is selected at the highest concentration of 0.125 μg/mL. We observed the highest number of significant differences in growth rates occurring at the highest concentration of Cefotetan at 0.125 μg/mL. The number of new mutations seems more favored at the middle concentrations of 0.0625 μg/mL. When taking into consideration the similarity matrix for Cefotetan we notice a decrease in similarity as the concentration increases; 66% when comparing 0.0312 μg/mL to 0.0625 μg/mL and a 56% similarity when comparing 0.0312 μg/mL to 0.125 μg/mL. Overall, the three adaptive landscapes have a 41% similarity.

*\*Cefotaxime*

The growth rates were measured in the presence of four different concentrations of Cefotaxime. We observed TEM-50 selected as the global optima for the two lowest concentrations of 0.04 μg/mL and 0.05 μg/mL, a double mutant being selected as the global optimum at 0.06 μg/mL, and a triple mutant being selected at the highest concentration of 0.123 μg/mL. The concentration that contains the highest number of significantly different growth rates was 0.05 μg/mL. As the concentration decreases with Cefotaxime, the number of new mutations selected also decreases. When looking at the similarity matrix for Cefotaxime, as the concentration difference increases, the similarity decreases with all four concentrations sharing 37.5% similarity. The highest percent similarity occurred between 0.05 μg/mL and 0.06 μg/mL of 78.1%.

*\*Cefepime*

The growth rates were measured in the presence of two different concentrations of Cefepime; 0.0312 μg/mL and 0.0156 μg/mL. We observed TEM-50 being selected as the global optimum for the lower concentration and a triple mutant being selected as global optimum at the higher concentration. There are more significantly different growth rates at the higher concentration of 0.0312 μg/mL. The percent similarity of these two landscapes is 75%.

*\*Amoxicillin*

While looking at the selective patterns of the growth rates in the presence of Amoxicillin at three different concentrations, we notice the global optimum changing from a double mutant being selected at the highest concentration of Amoxicillin at 1024 μg/mL, to a triple mutant being selected at 512 μg/mL, and a single mutant being selected at the lowest concentration of Amoxicillin recorded at 256 μg/mL. This is the first instance in which we noticed a single mutant being selected as the global optimum in any adaptive landscape. The concentration in which the most new mutations are favored is at 512 μg/mL. However, we observed the most significantly different growth rates at the highest concentration of 1024 μg/mL. When looking at the similarity matrix for Amoxicillin we notice an increase in similarity as the concentration difference increases; from 256 μg/mL to 512 μg/mL with 43.7% similarity and from 256 μg/mL to 2014 μg/mL with 53.1% similarity. A larger similarity occurs from 512 μg/mL to 1024 μg/mL with 65.6% similarity. We observe an overall similarity among all three landscapes of 31.3%.

*\*Ampicillin (8X)*

While looking at the selective patterns of the growth rates in the presence of Ampicillin at three different concentrations, we notice the global optimum at the highest concentration of 256 μg/mL being selected as TEM-50, a single mutant being selected as global optimum at 128 μg/mL, and a triple mutant being selected as global optimum at 64 μg/mL. We noticed at the highest concentration of 256 μg/mL there were the most significantly different growth rates. Taking into consideration the similarity matrices, we notice as the concentration difference increases, the similarity decreases. From 64 μg/mL to 128 μg/mL there is a similarity of 68.7% and from 64 μg/mL to 256 μg/mL 62.5% similarity. This decreases even further when comparing 128 μg/mL to 256 μg/mL with a 50% similarity. An overall similarity percentage of 40.6% recorded for the adaptive landscapes at all three concentrations.

**Note: The concentration of Ampicillin was 8 times more concentrated than others drugs used because of its low potency\***

Within all of the TEM-50 landscapes shown, the global optimum for each drug at each concentration had at least one inhibitor resistant mutation (either 1000 or 0001) when treated with an inhibitor and penicillin. While we created the complete adaptive landscapes, both inhibitor resistant mutations (IRM) and cephalosporin hydrolysis mutations (CHM) were being considered. Both have complicated interactions resulting in sign epistasis. This made it difficult to understand the effects of any mutation. To simplify these interactions and better explain the effects of IRM; we examined only the mutations involved in inhibitor resistant phenotypes separately. With these adaptive landscapes, we eliminated all reversions (backward arrows) and only kept the new mutations (forward arrows) in which there was selection for any allele that contained an inhibitor resistant mutation. Within the gene we are working with, there are only two inhibitor resistant mutants present; M69L (1000) and N276D (0001), as seen in Table 1.

Since we are only taking into consideration the beneficial inhibitor resistant mutations, we can more clearly see the effects of epistasis. Only the concentrations of the antibiotics used are stated because the inhibitors all remain at a constant concentration of 8μg/mL.

*Ampicillin + Sulbactam (SAM)*

We observed the selective patterns of the growth rates in the presence of Amoxicillin and Sulbactam at four different concentrations. We noticed the global optima contained at least one IRM in each of the different adaptive landscapes, with the triple mutant 1101 being selected twice at the two highest concentrations; 64 μg/mL and 32 μg/mL. TEM-50 was selected as global optimum at the lowest concentration of 8 μg/mL, and a double mutant being selected at 16 μg/mL. We observed the concentration that had the most significantly different growth rates among the new mutations selecting for inhibitor resistance was the highest at 64 μg/mL. When looking at the similarity matrix, we notice an overall increase in similarity as the concentration of Ampicillin increases; from 53.1% similar when comparing 8 μg/mL to 16 μg/mL, to 59.4% when comparing 8 μg/mL to 32 μg/mL, 16 μg/mL to 64 μg/mL and 32 μg/mL to 64 μg/mL. The larger the difference in concentration of Ampicillin, the more similar the adaptive landscapes seem to become.

