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James Dickey March 18, 2016

Mutation rates and the likelihood of acquired resistance: it's worse than you think

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Abstract

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Antibiotic resistance is an ever-growing public health concern. In acquired antibiotic resistance, the dominant population of infecting bacteria is susceptible to the treating antibiotic, and bacteria resistant to the drug emerge and ascend during the course of treatment, resulting in treatment failure. Therefore, being able to predict the likelihood that resistance will emerge during treatment of a bacterial infection is an important consideration as we try to preserve the usefulness of antibiotics. In the case of the antibiotic rifampin, which has long been associated with high rates of resistance, we suspected that resistance occurs at a higher frequency than we would normally predict. We posed the question: Can we predict the likelihood that resistance to single antibiotics will arise and ascend *in vitro* from estimates of the rate of mutation of resistance and the total number of cells in the culture? We developed two methods by which to predict this likelihood in *Staphylococcus aureus* to the antibiotics rifampin, fusidic acid, and streptomycin and tested these predications experimentally. We observed significantly higher frequencies of resistance in cultures containing rifampin. We noted a similar effect in cultures containing fusidic acid and streptomycin, but only under specific conditions. Additionally, when cultures containing antibiotics were left to incubate for several days, we noticed an accumulation of resistant mutants over time. We generated and tested a series of hypotheses to explain these higher than anticipated rates of resistance, as well as this accumulation effect.

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Introduction

Antibiotic resistance is an ever-growing concern worldwide, and combatting antibiotic resistance ranked among the CDC's top six public health focuses for 2016 (CDC press release). This thesis examines one source for antibiotic resistance, genetic mutation, and seeks to elucidate possible mechanisms for apparent elevated rates of resistance to a series of antibiotics. However, in its original form, this project was not intended to examine issues related to mutation rates. Initially, we set out to investigate the role of the number of ribosomal operons in bacteria and how this might affect antibiotic susceptibility. Early experiments utilized the RNA polymerase targeting antibiotic rifampin in an effort to observe interruptions with transcription. We immediately encountered problems with resistance arising in cultures with rifampin that prevented us from obtaining any useful observations. Evidently, antibiotic resistance does not only complicate clinical infections; it also creates problems in lab research seeking to fight the problem. Thus this thesis shifted focus from ribosomes to looking into this issue of resistance arising with alarmingly (and annoyingly) high frequency.

In the clinical setting, antibiotic resistance to a particular drug can generally be thought of in two ways: primary resistance, in which the infecting population is already resistant to the given antibiotic and for which treatment with the given antibiotic fails; and acquired resistance, in which resistant subpopulation(s) exist at relatively low frequencies in an otherwise susceptible infecting population. In the latter case, treatment with the antibiotic kills the dominant susceptible population, resulting in selection for the resistant population, allowing the resistant population to become dominant. In the case of acquired resistance treatment with a drug may or may not fail. A successful treatment relies on complex interactions between the drug, infecting bacteria, and the host immune system. In the event that treatment fails because of acquired resistance, the acquired resistance become a source of primary resistance.

Efforts have been made in recent years to reduce the frequency of drug resistant bacteria in the clinical setting. The CDC, for example, has a series of guidelines intended for use by hospitals, clinics, and physicians aimed at reducing the spread of resistant bacteria (Pollack and Srinivasan 2014). However, such guidelines are usually aimed at combating primary, and not acquired, resistance. In considering possible methods for reducing rates of acquired resistance, we pose the general question: Can we predict the likelihood of treatment failure due to the emergence and ascent of acquired resistance from the rate at which resistance is generated by mutation, the pharmacokinetics and pharmacodynamics of the antibiotic, the total number of bacteria in the infection, and the contribution of the innate and adaptive immune response? Addressing such a question would involve extensive testing and resources. In this study, we address the more specific and targeted question: Can we predict the likelihood that resistance to single antibiotics will arise and ascend *in vitro* from estimates of the rate of mutation of resistance and the total number of cells in the culture?

When considering the rate of mutation of a microorganism and the potential for this biological parameter to be inadequate as a predictor for total mutations, one is reminded of the heated debate over adaptive mutation in the 1990's. Proponents of the adaptive mutation hypothesis suggest that microorganisms such as bacteria or yeast are able to enhance mutation in a particular gene as a response to escape specific selective pressures. This is contrary to the established Darwinian view that the source of mutation, beneficial or otherwise, is random. This hypothesis gained much attention after a 1988 study by Cairns *et al.,* who observed increased numbers of *E. coli* mutants capable of utilizing lactose following an extended period of starvation. A flurry of other studies in support of the adaptive mutation hypothesis followed, primarily using limiting growth nutrients in bacteria or yeast (Cairns *et al.* 1991, Hall 1997, Foster 2000, Harris *et al.* 1994). One of these studies forayed into the realm of antibiotic resistance. Riesenfeld *et al*. (1997) showed using the classic Luria-Delbrük fluctuation test that *Escherichia coli* cultures exposed to super-minimum inhibitory concentrations (MIC) of ciprofloxacin

produced resistant colonies for several days following initial plating. Conventionally, mutation rates are calculated using the number of colonies that appear 1-2 days following plating. The authors of the study proposed that these early colonies were the result of pre-existing mutations, while contributing the accumulation of additional resistant colonies on subsequent days to adaptive mutation in non-dividing cells.

As with any good scientific controversy, the adaptive mutation debate spawned a series of works in stark refutation of the hypothesis. Many of the claims made by adaptive mutation proponents were shown to have other sources or mechanisms that followed the conventional view of Darwinian evolution (Sniegowski and Lenski 1995, Lenski and Mittler 1993, MacPhee 1993). In the years since the peak of this discussion, the adaptive mutation hypothesis has yet to gain strong support. And while recent work acknowledges the phenomenon of increased rates of mutation in non-dividing cells, such works generally seek to elucidate mechanisms that better fall in line with Darwinian principles (Morreall *et al.* 2015).

When examining how rates of mutation affect acquired resistance, and possible methods to prevent such resistance, the first practice that often comes to mind is the use of antibiotic combination therapy. This treatment approach utilizes two or more antibiotics at once to treat an infection. The belief is that if these antibiotics are of different classes and act independently, a bacterium must gain resistance mutations to both of these antibiotics simultaneously to escape death. Therefore, the theoretical likelihood of a bacterium generating resistance to two antibiotics is the product of the individual mutation rates of resistance to each antibiotic. In practice however, combination therapy is not so perfect, and there are other factors to consider. While combining drugs can have additive or synergistic effects in their antimicrobial actions, interactions that are antagonistic are potentially counterproductive to combination therapy. Preventing resistance is not particularly useful if treatment

fails altogether. The need to better understand mutation of resistance and other potential methods of reducing acquired resistance remains a prime topic of research.

Such ideas have been extensively studied in ciprofloxacin and other fluoroquinolones. Typically, resistance to fluoroquinolones requires two mutations. The ability, or lack thereof, of bacteria to gain both of the required mutations is similar to the dynamics of combination therapy. However, Drilica *et al*. (2001) has shown in *E. coli* that it is possible to gain these mutations one at a time, rather than simultaneously, in a stepwise fashion by gradually increasing the concentration of ciprofloxacin. This is further complicated by the fact that different fluoroquinolones may individually require two mutations each, but one of these required mutations will be mutually needed for both types of resistance. Thus, resistance to one fluoroquinolone may result in bacteria only needing one more mutation to gain resistance to a second fluoroquinolone. Therefore, the apparent mutation rate of resistance to a fluoroquinolone for the bacteria increases, resulting in higher frequencies of resistance.

