REPORT

ANTIBIOTIC RESISTANCE

Antibiotic tolerance facilitates the evolution of resistance

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Controlled experimental evolution during antibiotic treatment can help to explain the processes leading to antibiotic resistance in bacteria. Recently, intermittent antibiotic exposures have been shown to lead rapidly to the evolution of tolerance—that is, the ability to survive under treatment without developing resistance. However, whether tolerance delays or promotes the eventual emergence of resistance is unclear. Here we used in vitro evolution experiments to explore this question. We found that in all cases, tolerance preceded resistance. A mathematical population-genetics model showed how tolerance boosts the chances for resistance mutations to spread in the population. Thus, tolerance mutations pave the way for the rapid subsequent evolution of resistance. Preventing the evolution of tolerance may offer a new strategy for delaying the emergence of resistance.

Antibiotic-treatment failure is typically attributed to resistance. Many resistance mechanisms have been identified, including mutations that decrease the binding of the drug to its target and increased expression of efflux pumps (*1*). Resistance mutations result in a decrease in the effective concentration of the drug. The effect of such mutations is measured by the minimum inhibitory concentration (MIC), i.e., the lowest drug concentration needed to prevent visible growth of the microorganism. However, it has long been realized that other mechanisms can help bacteria survive antibiotic exposure (*2*). Nongrowing or slow-growing bacteria can survive bactericidal antibiotics that require active growth for killing. This property is known as "tolerance" (*3*). When the nongrowing phenotype occurs in only part of a clonal population, such as in biofilms, this subpopulation of "persisters" underlies treatment failure (*4*–*6*).

Recent studies (*7*–*9*) have shown that tolerance and persistence evolve rapidly under intermittent antibiotic exposure. When *Escherichia coli* populations were subjected to daily intermittent exposures to ampicillin, separated by intervals in fresh medium, the cultures became tolerant to ampicillin by acquiring mutations that extended their lag phase, i.e., the period before exponential growth is resumed after stationary phase, without any change in the MIC. The evolved mutants did not become resistant; they survived antibiotic treatment as long as they remained in the lag phase but were efficiently killed by ampicillin once growth resumed (*7*).

Whether tolerant strains that can evolve rapidly, impede or accelerate the evolution of antibiotic resistance is the subject of debate (*10*–*13*). To understand the interplay between resistance and tolerance, we evolved bacterial cultures using a slightly modified treatment protocol from (*7*) (fig. S1). We now used a lower dose of ampicillin (50 μg/ml), but which was still comparable to therapeutic doses and a fixed residual level during growth (*14*, *15*). We continued daily intermittent exposures until resistance was established as defined by clinical standards (*16*). Starting with three different *E. coli* strains, including an enteropathogenic (EPEC) strain (table S1), we found that 11 of the 14 cultures reached an MIC at least sevenfold greater than the MIC of the ancestral strains (Fig. 1A). Further analysis of the resistant cultures with whole-genome sequencing revealed that they all harbored mutations in the promoter of *ampC*, which codes for a beta-lactamase known to confer resistance to ampicillin when overexpressed (*17*–*19*) (Fig. 1B).

To elucidate the path leading to resistance, we analyzed the dynamics of MIC increase in batch cultures at each cycle of antibiotic treatment. Simultaneously, we performed phenotypic characterization using the ScanLag system (Fig. 2, A to C), which enables the measurement of the distribution of the lag period and growth of single colonies with an automated scanner setup (*20*). We observed an increase in MIC after 7 to 17 cycles (Fig. 2, D to F). Interestingly, after three to four cycles of antibiotic exposure, we observed that most bacteria showed delayed growth when plated on fresh medium (Fig. 2, G to I). As previously shown, delayed growth resulted from an extended lag time that conferred tolerance to the ampicillin treatment (*7*). Thus, the bacterial cultures seemed to have become tolerant several cycles before the emergence of resistance.

