Drug interactions modulate the potential for evolution of resistance

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Antimicrobial treatments increasingly rely on multidrug combinations, in part because of the emergence and spread of antibiotic resistance. The continued effectiveness of combination treatments depends crucially on the frequency with which multidrug resistance arises. Yet, it is unknown how this propensity for resistance depends on cross-resistance and on epistatic interactions—ranging from synergy to antagonism—between the drugs. Here, we analyzed how interactions between pairs of drugs affect the spontaneous emergence of resistance in the medically important pathogen Staphylococcus aureus. Resistance is selected for within a window of drug concentrations high enough to inhibit wild-type growth but low enough for some resistant mutants to grow. Introducing an experimental method for high-throughput colony imaging, we counted resistant colonies arising across a two-dimensional matrix of drug concentrations for each of three drug pairs. Our data show that these different drug combinations have significantly different impacts on the size of the window of drug concentrations where resistance is selected for. We framed these results in a mathematical model in which the frequencies of resistance to single drugs, cross-resistance, and epistasis combine to determine the propensity for multidrug resistance. The theory suggests that drug pairs which interact synergistically, preferred for their immediate efficacy, may in fact favor the future evolution of resistance. This framework reveals the central role of drug epistasis in the evolution of resistance and points to new strategies for combating the emergence of drug-resistant bacteria.

antibiotic resistance | drug combinations | epistasis | Staphylococcus aureus | mutant selection window

The widespread use of antibiotics pits clinical need against the reality of evolution (1–3). The clinical goal is to kill as many pathogenic bacteria as possible, or inhibit their growth to allow the immune system to gain the upper hand; but a drug that kills or inhibits the growth of susceptible pathogens confers a dramatic selective advantage to resistant lineages, eventually making the drug ineffective. Although major advances have been made in describing the impact of single drugs on bacterial resistance (3), it is still unclear how drugs in combination affect the evolution of resistance. Combinations of drugs may inhibit bacterial growth in complex ways, deviating from the neutral situation expected when the drugs do not interact (4–6). Compared with this null situation, drug combinations that interact to increase each other’s effects are termed “synergistic”; drugs whose combined effect is smaller than expected are termed “antagonistic” (4–7, 39) (Fig. 1D). We have previously shown that these epistatic drug interactions profoundly affect the selective advantage of a single horizontally transferred resistance allele (8). Here, we focus on the more complex scenario of the evolution of multidrug resistance by spontaneously occurring mutations.

In many infectious and noninfectious diseases, including HIV (9), tuberculosis (10), malaria (11, 12), and cancer (13), high rates of mutation confer resistance to individual drugs. Combination therapies are therefore used to increase the killing of single-drug-resistant strains or mutants. Unfortunately, multidrug resistance still arises: multiple mutations conferring resistance may accumulate, or a single mutation may confer resistance to several drugs (cross-resistance). We address here the frequency of such spontaneous resistant mutations in Staphylococcus aureus, one of the most worrisome multidrug-resistant bacteria (14). Although a major mode of resistance in S. aureus is horizontal gene transfer, resistance acquired vertically by spontaneous mutations is another concern and combination therapies aimed at preventing their emergence are frequently used (15, 16). The approach we develop using S. aureus as a model system is general in scope and can be applied to pathogens such as Mycobacterium tuberculosis, where resistance acquired during treatment by spontaneous mutations is critical.

Antibiotics impose a strong selection pressure on bacterial populations (17, 18): susceptible cells do not grow, and resistant cells already present in the population are selectively enriched. A commonly used measure of the potential to evolve resistance by spontaneous mutations is the size of the mutant selection window (MSW)—the range of drug concentrations where resistance is selectively enriched (19, 20). The MSW ranges from the minimum inhibitory concentration (MIC) that inhibits wild-type growth, to the mutant prevention concentration (MPC) where even very rare mutants are unlikely to grow (Fig. 1A). The frequency of resistance and the MSW of several clinically important individual drugs have been well characterized (21–26), and evidence suggests that the MSW of drug combinations can be smaller than the MSW of any of their individual drug constituents (27). Yet, a general relationship between the frequencies of cells resistant to combinations of drugs and the frequencies of resistance to each drug alone has not been established, and the effect of drug–drug interactions (epistasis and cross-resistance) on this relationship is not known.

We use both experimental and theoretical tools to explore how interactions between antibiotics impact the landscape on which selection can act. We develop a high-throughput system to measure frequencies of resistant S. aureus mutants over a matrix of concentrations of pairwise drug combinations, and apply this tool to three different types of drug pairs. Motivated by the diversity of results observed, we develop a quantitative theoretical model that makes clear the central role of drug epistasis and the impact of cross-resistance on the potential for evolution of resistance in multidrug environments. This model yields direct predictions for the impact of drug synergy on the emergence of resistance.