Drilica and colleagues have proposed a possible solution, which they call the mutation prevention concentration (MPC). They have shown using ciprofloxacin in *E. coli*, that maintaining a consistently high concentration in serum reduces rates of resistance *in vivo*. The idea behind this phenomenon is that in any given population of susceptible bacteria, there also exist subpopulations with intermediate levels of resistance at relatively low frequencies. Treatment with low or intermediate concentrations of an antibiotic would allow for selection for these resistant subpopulations, as their MICs would exceed that of the serum concentration. Keeping the concentration of antibiotic high and above the MICs of these resistant populations prevents these populations from ascending, as they are killed off with the susceptible population. Therefore, it is important to consider the effect that concentration can have on rates of mutation of resistance, particularly for single antibiotics.

In this study, we examine the likelihood that resistance to single antibiotics emerges in *Staphylococcus aureus* Newman and ask: Can we reliably predict this likelihood using estimates of the

rate of mutation of resistance and the total number of cells in the culture? We examined three antibiotics for which resistance arises via a single point mutation: rifampin, fusidic acid, and streptomycin. Rifampin, our inspiration for this project, is a broad spectrum bactericidal antibiotic that targets transcription via inhibition of the DNA-dependent RNA polymerase. It has long been associated with high rates of resistance and is now used almost exclusively in cases of *Mycobacterium tuberculosis*. Even then, it is only used in combination with other antibiotics (Goldstein 2014, Wishart *et al.* 2006). Fusidic acid is a bacteriostatic antibiotic that inhibits disassociation of bacterial elongation factor-G from the ribosome, thereby inhibiting translation (Chen *et al.* 2010, Wishart *et al* 2006). Beginning in the 1990's, fusidic acid saw widespread use as a topical treatment. High rates of resistance to fusidic acid were observed around this same time (Turnidge and Collignon 1999). Streptomycin was the first ever effective treatment developed for tuberculosis. It is a highly effective, broad spectrum bactericidal antibiotic that works by interfering with translation at the ribosome (Springer *et al.* 2001,Wishart *et al.* 2006).

We used the classic Luria-Delbrük fluctuation test to estimate the mutation rate, μ, of *S. aureus* to these three antibiotics. We attempted to predict the frequency at which resistance to each antibiotic would emerge using two different methods. The first method uses a simple statistical calculation. The second method uses a model in which we considered pharmacodynamic and pharmacokinetic parameters of each antibiotic in order to predict the likelihood that resistance will arise and ascend *in vitro*. We then tested our predictions and experimentally determined the frequency at which resistance emerges in a series of *in vitro* liquid cultures. From these results, we generated and experimentally tested multiple hypotheses to explain our observations.

Materials and Methods

Estimation of MIC

The minimum inhibitory concentration (MIC) of each antibiotic for *S.aureus* was determined using broth microdilution with Mueller-Hinton II (MHII) broth as the growth medium. Two-fold serial dilution was performed on 96-well plates with initial cell density ~$5x10^5$ cells/mL. Plates were incubated on shaker at 200 rpm and 37°C. After 24 hours, optical densities (OD) were measured at 630 nm using a Biotek microplate reader. The MIC was considered to be the concentration of antibiotic in the first well of the dilution series with an OD reading of <0.200.

Test for antibiotic resistance

Antibiotic resistance was determined using Kirby-Bauer disc diffusion assays. Standard bulk-purchased discs were used for rifampin and streptomycin (5 µg and 10 µg per disc, respectively). As fusidic acid discs were not readily available, 5 µg discs were prepared by transferring 5 µL of 0.1% fusidic acid solution onto a blank, sterile paper disc. Additionally, 5 µL of distilled water was added to the discs to allow for uniform diffusion of the fusidic acid solution throughout the disc.

Estimation of mutation rate

Mutation rates were estimated using the Luria-Delbrück fluctuation test. Twelve independent cultures *S. aureus* were grown overnight in 6 mL with initial densities of ~10^4 cells/mL. After 24 hours, four cultures were randomly selected from which estimates of the final cell density were obtained. The average of these four estimates was considered the overall cell density, *N*, to be used in our calculations to predict frequency of resistance. A known fraction of the volume each culture was plated onto an MHII agar plate containing either 10x or 100x the MIC of the antibiotic being tested. Plating was achieved using soft agar containing the same concentration of antibiotic as the plate (10x or 100x MIC). Soft agar

was used as opposed to spreading to ensure cell's complete exposure to the antibiotic. Taking the volume of culture plated divided by the total volume of culture gives the plating efficiency, p_e, a parameter necessary in calculating the mutation rate. In order to plate on rifampin, 0.1 mL of culture was added to 3 mL of antibiotic soft agar. Lower rates of mutation were expected for fusidic acid and streptomycin, and thus 1.0 mL of culture was added to 2mL of soft agar. The number of colonies on each plate was recorded after 24 hours. Mutation rates were calculated using the equations provided by Jones *et al*. (1994):

$$m_{med} = \frac{\frac{y}{p} - \ln(2)}{\ln\left(\frac{y}{p}\right) - \ln(\ln(2))} \quad (1)$$

$$m_0 = \frac{(1-p)\ln\left(\frac{z}{C}\right)}{p\ln(p)} \quad (2)$$

where *m* is the number of mutations per culture, *y* is the median number of colony forming units (cfu) on each plate, *p* is the plating efficiency (the proportion of culture volume plated), *z* is the number of cultures with zero cfu, and *C* is the total number of cultures tested. The median estimator (Equation 1) was used as the default estimator for calculating *m*. In the event that more than half of the cultures yielded zero cfu, the median number would be zero and the median estimator could not be used. In these cases, the zero estimator, (Equation 2), was used to calculate *m.* Once *m* was known, we calculated the mutation rate, μ, using the equation $\mu = m/N$ (Jones 1994).

Emergence of resistance

In order to measure the frequency at which resistance arises and ascends in culture for each antibiotic, 36 independent cultures were grown in 2mL MHII broth overnight from single colonies of *S aureus* Newman. After 24 hours, the stationary phase cell density was estimated in twelve randomly

selected cultures. The average of these estimates, $5x10^9$ cells/mL, was considered the cell density for all thirty-six cultures.

In 24 well macrotiter plates, these 36 independent cultures were used to inoculate 2mL wells of MHII broth containing 10x or 100x MIC rifampin, fusidic acid, or streptomycin. Half of the wells were then inoculated with an initial lower density while the other half were inoculated with a higher density. The initial densities used depended on the antibiotic in solution. Wells containing rifampin were inoculated with $5x10^6$ or $5x10^7$ cells/mL, while wells containing streptomycin or fusidic acid were inoculated with $1x10^7$ or $1x10^8$ cells/mL. We used lower inoculum densities for rifampin because we anticipated higher rates of resistance for this drug, and we hoped to avoid all cultures becoming resistant after a single day. In all, there were twelve combinations of initial cell densities and antibiotic concentrations: three antibiotics, each tested at two concentrations, with each of those concentrations tested at two initial cell densities. For each combination there were 36 2mL cultures, each inoculated with a different independent culture. Cultures were incubated at 37˚C on a shaker at 200 rpm. Over the course of four days cultures were monitored for changes in turbidity. Every twenty-four hours the number of turbid cultures for each category was noted. At the end of the experiment, three turbid cultures per cell density/antibiotic concentration, if any, were randomly selected and streaked out. Single colony isolates were tested to ensure cultures were indeed resistant

We developed two methods by which to predict the frequency at which resistance to given antibiotic arises and ascends in culture. The first method uses estimates of the mutation rate and density of cells in culture. We assume that the probability that no mutants will arise in culture follows a Poisson distribution. This probability then is given by:

$$\Pr(\text{zero mutants}) > 0 = e^{-\mu NV}$$

where μ is the mutation rate, N is the density of cells in culture in cells/mL, and V is the volume of culture in milliliters. We can subtract this value from 1, giving us the probability, P, of having at least one resistant mutant in a culture:

$$P = 1\text{-}e^{-\mu NV}$$

The expected proportion of resistant cultures is then equal to probability P, and the frequency of resistant cultures in n trials is nP rounded to the nearest integer.