The emergence of an extended lag time prior to the appearance of resistance could imply that this is the order in which mutations accumulate and spread through the population (*21*) (Fig. 2J). Our analysis, however, was performed on batch cultures that contained different clones. Therefore, the same sequence of events could also be attributed to independent competing lineages

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Table 1. Main mutations detected in whole-genome sequencing data and verified by Sanger sequencing. N.A., not applicable.

Strain	Seq ID	Genomic position	Mutation	Amino acid substitution	Gene	Annotation	Phenotype
MGY E7	CP019629	1,261,464	A>G	F138L (TTC→CTC)	*prsA*←	Ribose-phosphate pyrophosphokinase	Tolerance
MGY E7	CP019629	4,380,612	Insertion element IS2 (1330 bp)	N.A.	*ampC*←	*ampC* promoter	Resistance
KLY E1	CP008801.1	2,171,707–2,171,718	Δ12 bp	Δ89–92 deleted amino acid sequence ETIT	*metG*→	*metG* Methionyl-tRNA synthetase	Tolerance
KLY E1	CP008801.1	4,455,876	+A	N.A.	*ampC*←	*ampC* promoter	Resistance
EPEC E7	FM180568	2,326,669	G>A	G649D (GGC→GAC)	*metG*→	*metG* Methionyl-tRNA synthetase	Tolerance
EPEC E7	FM180568	4,726,849	+A	N.A.	*ampC*←	*ampC* promoter	Resistance

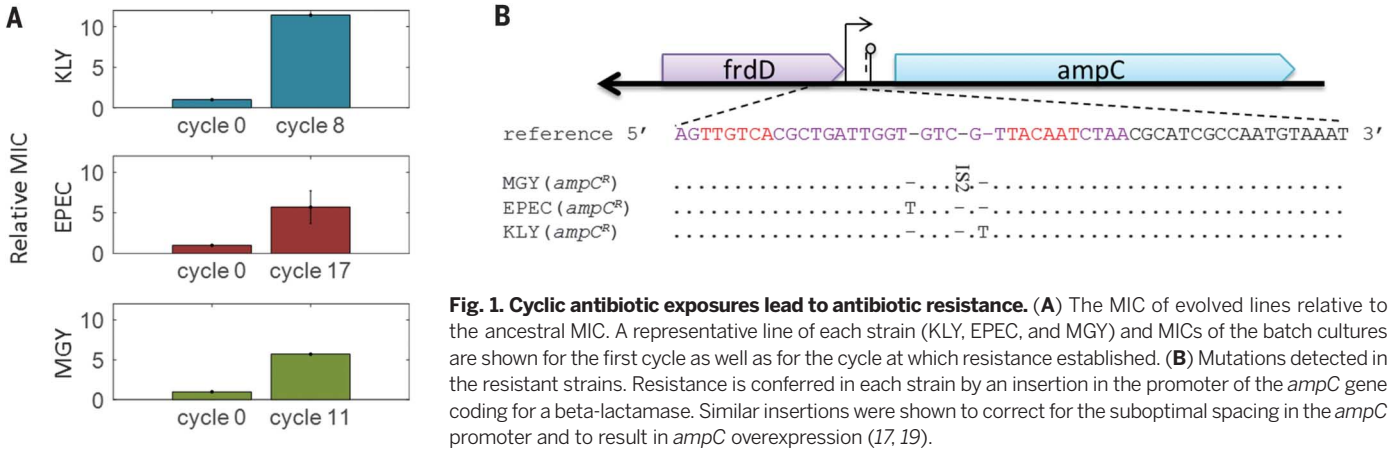

Fig. 1. Cyclic antibiotic exposures lead to antibiotic resistance. (**A**) The MIC of evolved lines relative to the ancestral MIC. A representative line of each strain (KLY, EPEC, and MGY) and MICs of the batch cultures are shown for the first cycle as well as for the cycle at which resistance established. (**B**) Mutations detected in the resistant strains. Resistance is conferred in each strain by an insertion in the promoter of the *ampC* gene coding for a beta-lactamase. Similar insertions were shown to correct for the suboptimal spacing in the *ampC* promoter and to result in *ampC* overexpression (*17*, *19*).

