**Title:**

**Adaptive Landscapes of Variant Mutant Alleles Change as Concentration of Antibiotic Change**

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**Abstract**

Most studies about the evolution of antibiotic-resistance are focused on selection for resistance at lethal antibiotic concentrations [[1](#_ENREF_1)], 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. High-resolution competition assays have shown that resistant bacteria are selected at relatively low concentrations of antibiotics [[1](#_ENREF_1)]. Sub-lethal concentrations of antibiotics are found widely 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 solved 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-lactams 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*.* As previously observed, the pathways change depending on the type of β-lactam. However, we also observed that the landscapes 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 likely accelerates the selection of numerous TEM variant genotypes within that environment.

**Introduction**

Bacteria are routinely exposed to a wide range of antibiotics that are present at a wide spectrum of concentrations due to the breakdown of antibiotics and their presence in agricultural runoff, wastewater, and food [[2](#_ENREF_2)]. This occurrence has been heavily documented [[3](#_ENREF_3),[4](#_ENREF_4)]. 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 [[5](#_ENREF_5)]. 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 [[6](#_ENREF_6)], [[5](#_ENREF_5)]. The antibiotics cannot be completely filtered out before being used for agriculture, which delivers low concentrations of antibiotics to crops and farmland [[6](#_ENREF_6)]. Also, some ranchers and farmers use antibiotics to promote lean muscle production in animals and counts for up 13% of antibiotic use [[3](#_ENREF_3),[4](#_ENREF_4)]. 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 [[7](#_ENREF_7)]. 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.

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 [[8](#_ENREF_8)]. 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 encode a β-lactamase is the *bla*TEM-1 gene [[9](#_ENREF_9)].

In 1963, the TEM β-lactamase (TEM-1) 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 170 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 [[10](#_ENREF_10)], 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. TEM-30, reported in 1992 [[11](#_ENREF_11)], 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 [[12](#_ENREF_12)], as the first Complex Mutant TEM (CMT), where both cephalosporin and inhibitor resistance appear simultaneously [[13](#_ENREF_13)]. 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 [[9](#_ENREF_9)]. We have created all 16 possible variations of those substitutions using site directed mutagenesis [[14](#_ENREF_14)].

Because of the widespread use of β-lactam antibiotics, there have been additional approaches that utilize a combination of mechanisms based on activators for β-lactamases such as clavulanic acid, sulbactam and tazobactam [[15](#_ENREF_15)]. 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 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. Within the TEM-50 gene, there are two substitutions that contribute to the inhibitor resistant phenotype, M69L (TEM-33) and N276D (TEM-84) [[15](#_ENREF_15)].

Epistasis, or non-additive interactions between mutations, plays a major role in antibiotic resistance. Epistatic interactions can be used to study topography of fitness landscapes and the dynamics of adaptation [[16](#_ENREF_16),[17](#_ENREF_17)]. One study shows these patterns of epistasis among large and small-effect beneficial substitutions occurring in TEM-1 [[18](#_ENREF_18)]. Schenk et al. found 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. It has been shown that epistasis occurs more frequently among mutations within the same gene [[19](#_ENREF_19)].

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 [[20](#_ENREF_20)]. Previous studies on GxE interactions have measured fitness on genotypes that differ by numerous unknown mutations and most recently [[21](#_ENREF_21)] investigated the effects of 26 genotypes in four environments measuring fitness relative to a common progenitor. Here we will study GxE interactions on sixteen genotypes that differ by up to four mutations with 10 different β-lactam antibiotics at three different concentrations using growth rates as a measurement of fitness.

To study within gene epistasis and genotype-by-environment effects on the adaptive landscape of the TEM-1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 look at how the concentration of β-lactam antibiotic affects the composition of each landscape by taking into consideration the ratio between new mutations (forward arrows) and reversions (backward arrows). With this information we are able to calculate similarity matrices to study how much each treatment, and each concentration, differs from one another. We also examine 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 variants of the substitutions that occur in TEM-50. After measuring the growth rates of the 16 genotypes, we created adaptive landscapes for each concentration of 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 results in the highest growth rate. In the case of each comparison, the arrows inclined towards TEM-50 signify selection for new substitutions and arrows inclined towards TEM-1 signify selection for reversions, depending on which growth rates are higher. Red arrows represent a significant difference between growth rates as determined by one-way ANOVA (p-value ≤ 0.05) and black 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.

