**Article**

**Title:**

**Adaptive Landscapes of Resistance Genes Change as Antibiotic Concentrations Change**

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**Abstract**

Most studies on the evolution of antibiotic-resistance are focused on selection for resistance at lethal antibiotic concentrations ([Hughes and Andersson 2012](#_ENREF_11)), which has allowed the detection of mutant strains that show strong phenotypic traits. However, solely focusing on lethal concentrations of antibiotics narrowly limits our perspective of antibiotic resistance evolution. New high-resolution competition assays have shown that resistant bacteria are selected at relatively low concentrations of antibiotics ([Hughes and Andersson 2012](#_ENREF_11)). This finding is important because sub-lethal concentrations of antibiotics are found widely in patients undergoing antibiotic therapies and in non-medical conditions, such as wastewater treatment plants, and food and water used in agriculture and farming. To understand the impacts of sub-lethal concentrations on selection, we measured thirty adaptive landscapes for a set of TEM β-lactamases containing all combinations of the four amino acid substitutions that exist in TEM-50 for 15 β-lactam antibiotics at multiple concentrations. We found that there are many evolutionary pathways within this collection of landscapes that lead to nearly every TEM-genotype that we studied*.* While it is known that the pathways change depending on the type of β-lactam, this study demonstrates that the landscapes also change dramatically as the concentrations of antibiotics change. Based on these results we conclude that the presence of multiple concentrations of β-lactams in an environment result in many different adaptive landscapes through which pathways to nearly every genotype are available.

**Introduction**

Bacteria are routinely exposed to a broad range of antibiotics that are present in a wide spectrum of concentrations due to their differential accessibility in the tissues of patients undergoing antibiotic therapy and in the environment outside the human body from the breakdown of antibiotics and their presence in agricultural runoff, wastewater, and food ([Gullberg, et al. 2011](#_ENREF_7)). This occurrence has been heavily documented ([Gustafson 1991](#_ENREF_8); [Wegener 2003](#_ENREF_25)). For example, antibiotics, along with other organic wastewater contaminants, have been found in 98.7% of water samples collected outside of suburban areas in the United States ([Kolpin, et al. 2004](#_ENREF_12)). Sub-lethal concentrations of antibiotics are present in wastewater throughout the world, ranging from 1μg/L to 64 μg/L in wastewater treatment plants and hospital effluent water supplies ([Watkinson, et al. 2009](#_ENREF_23)), ([Kolpin, et al. 2004](#_ENREF_12)). The antibiotics cannot be completely filtered out of the water before it is used for agriculture, which delivers low concentrations of antibiotics to crops and farmland ([Watkinson, et al. 2009](#_ENREF_23)). Also, some ranchers and farmers use antibiotics to promote lean muscle production in animals, which accounts for up to 13% of antibiotic use ([Gustafson 1991](#_ENREF_8); [Wegener 2003](#_ENREF_25)). Considering the extensive use of antibiotics in clinical and agricultural environments, it is not surprising to find evidence that sub-lethal concentrations of antibiotics are important selective pressures acting upon bacteria ([Blazquez, et al. 2012](#_ENREF_2)). There is abundant evidence that sub-lethal concentrations of antibiotics in the environment contribute to the increased frequency of antibiotic resistance mutations among microbial populations. Since sub-lethal concentrations of antibiotics have been established as important environmental selective pressures upon antibiotic resistant bacteria, we questioned how varying concentrations of β-lactam antibiotics would affect the genetic outcome of the evolving TEM resistance genes.

The β-lactam antibiotics were first introduced in 1943, with penicillin being the first. Since then, the world has been flooded with β-lactam antibiotics because of their high efficiency and low toxicity to the human body ([Guthrie, et al. 2011](#_ENREF_9)). 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 extensive exposure to β-lactam antibiotics, bacteria have evolved to produce an enzyme, called a β-lactamase, which has the ability to hydrolyze and inactivate the β-lactam ring of these antibiotics. One of the most frequently occurring genes in Gram-negative bacteria that encodes a β-lactamase is the *bla*TEM-1 gene ([Bradford 2001](#_ENREF_3)).

In 1963, the TEM β-lactamase (TEM-1) gene emerged among Gram-negative bacteria, and it rapidly increased in frequency to become the most frequent β-lactamase in most pathogenic Gram-negative populations. TEM β-lactamases have been found in *Escherichia coli* and other Gram-negative bacteria. The TEM resistance gene is a well-known model system. Among the TEM family members, TEM-1 is considered the wild type. Over 219 TEM variants have been found clinically, where forty-one have single amino acid substitutions and 89% have four or fewer amino acid substitutions. TEM-3, reported in 1987 ([Sirot, et al. 1987](#_ENREF_21)), was the first Extended-Spectrum β-lactamase (ESBL); as such it was able to hydrolyze extended spectrum β-lactams, in which cephalosporins are mainly categorized.

