**Title:**

**Rational Design of Antibiotic Treatment Plans**

Portia M. Mira1

Kristina Crona2

Devin Greene2

Juan C. Meza1

Bernd Sturmfels3

Miriam Barlow1

**Institutional Affiliations:**

1School of Natural Science, University of California, Merced

2 Department of Mathematics, American University

3 Departments of Mathematics, Statistics, and EECS, University of California, Berkeley

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**Abstract:**

**Introduction**

Antibiotic resistance is an inevitable outcome whenever antibiotics are used. There are many reasons for this: 1) As humans (also as eukaryotes), we are vastly outnumbered by bacteria in nearly all measures, including total population size, biomass, genetic diversity, emigration, and immigration [[1](#_ENREF_1)]; 2) bacteria can use horizontal gene transfer to share resistance genes across distantly related species of bacteria, including non-pathogens [[2](#_ENREF_2)]; 3) compared to humans, bacteria have relatively few vulnerable target sites [[3](#_ENREF_3)]; 4) microbes are the sources of nearly all antibiotics that are used by humans [[4](#_ENREF_4)]. Given the overwhelming numbers of bacteria, the limited number of target sites, the numerous ways that they can infect humans, and that they have been exposed to antibiotics for billions of years, resistance to antibiotics used by human populations is unavoidable.

Once resistance is present in a bacterial population, it is exceedingly difficult to remove for several reasons. If any amount of antibiotic is present in the environment, antibiotic resistance genes will confer a large fitness advantage [[5](#_ENREF_5)], and even when antibiotics are not present in an environment, the fitness costs for carrying and expressing resistance genes are small to non-existent [[6](#_ENREF_6)]. In addition to it being difficult to remove antibiotics from the environment [[7](#_ENREF_7)], if humans were to completely abandon the use of antibiotics, resistance would persist for years [[8](#_ENREF_8)].

Efforts to remove resistance genes from clinical environments by either discontinuing or reducing the use of specific antibiotics for some period of time, either through general reduction of antibiotic consumption or periodic rotations of antibiotics (cycling) have not worked in any reliable or reproducible manner [[9](#_ENREF_9)]; indeed it would have been surprising if they had worked [[10](#_ENREF_10),[11](#_ENREF_11)].

Since antibiotic resistance *is* unavoidable, it only makes sense to accept its inevitability and do the best we can within that framework. A reasonable approach is to rotate the usage of antibiotics. This has been implemented in many ways and there are recent studies to model the optimal duration, mixing vs cycling, and how relaxed antibiotic cycles may be and still function as planned [[12](#_ENREF_12),[13](#_ENREF_13)]. However, those models have not focused on developing a method for creating the ideal succession of antibiotics. In a previous publication [[14](#_ENREF_14)], we proposed that susceptibility to antibiotics could be restored by rotating consumption of multiple antibiotics that are a) structurally similar, b) inhibit/kill bacteria through the same target site, and c) result in pleiotropic fitness costs that reduce the overall resistance of bacteria to each other. We showed an anecdotal, proof-of-principle example [[14](#_ENREF_14)] of how this might work with a series of β-lactam antibiotics in which some would select for new amino acid substitutions in the TEM β-lactamase and others that would select reversions in TEM ultimately leading back to the wild-type (un-mutated) state.

Our current work is to identify β-lactam treatment plans that are the most likely to return a population expressing a small number of variant TEM enzymes to the wild-type state. The wild type TEM-1 and a handful of its descendants confer resistance to penicillins alone, while most of its descendants confer resistance to either cephalosporins or penicillins combined with β-lactamase inhibitors (inhibitor resistance), and a few confer resistance to both. Of the 194 TEM enzymes that have been identified clinically [[15](#_ENREF_15)], 174 (89.7%) differ from the wild type TEM 1 by at most four amino acid substitutions (see Table 1). Our choice of a system that includes four amino acid substitutions is based upon an apparent threshold for amino acid substitutions among functional TEM enzymes. The rarity of the co-existence of cephalosporin resistance and inhibitor resistance and the fact that no single substitution confers both phenotypes suggested that sign epistasis (i.e. reversals from beneficial to detrimental) exists as the substitutions that contribute to this dual phenotype are combined.

The ability to push an evolved TEM enzyme back to the wild type state would limit the range of antibiotics to which it could confer resistance. To embark upon our effort to determine the best way of doing this, we decided to create a model system based upon the enzyme TEM-50, which differs from TEM-1 by four amino acid substitutions. All four substitutions by themselves confer clearly defined resistance advantages in the presence of certain antibiotics. Additionally, TEM-50 is one of the few enzymes that simultaneously confers resistance to cephalosporins and inhibitor combined therapies.

**Results**

*From experimental data to mathematical models*

We first created all 16 variants of the four amino acid substitutions found in TEM-50 using site directed mutagenesis (Table 2). We then measured the growth rates of 12 replicates of *E.coli* DH5α-E expressing each variant in the presence of one of fifteen β-lactam antibiotics (Table 3).

There were 12 replicates for each sample in each antibiotic. We computed the mean growth rate (Table 4) and the variance of each sample, as well as the significance between adjacent variants that differed by one amino acid substitution. This was done using one-way ANOVA analysis.

