Epistasis between antibiotic tolerance, persistence, and resistance mutations

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Understanding the evolution of microorganisms under antibiotic treatments is a burning issue. Typically, several resistance mutations can accumulate under antibiotic treatment, and the way in which resistance mutations interact, i.e., epistasis, has been extensively studied. We recently showed that the evolution of antibiotic resistance in *Escherichia coli* is facilitated by the early appearance of tolerance mutations. In contrast to resistance, which reduces the effectiveness of the drug concentration, tolerance increases resilience to antibiotic treatment duration in a nonspecific way, for example when bacteria transiently arrest their growth. Both result in increased survival under antibiotics, but the interaction between resistance and tolerance mutations has not been studied. Here, we extend our analysis to include the evolution of a different type of tolerance and a different antibiotic class and measure experimentally the epistasis between tolerance and resistance mutations. We derive the expected model for the effect of tolerance and resistance mutations on the dynamics of survival under antibiotic treatment. We find that the interaction between resistance and tolerance mutations is synergistic in strains evolved under intermittent antibiotic treatment. We extend our analysis to mutations that result in antibiotic persistence, i.e., to tolerance that is conferred only on a subpopulation of cells. We show that even when this population heterogeneity is included in our analysis, a synergistic interaction between antibiotic persistence and resistance mutations remains. We expect our general framework for the epistasis in killing conditions to be relevant for other systems as well, such as bacteria exposed to phages or cancer cells under treatment.

antimicrobials | evolution of resistance | antibiotic persistence | synergy | killing assay

Tolerance and resistance are two different ways by which bacteria evade antibiotic treatment (1–3). Resistance is the inherited ability of microorganisms to grow in the presence of antibiotics, regardless of the duration of treatment. Resistance is achieved through different mechanisms such as efflux pumps or modification of the drug target (4) and is quantified by the minimum inhibitory concentration (MIC) (5) of antibiotic required to prevent the growth. Therefore, a more resistant strain has a higher MIC (Fig. 1 *A* and *B*). Note, however, that even a strain classified as "resistant"—i.e., has an MIC above the clinically defined breakpoint (6, 7)—may die if the antibiotic treatment is increased to above its MIC (Fig. 1*D*). The mutant prevention concentration (MPC) is the concentration required to prevent growth of resistant mutants evolved by a single mutation, i.e., it is above the MIC that can be acquired by single-step mutants (8–10). Single-step resistant mutants may have a very high MIC, but will still be killed by a treatment above the MPC. Guidelines for preventing the evolution of resistance suggest using doses above the MPC (11).

Tolerance, on the other hand, prolongs the duration of treatment that bacteria can sustain, for example by remaining dormant. Dormancy protects bacteria from the lethality of many types of antibiotics, whose action requires growth, such as beta-lactams and quinolones, as long as they remain dormant (Fig. 1*C*). Tolerance can be quantified by the minimum duration for killing (MDK_{99}); the time it takes to kill 99% of the culture at concentrations much higher than the MIC, where saturation of the killing efficacy is reached (Fig. 1*D*) (3, 12). A related phenomenon, called "antibiotic persistence," prolongs the duration of treatment that bacteria can sustain only for a subpopulation, even though the population is clonal. When monitoring the number of surviving bacteria versus time during treatment, two subpopulations can be observed: the majority of the bacterial population which is rapidly killed and a smaller subpopulation that persists for a much longer time, resulting in a biphasic killing curve (13, 14). When isolating cells from the persistent subpopulation, regrowing them and re-exposing to treatment, the same heterogeneous response to the drug is observed, indicating that the slower killing rate is not a result of a heritable change in the subpopulation. However, genetic mutations can increase (15) or decrease the size of the tolerant subpopulation (16). In the following, we refer to antibiotic persistence more succinctly as "persistence."

By evolving bacterial populations under intermittent antibiotic treatment, we previously found that tolerance and persistence promote the appearance of resistance (17). *E. coli* bacteria were subjected once a day for a few hours to a high concentration of a beta-lactam, above the MPC. First, bacteria evolved high tolerance by mutations in genes related to metabolism, without changing the MIC, as also observed in refs. 12, 18–20. Surprisingly, considering that the treatment was carried above the MPC in which single step mutants are expected to die, resistance mutations in the *ampC* gene fixated in the population. Indeed, the resistance level (MIC) of the mutants in our experiment was below the treatment concentration, but the combination with the tolerance mutations allowed the double mutants to survive the treatment nonetheless. However, no current description exists of the way resistance and tolerance mutations

Significance

The failure of antibiotic treatment is a major concern worldwide. Resistance is a main determinant in the survival of bacteria under antibiotics. However, it is often observed that bacteria become recalcitrant to antibiotics treatment, without developing resistance, a phenomenon termed "tolerance." Here we explored the interactions between tolerance and resistance mutations, both experimentally and theoretically, under killing conditions. We find that tolerance and resistance mutations interact synergistically, a finding that may be important for the design of more potent treatments.