Because of the widespread use of β-lactam antibiotics, there have been additional approaches to fight against β-lactamases that utilize a combination of mechanisms including inhibitors for β-lactamases such as clavulanic acid, sulbactam and tazobactam ([Chaibi, et al. 1999](#_ENREF_4)). To avoid β-lactamase activity, some antibiotics are given in conjunction with these β-lactamase inhibitors, resulting in a more effective treatment. These inactivators destroy the β-lactamase activity, which enhances the ability of the β-lactam to destroy the cell wall. An inhibitor-resistant TEM is a bacterial strain that produces an inhibitor-resistant enzyme that breaks down these β-lactamase inhibitors. TEM-30, reported in 1992 ([Vedel, et al. 1992](#_ENREF_22)), was the first inhibitor-resistant TEM (IRT), which means that it could continue to hydrolyze penicillins in the presence of a β-lactamase inhibitor. Cephalosporin resistance is usually separate from inhibitor resistance among TEM β-lactamases but TEM-50 was reported in 1997 ([Sirot, et al. 1997](#_ENREF_20)), as the first Complex Mutant TEM (CMT), where both cephalosporin and inhibitor resistance substitutions and phenotypes appear simultaneously ([Robin, et al. 2011](#_ENREF_17)).

Epistasis, or non-additive interactions between mutations, also play a major role in antibiotic resistance. Epistatic interactions can be used to study the topography of fitness landscapes and the dynamics of adaptation ([Kondrashov and Kondrashov 2001](#_ENREF_13); [Salverda, et al. 2011](#_ENREF_18)). Many studies show these patterns of epistasis among large and small-effect beneficial substitutions occurring in TEM-1 ([Barlow and Hall 2002](#_ENREF_1); [Weinreich, et al. 2006](#_ENREF_26); [Goulart, et al. 2013](#_ENREF_6); [Schenk, et al. 2013](#_ENREF_19)). It has been suggested ([Poon and Chao 2005](#_ENREF_15); [Watson, et al. 2011](#_ENREF_24)) that there are major epistatic interactions among the mutations within TEM-1 depending on which combination of the four mutations are present in the presence of just one antibiotic, cefotaxime. Poon and Chao showed that epistasis occurs more frequently among mutations within the same gene ([Poon and Chao 2005](#_ENREF_15)). Due to the delayed emergence of CMT type TEMs, we anticipated that epistasis (non-additive interactions between substitutions) and sign epistasis (when substitutions change from being beneficial to detrimental and vise versa) would be dominant features of the TEM-50 adaptive landscape. In this study, we focus on TEM-50, which is one of the clinically isolated variants with four substitutions ([Bradford 2001](#_ENREF_3)). We have created all 16 possible variations of those substitutions using site directed mutagenesis ([Goulart, et al. 2013](#_ENREF_6)).

The environment can also affect the fitness contributions of substitutions and their epistatic interactions. Genotype-by-Environment (GxE) interactions are defined as the change in the performance of two or more genotypes measured in two or more environments. Changes in rank order for different genotypes and changes in the magnitude of genetic, environmental and phenotypic variances can be evident between environments ([Gillespie and Turelli 1989](#_ENREF_5)). Previous studies on GxE interactions have measured fitness on genotypes that differ by numerous unknown mutations and most recently ([Remold and Lenski 2001](#_ENREF_16)) investigated the effects of 26 genotypes in four environments measuring fitness relative to a common progenitor. Here we present a study of GxE interactions on sixteen genotypes that differ by up to four substitutions with 10 different β-lactam antibiotics at three different concentrations using growth rates as a measurement of fitness. Other studies have used minimum inhibitory concentrations (MICs) ([Weinreich, et al. 2006](#_ENREF_26" \o "Weinreich, 2006 #2507)) to determine rough measurements of relative fitness. While MICs are clearly useful in this regard, in 2008, ([Mroczkowska and Barlow 2008](#_ENREF_14" \o "Mroczkowska, 2008 #2471)) showed that growth rates are a more sensitive measurement of resistance than MICs.

There are ~105 different β-lactams (http://www.whocc.no/atc\_ddd\_index/) that have selected for the resistance phenotypes contributed by the approximately 219 TEM genotypes (http://www.lahey.org/Studies/temtable.asp) that exist today. The specific effects of each β-lactam and the within-gene epistatic interactions have surely shaped the evolutionary landscapes of the TEM family. However, they have not been thoroughly investigated. At the most basic level, we reasoned that there might be differences in adaptive landscapes as antibiotic concentrations changed. Additionally we reasoned that changes in concentration of antibiotics are a biologically relevant occurrence worthy of investigation because 1) β-lactam concentrations may vary widely throughout the tissues of a patient undergoing β-lactam therapies and 2)β-lactam concentrations can also change rapidly as antibiotics waste breaks down in the environment.