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Fig. 1. Schematic illustration of the MIC and MPC, and their extensions to multidrug environments. (A) Selection for resistance occurs primarily within the mutant selection window (MSW)—drug concentrations ranging from the MIC (dashed blue) inhibiting wild-type growth to the MPC (dashed red) above which the frequency of resistance ($F_{R}$) drops to nondetectable values. (B) In drug combinations, these notions extend to the MIC line (blue) and the MPC line (red). Resistance frequency [$F_{XY}(C_X, C_Y)$, gray surface] is a function of the two-drug dosage and depends on the frequencies of resistance to the individual drugs, cross-resistance, and epistatic interactions. "Effective drugs," obtained by combining $X$ and $Y$ at fixed proportions, are geometrically represented by lines extending from the origin (thick black line at angle $\theta$), from which their MSW is defined (double-headed arrow). (C) The MSW of these effective drugs is plotted against the ratio of the drug combination (represented by $\theta$); the smallest of these windows characterizes the drug combination's potential to limit the emergence of resistance. (D) The shape of the MIC line of two drugs defines their epistatic interactions: a linear line ($F_{XY} = 1$) but not in the synergistic case ($F_{XY} < 1$). In both cases, frequencies of resistance to the individual drugs alone are the same ($F_X = 1$, $F_Y = 1$), illustrating that $F_{XY}$ is not in general equal to $F_X F_Y$ but rather depends critically on epistatic interactions.

**Results**

**Extending the MPC to Antibiotic Combinations Necessitates Consideration of Drug Epistasis.** Resistant mutants may appear spontaneously because of replication errors at frequencies typically lower than $1 \times 10^9$ cells. The frequency of those mutants and the drug window within which they survive characterize the potential of the population to evolve resistance and is represented by the curve $F_X(C_X)$—the frequency of mutants that resist concentration $C_X$ of drug X (Fig. 1A). This curve typically presents plateaus with sharp drops, indicating the existence of subpopulations of resistant cells. The drug concentration at which the frequency drops to nondetectable levels is the MPC, defined here as $F_X < 10^{-9}$ (see Materials and Methods). The mutant selection window (MSW) of a drug extends from the MIC to the MPC. To allow comparison between different antibiotics, we normalize drug concentrations to their respective MICs (note, though, that the absolute values of the MICs in $\mu$g/ml are also of direct clinical importance due to drug toxicity). The size of the MSW is then (MPC - MIC)/MIC (for instance, if the MIC is 1 $\mu$g/ml and MPC = 100 $\mu$g/ml, MSW = 100).

By analogy with resistance to a single drug, we introduce the surface $F_{XY}(C_X, C_Y)$, the frequency of cells that can grow in an environment containing a combination of the two drugs X and Y at the concentrations $C_X$ and $C_Y$ (Fig. 1B). As in the single-drug case, $F_{XY}$ is likely to exhibit plateaus: the first drop in frequency occurs as wild-type growth is inhibited, and defines the MIC line. The MPC line bounds the region where resistant mutants are unlikely to occur ($F_{XY} < 10^{-9}$). The drugs can be combined in different proportions to effectively produce "single" drugs, represented geometrically by linear lines extending from the origin in the drug concentrations plane (Fig. 1B, black line at angle $\theta$ corresponding to the drug ratio). These effective drugs have their own MSW, according to the geometric points of intersection with the MIC and MPC line. The smallest MSW obtained over all of the combinations of X and Y characterizes the potential of the drug pair X–Y for limiting the evolution of resistance (Fig. 1C, arrow).

The simplest approach in the absence of information on how the drugs interact would assume the frequency of resistance to the drug combination is the product of the frequencies of resistance to each of the individual drugs, $F_{XY}(C_X, C_Y) = F_X(C_X) F_Y(C_Y)$ (12, 28). It is known that this expectation breaks down in the presence of mutations that confer resistance to X and Y simultaneously (cross-resistance), but we highlight here that $F_{XY}$ may also significantly depend on epistatic interactions between drugs. The effect of drug combinations on growth inhibition can deviate from the null situation expected by Loewe additivity (Fig. 1D Left) (5), defining synergistic or antagonistic epistasis [Fig. 1D and supporting information (SI) Fig. S1]. Consider, for example, an environment containing 0.75 MIC of drug X and of 0.75 MIC of Y (Fig. 1D, X). In the antagonistic case, the wild type is able to grow and therefore the frequency of resistance is effectively $1 \cdot F_X(0.75, 0.75) = 1$. In contrast, if the drugs interact synergistically, the wild type cannot grow and therefore $F_{XY}(0.75, 0.75) < 1$. The frequency of resistance to each drug alone is the same in both cases [$F_X(0.75) = F_Y(0.75) = 1$], but epistatic interactions dramatically affect resistance to the combination. This simple example illustrates that even in the absence of cross-resistance, the frequency of cells resistant to the combination is not trivially the product of the frequencies of cells resistant to each of the single drugs.