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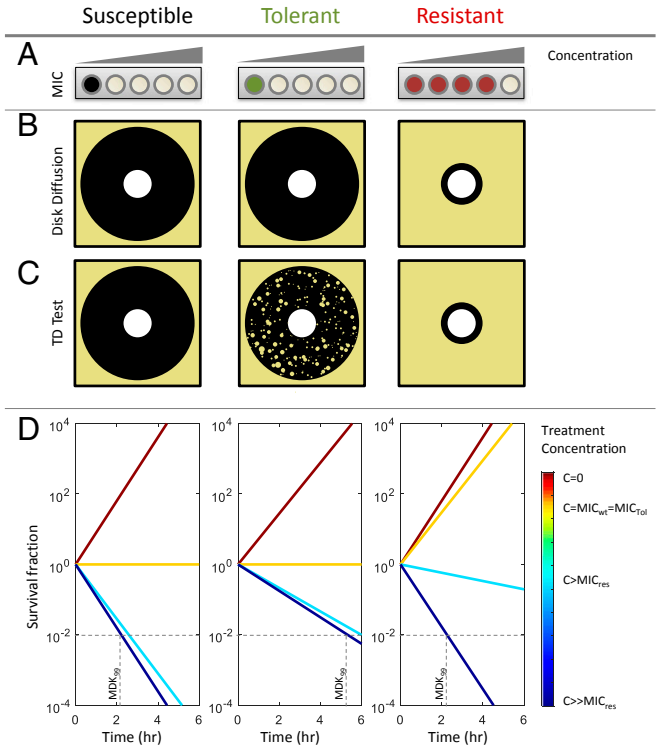

Fig. 1. A schematic illustration of the differences between *wt*, tolerant, and resistant strains. (*A*) The minimum inhibitory concentration (MIC) measured in gradually increasing concentration of antibiotics, identifying the minimum concentration that prevents growth. The *wt* and tolerant strains have the same MIC, while the resistant strain has a higher MIC. (*B*) Illustration of the disk diffusion assay. The black circle is the inhibition zone, where bacteria cannot grow, and indicates the MIC. The *wt* and tolerant strains have the same MIC, whereas the resistant strain, which has a smaller inhibition zone, has a higher MIC. (*C*) Illustration of the tolerance detection test (TDtest) performed after the disk diffusion assay (46): Once the antibiotic has diffused away from the inhibition zone, growth of surviving bacteria can be seen by adding glucose to the now empty disks. Only the tolerant bacteria have survived this duration of antibiotic treatment and can now form new colonies. Note that this phenotype is distinct from heteroresistance, where colony growth in the inhibition zone would occur already in *B* (before adding glucose). (*D*) Schematic survival curves of the *wt*, tolerant, and resistant strains at different treatment concentrations. When the concentration is zero, all strains grow. When the treatment is at $C = MIC_{wt} = MIC_{Tol}$, the *wt* and the tolerant strains neither grow nor die, but the resistant strain can grow. When the treatment is at $C > MIC_{res}$ all strains are killed but at different rates. When the treatment is at $C >> MIC_{res}$ the *wt* and the resistant strains are killed at similar rates, but the tolerant strain dies slower. The slower death rate at high concentrations is quantified by the MDK_{99} (3).

interact to enhance survival. Quantitative understanding the factors shaping the evolution of de novo resistance by mutation is a major goal (21).

Epistasis describes the effect of combining mutations that have an effect on the same phenotype (22, 23). An individual mutation arising on the background of another mutation could lead to a different phenotype, compared with when it occurs alone. Epistasis can provide information about the mechanisms related to these genes. When the phenotype affects fitness, the genetic interactions enable quantitative predictions of the evolutionary path (24–26). Fitness is typically measured by the relative abundance of individuals with a certain genotype after a period of growth (22, 27), but it can also be quantified by the growth rate (28, 29), by the relative progeny (30), or by the resistance level (31). The predicted neutrality of the double mutant is usually represented by a multiplicative function between the fitness of the corresponding single-mutant fitness values (32).