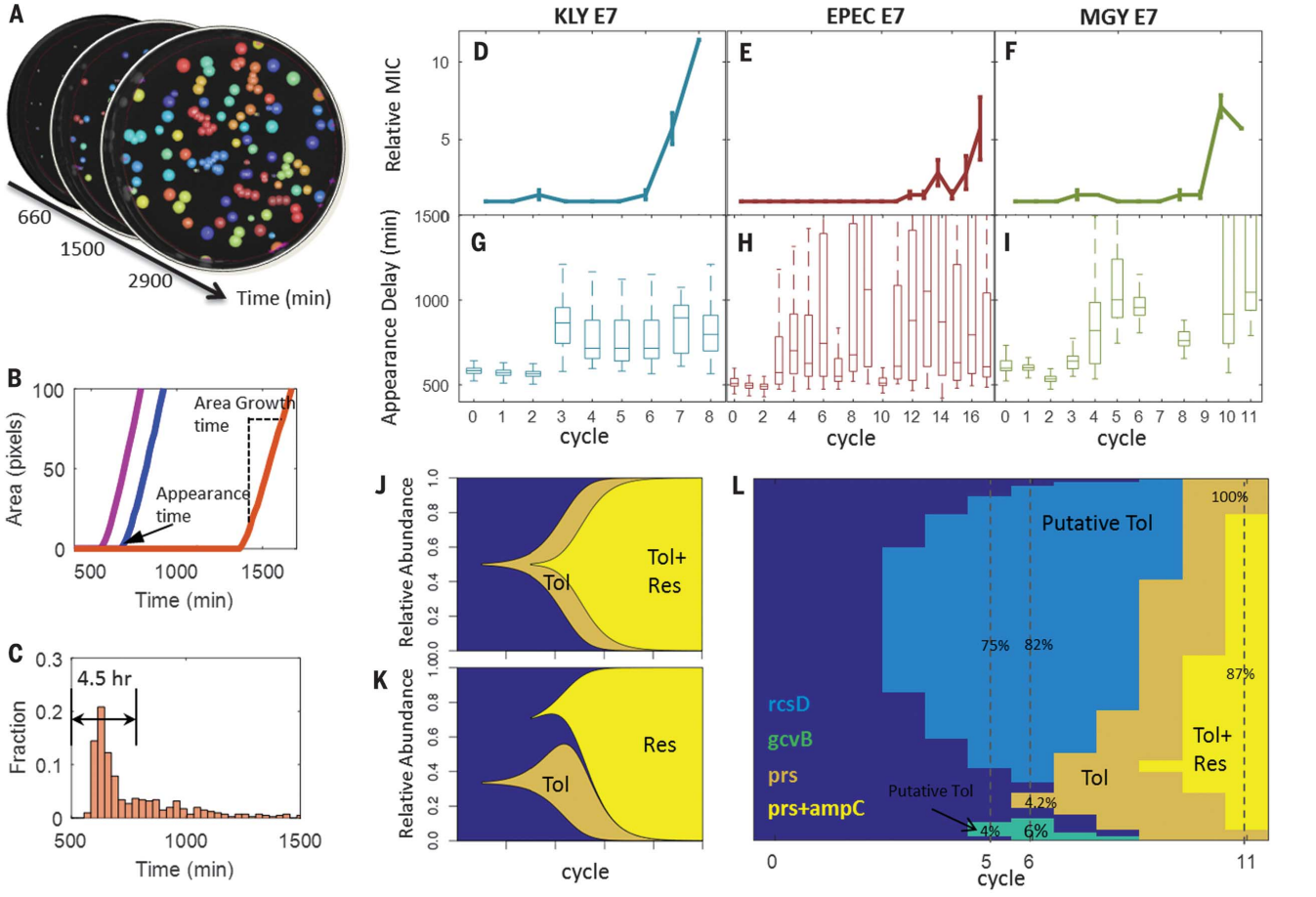

Fig. 2. Monitoring the population dynamics of resistance and tolerance. (**A** to **C**) Detection of colony appearance and growth time by ScanLag on the evolved persistent strain KLY *metG*^{T} *ampC*^{R}. (A) Automatic colonies-detection image. (B) Plot of the area of selected colonies over time. Each color represents a single colony. (C) Histogram of appearance-time distribution for the evolved persistent strain. Note the long tail of late appearance, which indicates growth impairment despite the absence of antibiotics. Bacteria with a lag phase longer than the 4.5 hours of ampicillin treatment would be able to survive under treatment. (**D** to **F**) MIC increase in evolving batch culture versus cycle number in strains KLY E7, EPEC E7, and MGY E7, respectively. (**G** to **I**) Appearance-time box plots of colonies in evolving batch culture versus cycle number in KLY E7, EPEC E7, and MGY E7, respectively. In all strains, the delay in the appearance of colonies due to extended lag time occurs several cycles before the MIC increase. (**J**) Schematic view of a possible scenario explaining the data presented in (D) to (I) in which tolerant (tol) mutants spread in the population and acquire a secondary resistance (res) mutation on top of the tolerant background. (**K**) Alternative scenario in which tolerant clones appear early in the culture, and resistant clones appear independently later on. (**L**) Measured frequencies of the tolerance and resistance mutations (*rcsD*, *gcvB*, *prs*, and *prs+ampC*) in batch culture of the MGY E7 line, cycles 0, 5, 6, and 11. Resistance is acquired in addition to the tolerant background, ruling out the scenario presented in (K).