To study within-gene epistasis and GxE effects on the TEM-1 to TEM-50 adaptive landscapes, we investigated the interactions of penicillins, cephalosporins, and β-lactamase inhibitors with 16 TEM genotypes to determine the combined effect of genotype and environment upon fitness outcomes. We looked at how the concentration of β-lactam antibiotics affects the composition of each landscape by taking into consideration the ratio between new substitutions (forward arrows) and reversions (backward arrows). With this information we were able to calculate similarity matrices to study how much each antibiotic treatment (a specific antibiotic at a given concentration), differs from the others that we tested. We also examined the global optimum within each landscape for each treatment. The global optimum is the genotype that has the highest growth rate (or can be considered the most fit) among all 16 genotypes.

**Results**

We measured the growth rates of the 16 genotypes that can be generated from all combination of the four substitutions in TEM-50. We verified that there were no spontaneous mutations occurring for the duration of the experiment by sequencing 50 samples of the genotype E104K after one experiment treated with cefprozil 128 µg/mL. We confirmed that, in fact, there were no spontaneous mutations except for one sample that accrued a synonymous mutation at the codon position 263.

After measuring the growth rates of the 16 genotypes, we created adaptive landscapes for each concentration of each β-lactam antibiotic. These adaptive landscapes compare the growth rates of strains expressing adjacent genotypes that differ by a single amino acid substitution and indicate the genotype that resulted in the highest growth rate (Figure 1). In the case of each comparison, the arrows point towards the higher growth rate. The arrows directed towards the genotype with more substitutions signify selection for new substitutions and arrows directed towards genotypes with fewer substitutions signify selection for reversions, depending on which growth rate was higher. Solid arrows represent a significant difference between growth rates as determined by one-way ANOVA (p-value ≤ 0.05) and dashed arrows represent no significant difference between genotypes (p-value ≥ 0.05). Using these arrows we identified evolutionary pathways in which adaptation occurs through either the acquisition of new substitutions or the loss of substitutions through reversions. In each instance, we assume that substitutions and reversions are only selected if their occurrence results in a higher growth rate than the previous genotype.

In each landscape (SI figures 1-10) we found that there is one genotype that proves to be the ‘most fit’, referred to as the global optimum. As the concentration of each antibiotic change, the global optimum also change in each β-lactam. Overall, TEM-50 appeared as the global optimum in 17% of all treatments (including twice in cefotaxime, and once in ampicillin, cefepime and ampicillin/sulbactam). Genotypes with three substitutions appeared as the global optima in 40% of all treatments, and at least once in each of the 15 β-lactams, sometimes at multiple concentrations. The genotype 1101, was the global optimum for four out of the nine penicillin/inhibitor treatments: Twice it appeared as the global optimum in ampicillin/sulbactam at 32/8 μg/mL and 64/8 μg/mL. It appeared once in amoxicillin/clavulanic acid at 512/8 μg/mL, and once in piperacillin/tazobactam at 512/8 μg/mL. This result was surprising because none of the genotypes with three substitutions have been clinically isolated, however they may exist in the environment where very low concentrations of β-lactams are more common.

Genotypes with two substitutions appeared as the global optima in 33% of all treatments and in all β-lactams except ampicillin and cefepime. Genotypes with one substitution appeared as the global optima in 10% of all treatments (only those with the β-lactams ampicillin, ceftazidime, or amoxicillin). TEM-1 did not appear as the global optimum in any of the treatments tested. However, it did appear within the top three genotypes for one treatment (Figure 2). Interestingly, we observed at least one pathway from the wild-type (TEM-1) to the global optimum in all landscapes, with the exception of amoxicillin at 1024 µg/mL.

Overall we found that the number of times the addition of an amino acid substitution was selected for was greater than the number of times a reversion of an amino acid substitution was selected (Table 1); 66.7% of the time the addition of an amino acid substitution was selected for in penicillin treatments and 80% in cephalosporin treatments. However, for the nine penicillin plus inhibitor treatments, the results were more variable (SI Figures 3-5). In four treatments, more amino acid substitutions were selected than reversions; in three treatments more reversions were selected than substitutions, and in two treatments, the number of selected substitutions and reversions were equal.

We then identified the three genotypes with the highest fitness in each landscape, and found that the genotypes 1101 and TEM-50 (1111) were selected the most frequently (Figure 2). Out of all treatments, these genotypes appeared among the top three genotypes the most frequently when treated with a penicillin/inhibitor. We also identified the three genotypes with the lowest fitness rankings in each landscape, and found that the genotypes 0111, 1011, and 1001 were the lowest ranked (Figure 2), with 0111 appearing in twenty-one landscapes among the lowest three rankings.

To validate the predictability of our adaptive landscapes, we performed competition experiments. A library of all 16 variant genotypes in approximately equal proportions was inoculated simultaneously in culture treated with cefotaxime at 0.123 µg/mL and incubated at 25 degrees for 22 hours. Twenty-nine samples from this culture were then sequenced to determine which genotypes displayed a competitive advantage in this environment. We found that 93% of the samples were ranked within the top 5 highest growth rate rankings, and 100% of the samples were ranked within the top 6 highest growth rates. These results support our findings within the adaptive landscapes.