These results are provided in Figures 1-15, where the arrows in the landscape maps connect pairs of adjacent enzymes. For each comparison of adjacent enzymes, we indicate the one whose expression resulted in the faster growth by directing the arrowhead towards that variant, and implying that evolution would proceed in that direction if the two variants occurred simultaneously in a population. In other words, the one indicated by the arrowhead would increase in frequency and reach fixation in the population, while the other would be lost. Red arrows indicate significance, and black arrows indicate differences that were not statistically significant by ANOVA, but that may still exist if a more sensitive assay was used.

We rank ordered the enzymes (Table 5) in each landscape diagram with a score from 1 to 16, with the enzyme promoting the fastest growth receiving a score of “1” and the enzyme with the slowest growth a score of “16”. This analysis shows that all enzymes have a score of 5 or better and a score of 13 or worse, in at least one landscape, indicating that there is abundant pleiotropy as antibiotic selective pressures change. That pleiotropy provides a basis for effectively alternating antibiotic to restore the wild type.

Based on the strong patterns of pleiotropy we observed, we reasoned that the choice and the succession of antibiotics were at least as important as other cycling considerations. We formalized our approach to optimal cycling as follows.

We start by considering the 15 antibiotics previously mentioned in Table 3: AMP, AM, CEC, CTX, ZOX, CXM, CRO, AMC, CAZ, CTT, SAM, CPR, CPD, TZP, and FEP. For each of these 15 antibiotics, we select exactly one TEM fitness landscape that exists at a specific concentration of the antibiotic. That landscape is a real  tensor whose entries are the growth rates we measured. Those growth rates depend upon the states of the four functionally important amino acid residues involved in the evolution of TEM-50. The indices  correspond to four possible amino acid substitutions and exist in either state 0, corresponding to no substitution at that site, or 1, which corresponds to an amino acid substitution that is involved in the resistance phenotype. We can identify with a vector of length 16 whose coordinates are indexed by .

The resulting 15 vectors, one for each antibiotic, are the rows in Table 4.

Our substitution model is a function  that assigns a transition matrix  to the fitness landscape for antibiotic . An entry of that matrix is denoted  .This represents the fixation probability for genotype  transitioning to genotype  in the presence of antibiotic . Recall that a transition matrix has nonnegative entries and its rows sum to 1. The rows and columns of  are labeled by , in some order that is fixed throughout. We require that our transition matrices respect the adjacency structure of the 4-cube, that is,  unless  and  are vectors in  that differ in at most one coordinate. Thus each row of  has at most 5 non-zero entries. In other words, we reasoned that resistant strains are most likely to be in competition with those that express resistance genotypes that are immediately adjacent (vary by a single amino acid substitution). In our model, each TEM has four variants that are adjacent, since there are four amino acid residues under consideration.

If  is a sequence of  antibiotics, then the matrix product is . Our goal is to maximize the matrix entry  for all 15 genotypes  other than 0000.

For each  this requires searching over all  antibiotic sequences of length .

*Finding optimal sequences of antibiotics*

We used two substitution models to determine the optimal (most probable) sequences of β-lactams for returning them to their wild type state. Briefly, the Correlated Probability Model (CPM) allows probabilities to be based upon the actual growth rates. It is given by applying formula (7) to the growth rates in Table 4. The Equal Probability Model (EPM) assumes that beneficial mutations

are equally likely and that only the direction of the arrows in Figures 1-15 is important. This means that the matrix entry  is  if genotype  has  outgoing arrows and there is an arrow from  to .

A visual summary of the highest probabilities seen in the 15 CPM transition matrices is provided in Figure 16. The CPM provides good estimates

if fitness differences between genotypes are small [[14](#_ENREF_14),[16](#_ENREF_16),[17](#_ENREF_17),[18](#_ENREF_18)]. The EPM has

been used in settings where only rank order (as in Table 5) is available [[19](#_ENREF_19)].

For all sequences of antibiotics of a fixed length (2,3,4,5, 6), we examined the probability that a given genotype is returned to the wild type state. The results are summarized in Figure 17 and Tables 8 and 9. We found that there was typically some increase in probability after allowing the addition of one more antibiotic to the treatment path than was strictly necessary to return to the wild type (0000).

However, beyond that addition, there were few cases where we saw major increases in the probability of returning to the wild type state (see Tables 7 and 8). The probabilities were similar using both the CPM and EPM models and tended to converge as the pathway lengths increased.

These results show the most likely paths for returning to the wild type state from various starting points.

Once returned to the wild type state, we identified cycles that would allow for alternation of antibiotics, and allow for some variation through amino acid substitution, but then rapidly return bacteria to the wild type state (Table 6). We found that in the most probable cases, the genotype varied by only one amino acid substitution before reverting back to the wild-type state. However, when treatment plans with lower probabilities are considered, we find that more amino acid substitutions in the genotype are allowed.

**Discussion**

In this study, we have developed an experimental approach for measuring pleiotropy and a mathematical approach for optimizing antibiotic cycling. These two methods provide the basic tools necessary for developing reasonable sequences of antibiotic therapies that should be able to restore susceptibility to select antibiotics. The experimental approach we developed is rapid and high throughput, and should be applicable to many species of resistant bacteria. The mathematical model we created expresses the problem of antibiotic resistance in general terms, and can therefore be applied to any resistance phenotypes conferred by related genotypes to identify the antibiotic rotations that have the highest probability of reversing the evolution of resistance.