**Experiments Show That Multidrug Resistance Variates Dramatically Between Drug Combinations.** To explore how resistance to combinations of drugs depends on resistance to each drug alone and interactions between the drugs (epistasis and cross-resistance), we designed an experimental setup to systematically measure the frequencies of resistance to more than a hundred different combinations of a given pair of drugs (see Materials and Methods and Fig. 2). We sampled the resistance surfaces of three drug pairs: fusidic acid–erythromycin (FUS-ERY), ciprofloxacin–ampicillin (CPR-AMP), and fusidic acid–amikacin (FUS-AMI). The drugs were chosen for their clinical relevance, diversity of mechanisms of action, and potential to evolve spontaneous resistance. The assayed drug pairs cover the range of epistatic interactions: the MIC lines, measured independently in liquid media on clonal wild-type populations, show synergy between FUS and ERY (epistasis parameter $\varepsilon = -0.1$; see Materials and Methods), antagonism between FUS and AMI ($\varepsilon = 0.3$), and no epistasis between CPR and AMP ($\varepsilon = 0.06$) (Fig 2C–E Insets). We measured resistance to each drug pair at $11 \times 11$ combined concentrations. Agar plates containing the relevant concentrations of drugs were prepared, inoculated with S. aureus at a range of inoculum sizes (from $10^{5}$ to $10^9$ cells per plate), and placed on scanners taking time-lapse high-resolution pictures every hour for 5 days (Fig. 2A). The images obtained were analyzed by an automated image-processing platform designed to count visible colonies on each plate (Fig. 2B) using a hundred different concentrations of a given pair of drugs (see Materials and Methods and Fig. 2). We sampled the resistance surfaces of three drug pairs: fusidic acid–erythromycin (FUS-ERY), ciprofloxacin–ampicillin (CPR-AMP), and fusidic acid–amikacin (FUS-AMI). The drugs were chosen for their clinical relevance, diversity of mechanisms of action, and potential to evolve spontaneous resistance. The assayed drug pairs cover the range of epistatic interactions: the MIC lines, measured independently in liquid media on clonal wild-type populations, show synergy between FUS and ERY (epistasis parameter $\varepsilon = -0.1$; see Materials and Methods), antagonism between FUS and AMI ($\varepsilon = 0.3$), and no epistasis between CPR and AMP ($\varepsilon = 0.06$) (Fig 2C–E Insets). We measured resistance to each drug pair at $11 \times 11$ combined concentrations. Agar plates containing the relevant concentrations of drugs were prepared, inoculated with S. aureus at a range of inoculum sizes (from $10^{5}$ to $10^9$ cells per plate), and placed on scanners taking time-lapse high-resolution pictures every hour for 5 days (Fig. 2A). The images obtained were analyzed by an automated image-processing platform designed to count visible colonies on each plate (Fig. 2B) using a hundred different concentrations of a given pair of drugs.

Our results show qualitatively different patterns of resistance to the three pairwise drug combinations. The frequency of cells resistant to combinations of FUS and AMI is comparable with that of cells resistant to AMI alone (Fig. 2E). By contrast, the frequency of cells resistant to combinations of CPR and AMP is significantly smaller than the frequencies of resistance to the same concentration of CPR alone, or AMP alone (Fig. 2D). The same is observed for FUS-ERY (Fig. 2C). Furthermore, mixing together FUS and ERY
leads to effective drugs whose MSWs can be up to one order of magnitude smaller than the MSW of FUS or ERY alone (Fig. 4 Insets). Thus, combinations of FUS and ERY can be found that significantly narrow the drug regime that selects for resistance. This effect is not observed for FUS-AMI or CPR-AMP. We find that the multiplicative model (i.e., \(F_{XY} = F_X F_Y\)) is unable to capture such diverse behaviors (Fig. S2), underscoring the need for a predictive model of resistance frequencies inclusive of epistasis and cross-resistance.

**Resistance Frequencies to Individual Drugs, Cross-Resistance, and Epistasis Determine the Frequency of Resistance to Multidrug Combinations.** The frequency of resistance \(F_X\) to a single antibiotic \(X\) derives from the makeup of the bacterial population at the time the antibiotic is introduced. We define \(p_X(x)\), the probabilistic density of cells whose MIC of drug \(X\) is exactly \(x\) (Fig. 3A); it derives from the frequency of resistance as \(p_X(x) = -dF_X(x)/dx\). Because the frequency of resistance to \(C_X\) of \(X\) is, by definition, the frequency of cells whose MIC is greater than \(C_X\) (Fig. 3B), we have

\[
F_X(C_X) = \int_{x>C_X} p_X(x)dx = \int_{x>0} \eta_X\left(\frac{C_X}{x}\right)p_X(x)dx, \tag{1}
\]

where \(\eta_X(z) = 1\) if \(z < 1\) and is 0 otherwise. In this equation, \(\eta_X\) characterizes the growth of one individual cell: for simplicity we consider only step functions, but \(\eta_X\) could also be smooth and account for natural variations in the response of isogenic cells.

In the case of two drugs \(X\) and \(Y\), the growth region of a bacterial cell is a function of the two-drug concentration, and is delimited by its MIC line \([\eta(C_X, C_Y) = 1\) if the cell can grow in \((C_X, C_Y)\), zero otherwise]. Earlier work (8) has shown that growth regions of wild type \((\eta_{XY})\) and resistant mutants \((\eta)\) tend to have a common shape, which characterizes the epistatic interactions between the drugs. We therefore approximate the region of growth of mutant cells in the population by a linear scaling of the wild type’s growth region (Fig. 3C inset; blue, wild type; red, a mutant). This approximation cannot be tested directly within our experimental data, but the fit of the resulting models to measured resistance frequencies supports its validity. The growth region of a bacterial cell whose MIC of drug \(X\) is \(x\) and whose MIC of \(Y\) is \(y\) is approximated by \(\eta(C_X, C_Y) = \eta_{XY}(C_X/C_Y, C_Y)\).