The second method to predict the expected frequency of resistant cultures uses a stochastic model, described in Appendix 1, to simulate a series of cultures from which we can estimate the likelihood that resistance will emerge during treatment with an antibiotic. Whereas our first method of prediction only takes into account the mutation rate and total number of cells, the model accounts for the killing of the susceptible bacteria population by the antibiotic. We experimentally determined a number of parameters to be used in the model: initial density of susceptible cells, N, the mutation rate, μ, and the maximum rate of kill, v, for each antibiotic.

When considering mutation rates for our predictions of the emergence of resistance, we assumed the most extreme scenario, and used the highest estimated mutation rate for 10x and 100x MIC in the simulations. We experimentally estimated the max rate of kill, v, for each antibiotic to be used in all simulations for that antibiotic, regardless of concentration. The density of cells N was estimated from the experimental cultures as previously described. For each of the twelve initial cell density antibiotic concentrations, we simulated 100 2mL cultures. We then calculated the proportion of cultures in which a resistant mutant emerged. We multiplied this proportion by 36 and rounded the result to the nearest integer. This was the expected number (frequency) of resistant cultures based on the simulation.

Estimation of max rate of kill

To use our model predicting the frequency at which resistance will ascend in culture, we required estimates of the maximum rate of kill for each antibiotic at each density/antibiotic concentration pairing. Four 10mL cultures were prepared for each antibiotic, one of each of the cell density/antibiotic concentration pairings describes in the emergence of resistance experiment. An additional control culture with no antibiotic was also incubated with initial density $1x10^7$ cells/mL. Cultures were inoculated with the appropriate density of cells and allowed to incubate in a water bath shaker at 37˚C for two hours. This pre-incubation was done to allow cells to enter the exponential growth phase, at which time the kill rate of the antibiotics should be highest. After two hours, the density of each culture was estimated and the appropriate amount of antibiotic was added to each culture to obtain 10x or 100x MIC. The density of each culture was then estimated one and two hours after the addition of antibiotic. The maximum rate of kill is given by:

$$\frac{\ln\left(\frac{D_{t2}}{D_{t0}}\right)}{t} = v$$

where D_{t2} is the density of cells at two hours after addition of antibiotic, D_{t0} is the density of cells immediately prior to the addition of antibiotic; t is the time between t_2 and t_0; in this case, two hours; and *v* is the maximum rate of kill. As estimates for the rates of kill were relatively poor, and we lacked the time to repeat this parameter estimation, the kill rate was taken to be the average of the four estimated maximum rates of kill for the four cell density- antibiotic concentration combinations of each antibiotic. As a result, the rate kill used in the model simulations was not changed when the concentration of antibiotic being simulated was changed. This assumption was considered acceptable, as the estimated mutation rates were different for the 10x and 100x MIC concentrations. This difference

in the mutation rates between concentrations should account for effects that different concentration of the antibiotics might have on resistance emerging in the simulation.

Results

MIC Estimates

The minimum inhibitory concentrations of *S. aureus* Newman were estimated to be: rifampin, 0.01 μg/mL; fusidic acid, 0.2 μg/mL; streptomycin, 7.5 μg/mL. These values were used to determine the 10x MIC and 100x MIC concentrations used in the Luria-Delbrük fluctuation test and emergence of resistance experiments.

Estimation of Mutation Rate

For all three antibiotics, we observed an accumulation of visible colonies over successive days of the fluctuation tests. Each antibiotic followed a different pattern of accumulation. On rifampin-containing plates, colonies appeared after 24 and 48 hours, with none or very few appearing on subsequent days. For fusidic acid, very few colonies appeared after one day; most plates yielded no colonies, leading to the use of the zero-estimator to calculate the mutation rate. After two days, substantially more colonies appeared. A dramatic increase was seen after three days of incubation, with further accumulation by the fourth day. Streptomycin followed a somewhat similar pattern. No or few colonies appeared on the first day, with a dramatic increase by day two. There was continued, although less dramatic, accumulation on days three and four. This pattern was observed in both 10x and 100x MIC for each antibiotic. Not unexpectedly, the actual colony numbers and extent accumulation was reduced in the 100x MIC plates. The mutation rates calculated from this experiment were those used in calculations to predict emergence of resistance, and are given in Table 1.

Antibiotic	Concentration (xMIC)	Estimated Mutation Rate, μ			
		Day 1	Day 2	Day 3	Day 4
RIF	10x MIC	4.4E-09	6.4E-09	6.9E-09	6.9E-09
	100x MIC	2.0E-09	3.6E-09	3.8E-09	3.9E-09
FUS	10x MIC	4.4E-11	2.3E-09	9.9E-09	1.5E-08
	100x MIC	N/A	6.0E-10	6.6E-10	7.3E-10
STM	10x MIC	4.4E-11	1.4E-08	1.9E-08	1.9E-08
	100x MIC	N/A	1.2E-10	1.4E-10	1.4E-10

Table 1. Estimated mutation rates recalculated for changes in colony number each day. As the total number of colonies on each the selective antibiotic plates increased each day, the mutation rate was recalculated each day. For FUS 10x MIC and STM 10x MIC on Day 1, it was necessary to use the zero-estimator for mutation rate as more than half the plates for each antibiotic yielded zero colonies. For all other estimates, including FUS 10x MIC and STM 10x on Days 2-4, the median estimator was used to calculate mutation rate. No mutation rate could be obtained for FUS 100x MIC and STM 100x MIC Day 1, as no colonies appeared on any of the twelve plates at for either antibiotic at 100x MIC.

Exploration of colony accumulation

We first noted this accumulation effect in early runs of the fluctuation tests. As this effect was not anticipated, only days at which a substantial amount of colonies appeared for each antibiotic were counted and mutation rate recalculated. This led to colony counts and therefore mutation rate estimates for only one or two days for each antibiotic. The experiment was repeated for each antibiotic, with careful scrutiny and precise counting for all four days, yielding the estimates in Table 1. However, during this early run of the fluctuation test we considered the possibility that the pharmacodynamics might differ between colonies that arose on different days. In order to test this possibility, we randomly selected three 10x plates this run of the fluctuation test. For rifampin, we selected and compared colonies that arose on days one and two. For fusidic acid, we selected colonies that appeared on days two and three. For streptomycin, we only selected colonies that arose on day two. As these isolates were taken from this early run of the fluctuation test, we had not recorded colonies that arose on days three or four in streptomycin, and thus could not test them; no colonies had appeared on day one. Six colonies were isolated from each plate: three for each day being compared (only three per plate for

streptomycin). The MIC's of single colony isolates streaked from each of these colonies were then determined. We looked for any trend that may have correlated with the day on which the colony arose.

A complete list of the MIC's from these isolates is given in Appendix 2 but described here. In rifampin, there were no clear differences between the colonies that arose on days one or two, and we observed a wide range of at least six different MICs. In fusidic acid, colonies that appeared on the second day had MICs of 50 μg/mL or 100 μg/mL. However, only three of the nine day one isolates had MIC's of 100 μg/mL, and they were all isolated from the same plate. Colonies that appeared on the third day all had MIC's of 6.25 μg/mL. This was true for all isolates from all three randomly selected fusidic acid plates. Interestingly, all colonies isolated from streptomycin plates gave MIC's of 60μg/mL. This is particularly remarkable, as all of the isolates were taken from plates containing 75 μg/mL of streptomycin. If this concentration were selecting for resistance as we would expect, the MIC's of the colony isolates should be greater than 75 μg/mL of streptomycin. We were able to rule out problems with the streptomycin stock solution as an explanation to this problem, as our controls of wildtype *S. aureus* Newman yielded the normal MIC of 7.5 μg/mL.