We have implemented this model and show that it can be used to identify antibiotic treatment pathways that reverse the evolution of the TEM β-lactamase. Due to the specific phenotypes we measured, our exact results are probably most applicable in a clinical environment where both outpatient and inpatient treatments are implemented. Determining the phenotypes of strains in even more antibiotics would likely expand the scope of possible treatment plans to a wider range of clinical environments populations.

Whenever an antibiotic treatment plan is considered, the evolution of resistance in response to the antibiotics used in the treatment plan should be considered. We have presented reasonable methods for doing this and present results which may have some immediate utility to physicians.

Methods

Experimental methods

*Strains and Cultures*

We expressed 16 mutant constructs of the blaTEM gene in plasmid pBR322 from strain DH5-αE. The 16 variants differ at all combinations of four amino acid residues and have been previously described [[14](#_ENREF_14)]. We grew them overnight (16 hours) in standing cultures and diluted them to a concentration of 1.9X105 as described elsewhere [[14](#_ENREF_14)].

We transferred 80 µl of each culture to a 384-well plate with one genotype present in each of the 16 rows. The first 12 wells of each row were antibiotic free (controls) and the last 12 wells contained a single antibiotic at an inhibitory, sublethal concentration

After plating, a membrane is placed over the plate and simultaneously incubated/measured in the Eon Microplate Spectrophotometer at a temperature of 25.1°C for 22 hours. This relatively cool (<37º) temperature is used because degradation of the antibiotics is much slower, while the growth rate of the bacteria is still sufficient to capture the complete exponential period of growth over the duration of the experiment. Overall, we have found that a temperature ~25ºC yields more reliable and consistent measurement of growth rates in the presence of antibiotics.

Measurements of cell density (light scattering) at a wavelength of 600 nanometers were automatically collected every 20 minutes after brief agitation to homogenize and oxygenate the culture.

*Growth Rates*

The data obtained from the microplate spectrophotometer is exported to the GrowthRates program to derive the growth rates. In essence, by measuring the optical density at frequent intervals the GrowthRates program can estimate the growth rate, , through a linear regression algorithm fitting the data from the exponential growth phase. Details can be found in [[20](#_ENREF_20)] in the section entitled “The Growth Curve” located on pages 233-4. The output of this program for the data we collected was a list  of 15 tensors, each of format. These are the rows in Table 4. So if  is a genotype, then  is the fitness of genotype  in the presence of antibiotic . This fitness is a growth rate, so we are here using the letter for a quantity often denoted by.

One-Way Analysis of Variance (ANOVA) was then used to compare the means of the growth rates obtained, and to determine if there were significant differences between the growth rates of adjacent genotypes.

Derivation of fixation probabilities

Once the growth rates have been determined under various experimental conditions, the next step is to use them to compute fixation probabilities.

???

These probabilities can be computed through the use of selection coefficients. The key step is to note that the selection coefficient can be described in terms of the experimentally derived growth rates, which in turn can be used to compute the desired fixation probabilities of mutations.

*Selection Coefficients*

Consider a population with non-overlapping generations. In this case, starting from an initial population size, , the population size, , after some number of generations, ,can be given by



(1) .

Here, the *absolute fitness* is a measure of the expected reproductive success and could be viewed as the probability of survival or the fraction of individuals that survive in the next generation. In a population where the wild-type has absolute fitness , and a single mutant has absolute fitness the *relative fitness* can be defined by



.

The selection coefficient (Gillespie 2004) can now be defined by

,

and can be thought of as a measure of fitness of the single mutant relative to the wild-type. If s is positive then the mutant is more fit than the wild-type, whereas if s is negative, then the mutant is less fit.

To make the connection between the selection coefficient and the growth rates, the continuous version of equation (1), can be written as



where is the generation time. It is well known that populations undergoing exponential growth obey Malthus' law, which states that the population at any point in time is given by:



.

Here the constant, , is known as the continuous growth rate specific to the population under consideration and is time. Comparing these two equations,



(2) ,

which relates between the growth rates and the absolute fitness of a mutant.

We now make the assumption that the selection coefficient,, is small such that we can use the approximation , from which it follows that



(3) .

Using equations (2-3), it follows that we can approximate the selection coefficient by

(4) ,

which provides us with the desired relationship between the growth rates and the selection coefficient (assuming that the selection coefficient is sufficiently small).

*Transition Matrices*

The final step involves the computation of the fixation probabilities from the growth rates, which can be substituted for selection coefficients as follows. We use two different models (CPM and EPM) for determining probabilities of mutational trajectories [[17](#_ENREF_17)] .

Correlated Probability Model (CPM): Suppose that the population is dominated by a particular genotype. If the genotype has  beneficial mutational neighbors, with selection coefficients , then one method for estimating the probability for the mutation *j* can be given by

(5) .

Equation(5) is well established, and was originally given in [[17](#_ENREF_17)].

If one now applies (4), the probability for the mutation equals



(6) 

In terms specified for the tensors representing each landscape given in Results under the subheading “*From experimental data to mathematical models”* 2),

(7) 

Equal Probability Model (EPM): According to the EPM model, the probabilities are equal for all beneficial mutations, so that one needs the fitness graphs only for computing the probabilities. The matrix entry  is  if genotype  has  outgoing arrows and there is an arrow from  to .