Next, by analogy to the single drug case, \(p_{XY}(x, y)\) is the density of cells in the population whose MIC of drug \(X\) alone is \(x\) and whose MIC of drug \(Y\) alone is \(y\) (Fig. 3C)—note that it does not contain information on the ability of cells to grow in combinations of these drugs, which is carried by \(\eta_{XY}\). With these definitions, the frequency of resistance to a given combination \((C_X, C_Y)\) of drugs \(X\) and \(Y\) is given by (Fig. 3D)

\[
F_{XY}(C_X, C_Y) = \int_{x,y>0} \eta_{XY}\left(\frac{C_X}{x}, \frac{C_Y}{y}\right)p_{XY}(x,y)dxdy. \tag{2}
\]

Unlike the MIC line \((\eta_{XY})\), which is experimentally measured in liquid media, the density of population \(p_{XY}\) cannot easily be measured. We have built a simple model where \(p_{XY}\) depends only on \(p_X\) and \(p_Y\) (measured as the derivatives of \(F_X\) and \(F_Y\)), and on cross-resistance between the drugs. When cross-resistance is absent, mutations conferring resistance to \(X\) or \(Y\) are independent: the density of population is \(p_{XY}^{\text{inde}}(x, y) = p_X(x)p_Y(y)\). Conversely, the mechanisms of resistance could be extremely correlated, as would

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**Fig. 2.** Measurement of resistance frequencies to pairwise drug combinations. (A) Frequencies of resistance of *S. aureus* to each of 11 \(\times\) 11 concentrations of a drug pair measured by counting colonies arising on agar plates (see Materials and Methods). Example colony images are shown for the pair of antibiotics ERY-FUS at the final time point (5-day incubation) for the most concentrated cell inoculum \((10^6\) cells per well). (B) Individual colonies detected by a custom image-processing platform—here, in false colors, on the plate labeled by an asterisk in A. (C-E) Measured frequencies of resistance (filled circles, or open circles if no resistant colonies appeared) plotted against the two-drug concentrations for FUS-ERY, CPR-AMP, and FUS-AMI. A standard polynomial interpolation surface is shown together with the points (gray surface). The MIC lines, measured independently in liquid media, define the nature of the epistatic interactions between the drugs (blue line, Insets). The MPC lines (red) represent the regions of drug concentrations above which no mutants appear.
be the case if the “two” drugs were in fact the same: this corresponds to a density $p_{XY}^{\text{Correl}}$ that depends only on $p_X$ and $p_Y$ (mathematically defined as the density that maximizes the correlation between resistance to $X$ and $Y$ under the constraints $p_X(y) = f_{X>0} p_X(x, y)dy$ and $p_Y(x) = f_{Y>0} p_Y(x, y)dy$; see SI Text). In general, some mutations confer resistance to only one of the drugs, and others confer resistance to both drugs at once: we model $p_{XY}$ as a linear combination between the two extreme cases, $p_{XY} = p_X^{\text{Indep}} + (1 - \xi)p_Y^{\text{Indep}}$. The parameter $\xi$ reflects cross-resistance, absent when $\xi = 0$ and maximal when $\xi = 1$.

This constitutes a model that quantifies exactly how drug epistasis, cross-resistance between drugs, and single-drug resistance determine the frequency of cells resistant to any combination of the drugs $X$ and $Y$. The densities $p_X$ and $p_Y$ are estimated by the derivatives of the measured frequencies of resistance to the individual drugs $F_X, F_Y$ (Eq. 1). They enable the construction of $p_{XY}^{\text{Indep}}$ and $p_{XY}^{\text{Correl}}$, which, tuned by the cross-resistance $\xi$, produce $p_{XY}$. The MIC line of the wild-type is a direct measurement of $p_{XY}$. Finally, $p_{XY}$ and $p_{XY}^{\text{Indep}}$ predict the frequency of resistance $F_{XY}$ to any combined concentration of the drugs $X$ and $Y$ (Eq. 2).

The Theoretical Model Captures the Experimental Results. We next compared the predictions of this model with our experimental results. Frequencies of resistance to the single drugs alone ($F_X, F_Y$) were measured together with the whole surface. The MIC line of each drug pair ($p_{XY}$) was directly measured in liquid media. Cross-resistance ($\xi$) is the only free parameter and was estimated for each drug pair by a least-squares fitting of predicted to experimental frequencies of resistance. The drug pair FUS-AMI shows strong cross-resistance ($\xi = 0.3$), whereas the drug pairs CPR-AMP and FUS-ERY show almost no cross-resistance ($\xi < 10^{-2}$). Our experimental results on resistance to combinations of drugs are well captured by the model (Fig. 4). This framework successfully accounts for very different behaviors in terms of epistatic interactions and cross-resistance.

**Theory Predicts That Synergistic Epistasis Favors Resistance.** We use this framework to weigh the impact of drug interactions on the evolution of resistance. Cross-resistance increases the MSW of drug combinations; with increased cross-resistance, mutants resistant to both drugs individually occur at frequencies high enough to appear in the population and contribute to the MSW of drug combinations (Fig. S3). The impact of epistasis, however, is less obvious. We present here the simplest model exhibiting the mechanisms by which epistasis affects the potential to evolve resistance to a combinations of drugs. For simplicity, we assume no cross-resistance; the combined impact of cross-resistance and epistasis is presented in Fig. S3.