While we created the complete adaptive landscapes, we considered both inhibitor resistant substitutions and cephalosporin hydrolysis substitutions. We observed that combinations of these two types of substitutions routinely result in sign epistasis. Throughout all 30 landscapes, we found sign epistasis in the majority of β-lactam antibiotic treatments (Table 2). There were just four cases in which no sign epistasis occurred and all were in penicillin/inhibitor treatments. We observed that the genotype 0010 had all detrimental effects in the treatments piperacillin/tazobactam (512/8 µg/mL and 128/8 µg/mL) and ampicillin/sulbactam (64/8 µg/mL). Also the genotype 1000 had all beneficial effects in the treatment ampicillin/sulbactam (8/8 µg/mL). Despite the exceptions it is clear that for the combining of inhibitor resistant substitutions with cephalosporin hydrolysis substitutions within the TEM gene, sign epistasis is the rule rather than the exception.

We then questioned what effect the change in concentration of antibiotics would have on the selection of each substitution. The growth rates of each genotype were plotted against concentration for each treatment and the slope was measured. We found that 73.5% of the slopes were negative, indicating that the overall growth rate decreased as the concentration of the treatment increased. Out of the remaining 26.5% of positive slopes, 66% of them appeared in treatments containing penicillins (SI Table 2, SI Figure 11 and SI Figure 12).

To ensure that these comparisons were valid, we performed one-way ANOVAs between the growth rates for each genotype across antibiotic concentrations. Significant (p-values ≤ 0.05) from this analysis indicate that there is a significant difference between growth rates at those different antibiotic concentrations for individual genotypes in most antibiotics. We found that 80% of comparisons were significantly different in cephalosporins, 47% of comparisons were significantly different in penicillins, and 42% of comparisons were significantly different in penicillin inhibitor combinations (SI Table 3). When we aggregated all genotypes, we found that one-way ANOVA analysis for each treatment across all concentrations indicated that each concentration was significantly different (p-value ≤ 0.05) than other concentrations within the same treatment, except for the two penicillin + inhibitor treatments (SAM and TZP) (p-value ≥ 0.05).

After considering all thirty treatments, we found that the ratios of selected substitutions and selected reversions changed as both the type of antibiotic and the concentration of the antibiotic changed. New substitutions outnumber reversions in 63.3% of the treatments; reversions outnumber new substitutions in 23.3% of the treatments. The frequency of new substitutions is equal to the frequency of reversions in 13.3% of the treatments. In 50% of the ten β-lactam antibiotics, there was a shift from an overall tendency for substitutions to be selected over reversions to a pattern where reversions were selected for over substitutions as the concentration of antibiotic was changed. These results indicated that changes in the concentrations of β-lactam antibiotics have almost as large of an effect as changing the type of β-lactam antibiotic (Table 1).

We further investigated the variation in ratios across concentrations by creating similarity matrices (Tables 3 and 4). In each matrix, we calculated the percent similarity of arrow direction among treatments. The overall range of similarity scores was 41%-84% similar when concentrations were changed but the β-lactam antibiotic remained the same. The range of similarity scores between treatments where the β-lactam varied and the concentration of antibiotic remained constant was for comparison of two cephalosporins (ceph vs. ceph) 50-69% and for comparison of a penicillin with a penicillin + inhibitor treatments were (pen vs. pen inh) 50%-63%. These numbers are well within the range of similarity between treatments when concentration is varied, but the β-lactam remained constant (41%-84%). This result indicates that changes in the concentrations of β-lactam have as large of an effect on evolutionary outcome as changes in the types of β-lactam.

Additionally, we performed a more conservative analysis, since many of the arrows in the adaptive landscapes were not significant. We computed the average similarity matrix across all 30 treatments comparing the position of growth rate mean of each individual genotype to the grand mean (SI Table 4). We only counted changes for the fitness effect of a genotype when it moved across the grand mean. The similarity scores ranged from 6% similar to 100% similar when comparing each treatment with the other 29. When looking among the same type of β-lactam antibiotic, the similarity ranged from 44%-100% with penicillin + inhibitors, 63%-88% with penicillins, and 25%-94% with cephalosporins. When comparing across different types of β-lactam antibiotics (i.e. penicillin + inhibitors, penicillins, cephalosporins), the similarity decreased (6%-88%), with penicillin + inhibitor and penicillins being least similar to cephalosporins (6% similar). While there is less of a tendency for the fitness effects of genotypes to change across concentrations using this metric, that tendency is still apparent.

**Discussion**

We considered four substitutions within the TEM-50 gene and have analyzed 30 adaptive landscapes generated at multiple concentrations of ten different antibiotics. We found that GxE interactions are numerous and complex. As each of the treatments change, whether by type or concentration, not only do the similarity scores between landscapes change, but the global optima likewise change.

Different antibiotic types and concentrations select for different genotypes. While cephalosporins and penicillins tend to select for new substitutions, penicillin + inhibitor combinations tend to select for reversions (Table 1). Interestingly, across all landscapes some substitutions are selected in many environments while others are not. This indicates that GxE effects may be stronger for some substitutions than for others depending on which treatments they are exposed to. GxE effects can also be seen clearly through consideration of changes in the global optimum in each landscape.

