**Introduction**

Most of what we know today about the evolution of antibiotic-resistance is focused on the selections of lethal drug concentrations that allow the detection of rare mutants with strong phenotypes (4). 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 selection of resistant bacteria occurs at extremely low antibiotic concentrations (3). In natural and clinical settings bacterial pathogens are exposed to a wide range of antibiotic concentrations, often associated with non-medical use of antibiotics (4). It is very important that we begin to focus on a variety of concentrations of antibiotics rather than just the lethal concentrations that we thought had the biggest impact on the evolution of resistance genes. Here we will show that there are many evolutionary pathways within the resistance gene *blaTEM-50* depending on the concentrations of antibiotic treatment. We will be using β-Lactams, which are the most important antibiotic family because of their wide spectrum of efficiency, their bactericidal activity, and their low toxicity (5).

**Materials and Methods**

*Strains and Cultures*

We used 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 p-buffer stocks and incubated them in 5 mL of Luria Broth with Tetracycline (5mL tetracycline/ 1 Liter of LB). In order to get the optimum number of bacterial cells, which is 1.9×105 cells per mL of broth, in a culture we used the equation:

×V1 = 1×105  × V2

1.9×108 is the expected number of cells per mL,

V1 is the volume of cells we are solving for,

V2 is the volume of broth we are using for the experiment, 5mL here,

O.D. is the apparent absorption of the culture.

We took the O.D of 200μL samples of each strain in the Eon Microplate Spectrophotometer 96 well plate. With these we calculated the volume of culture needed to transfer to the fresh 5mL Mueller Hinton broth to run the experiment.

For example, O.D. for a TEM-1 strain is 0.218

(1.9×108)×0.218×V1 = 1×105 × 5

4.14×107 × V1= 5× 105

V1=0.012 Liter

Or V1 = 12μL

12μL of culture would be transferred over to 5mL Mueller Hinton broth for the experiment.

Once we have the volumes of each culture, we transferred them into the new Mueller Hinton broth. On a 384-well plate, we transfer 80μL of each strain into the first 12 wells as the controls, and the last twelve wells as the experiment containing the antibiotic. We made the antibiotic solution by dissolving 10.24 mg of antibiotic per 1 mL of solvent (either p-buffer or water depending on the solubility of the drug). Based on Minimum Inhibitory Concentrations taken prior, we added the specific amount of antibiotic to the remaining cultures and transfer 80μL into the last twelve wells. This amount varied among the different antibiotics we used.

Once the samples have all been plated, a membrane is placed over the plate and placed in the Eon Microplate Spectrophotometer. The temperature set at 25.1°C for 22 hours. This microplate reader takes O.D. measurements at A600, every 20 minutes for the length of the experiment. The O.D. is taken from the measurement of the fraction of light that is absorbed by a solution. This absorption depends on the wavelength used; here we use a wavelength of 600 nm (2).

*Growth Rates*

We export the data obtained from the plate reader and run it through the ‘GrowthRates’ program, which 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.). This growth rate is the actual 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

Where N is the number of cells at time (t)

α is the first order growth rate constant, and is in reciprocal time units. (2)

So,

Integrating this equation from t=0 to t=t

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*

We used One-Way ANOVA’s 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, going from the wild type, TEM-1, to TEM-50. 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 each other and the experiment versus the controls to ensure our results were as accurate as possible.

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| Mutants | Binary Allele Code |
| TEM-1 | 0000 |
| M69L | 1000 |
| E104K | 0100 |
| G238S | 0010 |
| N276D | 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 | 1011 |
| G238S/N276D/M69L | 0111 |
| 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.

**Results**

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| **AMacintosh HD:Users:portia:Dropbox:TEM.50:Landscapes:CPR8Landscape copy.pdf** | **BMacintosh HD:Users:portia:Dropbox:TEM.50:Landscapes:CPR10Landscape copy.pdf** |
| **CMacintosh HD:Users:portia:Dropbox:TEM.50:Landscapes:CPR12.5Landscape copy.pdf** | |  |  |  | | --- | --- | --- | | **Concentrations** | **New Mutations: Significant/Total** | **Reversions: Significant/Total** | | 8 μg/mL | **5/21** | **1/11** | | 10 μg/mL | **5/15** | **13/17** | | 12.5 μg/mL. | **2/17** | **4/15** | |

**Figure 1:** Adaptive Landscapes for Cefprozil 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. d) Ratios of the number of new mutations favored over reversions for each concentration of Cefprozil. We can see that at 10μg/mL concentration there . Any higher concentration, or lower concentrations, the ratios decrease indicating fewer significant changes, whether it be new mutations or reversions.

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| **AMacintosh HD:Users:portia:Dropbox:TEM.50:Landscapes:CAZ0.0625Landscape copy.pdf** | **BMacintosh HD:Users:portia:Dropbox:TEM.50:Landscapes:CAZ.1Landscape copy.pdf** |
| **CMacintosh HD:Users:portia:Dropbox:TEM.50:Landscapes:CAZ1.25Landscape copy.pdf** | |  |  |  | | --- | --- | --- | | **Concentrations** | **New Mutations: Significant/Total** | **Reversions: Significant/Total** | | 0.0625μg/mL | **1/19** | **2/13** | | 0.1μg/mL | **11/19** | **6/13** | | 1.25μg/mL. | **14/18** | **9/14** | |

**Figure 2:** Adaptive Landscapes for Ceftazidime at various concentrations: a) 0.0625μg/mL, b) 0.1μg/mL, c) 1.25μg/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. d) Ratios of the number of new mutations favored over reversions for each concentration of Ceftazidime. We can see that the higher the concentrations of Ceftazidime the higher the ratio of significant new mutations.

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| **AMacintosh HD:Users:portia:Dropbox:TEM.50:Landscapes:CTT0.0312Landscape copy.pdf** | **BMacintosh HD:Users:portia:Dropbox:TEM.50:Landscapes:CTT0.0625Landscape copy.pdf** |
| **CMacintosh HD:Users:portia:Dropbox:TEM.50:Landscapes:CTT0.125Landscape copy.pdf** | |  |  |  | | --- | --- | --- | | **Concentrations** | **New Mutations: Significant/Total** | **Reversions: Significant/Total** | | 0.0312μg/mL | **7/18** | **9/14** | | 0.0625μg/mL | **1/21** | **4/11** | | 0.125μg/mL. | **10/14** | **12/18** | |

**Figure 3:** Adaptive Landscapes for Cefotetan at various concentrations: a) 0.0312μg/mL, b) 0.0625μg/mL, c) 0.125μg/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. d) Ratios of the number of new mutations favored over reversions for each concentration of Cefotetan. We can see that the higher the concentrations of Cefotetan, the more new mutations are favored as well as reversions.

**References:**

1. Hall, Barry G. Bellingham Research Institute. “*GrowthRates: A Program for Measuring Bacterial Growth Rates in the 21st Century”.*
2. Gaulart *et. Al.* PloS ONE. *“Designing Antibiotic Cycling Strategies by Determining and Understanding Local Adaptive Landscapes”* 2013
3. Hughes D, Andersson DI. SciVerse ScienceDirect. “Selection of resistance at lethal and non-lethal antibiotic concentrations”.SciVerse ScienceDirect 2012.
4. Gullberg E, Cao. S, Berg OG, Ilback C, Sandegren L, Hughes D, Andersson Ki: “Selection of resistant bacteria at very low antibiotic concentrations.” PLoS Pathog 2011.