We consider the case where only two mutations exist, one conferring resistance to drug $X$, the other to drug $Y$: small mutation frequencies preclude the appearance of double mutants, and the MSWs for drug $X$ and for drug $Y$ have the same size $M$. Only epistasis between the drugs $X$ and $Y$ varies, and is quantified by the real number $\epsilon$, which parameterizes the MIC line as $x^{10^0} + y^{10^0}$. Absence of epistatic interactions is represented by $\epsilon = 0$, while antagonism and synergy correspond to $\epsilon > 0$ and $\epsilon < 0$, respectively. Fig. 5A and B shows the frequencies of resistance to combinations of $X$ and $Y$ for synergistic or antagonistic epistasis. In both cases, $1:1$ ($\theta = 45^\circ$) is the ratio of $X$ and $Y$ for which the effective drug has the smallest MSW, defining $\text{MSW}_{XY}$. Analytical expressions for the corresponding $M_{XY}$ (dashed line) and $\text{MPC}_{XY}$ (solid line) of this “best” effective drug are geometrically derived and a closed form of the smallest MSW, $\text{MSW}_{XY} = (\text{MPC}_{XY} - \text{MIC}_{XY})/\text{MIC}_{XY}$, is obtained:

$$\text{MSW}_{XY} = \left(\frac{2}{1 + (M + 1)^{10^0}}\right)^{10^{-\epsilon}} - 1. \tag{3}$$
Discussion

Current clinical practice emphasizes the use of multidrug treatments primarily to increase the spectrum of activity (29–33), to increase efficacy (34), and, in some pathogens, to decrease the likelihood of the emergence of resistance (29). Clinicians generally prefer synergistic drug pairs when prescribing combination treatments to broaden the spectrum but usually do not consider the effect of drug epistasis on resistance (29). Our results imply that synergistic drug pairs may favor the evolution of resistance. In contrast, largely overlooked antagonistic drug combinations may suppress the emergence of resistance. This study, designed to explore the mostly uncharted territory of resistance to combinations of drugs, comprises drug pairs with different epistasis, different degrees of cross-resistance, and different frequencies of resistance to single drugs. Our theoretical prediction above can be validated experimentally by comparing pairs of drugs with different epistasis but with similar degree of cross-resistance, and similar single-drug MSW. Future screens focusing on finding such pairs could be designed in light of this model.

We focused on the frequency of spontaneous mutations conferring resistance in multidrug environments and emphasized the drug window selective for resistance. We note that this measure of propensity for evolution of resistance does not include other factors that may affect the emergence and spread of drug-resistant pathogens, such as variation in the drug concentration, natural variability in the response of isogenic cells, or the pharmacodynamics of the particular antibiotics (35). The dynamics of antibiotic treatment could lead to rounds of selection and adaptation: resistance may be acquired during the course of treatment. Temporal variations in drug dosage and nongenetic phenotypic tolerance may substantially
increase the likelihood of emergence and rate of evolution of resistance. For instance, persisters cells may remain dormant long enough for the antibiotic to decay, enabling adaptation and subsequent selection (36, 40). Furthermore, in natural and clinical settings, resistance acquired through horizontal transfer may play a major role in the evolution of resistance. Examining the impact of drug–drug interactions on these factors central to the development of drug resistance is a promising avenue for future research.

In conclusion, we present an experimental–theoretical framework that offers a quantitative, unified understanding of how resistance evolves across individual drugs, cross-resistance, and epistatic interactions affect the propensity for resistance in multidrug combinations. Importantly, our results suggest that antagonistic combinations may narrow the range of drug concentrations where resistance is selected for. In contrast, synergistic drug combinations, typically preferred in clinical settings, may in fact favor the evolution of resistance even though they increase killing efficiency. Our results indicate that drug interactions could be central to a tradeoff between immediate efficacy and the future prevention of resistance.

Materials and Methods

Bacteria and Antibiotics. We used a streptomycin-resistant S. aureus strain Newman NCTC 8178 (37). Growth media was liquid or agar Luria broth (supplemented, as indicated, with one or two of five different antibiotics (see Table S1)). A single colony (starting from a single cell) was inoculated in LB liquid and grown overnight; frozen aliquots of this culture were kept at −80°C. All experiments were initiated from a freshly thawed aliquot from this single batch.

MIC Line. The MIC line of a drug pair was measured by a standard overnight growth assay in liquid media, inoculating −10^5 wild-type cells in each of 6 wells (Costar plate; 150 μL per well) forming a 12 × 8 gradient of drug concentration (dilutions of 2/3 to 9/10). The MIC line was defined as the line separating regions of growth and no growth (practically, the contour line of optical density 0.1). The shape of the MIC line was used to define the function γ for each specific drug pair (Fig. 2 C–E, Insets). The sign of the epistatic interactions was determined by fitting γ with the function xα+ yβ + γ = 1; synergy, additivity and antagonism correspond respectively to negative, null, and positive values of γ.