*Piperacillin + Tazobactam (TZP)*

We observed the selective patterns of the growth rates in the presence of Piperacillin and Tazobactam at three different concentrations. We noticed the global optima contained at least one IRM in each of the different adaptive landscapes, with a double mutant being selected at the two lowest concentrations of 128 μg/mL and 256 μg/mL. A triple mutant was selected as the global optimum at the highest concentration of 512 μg/mL. In all three adaptive landscapes, we observed just two to three significantly different growth rates among the new mutations. When looking at the similarity matrix for TZP, we noticed an overall increase in similarity as the difference in concentration increased; from 71.8% similarity when comparing 128 μg/mL and 256 μg/mL to 78.1% similarity when comparing 128 μg/mL and 512 μg/mL.

*Amoxicillin + Clavulanic Acid (AMC)*

The growth rates were measured in the presence of two different concentrations of Amoxicillin with Clavulanic Acid. We observed a triple mutant being selected as global optimum at the lower concentration of 512 μg/mL and a double mutant being selected as global optimum at the higher concentration of 1024 μg/mL. In both adaptive landscapes, we observed the global optimum containing at least on IRM. The percent similarity of these two landscapes is 40.6%.

Finally, we see that the variation in landscapes change as the concentrations of antibiotics change. This holds true for all twelve antibiotic treatments and three antibiotic plus inhibitor treatments. Depending on the treatment, the percent similarity either increases or decreases as the difference in concentration of antibiotic increase. We also observed at least one pathway that leads from the wild-type (TEM-1) to the global optimum (highlighted with bold arrows) in all landscapes.

**Discussion**

To study the effects of epistatic interactions within these IRT’s, we needed to look at the adaptive landscapes and only focus on the pathways that show preference to a mutant containing an inhibitor-resistant mutation (IRM), i.e. arrows pointing toward a mutant containing an IRM. If an allele had a mutation in the first location (1000) or last location (0001) with an arrow pointing toward it, that signified the higher mutant is selected under the specific antibiotic conditions, over the ancestral mutant. This indicated that those inhibitor resistant mutations are beneficial to the cell rather than detrimental**.**

When treated with an inhibitor and penicillin, the global optimum for each drug at each concentration had at least one inhibitor resistant mutation in all landscapes. This signified there could be small trade offs, or compensatory effects of mutations. Sign epistasis causes mutations to become compensatory. Depending on which drug and concentration is used we can see that the same mutation has different effects. For example, in the landscape with Amoxicillin plus Clavulanic Acid, we can see the IRT-5 (binary 1000) being beneficial when treated at 1024 μg/mL, but detrimental when Amoxicillin is lowered to 512 μg/mL. This is also evident for the mutant that contains the inhibitor resistant mutation in both locations (1001). In Amoxicillin 1024 μg/mL this mutant shows to be the global optimum, however, when Amoxicillin is lowered to 512 μg/mL it shows to not be beneficial at all when compared to the more wild type strains, also demonstrated in AMC treatment. When looking at the top three optima in each landscape, we noticed a selection for the alleles 1101 and TEM-50 (1111). Out of all treatments, these alleles appeared the most when treated with an inhibitor and penicillin.

We also noticed that depending on the types of antibiotics used, the selection for new mutations versus reversions varied. We see that Cephalosporins tend to select for new mutations over reversions. Penicillins (and penicillins plus inhibitors) do not show this based off of the rations of new mutations: reversions (Table with ratios) and confirmed by T-tests of these ratios.

Also, conserved mutations in different environments can be seen within these landscapes. The same mutations co-occurring appeared to be the global optimum among many antibiotics, but only at some concentrations. Here we see the mutant strain 1101, appearing as the most fit mutant in four out of the nine treatments of penicillin plus inhibitor: Two appear in SAM at 32μg/mL and 64μg/mL, one in AMC 512 μg/mL, and one in TZP 512μg/mL.

The presented data suggests different antibiotic concentrations select for different alleles within *bla*TEM-50. Taking into consideration the beneficial IRT’s and global optimums, we can see that there is not a definite pattern among the change in concentration, and change in adaptive landscapes. The global optimum consistently carries at least one of the two inhibitor resistant mutations (for treatments with inhibitors), which we do not see in the adaptive landscapes of *bla*TEM-1 when inhibitors are not used. This shows that the use of inhibitors alongside antibiotics selects for inhibitor resistant mutants. We also noticed that in treatments with penicillins used only (Amoxicillin and Ampicillin) single mutants were selected as global optima. This only occurs with one treatments of a cephalosporin. \*\*

We can see how multiple concentrations that are present in the environment accelerate the evolutionary possibilities.

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| **AMacintosh HD:Users:portia:Dropbox:TEM.50:TEM-50 Landscapes:CPR:CPR8Landscape copy.pdf** | **BMacintosh HD:Users:portia:Dropbox:TEM.50:TEM-50 Landscapes:CPR:CPR10Landscape copy.pdf** |
| **CMacintosh HD:Users:portia:Dropbox:TEM.50:TEM-50 Landscapes:CPR:CPR12.5Landscape copy.pdf** | **DMacintosh HD:Users:portia:Dropbox:TEM.50:TEM-50 Landscapes:CPR:CPRComposite.pdf** |

**Figure 1:** Adaptive Landscapes for Cefprozil (CPR) at various concentrations: A) 8μg/mL, B) 10μg/mL, C) 12.5μg/mL. Forward arrows signify new mutations and backward arrows signify reversions. Red arrows represent significance with a p-value ≤ 0.05. Black arrows represent non-significance, p-value ≥ 0.05. The global optimum allele is highlighted in red D) Composite of all concentrations, showing only the arrows that remain in the same direction throughout the three concentrations.

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