Emergence of Resistance

The number of cultures in which resistance emerged each day across the four days of the experiment are given in Figure 1, with a table of the numbered counts the graphs display given in Appendix 2. The degree at which cultures accumulated varied between categories, and while there was not clear pattern at which resistant cultures accumulated, it was apparent that new resistant cultures appeared after the first day in all cases. For our analysis, we considered the number of resistant cultures at the start of the experiment (after one day of incubation) and at the end of the experiment (after four days of incubation) for comparisons to our two prediction methods.

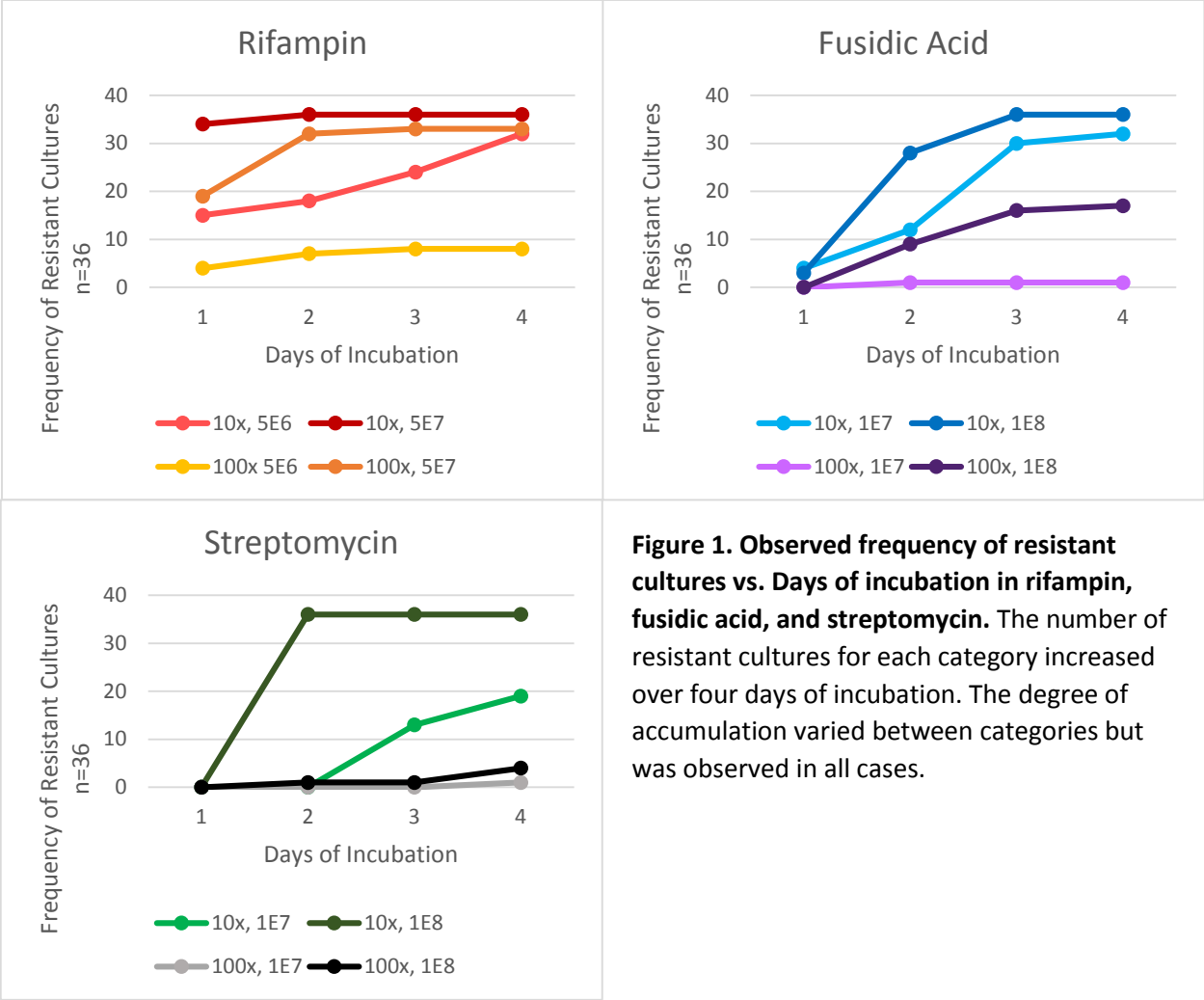

Figure 1. Observed frequency of resistant cultures vs. Days of incubation in rifampin, fusidic acid, and streptomycin. The number of resistant cultures for each category increased over four days of incubation. The degree of accumulation varied between categories but was observed in all cases.

Using the mutation rates from Table 1, we calculated the anticipated frequency of resistance that would occur in two different densities of *S. aureus*, using both 10x and 100x MIC of each antibiotic via two different methods. As a conservative estimate, we used the highest estimate of mutation rate of resistance to the antibiotic for the antibiotic and concentration being tested. Therefore, for all six cases (RIF 10x MIC, RIF 100x MIC, FUS 10x MIC, FUS 100x MIC, STM 10x MIC, and STM 100x MIC) we used the mutation rate calculated on Day 4 (Tab. 1).

In our first method, where the expected proportion of resistant cultures is given by *P,* we calculate the probability of having at least one resistant mutant in culture using the mutation rate and

total number of cells. Our second method uses a model simulation, described in Appendix 1, to simulate 100 cultures of each initial cell density and antibiotic concentration pairing. In this model, we consider not only the mutation rate and total number of cells, but also pharmacodynamic interactions of the antibiotic. We then took this simulated frequency and the probability *P* and found the expected number of resistant cultures to arise from 36 trials for each value. We compared these expected numbers of resistant cultures to the observed numbers of resistant cultures that arose after one day of incubation and after four days of incubation (Fig. 2). P-values were calculated using a two-sided binomial test.

In the case of rifampin, the frequency of resistant cultures was higher than expected by both predictors in all cell density-antibiotic concentration pairings after only one day of incubation. For these observed frequencies of resistance, all but one of these pairings (100x MIC, $5x10^7$ cells/mL) were significantly higher than both expected frequencies at $\alpha = 0.05$. While the observed frequency of resistance for 100x MIC, $5x10^7$ cells/mL was not significantly higher than that expected from the simulated frequency, it was significantly higher than the number predicted by the calculation using probability *P*. After four days of growth, the frequency of resistant cultures was significantly higher than both expected frequencies in all categories at $\alpha = 0.05$. In short, all but once scenario test resulted in higher frequencies of resistant cultures than we predicted using either method after only one day of growth. After four days, all scenarios examined had significantly higher frequencies of resistance than both of our predictions.