*Optimal antibiotic sequences and pathways of genotypes*

Let  denote the  transition matrix we derived for the antibiotic labeled . For any sequence  of  antibiotics, we consider the matrix product . This product is also a  transition matrix. Its entry in row *a* and column *b* is the fixation probability of genotype  mutating to genotype  under the antibiotic sequence . That probability is a sum of products of entries in the individual matrices , with one sum for each possible pathway of genotypes from  to . Our optimization algorithm enumerates all antibiotic sequences of length , and it selects all sequences that maximize the entry in row *a* and column *b* of the matrix product. In a subsequent step we then analyze these optimal antibiotic sequences, and for each such sequence, we extract the full list of genotype pathways that contribute.



We implemented this algorithm in the computer algebra software Maple, and we ran it for . The running time of the program is slow because of the exponential growth in the number of sequences. At present we do not know whether an efficient algorithm exists for solving our optimization problem for larger values of .

**Figure Legends**

Figures 1-15

These figures present a visual summary of the adaptive landscape 2x2x2x2 tensors in which each resistance phenotype conferred by each TEM genotype is enumerated. Arrows pointing upward represent addition of a mutation. Arrows pointing downward represent reversions. Red arrows indicate significance between adjacent growth rates as determined by one way ANOVA. Genotypes that confer the most resistance to each antibiotic are shown in red.

Figure 16

Each arrow is labeled by the drug or drugs corresponding to the maximal transition probability, taken over all 15 drugs. Each arrow is also labeled by the maximal probability. For the probability of a particular trajectory between genotypes, one multiplies the numbers on each arrow along the trajectory. Linear trajectories are possible. For example, from the starting point 1111, one can use the drug sequence: CEC, CAZ/CPR, TZP, AM across the trajectory 1111, 1110, 0110, 0100, 0000 for a probability of 0.44, since the numbers for the corresponding arrows are 1, 1, 1, 0.44. Circular trajectories are also possible. If we use CEC, CPR, TZP, AM, the probability for the trajectory 0000, 0100, 0110, 0100, 0000 is 0.44, since the numbers of the corresponding arrows are 1, 1, 1, 0.44.

Figure 17

Black arrows show transitions present in five step paths computed using both the CPM and the EPM. Red arrows signify transitions found only in optimum paths computed using the CPM whereas blue signify transitions only found using the EPM.

Figure 18

Black arrows show transitions present in six step paths computed using both the CPM and the EPM. Red arrows signify transitions found only in optimum paths computed using the CPM whereas blue signify transitions only found using the EPM.

**Table 1**

|  |  |
| --- | --- |
| Number of amino acid substitutions | Number of TEM enzymes |
| 1 | 53 |
| 2 | 53 |
| 3 | 37 |
| 4 | 31 |
| 5 | 10 |
| 6 | 2 |
| 7 | 2 |
| 8 | 0 |
| 9 | 0 |
| 10 | 1 |
| 11 | 1 |

**Table 2 Variant Enzymes Created, Binary Codes and Names of Enzymes Identified in Clinical Isolates**

|  |  |  |
| --- | --- | --- |
| **Number of Substitutions** | **Binary**  **Enzyme**  **Code** | **Variants with substitutions found in TEM-50** |
| 0 | 0000 | No substitutions  (TEM-1) |
| 1 | 1000 | M69L  (TEM-33) |
| 1 | 0100 | E104K  (TEM-17) |
| 1 | 0010 | G238S  (TEM-19) |
| 1 | 0001 | N276D  (TEM-84) |
| 2 | 1100 | M69L  E104K  (Not identified) |
| 2 | 1010 | M69L  G238S  (Not identified) |
| 2 | 1001 | M69L  N276D  (TEM-35) |
| 2 | 0110 | E104K  G238S  (TEM-15) |
| 2 | 0101 | E104K  N276D  (Not identified) |
| 2 | 0011 | G238S  N276D  (Not identified) |
| 3 | 1110 | M69L  E104K  G238S  (Not identified) |
| 3 | 1101 | M69L  E104K  N276D  (Not Identified) |
| 3 | 1011 | M69L  G238S  N276D  (Not identified) |
| 3 | 0111 | E104K  G238S  N276D  (Not identified) |
| 4 | 1111 | M69L  E104K  G238S  N276D  (TEM-50) |

**Table 3 Antibiotics used for this study**

|  |  |  |
| --- | --- | --- |
| **Antibiotic** | **FDA approval** | **Antibiotic Group** |
| Ampicillin (AMP) | 1963 | Aminopenicillin |
| Amoxicillin (AM) | 1972 | Aminopenicillin |
| Cefaclor(CEC) | 1979 | Cephalosporin |
| Cefotaxime (CTX) | 1981 | Cephalosporin |
| Ceftizoxime (ZOX) | 1983 | Cephalosporin |
| Cefuroxime (CXM) | 1983 | Cephalosporin |
| Ceftriaxone(CRO) | 1984 | Cephalosporin |
| Amoxicillin + Clavulanic acid (AMC) | 1984 | Penicillin derivative +  β-Lactamase inhibitor |
| Ceftazidime (CAZ) | 1985 | Cephalosporin |
| Cefotetan (CTT) | 1985 | Cephalosporin |
| Ampicillin + Sulbactam (SAM) | 1986 | Penicillin derivative +  β-Lactamase inhibitor |
| Cefprozil (CPR) | 1991 | Cephalosporin |
| Cefpodoxime (CPD) | 1992 | Cephalosporin |
| Pipercillin + Tazobactam (TZP) | 1993 | Penicillin derivative +  β-Lactamase inhibitor |
| Cefepime(FEP) | 1996 | Cephalosporin |