One central question is whether a combination of two different mutations will result in a bigger or smaller effect on the phenotype than expected from each mutation alone. To define the "expected" effect, which is also known as the "neutral" interaction, models of the combined effect of mutations on a phenotype need to be assumed, and several ways to define neutrality between mutations have been proposed (31–35).

In this work, we would like to examine the genetic interaction under killing conditions (i.e., above the MPC), by measuring the abundance of surviving bacteria after a bactericidal antibiotic treatment. Bacteria can survive transient exposure to antibiotics using different mechanisms described above, and the question is how a combination of those mechanisms affects survival. Our first goal is to derive a model for the expected effect of the genetic interaction between two types of mutations that have been observed under intermittent antibiotic treatment, namely resistance and tolerance mutations. Our second goal is to determine whether the interaction between resistance and tolerance mutations observed in the survival of evolved strains was stronger or weaker compared with our expectation (36). We also extend the analysis to include heterogeneity in tolerance, namely persistence. We find that tolerance and resistance mutations interact with a small synergistic effect. To assess the generality of our observations, and further extend our previous work, we present a similar analysis of a different type of tolerance, evolved under an antibiotic from a different class.

Results

Expected Interaction between Tolerance and Resistance Mutations. Most studies of epistasis analyze the combined effect of mutations on growth (28, 37, 38). In this study, we focus on the effect of mutations in killing conditions and measure fitness as the survival $S(t)$ after a period of killing, t. Here the killing conditions are taken as an antibiotic treatment at concentration, c, above the MPC and for duration t, but our analysis applies more generally to any transient killing conditions.

Phenomenological Models. The expected survival of the double mutant bearing a resistance (R) and a tolerance (T) mutation depends on the mechanism of interaction between these two mutations. Both tolerance and resistance result in higher survival under antibiotic treatment, but through very different pathways (39). To determine what the expectation is for the interaction between tolerance and resistance mutations, we can use the knowledge of their specific action on theoretical survival functions. Resistance levels are quantified by the MIC and it was shown that the dependence of the antibiotic concentration can be scaled by the MIC (40, 41). The main mechanisms for tolerance involve a slowdown of the killing due to a slowdown of an essential process in the bacteria, resulting in either a delay in growth (tolerance by lag) or a slower growth rate (tolerance by slow growth) (3). For each of these modes of tolerance, we present below a simple model, from which we extrapolate a general expected model for the interaction of tolerance with resistance.

Tolerance by lag protects bacteria from antibiotic treatment of duration t, as long as t is smaller than the lag time duration T_{lag} (3, 12). For longer treatment duration, the survival depends on the concentration, c, of the antibiotic scaled by the MIC of the strain, and ψ_{max} is the maximal growth rate;

$$S(c,t) = \begin{cases} 1 & t \le T_{lag} \\ e^{\psi_{max}\left(1-\frac{c}{MIC}\right)\cdot\left(t-T_{lag}\right)} & t > T_{lag} \end{cases}. \quad [1]$$

The typical timescale for killing is T_{lag} and mutations that increase the lag time will result in higher tolerance by lag. Note that the exponent is separable into a multiplication of two

functions—one is concentration related and the other is time related; i.e.,

$$S(c,t) = e^{f(c,MIC)\cdot g\left(t,T_{lag}\right)}. \quad [2]$$

If the mechanism for tolerance is not a delay in growth due to a precise lag, but rather a typical timescale to exit the lag phase or a reduced growth rate (tolerance by slow growth) (3), then the survival function will be scaled by the typical timescale for killing, namely the MDK_{99} (MDK from here on) resulting in:

$$S(c,t) = S\left(\frac{c}{MIC}, \frac{t}{MDK}\right). \quad [3]$$

A widely used phenomenological description of survival under antibiotic treatments is the Zhi model (42, 43):

$$S(c,t) = e^{\psi\cdot t}\ ,\ \psi(c) = \psi_{min}\cdot\frac{1-\left(\frac{c}{MIC}\right)^k}{\frac{\psi_{min}}{\psi_{max}}-\left(\frac{c}{MIC}\right)^k}, \quad [4]$$

which can also be rewritten in terms of the MDK and the MIC (3), as $MDK_{99} = \ln(0.01)/\psi_{min}$. It has been shown that in the case of tolerance by slow growth, the maximal growth rate is proportional to the rate of killing; i.e., $\psi_{min}/\psi_{max} = \alpha$ (constant) (44, 45). This leads us to a function of the form in Eq. **3**, and once again the exponent in Eq. **4** is separable into a multiplication of two functions, one concentration related and the other time related, as in Eq. **2**. In the case of tolerance by lag, the Zhi model is also separable under certain conditions (*SI Appendix*).