For fusidic acid, the 100x MIC, $1x10^7$ cells/mL cultures performed as expected. Based on the expected frequencies for this category, we anticipated no resistant cultures. No resistant cultures were observed after one day, and only one was observed after four days. This one culture was not statistically

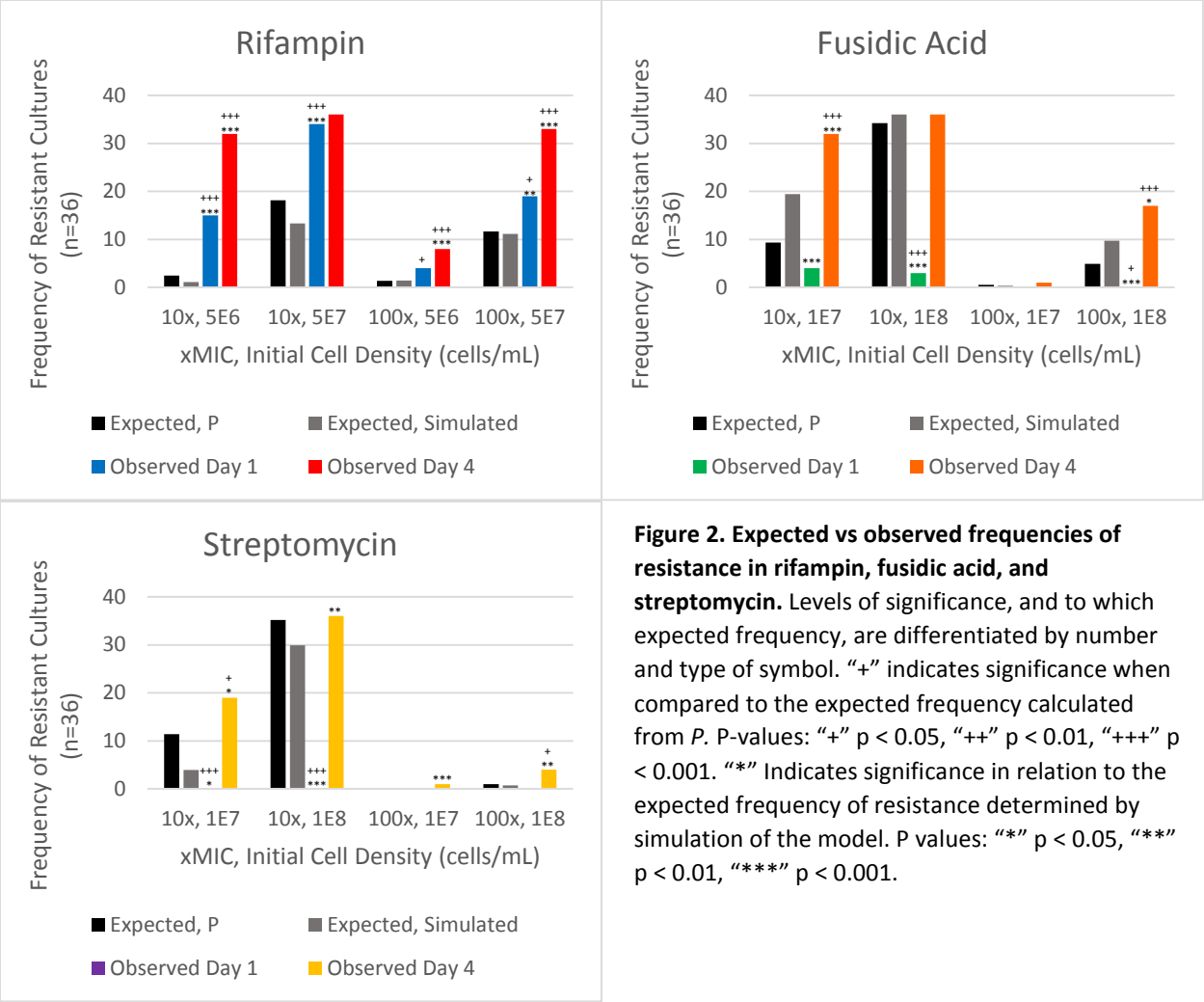

Figure 2. Expected vs observed frequencies of resistance in rifampin, fusidic acid, and streptomycin. Levels of significance, and to which expected frequency, are differentiated by number and type of symbol. "+" indicates significance when compared to the expected frequency calculated from *P.* P-values: "+" $p < 0.05$, "++" $p < 0.01$, "+++" $p < 0.001$. "*" Indicates significance in relation to the expected frequency of resistance determined by simulation of the model. P values: "*" $p < 0.05$, "**" $p < 0.01$, "***" $p < 0.001$.

significant from zero. Interestingly, the frequency of resistance was significantly lower at day one in all three other categories. This is not all that surprising, as we assumed the highest possible estimated mutation rate, obtained only after multiple days of colony accumulation, to calculate our expected frequencies. After four days of incubation however, the 10x MIC, $1x10^7$ cells/mL and 100x MIC, $1x10^8$ cells/mL cultures both had significantly higher numbers of resistant cultures than expected by both predictors. The 10x MIC, $1x10^8$ cells/mL cultures performed as expected by both predictors, with all 36 cultures becoming resistant.

In streptomycin, we saw a similar phenomenon. We saw no resistant cultures across all categories after one day of incubation. This was expected for the 100x MIC cultures. However, in 10x MIC cultures this was significantly fewer than expected by both predictors. Again, this was not that surprising as we used the highest estimated mutation rate in our predictions. By day four however, we observed significantly higher frequencies of resistance in the 10x MIC, $1x10^7$ cells/mL and 100x MIC, $1x10^8$ cells/mL cultures than we would expect from either predictor. Additionally, we observed a frequency of resistance in cultures with 10x MIC and $1x10^8$ cells/mL that was significantly higher than expected based on our model simulation, but not from our simple calculation.

Here we have noted that we observed significantly lower frequencies of resistance on day 1 in both streptomycin and fusidic acid cultures. We do not consider this to be an indication of any problem with our predictors or observed frequencies of resistance, as the mutation rates used to predict the expected frequencies were only obtained after one to four days of allowing colonies to accumulate during the fluctuation test used to estimate mutation rates. If these mutants, only appear after multiple days of incubation, then the result of significantly fewer resistant cultures appearing at day one is not unexpected.

Multiple Day Fluctuation Experiment

We hypothesized that the accumulation of cultures might be entirely due to pre-existing resistance mutations, and that the there was a delay in the expression of the resistant phenotype. In order to test this hypothesis, we ran a variation of the fluctuation experiment. Six 30mL cultures of *S. aureus* were incubated for 96 hours. Every 24 hours, a fluctuation test was on 10x MIC of each antibiotic, and performed with the same plating efficacies outlined in the materials and methods. Colonies were counted on each plate for four days.

If there was indeed a delay in the expression of the phenotype, we would expect the apparent mutation rate calculated from Day 1 colonies at all time points to increase with time, as this would have allowed the resistant phenotype to accumulate while cells were at stationary phase density. The mutation rate based on day one colonies did not change over the course of 96 hours. Furthermore, mutation rate estimates for each of the subsequent days did not show any changes (Figure 3).