We hypothesized that overall selection for the fixation the four substitutions we considered would increase as the concentration of antibiotic increased. To test this hypothesis, we counted the number of times that the addition of an amino acid substitution was selected versus the number of times that reversion of an amino acid substitution was selected.

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 penicillin plus inhibitor treatments, the number of times a reversion of an amino acid substitution occurred was greater than the number of times the addition of an amino acid substitution occurred; 44.4% to 33.3%. The penicillin plus inhibitor treatments also differed in such that 22.3% of the treatments resulted in the number of addition of amino acid substitutions was equal to the number of reversions.

After considering all thirty treatments performed, we have found that the ratios from the number of times the addition of an amino acid substitution was selected and the number of times a reversion of an amino acid substitution was selected changed as both the treatment changed and the concentrations within treatment changed.

We further investigated the variation in ratios across concentrations by creating similarity matrices (Table 2). In each matrix, we calculated the percent similarity of arrow direction among the concentrations of antibiotics. From the similarity matrices, we found that 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 β-lactam. To understand the basis for those similarities and differences, we considered each set of landscapes separately.

In each landscape (SI figures 1-10) there are different genotypes that prove to be the ‘most fit’, referred to as the global optimum. As the concentration of each antibiotic changed, the global optimum also changed 100 % of the time. Overall, TEM-50 appeared as the global optimum in 17% of all treatments (including twice in CTX, and once in AMP, FEP and SAM). Triple substitutions appeared as the global optimum in 40% of all treatments, at least once in each of the 15 β-lactams and some at multiple concentrations. Double substitutions appeared as the global optimum in 33% of all treatments in all β-lactams except AMP and FEP. Single substitutions appeared at the global optimum in only 10% of all treatments (including AMP, CAZ, and AM). TEM-1 did not appear as the global optimum in any of the treatments tested (Figure 1). While considering the top three optima in each landscape, we noticed that the genotypes 1101 and TEM-50 (1111) were selected the most frequently. Out of all treatments, these genotypes appeared the most when treated with an inhibitor and penicillin. The genotype 1101, was the global optimum in four out of the nine treatments of penicillin/inhibitor combinations: Two appear as global optimum in SAM at 32μg/mL and 64μg/mL, one in AMC 512 μg/mL, and one in TZP 512μg/mL.

Within all of the TEM-50 landscapes shown, the global optimum for each at each concentration had at least one inhibitor resistant mutation (binary either 1000 or 0001) when treated with an inhibitor and β-lactam. While we created the complete adaptive landscapes, we considered both inhibitor resistant mutations and cephalosporin hydrolysis mutations. We observed that combinations of these two types of substitutions result in abundant sign epistasis. To simplify these interactions and better explain the effects of Inhibitor resistance substitutions, we examined these mutations separately. With these adaptive landscapes, we eliminated all reversions (backward arrows) and only kept the new substitutions (forward arrows) in which there was selection for any genotype that contained an inhibitor resistant mutation (SI Figures 3-5). With these stringent restrictions we still found that there was a pathway towards each global optimum, which shows that β-lactamase inhibitors are an important selective pressure in each of these landscapes.

Finally, we see that the variation in landscapes change as the concentrations of β-lactams change. This holds true for all twelve β-lactam treatments and three β-lactam plus inhibitor treatments. Depending on the treatment, the percent similarity either increases or decreases as the difference in concentration of β-lactam increase (Table 2) and the ratios of new substitutions versus reversions differ in each treatment (Table 1). 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.

We observed at least one pathway between the wild-type (TEM-1) and the global optimum in all landscapes.

**Discussion**

We have considered four substitutions within TEM-50 gene and multiple concentrations of fifteen antibiotics. When treated with an inhibitor and penicillin, the global optimum for each at each concentration had at least one inhibitor resistant mutation in all landscapes. This signified that there could be small trade offs, or compensatory effects of mutations. Sign epistasis causes mutations to become compensatory. Depending on which antibiotic and what concentration is used we can see that the same substitution can have different effects. For example, in the landscape with Amoxicillin plus Clavulanic Acid, we can see the genotype 1000 being beneficial when treated at 1024 μg/mL, but detrimental when Amoxicillin is lowered to 512 μg/mL. This is also evident for genotype 1001, which exclusively contains inhibitor resistance substitutions. In Amoxicillin 1024 μg/mL this genotype 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.