While it may not be surprising that the overall tendency to select for new substitutions or reversions would change between β-lactam treatments, we were quite surprised that the overall effects of the genotypes under investigation could become substantially more detrimental or substantially more beneficial simply as the antibiotic concentration changes. In addition, the global optima change as the type of β-lactam or as the concentration of a β-lactam changes. TEM-50 is the most frequent global optimum (40% of the time) throughout all 30 landscapes, whereas TEM-1 does not appear at all as a global optimum. It is unsurprising that TEM-1 is not the global optimum in these landscapes because this study is focused much more upon penicillin + inhibitor combinations and cephalosporins than upon the early generation penicillins that TEM-1 hydrolyzed throughout the 1960’s and 1970’s.

When we considered just the penicillin + inhibitor treatments, the global optima consistently carried at least one of the two inhibitor resistant substitutions in penicillin + inhibitor treatments. This signifies that β-lactamase inhibitors are an important selective pressure. In treatments where penicillins were used alone (amoxicillin and ampicillin), single substitutions were selected as global optima. This can be explained because amoxicillin and ampicillin are some of the earlier β-lactam antibiotics and early TEM β-lactamases are able to hydrolyze them very well.

Interestingly, we found pathways throughout all of these adaptive landscapes that lead to the global optima, except for one (amoxicillin at 1024 µg/mL). For the treatments that selected TEM-50 as the global optimum (cefepime 0.0156 µg/mL, cefotaxime 0.04 and 0.05 µg/mL, and, amoxicillin/sulbactam 8/8 µg/mL, ampicillin 2,048 µg/mL), there are multiple pathways through the landscapes from TEM-1 to TEM-50, which pass through many of the other genotypes. However, for the treatments that did not select TEM-50 as the global optimum, there were no complete pathways from TEM-1 to TEM-50. Although in each of those landscapes, pathways from TEM-1 to the global optimum existed for every treatment except amoxicillin 1024 µg/mL. This is interesting because TEM-1 is often thought to be most efficient at hydrolyzing early penicillins such as amoxicillin. This may not be the case at every concentration, but there may not be a path to the optimum so TEM-1 may persist even in those conditions.

We also noticed many other examples of GxE interactions. Depending on which β-lactam antibiotic and what particular concentration was used, the same substitution can have different effects. For example, in the landscapes with amoxicillin + clavulanic acid (SI Figure 4), we note that the genotype 1000 was beneficial when treated at 1024/8 μg/mL amoxicillin + clavulanic acid, but detrimental when amoxicillin is lowered to 512 μg/mL. This is also evident for genotype 1001, which exclusively contains inhibitor resistant substitutions. In the amoxicillin + clavulanic acid treatment at 1024/8 μg/mL this genotype is the global optimum, however, when amoxicillin is lowered to 512 μg/mL it is not beneficial at all when compared to adjacent genotypes. There are many similar examples throughout each of the adaptive landscapes (SI Figure 1-10).

Another interesting finding was abundant sign epistasis within all thirty adaptive landscapes as well as when looking at the position of each genotype mean compared to the grand mean. A good example of this is the substitutions E104K (0100) and G238S (0010). The high fitness of G238S is also seen in Schenk et. Al 2012, M Camps (Reference?) Both do exceptionally well individually, even more so when they are combined to the doublet E104K/G238S (0110). However when a third substitution is added N276D (0001), creating the genotype G238S/N276D/E104K (0111), the number of genotype means drop from 24 to only 5 above the grand mean. For the examples of substitutions in environments for which sign epistasis did not exist, it was likely because the treatment highly selected for inhibitor-resistant phenotypes.

Surprisingly, we also found that concentration seems to have as large of an effect on variance between adaptive landscapes as changes in the type of β-lactam. This result can be seen in (SI Table 3) where 80% of cephalosporins, 47% of penicillins and 42% of penicillin + inhibitor treatments are significantly different when comparing each genotype across concentrations for each treatment. This result means that the degradation of antibiotics in the environment may contribute as much to the evolution of variant genotypes of resistance genes as the introduction of new β-lactams.

We have been able to show that an abundance of readily available evolutionary trajectories appear across antibiotic type and concentration which show that varied, residual concentrations of antibiotics can select many evolutionary outcomes within the TEM β-lactamase gene . We have been able to show this using growth rate as a more sensitive measurement of fitness when compared to MICs (Barlow Mroczkowska###). This is important because antibiotic concentrations fluctuate in vivo and are present in the environment and by using growth rates, we are able to see the effect that antibiotic concentration has on the genotypes within TEM β-lactamase, something which MIC-measurements can fall short. Future studies will determine whether this pattern holds across other antibiotic resistance genes and to what extent the evolutionary potentials of resistance genes are expanded through residual amounts of antibiotic in the environment.

**Materials and Methods**

*Strains and Cultures*

We expressed all 16 TEM variant genotypes from the pBR322 plasmid ([Goulart, et al. 2013](#_ENREF_6)) in E. coli strain DH5-αE. We incubated them in 5 mL of Luria Broth with Tetracycline (5mL tetracycline/ 1 Liter of LB) overnight in oxygen limited cultures and then diluted them to a concentration of 1.9 X 105 cells per mL .