**Table 4 Average Growth Rates ( x 10-3): the rows are the fitness landscapes**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **0000** | **1000** | **0100** | **0010** | **0001** | **1100** | **1010** | **1001** |
| AMP | 1.85 | 1.57 | 2.02 | 1.95 | 2.08 | 2.10 | 0.05 | 2.17 |
| AM | 1.78 | 1.72 | 1.45 | 2.04 | 1.78 | 1.56 | 1.80 | 2.01 |
| CEC | 2.26 | 0.23 | 2.40 | 2.15 | 2.00 | 2.15 | 2.24 | 0.17 |
| CTX | 0.16 | 0.18 | 1.65 | 1.93 | 0.085 | 0.225 | 1.97 | 0.14 |
| ZOX | 0.99 | 1.11 | 1.70 | 2.07 | 0.81 | 1.12 | 1.89 | 1.17 |
| CXM | 1.75 | 0.42 | 2.94 | 2.07 | 1.70 | 2.02 | 1.91 | 1.57 |
| CRO | 1.09 | 0.83 | 2.88 | 2.55 | 0.29 | 1.41 | 3.17 | 0.54 |
| AMC | 1.44 | 1.42 | 1.67 | 1.06 | 1.57 | 1.38 | 1.54 | 1.35 |
| CAZ | 2.13 | 0.29 | 2.04 | 2.62 | 2.66 | 2.63 | 1.60 | 0.58 |
| CTT | 2.13 | 3.24 | 3.29 | 2.80 | 1.92 | 0.55 | 2.88 | 2.97 |
| SAM | 1.88 | 2.20 | 2.46 | 0.13 | 2.53 | 2.50 | 2.31 | 2.57 |
| CPR | 1.74 | 1.55 | 2.02 | 1.76 | 1.66 | 0.22 | 0.17 | 0.26 |
| CPD | 0.60 | 0.43 | 1.76 | 2.60 | 0.25 | 0.64 | 2.65 | 0.39 |
| TZP | 2.68 | 2.71 | 3.04 | 2.43 | 2.91 | 2.45 | 0.17 | 2.50 |
| FEP | 2.59 | 2.07 | 2.44 | 2.39 | 2.57 | 2.74 | 2.96 | 2.45 |
|  |  |  |  |  |  |  |  |  |
|  | **0110** | **0101** | **0011** | **1110** | **1101** | **1011** | **0111** | **1111** |
| AMP | 2.03 | 2.43 | 2.20 | 0.09 | 2.32 | 0.08 | 0.03 | 2.82 |
| AM | 1.18 | 1.75 | 1.54 | 1.77 | 2.25 | 2.01 | 0.06 | 2.05 |
| CEC | 0.002 | 2.65 | 1.85 | 2.64 | 0.10 | 0.09 | 0.21 | 0.52 |
| CTX | 2.30 | 2.35 | 0.14 | 0.12 | 0.09 | 0.20 | 2.27 | 2.41 |
| ZOX | 2.14 | 2.68 | 2.01 | 1.10 | 1.11 | 0.68 | 2.69 | 2.59 |
| CXM | 2.92 | 1.94 | 2.17 | 1.59 | 1.68 | 2.75 | 3.27 | 2.92 |
| CRO | 2.73 | 3.04 | 0.66 | 2.74 | 0.75 | 1.15 | 0.44 | 3.23 |
| AMC | 0.07 | 1.46 | 1.63 | 1.31 | 1.91 | 1.59 | 0.07 | 1.73 |
| CAZ | 2.92 | 2.69 | 2.76 | 2.89 | 2.68 | 1.38 | 0.25 | 2.56 |
| CTT | 3.08 | 0.59 | 2.89 | 3.19 | 3.18 | 0.89 | 3.51 | 2.54 |
| SAM | 0.08 | 0.09 | 2.44 | 2.53 | 3.00 | 2.89 | 0.09 | 3.45 |
| CPR | 2.04 | 1.79 | 2.05 | 1.81 | 0.24 | 0.22 | 2.18 | 0.29 |
| CPD | 2.91 | 3.04 | 1.47 | 0.96 | 0.99 | 1.10 | 3.10 | 3.27 |
| TZP | 2.53 | 0.14 | 3.31 | 0.61 | 2.74 | 0.09 | 0.14 | 0.17 |
| FEP | 2.65 | 2.83 | 2.81 | 2.80 | 2.86 | 2.63 | 0.61 | 3.20 |