Frequency of Resistance. We measured the frequency of resistance with a resolution spanning nine orders of magnitude, across an 11 × 11 grid of drug concentrations. For each two-drug concentration we used one six-well plate (Becton Dickinson Multiwell), and poured 7 mL of agar supplemented with the selected drug concentration of drugs (e.g., Fig. S5). After agar solidification, each of the six wells was inoculated with a different number of bacterial cells (approximately 10^5.5, 10^6, 10^6.5, 10^7, and 10^8). The plates were then incubated on scanners (see below) in a controlled environmental room at 30°C and 70% relative humidity for 5 days, a duration optimized for the detection of resistant colonies by our custom software (Fig. 56; effect of the incubation time).

We defined the MIC as the smallest concentration of antibiotics that, as the total number of mutants in all wells of the relevant drug concentrations with countable colonies (typically, < 500 cfu per well) divided by the total number of colonies plated on these specific wells. The MPC (respectively MLC line) bounds the drug concentrations where at least one growing colony was observed. This corresponds here to frequencies of resistance greater than 10^−2, while the standard definition of the MPC is usually FX > 10^−9 (20, 38); our measurements of the MSW may differ from those obtained by the standard method. The use of frozen cell aliquots prepared from the same single culture eliminates much of the Luria–Delbrück fluctuations. Some additional fluctuations due to the last 100-fold amplification step are still present (Fig. 57).

Scanner and Imaging Platform. We built an array of 30 office scanners (Epson Perfection 3170/3490) controlled by one computer. Five plates were placed in each scanner. The scanners were programmed to take time-lapse pictures of the plates at 600 dpi every hour for 5 days. We built an image-analysis platform in MATLAB (MathWorks) to count the number of colonies arising in each plate. The platform detects single colonies larger than one-tenth of a millimeter and tracks their growth by using a custom contour-detection algorithm based on contract gradients (Fig. S2).

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7. Newman NCTC 8178 (37). Growth media was liquid or agar Luria broth (LBSupplemented, as indicated, with one or two of five different antibiotics (see Table S1)). A single colony (starting from a single cell) was inoculated in LB liquid and grown overnight; frozen aliquots of this culture were kept at −80°C. All experiments were initiated from a freshly thawed aliquot from this single batch. 
SI Text

A General Framework for the Impact of Multiple Interacting Stressors on a Mixed Population. In this article, we derived an expression for the frequency of resistance to combinations of two drugs (Eq. 2) in the case of spontaneous mutations. We showed that the frequency of resistance \( F_{XY} \) is completely characterized by two parameters: \( \eta_{XY} \), which describes how the two interacting drugs impact the growth of one individual cell, and \( p_{XY} \), which describes the structure of the population in terms of resistance to each drug alone. Here, we extend this result to describe in a general way the impact of multiple interacting stressors in a mixed population.

Let us consider \( n \) environmental stressors denoted by \( X_1, \ldots, X_n \), whose values are positive numbers, in analogy with drug concentrations. We aim to describe the effect of those stressors on a population \( \Omega \) in terms of one given phenotypic trait—in this article, growth and its inhibition. An individual from this population placed in a given environment either possesses the phenotypic trait or does not—for instance, a cell in a medium grows or does not grow. To each individual \( \omega \) in \( \Omega \), we associate the value \( \eta(\omega, x) \) of the phenotypic trait under the stress \( x = (x_1, \ldots, x_n) \); this value is one if \( \omega \) possesses this trait, and zero if it does not. In the general case, an individual’s response to stress can bear a stochastic component, as for the case of persister cells in isogenic populations. This can be directly accounted for in this framework by considering \( \eta(\omega, \cdot) \) as a probability density, rather than as a binary variable. With these definitions, the frequency of the trait in the population under stress is given by

\[
F(x_1, \ldots, x_n) = \int_{\omega \in \Omega} \eta(\omega, x_1, \ldots, x_n) d\omega. \quad [S1]
\]

Large groups of individuals in the population may possess the same response to stress. We therefore describe the population \( \Omega \) relative to the behavior of individuals under stress as follows. It is possible to find a measurable set \( I \) of ordered indexes and typical individuals \( \{\omega_i \in \Omega, i \in I\} \), such that for any individual \( \omega \in \Omega \), there is an index \( i \in I \) for which under any stress, \( \omega \) behaves like \( \omega_i \); \( \forall x = (x_1, \ldots, x_n) \in \mathbb{R}^n, \eta(\omega, x) = \eta(\omega_i, x) \). We define in this case \( \eta(.) = \eta(\omega_i, \cdot) \). We then associate to any index \( i \in I \) the proportion \( p(i) \) of individuals in \( \Omega \), which behave like \( \omega_i \). We naturally have \( \int_{\Omega} p(i) di = 1 \). The equation for the frequency of the trait then becomes

\[
F(x_1, \ldots, x_n) = \int_{i \in I} \eta(x_1, \ldots, x_n) p(i) di. \quad [S2]
\]

The quantity \( p \) describes the population \( \Omega \) in terms of its response to each stress alone, while the quantities \( \eta \) describe the way the stressors together interact and impact one individual’s phenotype, analogous to the epistasis between drugs. In other words, \( I \) tells how many different responses to stress exist in the population, \( p \) tells how frequent those responses are in the population, and \( \eta \) tells how the combined stresses impact one individual. These are the three relevant, sometimes measurable, quantities that are necessary and sufficient to describe the response of a population to multiple interacting stressors.