We also show how genotype-by-environment interactions appear in the TEM-50 gene. As the treatments change, whether by antibiotic type or concentration, the most successful genotypes change in each case. For example, we noticed that depending on the types of antibiotics used, selection for new mutations versus reversions vary: cephalosporins tend to select for new substitutions over reversions whereas penicillins and penicillins plus inhibitors do not (Table 1). Across all landscapes, some substitutions are selected in many environments and others are not, indicating that sign epistasis effects may be stronger for some substitutions than others.

The presented data suggest that different antibiotic concentrations select for different genotypes. However there is no predictable pattern among the changes in concentration or adaptive landscapes. The global optima consistently carry at least one of the two inhibitor resistant substitutions in treatments with β–lactamase inhibitors; in treatments where penicillins were used alone (Amoxicillin and Ampicillin) single substitutions were selected as global optima of ???.

An abundance of readily available evolutionary trajectories across concentrations of antibiotics show that varied, residual concentrations of antibiotics can, and likely do accelerate the evolution of the TEM β-lactamase. Future studies will show whether this pattern holds across other antibiotic resistance genes and to what extent the evolutionary potentials of resistance genes are expanded through antibiotic containing waste.

**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 [[14](#_ENREF_14)]. 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, with 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, the 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 antibiotic). 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 [[22](#_ENREF_22)].

*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. 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 the natural log of the O.D. [[22](#_ENREF_22)]. 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). (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 [[22](#_ENREF_22)].

*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 genotypes. We compared each of the genotypes with those that had just one substitution different from each other, going from the wild type, TEM-1, to TEM-50 (Table 3). We were working with a 95% confidence interval, which translates to 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.

|  |  |  |
| --- | --- | --- |
| **Penicillins** | **Concentration (μg/mL)** | **F: B** |
| **Amoxicillin** | 1024 | 10:22 |
|  | 512 | 17:15 |
|  | 256 | 13:19 |
|  |  |  |
| **Ampicillin 8X** | 256 | 22:10 |
|  | 128 | 18:14 |
|  | 64 | 20:12 |
|  |  |  |
| **Pen + Inhibitors** | **Concentration (μg/mL)** | **F: B** |
| **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)** | **F: B** |
| **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: reversion, for each antibiotic treatment and concentration used. Antibiotics in first column, concentration in μg/mL in the second column, and ratio in third column.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| A) SAM   |  |  |  |  |  | | --- | --- | --- | --- | --- | |  | **8** | **16** | **32** | **64** | | **8** | - | 53.1% | 59.4% | 50% | | **16** |  | - | 53.1% | 59.4% | | **32** |  |  | - | 59.4% | | **64** |  |  |  | - | | B) TZP   |  |  |  |  | | --- | --- | --- | --- | |  | **128** | **256** | **512** | | **128** | - | 71.8% | 78.1% | | **256** |  | - | 56.2% | | **512** |  |  | - | |
| C) CPR   |  |  |  |  | | --- | --- | --- | --- | |  | **8** | **10** | **12.5** | | **8** | - | 56% | 68% | | **10** |  | - | 68% | | **12.5** |  |  | - |   Composite: 44% | D) CTT   |  |  |  |  | | --- | --- | --- | --- | |  | **0.0312** | **0.0625** | **0.125** | | **0.0312** | - | 66% | 56% | | **0.0625** |  | - | 59% | | **0.125** |  |  | - |   Composite: 41% |
| E) CAZ   |  |  |  |  | | --- | --- | --- | --- | |  | **0.0625** | **0.1** | **0.125** | | **0.0625** | - | 75% | 68% | | **0.1** |  | - | 84% | | **0.125** |  |  | - |   Composite: 66% |  |

**Table 2:** 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. 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.

|  |  |  |
| --- | --- | --- |
| **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 3:** 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.

**Figure 1:** Bar plot that depicts the frequency of each TEM-50 variant appearing as one of the tope three Global Optima across all concentrations of the 15 β-lactam treatments.