This property of separability, which stems from more general considerations (*SI Appendix*), allows for computation of the expected survival of the double mutant, S_{RT}, given that the survivals of each mutant and of the *wt* are known (*SI Appendix*):

$$log(S_{RT}) = \frac{log(S_R)\cdot log(S_T)}{log(S_{wt})}. \quad [5]$$

Experimental deviation from this expected model can be quantified by the epistasis measure ε given by:

$$\varepsilon = log\left(S_{RT}^{experiment}\right) - \frac{log(S_R)\cdot log(S_T)}{log(S_{wt})}. \quad [6]$$

We show in *SI Appendix* that this measure of epistasis is not specific to our models for tolerance (Eqs. **1** and **3**) and holds for a whole class of general survival functions.

Note that this epistasis measure is analogous to a known measure of epistasis derived for impaired growth and termed the "product model" (32). The derivation above shows that the product model is the expected way tolerance and resistance mutations affect survival. In the absence of other mechanisms, ε as defined by Eq. **6** is expected to be equal to zero.

Experimental Effect of Tolerance by Lag and Resistance. We now turn to the analysis of the interactions between mutations that occurred during our experimental evolution with two different lines of *E. coli* (KLY E1, MGY E7; *SI Appendix*, Table S1) under intermittent ampicillin treatment (17). Each line evolved tolerance rapidly by mutations in genes involved in metabolism, resulting in tolerant strains (KLY $metG^T$ and MGY prs^T, respectively). Subsequently, each line evolved resistance by acquiring a mutation in the beta-lactamase *ampC*, on the background of the tolerance mutation (KLY $metG^T$ $ampC^R$ and MGY prs^T $ampC^R$, respectively). To measure the epistasis between the tolerance mutation and the resistance mutation in each strain, we also created strains that harbor the resistant mutation alone (KLY $metG^{wt}$ $ampC^R$ and MGY prs^{wt} $ampC^R$, respectively; *SI Appendix*).

Phenotypic characterization of each strain showed that the resistance mutations resulted in a higher MIC, whereas the tolerance mutations resulted in a longer lag time (17) (*Materials and Methods*). As noted in ref. 17 the KLY $metG^T$ strain is heterogeneous in its tolerance, namely has a high persistence phenotype (Fig. 2*A*). We will first ignore this heterogeneity and focus on the global tolerance of this strain. A more precise calculation that takes into account tolerance heterogeneity, namely persistence, is done as a second step.

The independence of resistance and tolerance phenotypes can be seen by comparing the levels of tolerance or resistance in the double mutant to those of the strains that harbor a single mutation: the MIC of the double mutant for tolerance and resistance is identical to that of the resistant mutant (Fig. 2*B*), and the lag time distribution of the double mutant is identical to that of the tolerant mutant. These distinct phenotypes can be visualized using the recently developed TDtest method (46) (Fig. 2 *C*–*F*) and using ScanLag (47), for the lag distribution analysis (Fig. 2*A*). We now turn to evaluate the effect of combining these two phenotypes on the survival under antibiotics.