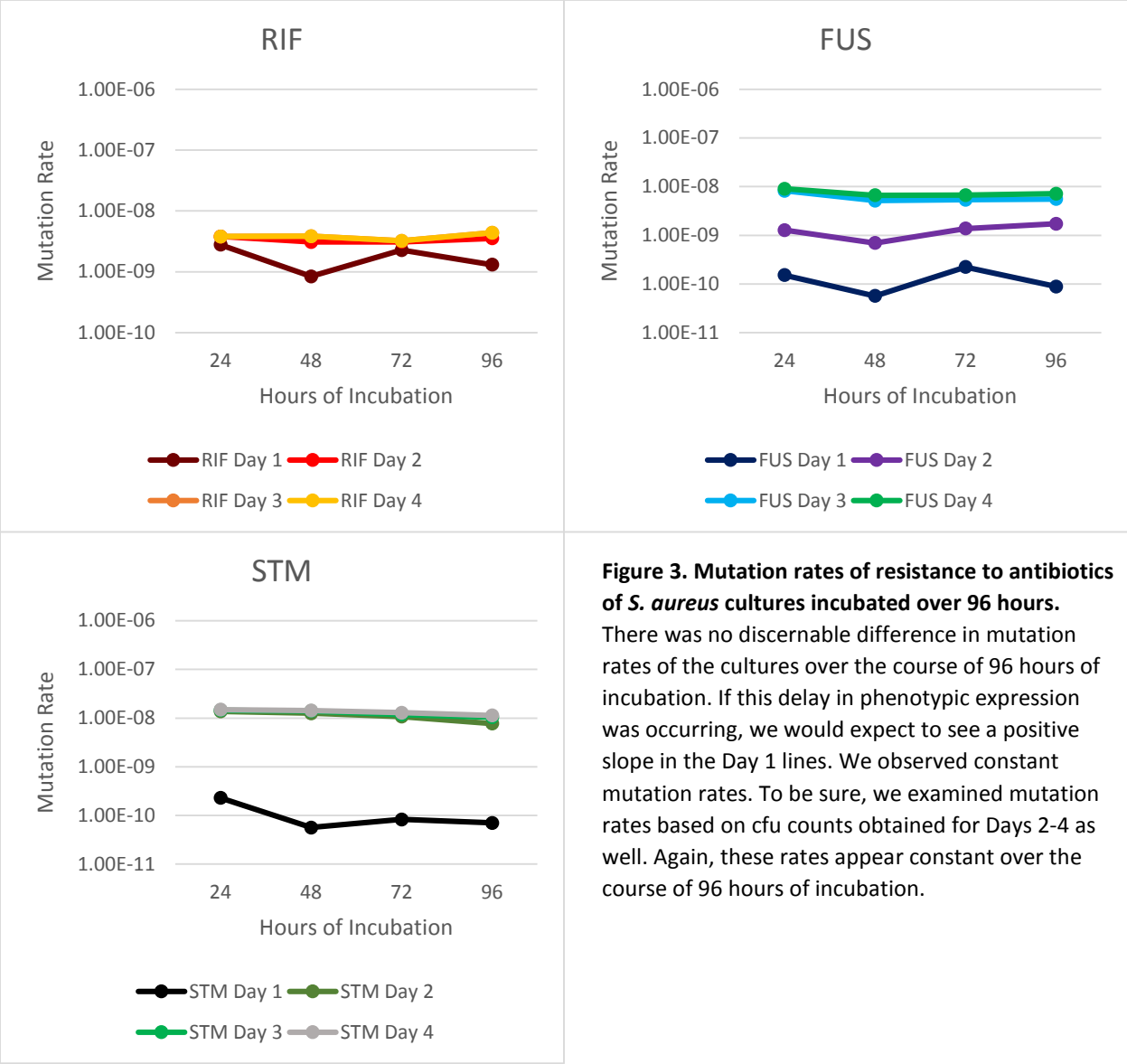

Figure 3. Mutation rates of resistance to antibiotics of *S. aureus* cultures incubated over 96 hours. There was no discernable difference in mutation rates of the cultures over the course of 96 hours of incubation. If this delay in phenotypic expression was occurring, we would expect to see a positive slope in the Day 1 lines. We observed constant mutation rates. To be sure, we examined mutation rates based on cfu counts obtained for Days 2-4 as well. Again, these rates appear constant over the course of 96 hours of incubation.

Test for Antibiotic Mutagenesis

As a potential source of the increased frequency of resistance of *S.aureus* to the tested antibiotics, we hypothesized that the antibiotics themselves might be mutagenic. That is, exposure of *S. aureus* to the antibiotics during growth increased the mutation rate, hence the higher than anticipated frequencies of resistance in liquid culture. To test this hypothesis, we took three of our resistant isolates from the exploration of the colony accumulation: RIF^R, MIC = 2.5 µg/mL; FUS^R, MIC= 6.25 µg/mL, and STM^R, MIC = 60 µg/mL. Using these mutant strains, we conducted an altered version of our Luria-Delbrück fluctuation test. In this case however, we grew up the twelve independent cultures of each of the mutant strain in the presence of the antibiotic they were resistant to. RIF^R and FUS^R cells were each grown up in 10x the wildtype MIC of rifampin and fusidic acid, respectively. STM^R did not initially grow in the presence of 10x or 5x the wildtype MIC of streptomycin. This could potentially be due to underestimation of the STM^R MIC to streptomycin. As the MIC estimation for the mutant was done with a single starting concentration using a two-fold dilution, the true MIC of this mutant could be as high as 60 µg/mL or as low as 31 µg/mL. If it was indeed on the lower end of this range, the strain would be unable to grow in 5x the wildtype MIC (37.5 µg/mL) or higher. To adjust for this, we grew up the STM^R mutant in 1x the wildtype MIC of streptomycin. As the goal of the experiment was simply to expose *S. aureus* cells to the antibiotic, this concentration was deemed sufficient.

The twelve independent cultures of each resistant strain were then plated twice. Each plate contained 10x the MIC of one of the two antibiotics not present in the growth media: the RIF^R cultures were plated on 10x fusidic acid and streptomycin, FUR^R cultures were plated on 10x MIC of rifampin and streptomycin, and the STM^R cultures were plated on 10x MIC of rifampin and fusidic acid. As with our standard Luria- Delbrück fluctuation test, cell density for each set of twelve cultures was estimated by averaging the estimated densities of four of the twelve cultures, selected at random. Colonies were counted each day for four days after plating, and the mutation rate was recalculated for each day. There

was no clear difference in the mutation rates of the strains of *S. aureus* exposed to the antibiotics (Table 2).

Strain	Antibiotic in plate (10x MIC)	Estimated Mutation Rate, μ			
		Day 1	Day 2	Day 3	Day 4
RIF^R	FUS	1.0E-11 (4.4E-11)	3.7E-10 (2.3E-9)	2.8E-9 (9.9E-9)	8.6E-9 (1.5E-8)
	STM	N/A (4.4E-11)	2.3E-8 (1.4E-8)	2.6E-8 (1.9E-8)	2.6E-8 (1.9E-8)
FUS^R	RIF	1.1E-9 (4.4E-9)	6.2E-9 (6.4E-9)	6.8E-9 (6.9E-9)	7.1E-9 (6.9E-9)
	STM	9.7E-12 (4.4E-11)	1.8E-8 (1.4E-8)	2.9E-8 (1.9E-8)	2.9E-8 (1.9E-8)
STM^R	RIF	7.9E-9 (4.4E-9)	8.1E-9 (6.4E-9)	9.0E-9 (6.9E-9)	9.4E-9 (6.9E-9)
	FUS	2.3E-10 (4.4E-11)	1.1E-9 (2.3E-9)	7.8E-9 (9.9E-9)	1.8E-8 (1.5E-8)

Table 2. Mutation rates of resistant strains exposed to antibiotic. The first number in each cell gives the mutation rate estimated for the resistant strain after exposure to the antibiotic. The value given in parentheses is the corresponding wildtype mutation rate from Table 1, given here for convenience in comparison. There was no distinct difference between the two mutation rates for any of the resistant strains.