After dilution, 80μL of each liquid culture cultures were transferred to a 384-well plate, each well holding a maximum of 100 μL. 12 replicates of each culture were then incubated with or without (control) antibiotic treatment. Once the samples have all been plated, a breathable membrane is placed over the plate and the plate is placed in the Eon Microplate Spectrophotometer. The temperature was set at 25.1°C and the experiment is ran for 22 hours. The O.D. measurements at 600 nanometers are read every 20 minutes for the entire 22 hours ([Hall, et al. 2013](#_ENREF_10))

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 antibiotic). The concentration of antibiotic used was based on Minimum Inhibitory Concentrations (MIC’s) taken prior.

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*Growth Rates*

The data obtained from the plate reader is exported and run through the ‘GrowthRates’ program, which calculates the growth rate based on the growth curve of each sample. The growth rates are calculated as the slope of the line at the exponential phase of each growth curve, because bacterial cultures grow exponentially, and the O.D increases as a function of the natural log of the O.D. ([Hall, et al. 2013](#_ENREF_10)). The 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). The equation (2) can also be written as

(2)

Integrating (2) 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 ([Hall, et al. 2013](#_ENREF_10)).

*Statistical Analysis*

A 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 growth rates of each genotype. We compared each of the genotypes with those that were adjacent, (differed by a single amino acid substitution), going from the wild type, TEM-1, to TEM-50 (Table 5). We were working with a 95% confidence interval, which translates to a p-value of less than or equal to 0.05. Not only did we compare the experimental data (the data obtained from just the treated genotypes), we also compared the growth rates from controls with the growth rates from the treated genotypes to confirm that the treated samples were in fact different than the non-treated samples, as well as compared the genotypess across concentration of each drug to ensure that the genotypes are significantly different across concentrations of the same antibiotic.

A complete set of growth rate data for each of the thirty treatments is provided in the Supplemental Information Table 1.

|  |  |  |
| --- | --- | --- |
| **Penicillins** | **Concentration (μg/mL)** | **S: R** |
| **Amoxicillin** | 1024 | 10:22 |
|  | 512 | 17:15 |
|  | 256 | 13:19 |
|  |  |  |
| **Ampicillin** | 2048 | 22:10 |
|  | 1024 | 18:14 |
|  | 512 | 20:12 |
|  |  |  |
| **Pen + Inhibitors** | **Concentration (μg/mL)** | **S: R** |
| **Piperacillin/ Tazobactam** | 8/512 | 15:17 |
|  | 8/256 | 13:19 |
|  | 8/128 | 12:20 |
|  |  |  |
| **Amoxicillin/ Clavulanic Acid** | 8/1024 | 16:16 |
|  | 8/512 | 16:16 |
|  |  |  |
| **Ampicillin/ Sulbactam** | 8/64 | 17:15 |
|  | 8/32 | 18:14 |
|  | 8/16 | 13:19 |
|  | 8/8 | 24:8 |
|  |  |  |
| **Cephalosporins** | **Concentration (μg/mL)** | **S:R** |
| **Cefprozil** | 12.5 | 17:15 |
|  | 10 | 15:17 |
|  | 8 | 21:11 |
|  |  |  |
| **Cefotetan** | 0.125 | 14:18 |
|  | 0.0625 | 21:11 |
|  | 0.0312 | 18:14 |
|  |  |  |
| **Cefotaxime** | 0.123 | 19:13 |
|  | 0.06 | 17:15 |
|  | 0.05 | 18:14 |
|  | 0.04 | 14:18 |
|  |  |  |
| **Ceftazidime** | 0.125 | 18:14 |
|  | 0.1 | 19:13 |
|  | 0.0625 | 19:13 |
|  |  |  |
| **Cefepime** | 0.0312 | 22:10 |
|  | 0.0156 | 22:10 |