This framework can potentially describe resistance of isogenic populations of bacteria to multiple compounds, or the adaptation of birds in urban environments where stressors include light, noise, and chemical environment. In our article, we focused on describing how the rather complex parameters (\( \eta \) and \( p \)) could be obtained from measures of simpler quantities pertaining to single stressors. In different situations, other measures and approximations may be relevant.

A Simple Copula-Based Model of Cross-Resistance. If \( \Omega \) is a population of cells and the stressors are chemical compounds, then \( p \) is a density of probability. In the case of only one drug \( X, p_X(x) \) is the probability that a cell from \( \Omega \) can grow in an environment containing up to (but not more than) \( x \) of \( X \) alone. In the case of two drugs \( X \) and \( Y, p_{XY}(x, y) \) is the probability that a cell from \( \Omega \) can grow in an environment containing up to (but not more than) \( x \) of drug \( X \) alone and can also in an environment containing up to (but not more than) \( y \) of drug \( Y \) alone. Equivalently, \( p_{XY}(x, y) \) is the probability that a cell has an MIC of \( X \) equal to \( x \), and an MIC of \( Y \) equal to \( y \). A relationship exists between the two- and one-drug cases: \( p_X \) and \( p_Y \) are the marginal densities of \( p_{XY} \), i.e., \( p_X(x) = \int_{\mathbb{R}^+} p_{XY}(x, y) dy \) and \( p_Y(y) = \int_{\mathbb{R}^+} p_{XY}(x, y) dx \). In general, for two given densities \( p_X \) and \( p_Y \), there are many probability densities \( p_{XY} \) of which they could be the marginals. We describe here a biologically relevant model capable of associating a single \( p_{XY} \) from two given \( p_X \) and \( p_Y \), and the level of cross-resistance between drugs \( X \) and \( Y \). We suppose that \( p_X \) and \( p_Y \) are given, and we ask to what extent the added knowledge of the degree of cross-resistance is enough to define uniquely \( p_{XY} \).

Cross-resistance denotes a situation where a single mutation confers resistance to both drugs at once. Cross-resistance can be measured by the correlation between resistance to \( X \) and resistance to \( Y \)—how much more likely is a cell to be resistant to a drug, knowing it is resistant to the other? In our framework, this correlation is expressed by

\[
r_{XY} = \frac{\int_{x>0} \int_{y>0} (x-\bar{x})(y-\bar{y}) p_{XY}(x,y) dx dy}{\sqrt{\int_{x>0} (x-\bar{x})^2 p_X(x) dx \int_{y>0} (y-\bar{y})^2 p_Y(y) dy}}. \quad [S3]
\]

This quantity is null when there is no correlation between resistance to \( X \) and resistance to \( Y \); in this case, \( p_{XY} \) is uniquely defined as \( p_{XY}^{\text{Indep}}(x, y) = p_X(x)p_Y(y) \). At the other end of the spectrum, correlation between the two drugs could be maximal: it was shown (1) that there exists a unique density \( p_{XY}^{\text{Corr}} \) that maximizes the correlation \( r_{XY} \) and whose marginal densities are \( p_X \) and \( p_Y \). Then, we model the function \( p_{XY} \) as a linear combination between the case exhibiting no cross-resistance and the case exhibiting full cross-resistance: \( p_{XY} = \xi p_{XY}^{\text{Corr}} + (1 - \xi) p_{XY}^{\text{Indep}} \). The parameter \( \xi \) tuning this linear combination is proportional to \( r_{XY} \) and is called in our study the degree of cross-resistance.

Existence and uniqueness of a solution to the maximization of \( r_{XY} \) given \( p_X \) and \( p_Y \) interestingly comes from a branch of mathematical finance, copula theory, which frames this problem in terms of probability distributions (2). The link between the
density $p$ and the distribution $F$ of a stochastic variable $z$ is $F(z) = \int_{z=0}^{\infty} p(u)\,du$. Copulas, which can be defined with precision (3), are loosely understood as function $C$ such that, for any marginal densities $F_X$ and $F_Y$, the function $H(x, y) = C(F_X(x), F_Y(y))$ defines a joint probability distribution. Fréchet and Hoeffding separately showed (4, 5) that the function $M = \min(F_X(x), F_Y(y))$, termed the Fréchet–Hoeffding upper bound, is the unique distribution which maximizes the correlation between $X$ and $Y$. Our density $p_{\text{Corr}}$ is the density of that Fréchet–Hoeffding upper-bound. The family $p_{XY} = p_{\text{Corr}}(x, y) + (1 - \mathbb{I}_{\text{Indep}}(x, y))$ is the density of a subclass of the Fréchet family of copulas (1). Many other families of copulas have been extensively studied in the second half of the 20th century from a purely mathematical standpoint: exploring their relevance to problems in biology could be insightful.