References

1. Hughes D, Andersson DI (2012) Selection of resistance at lethal and non-lethal antibiotic concentrations. Current opinion in microbiology 15: 555-560.

2. Gullberg E, Cao S, Berg OG, Ilback C, Sandegren L, et al. (2011) Selection of resistant bacteria at very low antibiotic concentrations. PLoS pathogens 7: e1002158.

3. Gustafson RH (1991) Use of antibiotics in livestock and human health concerns. Journal of dairy science 74: 1428-1432.

4. Wegener HC (2003) Antibiotics in animal feed and their role in resistance development. Current opinion in microbiology 6: 439-445.

5. Kolpin DW, Skopec M, Meyer MT, Furlong ET, Zaugg SD (2004) Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions. The Science of the total environment 328: 119-130.

6. Watkinson AJ, Murby EJ, Kolpin DW, Costanzo SD (2009) The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. The Science of the total environment 407: 2711-2723.

7. Blazquez J, Couce A, Rodriguez-Beltran J, Rodriguez-Rojas A (2012) Antimicrobials as promoters of genetic variation. Current opinion in microbiology 15: 561-569.

8. Guthrie VB, Allen J, Camps M, Karchin R (2011) Network models of TEM beta-lactamase mutations coevolving under antibiotic selection show modular structure and anticipate evolutionary trajectories. PLoS computational biology 7: e1002184.

9. Bradford PA (2001) Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clinical microbiology reviews 14: 933-951, table of contents.

10. Sirot D, Sirot J, Labia R, Morand A, Courvalin P, et al. (1987) Transferable resistance to third-generation cephalosporins in clinical isolates of Klebsiella pneumoniae: identification of CTX-1, a novel beta-lactamase. The Journal of antimicrobial chemotherapy 20: 323-334.

11. Vedel G, Belaaouaj A, Gilly L, Labia R, Philippon A, et al. (1992) Clinical isolates of Escherichia coli producing TRI beta-lactamases: novel TEM-enzymes conferring resistance to beta-lactamase inhibitors. The Journal of antimicrobial chemotherapy 30: 449-462.

12. Sirot D, Recule C, Chaibi EB, Bret L, Croize J, et al. (1997) A complex mutant of TEM-1 beta-lactamase with mutations encountered in both IRT-4 and extended-spectrum TEM-15, produced by an Escherichia coli clinical isolate. Antimicrobial agents and chemotherapy 41: 1322-1325.

13. Robin F, Delmas J, Machado E, Bouchon B, Peixe L, et al. (2011) Characterization of the Novel CMT Enzyme TEM-154. Antimicrobial agents and chemotherapy 55: 1262-1265.

14. Goulart CP, Mahmudi M, Crona KA, Jacobs SD, Kallmann M, et al. (2013) Designing antibiotic cycling strategies by determining and understanding local adaptive landscapes. PloS one 8: e56040.

15. Chaibi EB, Sirot D, Paul G, Labia R (1999) Inhibitor-resistant TEM beta-lactamases: phenotypic, genetic and biochemical characteristics. The Journal of antimicrobial chemotherapy 43: 447-458.

16. Kondrashov FA, Kondrashov AS (2001) Multidimensional epistasis and the disadvantage of sex. Proceedings of the National Academy of Sciences of the United States of America 98: 12089-12092.

17. Salverda ML, Dellus E, Gorter FA, Debets AJ, van der Oost J, et al. (2011) Initial mutations direct alternative pathways of protein evolution. PLoS genetics 7: e1001321.

18. Schenk MF, Szendro IG, Salverda ML, Krug J, de Visser JA (2013) Patterns of Epistasis between beneficial mutations in an antibiotic resistance gene. Molecular biology and evolution 30: 1779-1787.

19. Poon A, Chao L (2005) The rate of compensatory mutation in the DNA bacteriophage phiX174. Genetics 170: 989-999.

20. Gillespie JH, Turelli M (1989) Genotype-environment interactions and the maintenance of polygenic variation. Genetics 121: 129-138.

21. Remold SK, Lenski RE (2001) Contribution of individual random mutations to genotype-by-environment interactions in Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America 98: 11388-11393.

22. Hall BG, Acar H, Nandipati A, Barlow M (2013) Growth Rates Made Easy. Molecular biology and evolution.