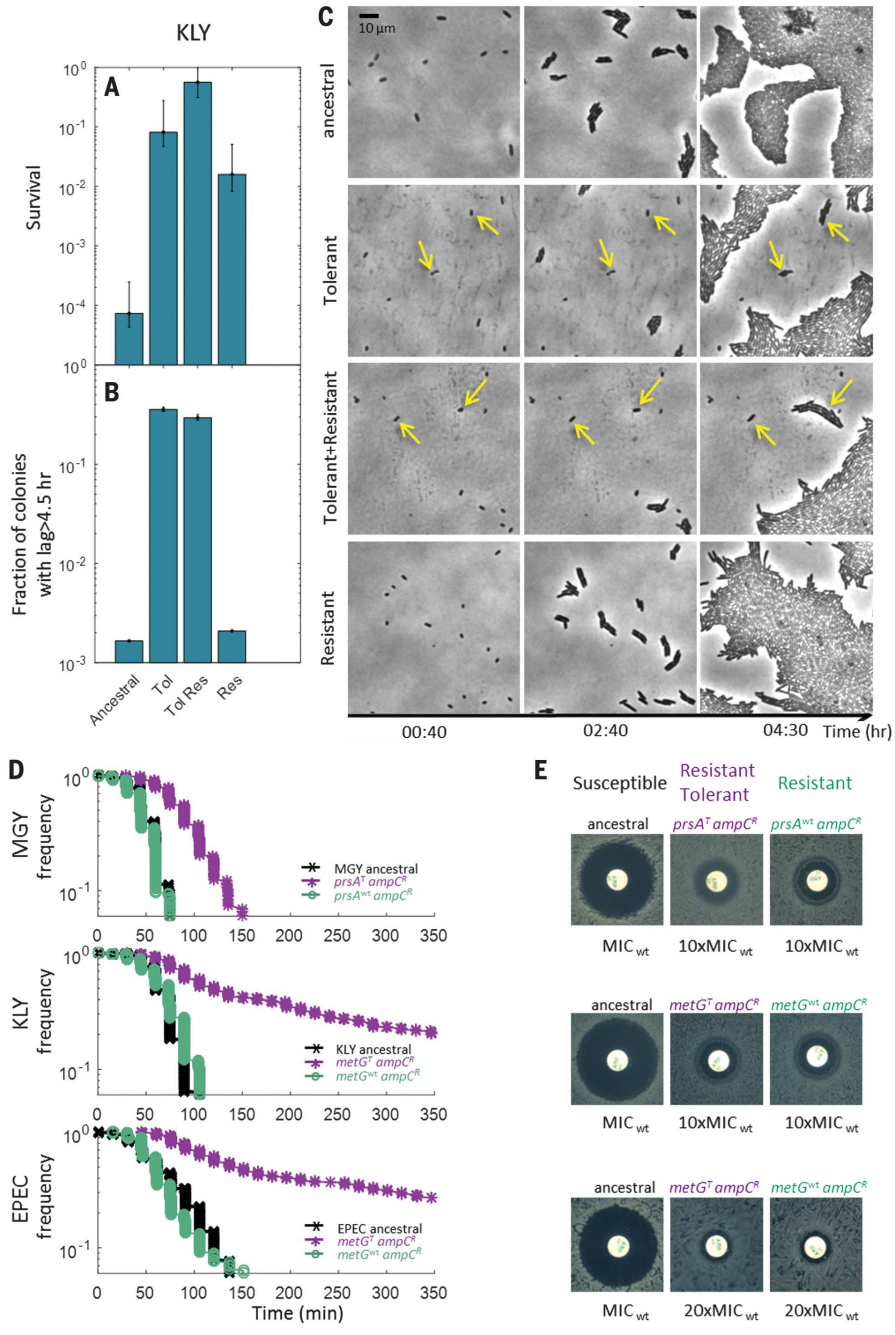

Fig. 3. The extended lag time underlies tolerance but does not affect resistance. (**A**) Survival fraction under 4.5 hours of ampicillin treatment when exposed during the lag phase for the ancestral (KLY), tolerant (KLY *metG*^{T}), tolerant+resistant (KLY *metG*^{T} *ampC*^{R}), and restored *metG wt* allele (KLY *metG*^{wt} *ampC*^{R}) strains. (**B**) Quantification of the growth delay as the fraction of colonies that exited the lag phase after more than 4.5 hours of ampicillin treatment for the strains shown in (A). The extended lag time of the tolerant mutants results in enhanced survival to treatment. The restored *wt* allele of *metG* restores the short *wt* lag but keeps the enhanced survival due to *ampC*. (**C**) Phase-contrast images of time-lapse microscopy of single-cell lag time in ancestral, tolerant, tolerant+resistant, and resistant-only strains. The yellow arrows mark single bacteria with extended lag times characteristic of the tolerant strains. (**D** and **E**) Restoring the *wt* tolerance-gene allele in the double mutants (tolerant+resistant) clones of strains MGY, KLY, and EPEC restores normal growth without decreasing the MIC. (D) ScanLag analysis of the appearance of colonies on plates presented as the fraction of colonies that were still undetected at designated times. As colonies start appearing, the fraction of undetected colonies decreases. Green indicates restoration of the tolerance mutation to the *wt* allele in the double mutants. (E) Disk-diffusion assays for the strains shown in (D) (disk diameter, 6 mm). Restoration of the *wt* alleles for the tolerance genes (*prsA* and *metG*) in the tolerant+resistant double mutants abolishes the growth delay without changing the MIC.

(Fig. 2K). To determine whether the resistance mutations appeared on the ancestral background or on the background of a tolerant strain, we isolated the first resistant clone that established in each population. Mutations above a frequency of 80% in the whole-genome sequencing of the batch cultures are listed in Table 1. We found that in addition to the resistance mutations in *ampC*, each clone bears an additional mutation in genes that had been previously mapped to the "tolerome" (*7*, *22*–*24*). Other mutations with putative association with the tolerome are listed in table S2.