**Table 5 Rank order of enzymes in each antibiotic (derived from Table 4)**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Antibiotic | 0000 | 1000 | 0100 | 0010 | 0001 | 1100 | 1010 | 1001 | 0110 | 0101 | 0011 | 1110 | 1101 | 1011 | 0111 | 1111 |
| AMP | 11 | 12 | 9 | 10 | 7 | 5 | 15 | 6 | 8 | 2 | 4 | 13 | 3 | 14 | 16 | 1 |
| AM | 8 | 11 | 14 | 3 | 7 | 12 | 6 | 4 | 15 | 10 | 13 | 9 | 1 | 5 | 16 | 2 |
| CEC | 4 | 12 | 3 | 7 | 9 | 8 | 5 | 14 | 6 | 1 | 10 | 2 | 15 | 16 | 13 | 11 |
| CTX | 11 | 10 | 7 | 6 | 16 | 8 | 5 | 12 | 3 | 2 | 13 | 14 | 15 | 9 | 4 | 1 |
| ZOX | 14 | 11 | 8 | 5 | 15 | 10 | 7 | 9 | 4 | 2 | 6 | 3 | 12 | 16 | 1 | 3 |
| CXM | 11 | 16 | 2 | 7 | 12 | 8 | 10 | 15 | 4 | 9 | 6 | 14 | 13 | 5 | 1 | 3 |
| CRO | 10 | 11 | 4 | 7 | 16 | 8 | 2 | 14 | 6 | 3 | 13 | 5 | 12 | 9 | 15 | 1 |
| AMC | 9 | 10 | 3 | 14 | 6 | 11 | 7 | 12 | 15 | 8 | 4 | 13 | 1 | 5 | 16 | 2 |
| CAZ | 10 | 15 | 11 | 8 | 6 | 7 | 12 | 14 | 1 | 4 | 3 | 2 | 5 | 13 | 16 | 9 |
| CTT | 12 | 3 | 2 | 10 | 13 | 16 | 9 | 7 | 6 | 15 | 8 | 4 | 5 | 14 | 1 | 11 |
| SAM | 12 | 11 | 8 | 13 | 5 | 7 | 10 | 4 | 16 | 14 | 9 | 6 | 2 | 3 | 15 | 1 |
| CPR | 7 | 9 | 3 | 6 | 8 | 13 | 16 | 11 | 2 | 5 | 1 | 4 | 12 | 14 | 15 | 10 |
| CPD | 13 | 14 | 7 | 6 | 16 | 12 | 5 | 15 | 4 | 3 | 8 | 11 | 10 | 9 | 2 | 1 |
| TZP | 6 | 5 | 2 | 10 | 3 | 9 | 12 | 8 | 7 | 15 | 1 | 11 | 4 | 16 | 14 | 13 |
| FEP | 10 | 15 | 13 | 14 | 11 | 7 | 2 | 12 | 8 | 4 | 5 | 6 | 3 | 9 | 16 | 1 |
| best value | 4 | 3 | 2 | 3 | 3 | 5 | 2 | 4 | 1 | 1 | 1 | 2 | 1 | 3 | 1 | 1 |
| worst value | 14 | 16 | 14 | 14 | 16 | 16 | 15 | 15 | 16 | 15 | 13 | 14 | 15 | 16 | 16 | 13 |

**Table 6. Maximum Probability Using CPM**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Starting  Genotype | 1 Step | # | 2 Step | # | 3 Step | # | 4  Step | # | 5 Step | # | 6 Step | # | Total  # |
| 1000 | 1.0 | 1 | 1.0 | 3 | 1.0 | 7 | 1.0 | 15 | 1.0 | 31 |  |  |  |
| 0100 | 0.444 | 1 | 0.444 | 5 | 0.444 | 25 | 0.444 | 124 | 0.444 | 627 |  |  |  |
| 0010 | 0.387 | 1 | 0.387 | 2 | 0.444 | 1 | 0.444 | 9 | 0.471 | 1 |  |  |  |
| 0001 | 0.287 | 1 | 0.287 | 1 | 0.559 | 2 | 0.559 | 8 | 0.635 | 2 |  |  |  |
| 1100 |  |  | 0.444 | 3 | 0.444 | 15 | 0.444 | 77 | 0.444 | 390 |  |  |  |
| 1010 |  |  | 0.534 | 1 | 0.534 | 7 | 0.592 | 1 | 0.592 | 8 |  |  |  |
| 1001 |  |  | 0.559 | 1 | 0.559 | 4 | 0.635 | 1 | 0.635 | 4 |  |  |  |
| 0110 |  |  | 0.444 | 1 | 0.444 | 8 | 0.444 | 53 | 0.444 | 322 |  |  |  |
| 0101 |  |  | 0.191 | 1 | 0.191 | 10 | 0.299 | 1 | 0.320 | 1 |  |  |  |
| 0011 |  |  | 0.354 | 1 | 0.354 | 6 | 0.384 | 1 | 0.384 | 12 |  |  |  |
| 1110 |  |  | - |  | 0.444 | 2 | 0.444 | 21 | 0.444 | 169 |  |  |  |
| 1101 |  |  | - |  | 0.274 | 1 | 0.274 | 10 | 0.444 | 4 |  |  |  |
| 1011 |  |  | - |  | 0.341 | 1 | 0.341 | 1 | 0.444 | 8 |  |  |  |
| 0111 |  |  | - |  | 0.207 | 1 | 0.242 | 1 | 0.444 | 2 |  |  |  |
| 1111 |  |  | - |  | - |  | 0.444 | 2 | 0.444 | 33 |  |  |  |