Discussion

Rates of mutation of resistance to antibiotics are an important factor to consider in the ongoing fight against antibiotic resistance. With cases of multi-drug resistant bacteria increasing and introduction of new antibiotics decreasing, we are increasingly at risk of losing otherwise useful drugs. Particularly bothersome in this context is the case of rifampin. By most measures, rifampin is a great drug. It is a broad spectrum antibiotic that is effective at relatively low concentrations. Add to this that rifampin penetrates most tissues very effectively, it would seem that this is a fantastic antibiotic. And yet, it is used almost exclusively for tuberculosis treatment. High rates of resistance to this drug have been recorded for decades, leading to its sparing use and usually only in combination therapy (Goldstein

2014). This issue of high rates of resistance goes beyond complicating clinical treatment of infections. Resistance to this drug emerging during research can make studying the pharmacodynamics and pharmacokinetics of rifampin difficult, frustrating physicians, scientists, and undergraduates simply trying to write an honors thesis. We suspected that rifampin resistance was not only occurring at high rates, but that these increased rates of resistance were higher than we would anticipate using the conventional population biology approach of predicting numbers of mutants by μN. We extended our study to include fusidic acid and streptomycin, two antibiotics that are also effective against *S. aureus* and for which resistance evolves by a single mutation, to see if this phenomenon of high rates of resistance bears any generality.

Our experiments showed that in rifampin, *S. aureus* evolves resistance at a significantly higher frequency than we predicted, via two different methods, after only one day of growth. This difference was only increased as over subsequent days of incubation, more cultures became resistant. This higher than predicted frequency of resistance did not appear to be a general phenomenon. While we did observe significantly higher frequencies of resistance in both streptomycin and fusidic acid, it was only we paired lower antibiotic concentrations with lower cell densities and higher antibiotic concentrations with higher cell densities. Additionally, these significantly higher frequencies of resistant cultures were only achieved when cultures were allowed to incubate for two to four days. Cultures containing fusidic acid and streptomycin were almost always clear after a single day of incubation.

While we initially suspected that allowing these cultures and the selective antibiotic plates of the fluctuation tests to incubate more than 24 hours might yield some additional growth, we did not expect this accumulation occur to the extent we observed. An earlier study by Riesenfeld *et* al. (1997) that used ciprofloxacin observed a similar phenomenon and attributed it to adaptive mutation, claiming that colonies that arise early are the result of pre-existing mutants in the culture while colonies that arose later are the result of mutations generated in non-dividing cells. In our study, rather than attribute

the phenomenon with rifampin, fusidic acid, and streptomycin in *S. aureus* to adaptive mutation, we generated and tested a series of hypotheses in an effort to explain this accumulation of resistance.

The null case is that we can accurately predict the likelihood of resistance emerging using estimates of the mutation rate, μ, and total number of cells in culture, N. Our emergence of resistance in liquid cultures led us to reject this case for rifampin. While the effect was similar in cultures of fusidic acid and streptomycin, it was not as pronounced and occurred only in specific conditions. We cannot therefore completely reject the null hypothesis for these two antibiotics.

One proposed explanation for the increase of resistant mutants over time is that the antibiotic is decaying while in culture. Following the thinking of Drilica *et al* (2001), there may be resistant mutants whose MIC's are above the wildtype MIC but below the concentration of antibiotic added to culture. If these cells were persisting while the antibiotic was decaying, then it is simply waiting game for these resistant cells. They can persist until the antibiotic concentration drops below their MICs, at which point they can begin to grow and ascend in culture. While we did not explicitly test this possibility, we find it highly unlikely. Streptomycin and rifampin are known to be stable at 25˚C in liquid and structured media for up to 90 and 56 days, respectively (Creswell 1993, The Merck Index). Furthermore, the colonies obtained from different days of the rifampin and fusidic acid fluctuation tests all yielded MIC's higher than that of the concentration of antibiotic contained in the plates. These colonies would not require the antibiotic concentration to drop and can grow on our plates without any antibiotic decay. While the results of this same experiment in streptomycin gave a result in support of the antibiotic decay hypothesis, we still find this prospect unlikely given streptomycin's stability. With the proper equipment, examining the concentration of antibiotics in culture over time is a fairly straightforward task. Liquid chromatography and mass spectrometry are often used to quantify unknown concentrations of antibiotic (Niessan 1998). These methods could be used in future studies to accurately monitor for changes in antibiotic concentration as a test for antibiotic degradation in bacteria cultures.

As another alternative hypothesis, we proposed that there may be a delay in expression of the resistant phenotype. In this scenario, we assume all resistant mutants are already present at the time of antibiotic exposure. However, as the mutation must translate into mutant proteins or ribosomes to confer the resistance phenotype, it could be that these cells are not producing adequate amounts of the mutant protein or ribosome to escape selection. We proposed that these cells could persist or grow slowly until enough mutant proteins or ribosomes have accumulated in a cell, allowing that cell to eventually exhibit the resistance phenotype and ascend in culture. Our results from the multiple day fluctuation test are evidence to reject this hypothesis. By allowing the culture to sit at stationary phase density for several days, this should have allowed cells to accumulate a resistance phenotype. These cells should appear sooner during a fluctuation test, resulting in increased mutation rate estimates based on Day 1 or Day 2 colony counts. We saw no increase in mutation rate. We acknowledge however that this is not sufficient to completely reject this hypothesis. Another possible test of this hypothesis would be to repeat this experiment, but to allow cells to remain in the exponential growth phase, rather than the stationary phase. Allowing cells to growth for extended periods rather than remain non-growing at stationary phase may yield a different result than we observed here.

We considered that the antibiotics are mutagenic and are increasing the general rate of mutation in a cell and rejected this possibility based on our experiments. Resistant strains exposed to each antibiotic did not have increased rates of mutation to the two other antibiotics. If exposure to the antibiotic increases the general mutation rate for *S. aureus*, we should have observed elevated rates of mutation for these other antibiotics.

In conclusion, we observed significantly higher frequencies of resistance in cultures of *S. aureus* exposed to 10x and 100x MIC of rifampin. We observed a similar effect with fusidic acid and streptomycin, but only after cultures were incubated for two to four additional days. For rifampin, we rejected the null hypothesis: we were unable to accurately predict the frequency at which resistance to

the antibiotic emerged based on the mutation rate and total number of cells. We were not able to reject the null hypothesis for fusidic acid and streptomycin, leading us to believe that this phenomenon may not be general. While we acknowledged that resistant mutations occur more frequently than anticipated by the mutation rate, we were able to reject the possibility that these antibiotics are mutagenic. However, more work is needed to elucidate the mechanisms responsible for the elevated rate of resistance to rifampin and the accumulation effect seen in all three antibiotics.

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Appendices

Appendix 1. Model for the emergence of resistant mutants in media with the selecting antibiotic

The purpose of this semi stochastic model is to estimate the likelihood that resistant mutants will emerge in cultures of susceptible bacteria treated with antibiotics. It can be used for a batch culture, as in the experiments we are performing, or continuous (chemostat) culture. There are two populations of bacteria, N1 and N2 cells per ml, each of which can produce non-replicating, antibiotic refractory persisters, N1P and N2P. We assume Hill functions (REGOES et al. 2004) for the antibiotic-concentration dependent growth rates of N1 and N1,

$$\psi_i(A,R) = \left(\psi_{MAXi} - (\psi_{MAXi} - \psi_{MINi}) \left[\frac{\left(\frac{A}{ZMIC_i} \right)^{\kappa i}}{\left[\left(\frac{A}{ZMIC_i} \right)^{\kappa i} - \frac{\psi_{MINi}}{\psi_{MAXi}} \right]} \right] \right) \frac{R}{(R+k)}$$

where i = 1 or 2, A is the concentration of the antibiotic, ψ_{MAXi} and ψ_{MINi} are, respectively the maximum and minimum growth rates, $\psi_{MAXi} > 0$, $\psi_{MINi} < 0$. The parameter κi (kappa) is a parameter that determines the shape of the Hill function, $ZMIC_i$ the concentration of the antibiotic where the bacteria are neither growing or dying, R the concentration of a limiting resource, k, the Monod parameter, the concentration of the limiting resource when the maximum growth rate in the absence of antibiotics is $\psi_{MAXi}/2$.