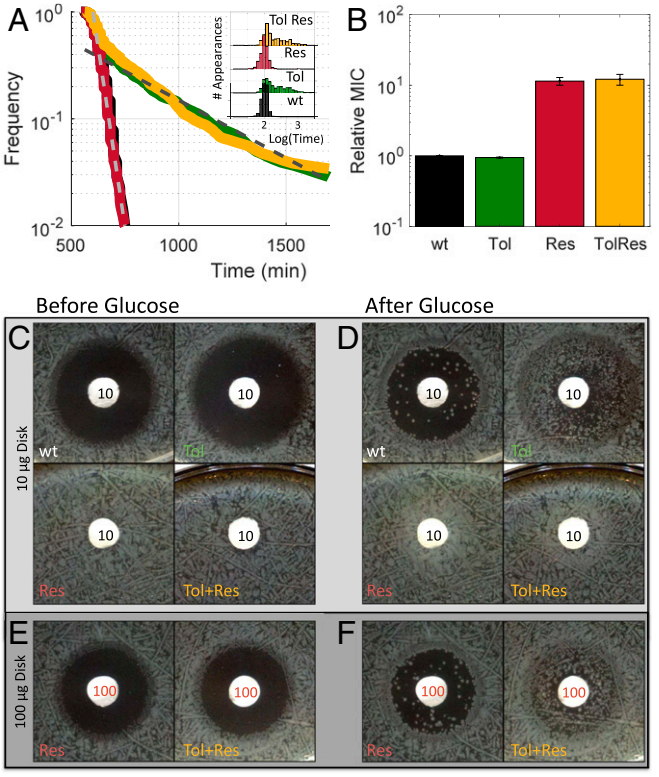

Fig. 2. Phenotypic characterizations of the *wt* (KLY), single mutants, and double mutants for resistance and tolerance. (*A*) ScanLag analysis of the lag time distribution, presented as the fraction of colonies not yet detected when plated on antibiotic-free medium. The tolerant (green) and resistant + tolerant (yellow) strains have prolonged lag, relative to the *wt* (black) and resistant (red) strains. (*Inset*) The same data showed as appearance distributions with log(Time) *x* axis. Note that the tolerant mutants in this example have a bimodal distribution of lag time, leading to persistence. (*B*) The relative MIC, as measured with antibiotic serial dilution method. (*C*) Resistance visualization using the disk diffusion assay with 10-μg-ampicillin disks. (*D*) Tolerance visualization with the TDtest (46), see Fig. 1*D*. (*E*) Scaling of the resistance level with MIC. Same as *C* and *D*, but with a 10-fold higher amount of ampicillin. Note that the inhibition zone of the *wt* with 10 μg ampicillin is similar to that of the resistant mutant with 100 μg ampicillin. (*F*) After the TDtest: the tolerant + resistant strain can be distinguished by the higher survival in the inhibition zone, as in *D*.

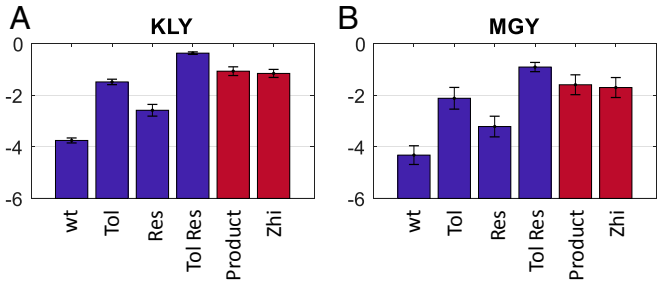

Fig. 3. Experimental results of the fitness of each mutation separately as well as the double mutants for two evolved strains. Bacteria were evolved by cyclic exposure at lag phase, resulting first in tolerance by lag and eventually in resistance. The bar graph shows the measured survival fraction (blue) of each of the two mutants—KLY E1 (*A*) and MGY E7 (*B*), and the expected survival (red) calculated with the product model or the Zhi model. The higher survival fraction in the experimental data shows that tolerance and resistance have mild positive epistatic interactions according to the product model. Error bars denote the SE. Tolerant, resistant, and tolerant + resistant mutants are denoted as Tol, Res, and TolRes, respectively. The expected survival from the product model and the Zhi model are denoted as Product and Zhi, respectively.

Measuring Epistasis between Tolerance by Lag and Resistance. To evaluate the epistasis between tolerance and resistance mutations in the evolved strains, we performed killing assays for each of the strains bearing either one or two mutations and measured the survival in ampicillin after 4.5 h of exposure, the killing conditions to which the evolved strains were exposed during the experimental evolution protocol. The concentration of ampicillin in this protocol is close to the clinically relevant one. It is high for the *wt* and tolerant strains ($c >> \mathrm{MIC}$), but "intermediate" for the resistant strains ($c > \mathrm{MIC}^{\mathrm{res}}$), and therefore kills the resistant strains at a slower rate, similar to the cyan lines in Fig. 1*D*.

Knowing the effect of each mutation separately allowed us to calculate the expected survival of the double mutants, assuming either the product model or the Zhi function (Fig. 3). We note that the two give similar results. For two evolved lines initiated from different ancestral *E. coli* strains (KLY and MGY), we found that the measured survival of the double mutant is close to the expected survival according to the product model (Eq. **6**), with an additional positive interaction, suggesting a synergistic effect between tolerance and resistance mutations in the KLY evolved strain (P = 1e-3, n = 11) and for the MGY strain (P = 0.03, n = 6). Similar calculations assuming the Zhi function led to similar results, i.e., positive epistasis is observed (Fig. 3). To extend our measurements to a pathogenic strain of *E. coli*, we repeated the survival assays in EPEC, its evolved derivatives (17) and reconstructed resistant mutant. Similar results were obtained (*SI Appendix*, Fig. S1). We conclude that for the conditions measured, the double mutants behave close to the expectation from neutral interaction models with an additional positive epistasis that can be detected in the interaction between tolerance and resistance mutations in our strains.