**Table 7. Maximum Probability Using EPM**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Starting Genotype | 1 Step | # | 2 Step | # | 3 Step | # | 4 Step | # | 5 Step | # | 6 Step | # | Total  # |
| 1000 | 1.0 | 1 | 1.0 | 3 | 1.0 | 7 | 1.0 | 15 | 1.0 | 31 |  |  |  |
| 0100 | 0.333 | 1 | 0.333 | 5 | 0.333 | 28 | 0.333 | 145 | 0.389 | 1 |  |  |  |
| 0010 | 0.333 | 1 | 0.333 | 8 | 0.333 | 13 | 0.333 | 25 | 0.375 | 6 |  |  |  |
| 0001 | 0.500 | 1 | 0.500 | 1 | 0.667 | 4 | 0.667 | 8 | 0.667 | 12 |  |  |  |
| 1100 |  |  | 0.333 | 6 | 0.333 | 24 | 0.389 | 1 | 0.389 | 4 |  |  |  |
| 1010 |  |  | 0.500 | 1 | 0.500 | 7 | 0.583 | 1 | 0.583 | 8 |  |  |  |
| 1001 |  |  | 0.667 | 2 | 0.667 | 4 | 0.667 | 6 | 0.667 | 8 |  |  |  |
| 0110 |  |  | 0.333 | 1 | 0.333 | 8 | 0.333 | 56 | 0.333 | 352 |  |  |  |
| 0101 |  |  | 0.250 | 1 | 0.250 | 6 | 0.333 | 4 | 0.448 | 1 |  |  |  |
| 0011 |  |  | 0.417 | 1 | 0.417 | 6 | 0.500 | 2 | 0.500 | 14 |  |  |  |
| 1110 |  |  | - |  | 0.333 | 2 | 0.333 | 21 | 0.333 | 175 |  |  |  |
| 1101 |  |  | - |  | 0.250 | 1 | 0.264 | 1 | 0.333 | 8 |  |  |  |
| 1011 |  |  | - |  | 0.333 | 2 | 0.333 | 1 | 0.361 | 1 |  |  |  |
| 0111 |  |  | - |  | 0.208 | 1 | 0.208 | 10 | 0.333 | 2 |  |  |  |
| 1111 |  |  | - |  | - |  | 0.333 | 2 | 0.333 | 33 |  |  |  |

**Table 8. CPM transitions and antibiotics from optimal treatment plans**

|  |  |  |  |
| --- | --- | --- | --- |
| **Mutations** | **Drugs associated with transitions in optimal paths (probability)** | **Reversions** | **Drugs associated with transitions in optimal paths (probability)** |
| 0000-1000 | CTT(0.38) | 1111-1110 | CEC(1.0) |
| 0000-0100 |  | 1111-1101 |  |
| 0000-0010 |  | 1111-1011 |  |
| 0000-0001 |  | 1111-0111 |  |
| 1000-1100 |  | 1110-1100 | AMC, AMP, CTX, CXM, TZP(0.49), ZOX |
| 1000-1010 |  | 1110-1010 | AMC, AM, CPD, CRO(0.47), CTX, CXM, FEP, ZOX |
| 1000-1001 |  | 1110-0110 | AMP, CAZ(1.0), CPD, CPR(1.0), CTX, CXM, TZP, ZOX |
| 0100-1100 | SAM(1.0) | 1101-1100 |  |
| 0100-0110 | CPR(1.0) | 1101-1001 |  |
| 0100-0101 |  | 1101-0101 |  |
| 0010-1010 | CRO(0.78) | 1011-1010 | CRO(0.49), CYX |
| 0010-0110 | CRO | 1011-1001 |  |
| 0010-0011 |  | 1011-0011 |  |
| 0001-1001 | AM(1.0), CTT, SAM(1.0) | 0111-0110 | TZP(0.42) |
| 0001-0101 |  | 0111-0101 |  |
| 0001-0011 |  | 0111-0011 | TZP(0.57) |
| 1100-1110 | CEC,CRO | 1100-1000 | CTT |
| 1100-1101 |  | 1100-0100 | CEC, CRO, CTX(1.0), CXM(1.0), ZOX(1.0) |
| 1010-1110 | CEC(1.0),CTT | 1010-1000 | CTT(0.53) |
| 1010-1011 |  | 1010-0010 |  |
| 1001-1101 |  | 1001-1000 | CPR, CTT(0.56) |
| 1001-1011 |  | 1001-0001 | CPR |
| 0110-1110 | CEC, CRO, FEP(1.0) | 0110-0100 | CEC, CRO, TZP(1.0) |
| 0110-0111 |  | 0110-0010 |  |
| 0101-1101 |  | 0101-0100 | CXM(0.44) |
| 0101-0111 | CXM | 0101-0001 |  |
| 0011-1011 | CRO | 0011-0010 | AM, CRO(0.79) |
| 0011-0111 |  | 0011-0001 | AM, CTT |
| 1110-1111 | AMC, AMP, AM, CPD, CRO, CTX, CXM, FEP, SAM(1.0), ZOX | 1000-0000 | CPR(1.0) |
| 1101-1111 | FEP(1.0),SAM(1.0) | 0100-0000 |  |
| 1011-1111 | CRO,CTX, CXM(1.0), SAM(1.0) | 0010-0000 | AM, FEP |
| 0111-1111 | CPD(1.0) | 0001-0000 | FEP |