Further analysis of previous cycles of antibiotic exposure for the batch cultures in which the tolerant+resistant mutants evolved revealed that the same tolerance mutations had been present prior to the appearance of the *ampC* resistance mutations. Thus, resistance appeared as a second mutational event in addition to the tolerance mutation (Fig. 2, J and L). We note that in a tolerant clone (KLY *metG*^{T}), only a subpopulation became tolerant, a phenomenon called persistence (fig. S2) (*4*, *5*, *22*). The survival advantage of the persister subpopulation was enough to enable the subsequent establishment of resistance.

Analysis of the phenotypic effect of the tolerance and persistence mutations in clones that do not bear *ampC* resistance mutations revealed that they increased survival by extending the lag time of the culture (Fig. 3, A to C). Restoring the wild-type (*wt*) alleles of the tolerance mutations in the evolved tolerant+resistant clones abolished the extended lag period (Fig. 3D and fig. S3) without affecting the MIC (Fig. 3, D to E). These results enabled us to reconstruct the evolutionary trajectory that led to the establishment of these mutations in the population (Fig. 4A). The trajectory leading to increased survival first went through mutation endowing tolerance, which improved the survival of the ancestral strain by more than an order of magnitude (Fig. 4A, blue arrow). Survival was amplified by a subsequent resistance mutation in *ampC* that increased the MIC by a factor of 10 (Fig. 4A, red arrow, and fig. S4). By initiating the evolutionary protocol in parallel for tolerant strains as well as *wt* strains, we observed that the resistance mutations established faster on the tolerant background (Fig. 4B).

Why did resistance mutations in our experiments always occur on the background of tolerance or persistence mutations? In general, the probability for establishment of a mutation in the cyclic antibiotic-exposure protocol, P_{est}, depends on two factors (*25*, *26*): (i) the probability of the occurrence of the mutation and (ii) the probability that this mutation is not lost during the antibiotic-exposure phase (*15*).

$$P_{est} = \sum_{t=0}^{T-1} \mu N_{min} 2^{t} \left[1 - (1 - S_R)^{2^{T-t}}\right] \quad (1)$$

Here, $N_{min}2^{t}$ is the population size at generation t, T is the total number of generations, μ is

mutation rate, and S_R is the survival rate of a resistant mutant. One reason for the rapid establishment of the tolerance mutations is that they occur more frequently than the resistance mutations (larger μ), owing to a larger target size (*22*, *23*). Indeed, mutations leading to high ampicillin resistance are mostly restricted to the *ampC* promoter, whereas tolerance mutations are observed in several genes and in different locations in those genes. Furthermore, we observed that the survival advantage conferred by resistance mutations under the high-level ampicillin treatment was comparable to that of the tolerance mutations (Fig. 4A). Despite the increase in MIC conferred by the *ampC* resistance mutations (*16*), and their classification as "resistant" according to clinical standards (*16*), their MIC is still below the treatment exposure and results in partial resistance. Hence, tolerance mutations dominate the population after a few cycles (*15*).

As predicted in (*10*), we observed that tolerance supports the continued survival of the bacterial population. The reservoir of tolerant bacteria extends the window of opportunity for rarer mutations to occur (*11*). In addition, we found that tolerance specifically enhances the establishment of resistance mutations by the epistasis between tolerance and partial resistance to the treatment. Using the experimental values (table S3 and figs. S5 and S6), we calculated the probability for the establishment of a resistance mutation if it occurs in a tolerant background as $P_{est}^{R/tol}$ or in the *wt* background as $P_{est}^{R/wt}$. Our analysis shows that without tolerance, the *ampC* mutations would most often be lost during antibiotic treatment and that more than 100 cycles of antibiotic exposure would be needed for these partial-resistance mutations to establish in the population (*15*) (Fig. 4C). In other words, the tolerant background enables the establishment of a resistance mutation by lowering its probability of getting lost during the antibiotic treatment (fig. S7). Stochastic simulations of the full experimental protocol confirm this result (fig. S8). This analysis suggests that partial-resistance mutations constantly arise in a population but are rapidly lost despite an increase in MIC and that their chances of spreading in the population are significantly enhanced on a tolerant background (fig. S9). We found that the effect of tolerance is particularly important at concentrations of antibiotics high enough to prevent occurrence of full resistance, i.e., at concentrations that have been defined as the mutant-prevention concentrations (MPCs) (*27*). At lower concentrations, fully resistant, single-step mutants may appear and dominate the population, even in the absence of tolerance (Fig. 4D).