As in (STEWART AND LEVIN 1973) we assume the bacteria consume the limiting resource at a rate proportion to their growth rate, and a conversion efficiency constant e μg, which is the amount of resource needed to produce a new cell. Non-replicating persisters are generated at a rate g per cell per hour from the N1 and N2 populations and return to those replicating populations at a rate of h per cell per hour. For the batch culture system considered here, the only resources available and antibiotics present those at the start of the experiment, respectively R(0) and A(0), and there is no flow through the system, w=0. The effective concentration of the antibiotic can decay at a rate of da per hour. We assume that N2 is less susceptible to the antibiotic than N1 and can be generated from N1 by mutation, which occurs at rate of μ per cell per hour. We neglect mutation from N2→N1.

With these definitions and assumptions, the rates of change in the densities of the bacteria and concentrations of resources and antibiotics are given by,

$$\frac{dR}{dt} = w(C-R) - e(\psi_1(A,R)N1 + \psi_2(A,R)N2)$$

$$\frac{dA}{dt} = -A(w+da)$$

$$\frac{dN1}{dt} = \psi_1(A,R)N1 - \mu N1 - gN1 + hN1P - wN1$$

$$\frac{dN2}{dt} = \psi_2(A,R)N2 + \mu N1 - gN2 + hN2P - wN2$$

$$\frac{dN1P}{dt} = gN1 - hN1P - wN1P$$

$$\frac{dN2P}{dt} = gN2 - hN2P - wN2P$$

In our simulation we assume that mutation is generated by a Monte Carlo process. When a random number x (0<x<1) from a rectangular distribution, is less than μ*N1*vol*Δt where Δt is the step sizes (an Euhler simulation) 1/(Δt*Vol) cells enter the N2 population and are removed from the N1.

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The Berkeley Madonna™ program for this simulation follows.

```
{Antibiotic treatment model Continuous culture}
{Two competing Populations with different PD parameters}
{Persisters generated and flow in and out of the N1 and N2 populations}
```

{Antibiotic treatment model Continuous culture}
{Two competing Populations with different PD parameters}
{Persisters generated and flow in and out of the N1 and N2 populations}

```
METHOD EULER

STARTTIME = 0
STOPTIME=200
DT = 0.001
```

```
init N1=1E8 {Bacteria Population 1}
init N2=1E8 {Bacteria Population 2}
init N1P =0 {Persisters 1}
init N2P =0 {Persisters 2}
{assume persisters are completely refractory to the anibiotic but don't grow}
init A=10 {Concentration of the antibiotic}
init R=100 {Initial resource concentration}
init TT=0 {Index for the addition of antibiotics}

{Population growth and Hill function parameters}
vmax1 =1.2 {maximun growth rate pop1}
vmax2= 1.2 {maxium growth rate pop2}
vmin1 =-0.1 {minimum growth rate Pop1}
vmin2 =- 0.1 {minimum growth rate Pop2}
k=1 {Monod constant}
KS1=1 {Kappa Pop 1}
KS2=1 {Kappa Pop 2}
MIC1=1 {MIC Pop 1}
MIC2=1 {MIC Pop 2}
ref1=0.5 {Low density cut off Pop1}
ref2=0.5 {Low density cut off Pop2}
mu=1E-8 {mutation rate per cell per hour N1-->N2}
vol = 10 {Volume of the culture}
g=1e-5 {Rate of production of peristers}
h= 1e-5 {Rate of resturn to active state}

w =0.1 {Flow rate}
Amax = 10 {Antibiotic 1 maximum dose }

Dose = 10 {Dosing interval}
da=0 {antibotic decay rate}
C=1000 {Reservoir concentration of the resource}
e=5e-7 {Conversion efficiency}


psi=R/(R+k) {Resource function}
{Hill Functions}
HS1=((VMax1- Vmin1)*(A/MIC1)^ks1)/((A/MIC1)^ks1-Vmin1/vmax1)
HS2=((VMax2- Vmin2)*(A/MIC2)^ks2)/((A/MIC2)^ks2-Vmin2/vmax2)

{Net Growth Rates}
G1=(Vmax1-HS1)*psi
G2=(Vmax2-HS2)*psi

NT=N1+N2

{Differential Equations}
```

```
d/dt (A)=-A*(w+da)+Add {Change in antibiotic concentration}
d/dt (R) = w*(C-R) - e*(N1*G1+N2*G2) {Change in resource concentration}
d/dt (N1)=G1*N1*Y1-g*N1+h*N1P- w*N1-N2ADD/vol {Change in the density of population 1}

d/dt (N1P)=g*N1-h*N1P-w*N1P
d/dt (N2P)= g*N2-h*N2P - w*N2P

d/dt (N2)=G2*N2*Y2+g*N2+h*N2P-w*N2 +N2ADD {Change in the density of population 2}
d/dt (TT) = 1 - HT/dt {Change in the time index}

{Antibiotic dosing}
Add= IF TT> dose THEN AMAX/dt ELSE 0
HT= IF TT> dose  THEN TT ELSE 0
{Cut off}
Y1=IF N1<0.5 THEN 0 ELSE 1
Y2=IF N2<0.5 THEN 0 ELSE 1

{Mutation}
ran = RANDOM (0,1)
N2ADD= If ran < N1*mu*dt*vol THEN 1/(DT*Vol) ELSE 0
```

Appendix 2. Table of observed frequencies of resistant cultures over four days of incubation

Antibiotic	[AB] (x MIC)	N (cells/mL)	Number of Resistant Cultures (n=36) Days of Incubation			
			1	2	3	4
RIF	10x	5.00E+06	15	18	24	32
		5.00E+07	34	36	36	36
	100x	5.00E+06	4	7	8	8
		5.00E+07	19	32	33	33
FUS	10x	1.00E+07	4	12	30	32
		1.00E+08	3	28	36	36
	100x	1.00E+07	0	1	1	1
		1.00E+08	0	9	16	17
STM	10x	1.00E+07	0	0	13	19
		1.00E+08	0	36	36	36
	100x	1.00E+07	0	0	0	1
		1.00E+08	0	1	1	4

Note: A graphical representation of this table is given in Figure 1.

Appendix 3. Table of estimated MICs of colonies isolated from fluctuation test

RIF			FUS			STM		
Culture No.	Day Colony Appeared	MIC	Culture No.	Day Colony Appeared	MIC	Culture No.	Day Colony Appeared	MIC
1	1	2.5	7	1	50	1	2	60
	1	2.5		1	50		2	60
	1	5		1	50		2	60
	2	0.3125		2	6.25	5	2	60
	2	10		2	6.25		2	60
	2	>10		2	6.25		2	60
5	1	0.625	8	1	50	10	2	60
	1	2.5		1	50		2	60
	1	>10		1	50		2	60
	2	>10		2	6.25			
	2	>10		2	6.25			
	2	N/A		2	6.25			
12	1	5	11	1	100			
	1	>10		1	100			
	1	>10		1	100			
	2	N/A		2	6.25			
	2	N/A		2	0.1			
	2	N/A		2	6.25			
WT 1		0.039		WT 1	0.2	WT 1		7.5
WT 2		0.039		WT 2	0.8	WT 2		7.5
WT 3		0.039		WT 3	0.2	WT 3		7.5

Note: Culture No. simply indicates the number randomly assigned to cultures during the experiment as a means to keep the experiment organized. As the fluctuation tests these colonies were isolated from were each run separately, cultures sharing the same number are not related. They happened to be assigned the same number for different experiments. Additionally, for rifampin cultures 5 and 12, N/A indicates the colony did not streak out properly. Rather than spend the time and resources to grow these cultures again, they were excluded from the experiment.