Epistasis of Resistance with Other Forms of Tolerance. To test whether our results can be generalized to other forms of tolerance and for other antibiotics, we performed evolution experiments using a different protocol and antibiotic. In this protocol, the ancestral strain, MGCH (*SI Appendix*), was exposed cyclically during exponential growth to norfloxacin, a fluoroquinolone. After two such cycles, a tolerant strain, $\mathrm{MGCH}^{\mathrm{T}}$, was identified (*SI Appendix*). Tolerance was confirmed by phenotypic testing, showing that it has orders of magnitude higher survival than its ancestral strain under norfloxacin, without any increase in its MIC (Fig. 4 *A* and *B*). Analysis of the growth of the mutant strain under the microscope (Fig. 4*C*) revealed that the tolerance was due to a lower growth rate, rather than a longer lag (Fig. 4 *D* and *E*).

In these strains (*wt* and tolerant), we constructed a known norfloxacin resistance mutation [*gyrA* S83L (48)] to allow us to test epistasis in a similar manner to that of the tolerance-by-lag strains. The strains were exposed to norfloxacin and survival was measured. Using the measurements of the *wt*, tolerant and resistant strains, we calculated the expected survival of the double mutant (according to both models, Fig. 4*F*), and once again found it to be similar to the measured survival, with a slight positive interaction. We therefore conclude that our results can be extended to different types of tolerance and different antibiotics.

The Effect of Antibiotic Persistence on Epistasis. So far, we have ignored corrections due to the potential heterogeneous response to antibiotics and assumed the same survival probability for all bacteria within a clonal culture. However, we do observe persistence in some of our evolved strains. For example, the $metG^{T}$ mutations on the KLY background confer tolerance only to about 5% of the population, resulting in a bimodal killing curve (Fig. 5*A*), a hallmark of persistence. In other words, the tolerance mutation has partial penetrance, resulting in a subpopulation of persister bacteria.

The product model, however, does not apply straightforwardly for persistence, since the heterogeneous survival cannot be described by a multiplicative function (*SI Appendix*). To correctly compute the epistasis factor between the resistant and tolerant