Our results indicate that tolerance plays a crucial role in the evolution of resistance in a bacterial population under cyclic exposures to high ampicillin concentrations. The key factors are that tolerance arises rapidly as a result of the large number of possible mutations that lead to it and that the combined effect of resistance and tolerance promotes the establishment of a partial-resistance mutation on a tolerant background. The initial mutations in the resistance gene that confer partial resistance, in this case in *ampC*, can lead to full resistance by additional mutations. Many studies have shown that several mutations are typically needed to confer high resistance (*28*, *29*) and that partial resistance will lead rapidly to full resistance in vitro (*30*, *31*) and in vivo (*32*). However, high resistance that requires several mutations is typically achieved by a gradual increase in antibiotic concentrations (*29*, *33*, *34*). Here we show that even at concentrations above the MPC, the evolution of tolerance can lead to the fixation of partial-resistance mutations that substantially elevate the probability of full resistance. Indeed, in one of our lines, full resistance was attained by the sequential accumulation of two mutational events in the promoter region of *ampC* (fig. S10). Coevolution of tolerance and resistance factors, as suggested for the

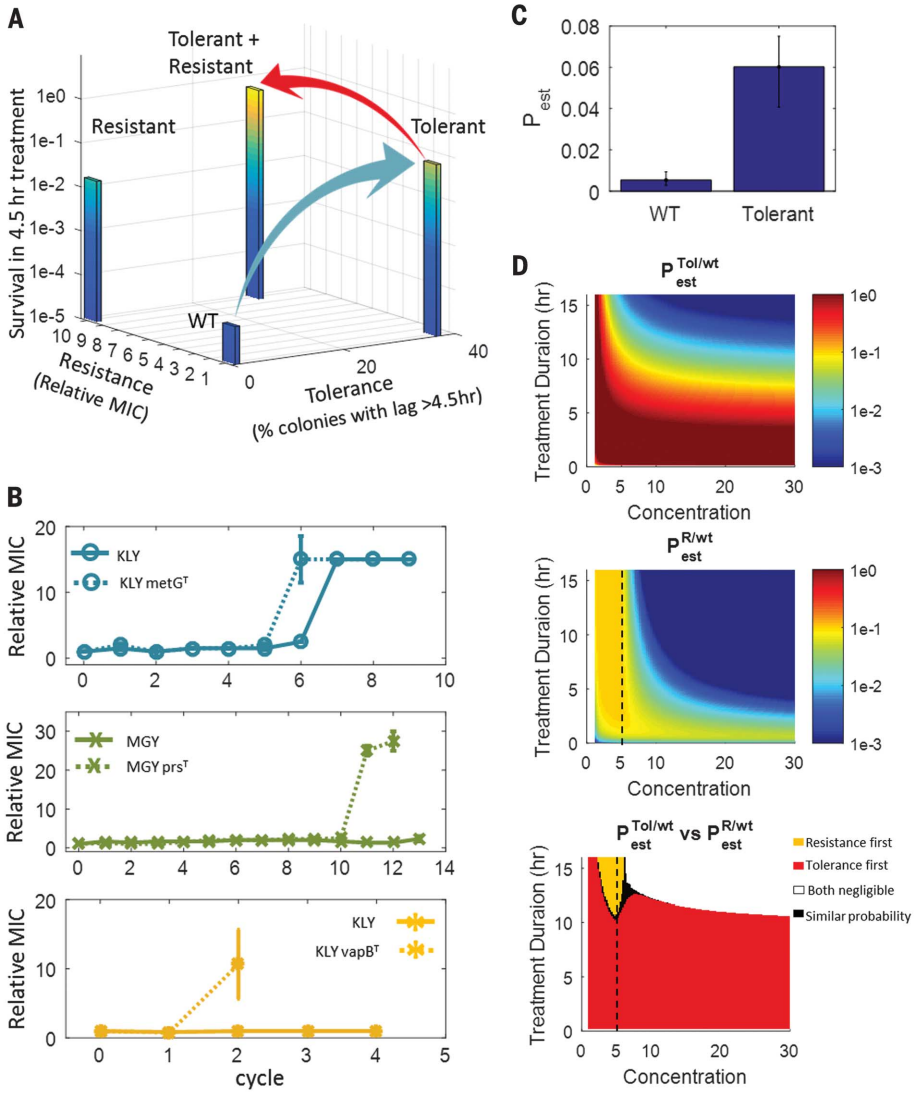

Fig. 4. Understanding the evolutionary trajectory for the establishment of tolerance before resistance. (**A**) Experimental evolutionary trajectory first passed through the high-tolerance peak (blue arrow) and then moved to resistance (red arrow). Height represents the survival under 4.5 hours of ampicillin treatment at 50 μg/ml. Resistance is shown relative to ancestral MIC; tolerance is shown as the percentage of colonies with a lag longer than 4.5 hours. (**B**) Evolution experiments with cultures started from a tolerant strain (dashed lines) evolved resistance earlier than the *wt* strain (solid lines). Top panel, strains KLY and KLY *metG*^{T}; middle panel, strains MGY and MGY *prs*^{T}; bottom panel, strains KLY and KLY *vapB*^{T} [without residual ampicillin (*14*)]. (**C**) Enhancement of the probability to establish resistance in tolerant background. Simulation results for P_{est}^R for a wild-type (WT) or a tolerant background. (**D**) Probability of establishment of typical tolerance mutations (top panel) or typical resistance mutations (middle panel) for different treatment durations and concentrations of antibiotic. The dashed line in the middle panel marks the MPC. The bottom panel shows the regions in which each mutation is more likely to establish.