**Table 1**: List of the ratios, new substitutions (S): reversions (R), for each antibiotic treatment and concentration used. Antibiotics listed in the first column, concentration of antibiotic in μg/mL in the second column, and ratio of new substitutions (S): reversions(R) in third column.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| β-lactam Treatment | Concentration (µg/mL) | Substitutions | | | |
| 1XXX | X1XX | XX1X | XXX1 |
| Cefotetan | 0.125 | 2/6 | 4/4 | 6/2 | 6/2 |
| 0.0625 | 3/5 | 6/2 | 6/2 | 6/2 |
| 0.0312 | 6/2 | 7/1 | 2/6 | 2/6 |
| Ceftazidime | 0.125 | 2/6 | 5/3 | 7/1 | 4/4 |
| 0.1 | 2/6 | 6/2 | 6/2 | 5/3 |
| 0.0625 | 5/3 | 4/4 | 5/3 | 5/3 |
| Cefepime | 0.0312 | 5/3 | 5/3 | 7/1 | 5/3 |
| 0.0156 | 5/3 | 5/3 | 6/2 | 5/3 |
| Cefprozil | 128 | 4/4 | 6/2 | 3/5 | 4/4 |
| 100 | 1/7 | 5/3 | 5/3 | 4/4 |
| 80 | 2/6 | 7/1 | 6/2 | 5/3 |
| Cefotaxime | 0.123 | 5/3 | 5/3 | 7/1 | 2/6 |
| 0.06 | 4/4 | 4/4 | 7/1 | 2/6 |
| 0.05 | 4/4 | 5/3 | 7/1 | 2/6 |
| 0.04 | 1/7 | 6/2 | 5/3 | 2/6 |
| Ampicillin | 256 | 4/4 | 7/1 | 4/4 | 7/1 |
| 128 | 5/3 | 6/2 | 4/4 | 3/5 |
| 64 | 4/4 | 6/2 | 4/4 | 6/2 |
| Amoxicillin | 1024 | 4/4 | 3/5 | 2/6 | 2/6 |
| 512 | 6/2 | 2/6 | 3/5 | 6/2 |
| 256 | 3/5 | 4/4 | 3/5 | 3/5 |
| Ampicillin/ Sulbactam | 64/8 | 4/4 | 6/2 | 0/8\* | 7/1 |
| 32/8 | 5/3 | 6/2 | 1/7 | 6/2 |
| 16/8 | 4/4 | 2/6 | 1/7 | 6/2 |
| 8/8 | 8/0\* | 5/3 | 4/4 | 6/2 |
| Piperacillin/ Tazobactam | 512/8 | 5/3 | 7/1 | 0/8\* | 3/5 |
| 256/8 | 4/4 | 5/3 | 1/7 | 3/5 |
| 128/8 | 2/6 | 7/1 | 0/8\* | 3/5 |
| Amoxicillin/Clavulanic Acid | 1024/8 | 6/2 | 3/5 | 2/6 | 3/5 |
| 512/8 | 5/3 | 4/4 | 2/6 | 5/3 |

**Table 2:** Ratios of beneficial over detrimental effects of each of the four substitutions within TEM-50 (using binary code). The β-lactam antibiotic treatments listed on the left, followed by the concentration in µg/mL. Asterisks represent the genotypes that resulted in no sign epistasis in a specific treatment.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| A) SAM   |  |  |  |  |  | | --- | --- | --- | --- | --- | |  | **8 µg/mL** | **16 µg/mL** | **32 µg/mL** | **64 µg/mL** | | **8 µg/mL** | - | 53% | 59% | 50% | | **16 µg/mL** |  | - | 53% | 59% | | **32 µg/mL** |  |  | - | 59% | | **64 µg/mL** |  |  |  | - | | Composite: 34.3% | | | | | | B) TZP   |  |  |  |  | | --- | --- | --- | --- | |  | **128 µg/mL** | **256 µg/mL** | **512 µg/mL** | | **128 µg/mL** | - | 72% | 78% | | **256 µg/mL** |  | - | 56% | | **512 µg/mL** |  |  | - | | Composite: 50% | | | | |
| C) CPR   |  |  |  |  | | --- | --- | --- | --- | |  | **8 µg/mL** | **10 µg/mL** | **12.5 µg/mL** | | **8 µg/mL** | - | 56% | 68% | | **10 µg/mL** |  | - | 68% | | **12.5 µg/mL** |  |  | - |   Composite: 44% | D) CTT   |  |  |  |  | | --- | --- | --- | --- | |  | **0.0312 µg/mL** | **0.0625 µg/mL** | **0.125 µg/mL** | | **0.0312 µg/mL** | - | 66% | 56% | | **0.0625 µg/mL** |  | - | 59% | | **0.125 µg/mL** |  |  | - |   Composite: 41% |
| E) CAZ   |  |  |  |  | | --- | --- | --- | --- | |  | **0.0625 µg/mL** | **0.1 µg/mL** | **0.125 µg/mL** | | **0.0625 µg/mL** | - | 75% | 68% | | **0.1 µg/mL** |  | - | 84% | | **0.125 µg/mL** |  |  | - |   Composite: 66% | F) CTX   |  |  |  |  |  | | --- | --- | --- | --- | --- | |  | **0.04 µg/mL** | **0.05 µg/mL** | **0.06 µg/mL** | **0.123 µg/mL** | | **0.04 µg/mL** | \_ | 63% | 66% | 53% | | **0.05 µg/mL** |  | \_ | 78% | 59% | | **0.06 µg/mL** |  |  | \_ | 69% | | **0.123 µg/mL** |  |  |  | \_ | | Composite 37.5% | | | | | |
| G) AMP   |  |  |  |  | | --- | --- | --- | --- | |  | **64 µg/mL** | **128 µg/mL** | **256 µg/mL** | | **64 µg/mL** | \_ | 69% | 63% | | **128 µg/mL** |  | \_ | 50% | | **256 µg/mL** |  |  | \_ | | Composite 40.6% | | | | | H) AM   |  |  |  |  | | --- | --- | --- | --- | |  | **256 µg/mL** | **512 µg/mL** | **1024 µg/mL** | | **256 µg/mL** | \_ | 44% | 53% | | **512 µg/mL** |  | \_ | 66% | | **1024 µg/mL** |  |  | \_ | | Composite 31.3% | | | | |
| I) FEP   |  |  |  | | --- | --- | --- | |  | **0.0312 µg/mL** | **0.0156 µg/mL** | | **0.0312 µg/mL** | \_ | 75% | | **0.0156 µg/mL** |  | \_ | | Composite: 75% | | | | J) AMC   |  |  |  | | --- | --- | --- | |  | **512 µg/mL** | **1024 µg/mL** | | **512 µg/mL** | \_ | 41% | | **1024 µg/mL** |  | \_ | | Composite: 40.6% | | | |