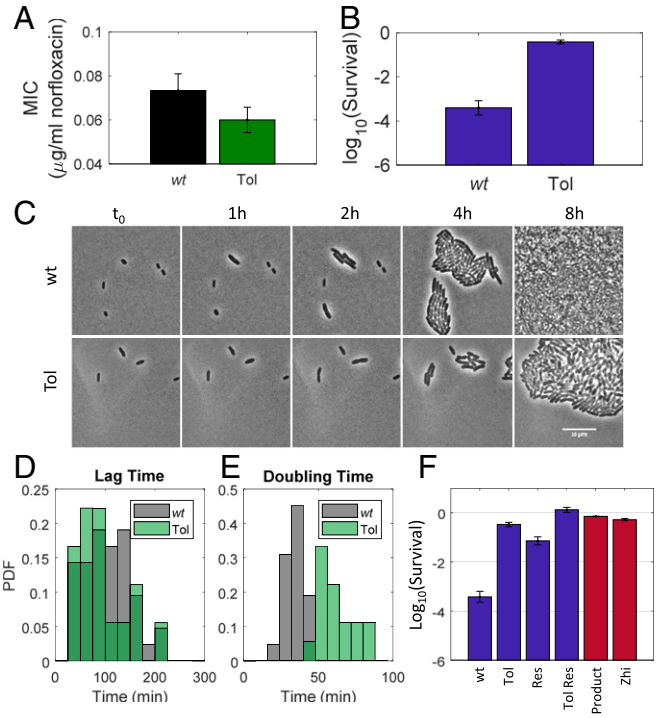

Fig. 4. Characterization of tolerance-by-slow-growth mutant. (*A*) MIC of the tolerant mutant ($\mathrm{MGCH}^{\mathrm{T}}$), no significant difference from that of the *wt* (P = 0.5, n = 4). (*B*) Higher survival of the tolerant mutant under norfloxacin (P = 0.03, n = 4). (*C*) Phase-contrast microscopy images of the ancestral and tolerant-by-slow-growth mutant (scale bar: 10 μm). (*D* and *E*) Data extracted from microscopy assay, using (monolayer) microcolony area as a proxy for biomass. Lag time is similar (P = 0.9, n = 60), while doubling time is significantly different (P = 5e-10, n = 60). (*F*) Epistasis between the tolerance and resistance mutations. The higher survival fraction in the experimental data shows that these tolerance-by-slow-growth and resistance mutations also have mild positive epistatic interactions, according to the product model (P = 0.05, n = 3). Tolerant, resistant, and tolerant + resistant mutants are denoted as Tol, Res, and TolRes, respectively. The expected survival from the product model and the Zhi model are denoted as Product and Zhi, respectively.

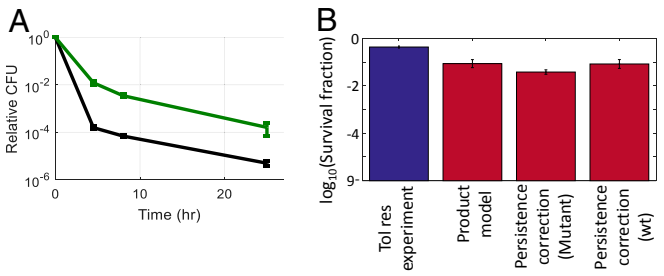

Fig. 5. Effect of persistence on epistasis. (*A*) Killing curve assay of the KLY *wt* (black) and high antibiotic persistence (green) strains. Persistence level can be observed in both, but is 2 orders of magnitude higher in the mutant. (*B*) Survival of the double mutant as measured experimentally (blue) and calculated (red). The expected survival from the product model is calculated as in Fig. 3. The expected survival with evolved high persistence correction (persistence correction [mutant]) is significantly lower than the expected survival calculated by the product model with the same survival rates (P = 0.03, n = 6). In this case the synergistic interaction is even stronger. This strain also has low basal amount of persistence in the *wt* and the resistant. If we take into consideration that the *wt* and the resistant survival is slightly higher because of the persistence, we can subtract it and then calculate the expected survival of the double mutant with *wt* persistence correction (persistence correction [*wt*]).

mutation in this strain, we need to take into account the effect of persistence. Therefore, we explicitly computed the effect of persistence on the survival function of the evolved strain. The result of this correction is a lower expected survival (*SI Appendix*) (Fig. 5*B*). In other words, the correction for persistence reduces the expected survival of the double mutants compared with the previously used product model, making the observed synergy even bigger. We conclude that the synergy observed in the positive epistasis between the *metG* persistence mutation and the resistance mutation is not due to the correction for heterogeneity of the evolved strain.

Another possible correction to the survival function may come from the persistence level of the *wt* strain itself, as the survival of the *wt* enters the calculation of the product model. The *wt* ancestral strains have been shown to harbor a small percentage (~0.1%–0.01%) of persister cells that are more tolerant (Fig. 5*A* and ref. 12). Similarly to the calculation above, we computed the correction to the epistasis due to the persistence in the *wt* strains. We measured the persistence level and explicitly wrote the survival function for persistence in the *wt* strain. We find that the correction is very small and cannot account for the positive epistasis that we measure (Fig. 5*B*).

We conclude that the corrections due to persistence in the interaction between tolerance and resistance mutations are small and do not account for the observed synergism.

Discussion

In this work, we have derived an expected model for the genetic interaction between tolerance and resistance mutations under killing conditions. We have shown that our experimental measurements of the epistasis in several different double mutants are higher than the expectation of the model, showing a small but significant synergistic interaction between resistance and tolerance. Similar results were obtained for different forms of tolerance and under antibiotics from different classes. We have extended our model to account for heterogeneity due to persistence. We conclude that an additional mechanism is responsible for the observed synergy. A possible mechanism, which could contribute to synergy between tolerance and resistance, is when resistance factors are more effective when the cell is dormant or slow growing. For example, in ref. 49, efflux pumps are more expressed in the persister cells, leading to more effective pumping out of the drug. Thus, in addition to the direct protective effect of slow growth against antibiotics that target growing bacteria, the up-regulation of the resistance factor by slow growth can result in a synergistic protection.

We note that to get a clean analysis, we focus here on mutations which contribute either to tolerance or to resistance, but not to both simultaneously. Resistance mutations may have a direct effect on the tolerance level as well, for example, if they are accompanied by a growth defect. In most cases, this effect is small compared with typical tolerance mutations observed.