fumerate reductase operon (*FRD*) and *ampC* (*35*), may indicate how often tolerance has led to resistance.

Notably, two of the *ampC*-promoter mutations that we mapped in our evolved strains were found in clinical isolates (*18*). However, the emergence of high resistance preceded by the acquisition of tolerance would typically go undetected, as clinical isolates are routinely tested for MIC but not for tolerance. The detection of tolerance in clinical settings (*36*) may be crucial for determining the regimen and type of antibiotics required to prevent the subsequent emergence of resistance. Analyses similar to ours for other antibiotics and pathogens should shed light on the generality of the effect.

We have reported here enhancement by tolerance of the subsequent evolution of resistance in bacterial populations under cyclic antibiotic treatments. The epistasis we describe between tolerance and resistance is quite generic and should not depend on the mode of appearance of the mutation, whether evolved de novo or acquired horizontally, nor should it be specific to ampicillin, as tolerance increases survival to several classes of antibiotics. Finally, the importance of tolerance for the subsequent evolution of resistance indicates that new drugs (*37*, *38*), or drug combinations, that decrease tolerance may impede the evolution of resistance.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/355/6327/826/suppl/DC1
Materials and Methods
Figs. S1 to S10
Tables S1 to S5
References (*39*–*53*)

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Editor's Summary

Resistance on a background of tolerance

Bacteria survive antibiotic exposure either because they are quiescent when antibiotics are around in the highest concentrations (i.e., tolerance) or because they acquire active biochemical resistance mechanisms (i.e., resistance). Both tolerance and resistance involve the acquisition of mutations from the wild type. Levin-Reisman *et al.* used in vitro evolution experiments to show that populations of bacteria that become genetically resistant to the antibiotic ampicillin most quickly do so on a background of tolerance mutations (see the Perspective by Lewis and Shan). Because the probability of a tolerant organism surviving is higher, it has a greater chance of subsequently acquiring resistance mutations. Tolerance is often overlooked in the clinic but should in future be screened for and targeted more precisely to reduce the rates of acquired resistance.

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