**Table 9. EPM transitions and antibiotics from optimal treatment plans**

|  |  |  |  |
| --- | --- | --- | --- |
| **Mutations** | **Drugs associated with transitions in optimal paths (probability)** | **Reversions** | **Drugs associated with transitions in optimal paths (probability)** |
| 0000-1000 | CTT(0.38) | 1111-1110 | CEC(1.0) |
| 0000-0100 |  | 1111-1101 |  |
| 0000-0010 |  | 1111-1011 |  |
| 0000-0001 |  | 1111-0111 |  |
| 1000-1100 |  | 1110-1100 | AMC,CTT,CXM,TZP(0.49),ZOX |
| 1000-1010 | AM | 1110-1010 | AMC,AM,CPD,CRO(0.47),CTT,CXM,FEP,ZOX |
| 1000-1001 | AM(0/68) | 1110-0110 | AMP,CAZ(1.0),CPD,CPR(1.0),CTT,CXM,TZP,ZOX |
| 0100-1100 | SAM(1..0) | 1101-1100 | AMP,ZOX |
| 0100-0110 | CPR(1.0) | 1101-1001 | ZOX |
| 0100-0101 |  | 1101-0101 | CAZ(1.0) |
| 0010-1010 | CPD,CRO(0.78),CTT | 1011-1010 |  |
| 0010-0110 | CPD,CRO | 1011-1001 | AM |
| 0010-0011 |  | 1011-0011 |  |
| 0001-1001 | SAM(1.0) | 0111-0110 |  |
| 0001-0101 |  | 0111-0101 |  |
| 0001-0011 |  | 0111-0011 |  |
| 1100-1110 | AM,CEC,CRO | 1100-1000 | AMC,AM,CPR(0.28),TZP |
| 1100-1101 |  | 1100-0100 | CEC,CRO,CTT,CXM(1.0) ZOX(1.0) |
| 1010-1110 | CEC(1.0),CTT | 1010-1000 | CTT(0.53) |
| 1010-1011 |  | 1010-0010 |  |
| 1001-1101 |  | 1001-1000 | CEC,CPR,CTT(0.56) |
| 1001-1011 |  | 1001-0001 | CEC(0.97),CPR |
| 0110-1110 | CEC,CRO,FEP(1.0) | 0110-0100 | CEC,CRO,TZP(1.0) |
| 0110-0111 |  | 0110-0010 |  |
| 0101-1101 | AM | 0101-0100 |  |
| 0101-0111 |  | 0101-0001 | AM |
| 0011-1011 | SAM(1.0) | 0011-0010 |  |
| 0011-0111 |  | 0011-0001 | SAM |
| 1110-1111 | AMC,AMP,AM,CPD,CRO,CTT,CXM,FEP,SAM(1.0),ZOX | 1000-0000 | AM,CPR(1.0) |
| 1101-1111 | FEP(1.0),SAM(1.0) | 0100-0000 | AM(0.44) |
| 1011-1111 | AM | 0010-0000 |  |
| 0111-1111 | CPD(1.0) | 0001-0000 | CPR |

**Table 10. We will create a table similar to Table 9, but for 6 step paths.**

**[Do we want to leave in both tables, or have only one?]**

**[Do we want to make tables and figures for all path lengths and include them in supplemental information?]**

**Figure 1 AMP: Ampicillin 256 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:AMP8X:AMP8X256Landscape copy.pdf**

**Figure 2 AM: Amoxicillin 512 µg/mlMacintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:AM:AM512Landscape copy.pdf**

**Figure 3 CEC: Cefaclor 1 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CEC:CEC1Landscape copy.pdf**

**Figure 4 CTX: Cefotaxime 0.05 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CTX:CTX0.05Landscape copy.pdf**

**Figure 5 ZOX: Ceftizoxime 0.03 µg/mlMacintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:ZOX:ZOX0.03Landscape copy.pdf**

**Figure 6 CXM: Cefuroxime 1.5 µg/mlMacintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CXM:CXM1.5Landscape copy.pdf**

**Figure 7 CRO: Ceftriaxone 0.045 µg/mlMacintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CRO:CRO0.045Landscape copy.pdf**

**Figure 8 AMC: Amoxicillin/Clavulanate 512 µg/ml and 8µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:AMC:AMC512.8Landscape.pdf**

**Figure 9 CAZ: Cefazidime 0.1 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CAZ:CAZ.1Landscape copy.pdf**

**Figure 10 CTT: Cefotetan 0.312 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CTT:CTT0.0312Landscape copy.pdf**

**Figure 11 SAM: Ampicillin/Sulbactam 8 µg/ml and 8µg/mlMacintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:SAM:SAM8.8Landscape copy.pdf**

**Figure 12 CPR: Cefprozil 100 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CPR:CPR10Landscape copy.pdf**

**Figure 13 CPD: Cefpodoxime 2 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CPD:CPD2Landscape.pdf**

**Figure 14 TZP: Pipercillin / Tazobactam 8.12µg/ml and 8 µg.ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:TZP:TZP8.128Landscape copy.pdf**

**Figure 15 FEP: Cefepime 0.0156µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:FEP:FEP0.0156Landscape.pdf**

**Summary of Highest CPM probabilities**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:NewMutationProb.Drug.042814PM.pdf**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:ReversionProb.Drug.042814PM.pdf**

**Figure 17. Summary of Optimal Five Step Sequences (EPM and CPM)**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):ManuscriptFigures:5Cycle.M1.M2.pdf**

**Figure 18. Summary of Optimal Six Step Sequences (EPM and CPM)**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):ManuscriptFigures:6CycleM1.M2.pdf**

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