In this work, we studied treatment above the MPC and found a synergistic interaction between tolerance and resistance. However, if the treatment is carried at a lower concentration, then full resistance can be acquired, in which case the additional growth delay conferred by the tolerance mutation will only give the double mutant a disadvantage. Therefore, the epistasis between tolerance and resistance for treatments below the MPC is expected to be suppressive. At the other extreme, in treatment at very high concentrations, far above the MPC, resistance (achievable by a single mutation) will not offer any advantage, and the interaction in this case is expected to be neutral.

One could argue that the evolution of partial resistance at concentrations above the MPC is not that important, since these partially resistant bacteria will eventually die. However, it has been shown in several different laboratory evolution experiments that partial resistance can rapidly lead to the acquisition of full resistance by a second adaptive step (Fig. 6) (50–52). Indeed, when we continued the intermittent exposures on a line that started from a tolerant strain (17), it led first to partial resistance to antibiotics, followed by a second mutation that granted full resistance.

We have previously shown that resistance is more likely to occur on the background of tolerance (17), given the effect of the combined mutations on survival. The analysis presented here examines the interaction between the tolerance and resistance mutations, once acquired. As detailed in ref. 17, to compute the full effect of the interaction between resistance and tolerance mutations, the difference in the probability of each mutation to occur needs to be taken into account. Given the much larger target size for tolerance (12) than for resistance, the evolutionary path is dictated more by the probability of establishment than by the survival under the drug. Predicting the potential for evolving resistance is a crucial factor to determine before introducing a new drug (53). Future work may consider the effect of epistasis on the probability of establishment beyond the survival epistasis analysis presented here.

Finally, the general analysis of epistasis of resistant and tolerant mutations presented above is not limited to genetic tolerance and resistance, but also relevant for the interaction between resistant mutations and phenotypic tolerance, such as observed in biofilms (54), a major concern in recalcitrant infections. Moreover, the ability of cells to survive lethal treatments by tolerance and persistence has been shown to be relevant in the context of antifungals (55) as well as anticancer drugs (56). Therefore, we expect that the framework for the interaction between resistance, tolerance, and persistence mutations

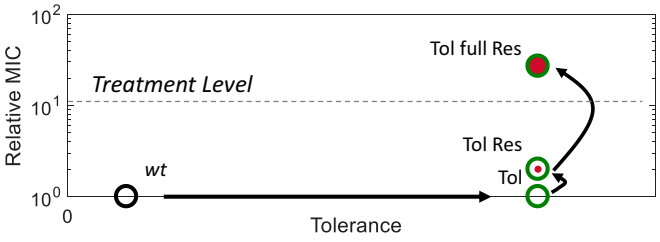

Fig. 6. Mutating from partial resistance to full resistance on a tolerant ancestral background. A schematic diagram of the evolved mutations. The black circle represents the *wt* strain, which acquired a tolerance mutation (MGY prs^T) (green circle). This strain then acquired a partial resistance mutation (point mutation in *ampC* region) resulting in a twofold increase in MIC (red dot in the green circle). Partial resistance was then followed by full resistance by an additional mutation in the *ampC* promoter (green circle filled with red).

presented here to be widely applicable for the study of drug resistance evolution.

Materials and Methods

Tolerance by Lag (tbl) Evolution Protocol (17). Evolution experiment: bacteria were first grown overnight (18 h at 37 °C with shaking in 1 mL LB Lennox medium [LBL, Sigma]), then diluted in fresh medium 1:100 and exposed to antibiotic treatment (50 μg/mL ampicillin at 37 °C with shaking for 4.5 h), and then the antibiotic was washed off (centrifugation twice for 20 min at 1,500 *g* at 4 °C), cyclically.

Tolerance by Growth (tbs) Evolution Protocol. Evolution experiment: bacteria were first grown in and maintained at exponential phase by subculturing at low OD (0.02), then exposed to antibiotic treatment in 96-well plates (4.5 μg/mL norfloxacin at 37 °C with shaking for 80 min), and then the antibiotic was washed off (centrifugation twice for 10 min at 1,200 *g* at 4 °C), cyclically.

Survival Assays. Bacteria were grown in LBL, exposed to bactericidal treatment for the time specified, and colony forming units were evaluated by colony counts on LBL agar or most probable number method (57).

TDtest. The TDtest was used as described in ref. 46.

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