**Table 3:** Similarity matrices for five treatments. A) Amoxicillin/Sulbactam B) Piperacillin/Tazobactam. The concentration for the inhibitors stays constant throughout at 8 μg/mL. C) Cefprozil D) Cefotetan and E) Ceftazidime F) Cefotaxime G) Ampicillin H) Amoxicillin I) Cefepime J)Amoxicillin/Clavulanic Acid. The concentration of antibiotic is across the top row and left columns in bold, units in μg/mL. The percentage of similarity among the adaptive landscapes for each comparison is shown, and represents the arrows that match in direction between the two concentrations being compared. The percentage of arrows that appear in each composite is also listed under the corresponding tables.

|  |  |  |
| --- | --- | --- |
| **CTT CAZ** | **0.125** | **0.0625** |
| **0.125** | 50% | 59% |
| **0.0625** | 53% | 69% |
|  |  |  |
| **TZP AMP** | **128** | **256** |
| **128** | 56% | 56% |
| **256** | 53% | 59% |
|  |  |  |
| **AM AMC** | **512** | **1024** |
| **512** | 60% | 53% |
| **1024** | 50% | 63% |

**Table 4:** Similarity matrices of different β-lactam antibiotics with similar concentrations. The concentrations of β-lactam antibiotics are µg/mL. For the penicillin plus inhibitor treatments, the concentration is just for the penicillin, the inhibitors are at a constant concentration of 8 µg/mL.

|  |  |  |
| --- | --- | --- |
| **Substitution** | **Isolated** | **Binary Allele Code** |
| TEM-1 | TEM-1 | 0000 |
| M69L | TEM-33 | 1000 |
| E104K | TEM-17 | 0100 |
| G238S | TEM-19 | 0010 |
| N276D | TEM-84 | 0001 |
| M69L/E104K | - | 1100 |
| M69L/G238S | - | 1010 |
| M69L/N276D | TEM-35 | 1001 |
| G238S/E104K | TEM-15 | 0110 |
| G238S/N276D | - | 0011 |
| N276D/E104K | - | 0101 |
| M69L/E104K/N276D | - | 1101 |
| M69L/E104K/G238S | - | 1110 |
| G238S/N276D/E104K | - | 0111 |
| G238S/N276D/M69L | - | 1011 |
| TEM-50 | TEM-50 | 1111 |

**Table 5:** Constructs containing all of the possible substitutions in *blaTEM-50.* The left column lists the substitutions 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. If the variant has been clinically isolated, the name is listed in the center column. The right hand column shows the binary allelic code we used to represent these variants. The number ‘1’ represents the substitution present and a ‘0’ represents the no substitution at that specific location. For example, M69L corresponds to Methionine being replaced by Leucine on the 69th position. The two substitutions included in this experiment that are inhibitor resistant TEM’s are denoted.

|  |  |
| --- | --- |
| **AMacintosh HD:Users:portia:Dropbox:TEM.50:TEM-50 Landscapes:CPR:CPR8Landscapedashed.pdf** | **BMacintosh HD:Users:portia:Dropbox:TEM.50:TEM-50 Landscapes:CPR:CPR10Landscapedashed.pdf** |
| **CMacintosh HD:Users:portia:Dropbox:TEM.50:TEM-50 Landscapes:CPR:CPR12.5Landscapedashed.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) 80μg/mL, B) 100μg/mL, C) 128μg/mL. Forward arrows signify new substitutions and backward arrows signify reversions. Solid arrows represent significance with a p-value ≤ 0.05. Dashed arrows represent non-significance, p-value ≥ 0.05. The global optimum genotype is highlighted in bold D) Composite of all concentrations, showing only the arrows that remain in the same direction throughout the three concentrations. We see a genotype with three substitutions as the optimum at the highest concentration and a genotype with two substitutions as optima at the two lower concentrations. We also observed the largest number of significant differences in growth rates at the middle concentration of 100 μg/mL, which was also the concentration where we observed the greatest number of significant improvements resulting from reversions.

**Figure 2:** Bar plot that depicts the frequency of each TEM-50 variant appearing as one of the top three maxima or bottom three minima across all concentrations of the 15 β-lactam treatments.

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