The case where the densities are finite sums of Dirac functions is of particular biological relevance because it signifies that the population of cells is made of only a finite number of subpopulations that differ in their response to antibiotics. In this case, as in our article, the densities are matrices. We showed (J.-B.M. and R.K., unpublished work) that a simple characterization of the density with maximum correlation holds:

$$p_{XY}^{\text{Corr}} = \text{density of Frechet–Hoeffding upper bound if and only if in any two-by-two submatrix of } p_{XY}^{\text{Corr}}, \text{ at least one of the two non-diagonal elements is null:}$$

$$\forall (i < j, k < l). p_{XY}^{\text{Corr}}(i, l) p_{XY}^{\text{Corr}}(j, k) = 0.$$

From this characterization, we obtain a simple algorithm (6) that gives $p_{XY}^{\text{Corr}}$. The algorithm takes in any stochastic matrix whose marginal densities are $p_X$ and $p_Y$, and outputs the unique density with same marginals and maximizes the correlation—$p_{XY}^{\text{Corr}}$.

**Algorithm.** $M$ is the input stochastic matrix.

- For all row indexes $i < j$, column indexes $k < l$:
  - If $M(i, l), M(j, k) = 0$:
    - Continue, as Eq. S4 is verified.
  - Else:
    - let $m = \min(M(i, l), M(j, k))$
    - let $N(i, l) = M(i, l) - m$, $N(j, k) = M(j, k) - m$, $N(i, k) = M(i, k) + m$, $N(j, l) = M(j, l) + m$. The marginals of $N$ and $M$ are identical, but the correlation between $X$ and $Y$ is higher with density $N$ than $M$.
    - run the algorithm again with $N$ instead of $M$.
- return $M$

The Multiplicative Model Implicitly Assumes Buffering and No Cross-Resistance. It is a commonly used approximation that the frequency of resistance to a combination ($C_X, C_Y$) of antibiotics $X$ and $Y$ equals the product of the frequencies of resistance to each drug alone: $F_X(C_X, C_Y) = F_X(C_X)F_Y(C_Y)$. This approximation, termed here “multiplicative model,” is unable to account for our experimental data (Fig. S2). We show here that the multiplicative model always assumes buffering epistatic interactions and no cross-resistance, which may explain why it fails to capture the frequency of resistance to drug combinations with diverse features.

Let us first consider the region of wild-type growth, which we characterize by the function $\eta_{XY}$. The points $(C_X, C_Y)$ where the wild type grows are such that $\eta_{XY}(C_X, C_Y) = 1$, and zero otherwise. This function can be deduced from the frequency of resistance: $\eta_{XY}(C_X, C_Y) = 1$ if $F_X(C_X) = 1$ and zero elsewhere. Since $F_X(C_X, C_Y) = F_X(C_X)F_Y(C_Y)$, this amounts to $\eta_{XY}(C_X, C_Y) = 1$ if and only if $F_X(C_X) = 1$ and $F_Y(C_Y) = 1$, which is the case only when $C_X \leq 1$ and $C_Y \leq 1$. Therefore, $\eta_{XY}(C_X, C_Y) = \eta(C_X)\eta(C_Y)$, where $\eta(x)$ was previously defined as equal to one when $x \leq 1$ and zero elsewhere (Eq. 1). This region where $\eta_{XY}(C_X, C_Y) = 1$ is the unit square: its boundary, the MIC line of the combination $X$–$Y$, is characterized by an epistasis coefficient $\xi = \infty$. In other words, the multiplicative model assumes that the drugs completely buffer each other—as long as each drug is below its own MIC, the wild type can grow.

Then, we show that the multiplicative model does fit in our model. Indeed, working on the expression of the frequency of resistance for the multiplicative model $F_{XY}(C_X, C_Y) = F_X(C_X)F_Y(C_Y)$, we obtain from Eq. 1:

$$F_{XY}(C_X, C_Y) = \int_{x,y=0}^{\infty} \eta \left( \frac{C_X}{x}, \frac{C_Y}{y} \right) p_X(x)p_Y(y)\,dx\,dy$$

and

$$F_{XY}(C_X, C_Y) = \int_{x,y=0}^{\infty} \eta_{XY} \left( \frac{C_X}{x}, \frac{C_Y}{y} \right) p_X(x)p_Y(y)\,dx\,dy.$$ 

Then, defining

$$p_{XY} = p_Xp_Y : F_{XY}(C_X, C_Y) = \int_{x,y=0}^{\infty} \eta_{XY} \left( \frac{C_X}{x}, \frac{C_Y}{y} \right) p_{XY}(x,y)\,dx\,dy.$$ 

This expression shows that the multiplicative model is contained in our framework (Eq. 2). The multiplicative model always assumes buffering epistatic interactions, given by $\eta_{XY}(C_X, C_Y) = \eta(C_X)\eta(C_Y)$. Since $p_{XY} = p_Xp_Y$, this model always assumes no cross-resistance ($\xi = 0$). We have seen in our study that the behavior in terms of epistasis and cross-resistance of combinations of two drugs can be very diverse, ranging from synergy to suppression, and degrees of cross-resistance going from null to 1. The multiplicative model cannot account for those features.

Fig. S1. Drug epistasis with respect to growth inhibition. Epistasis between drugs is marked by an inhibition of wild-type growth that deviates from a null expectation based on the effect of each drug alone. The shape of the MIC line, which bounds the region of wild-type growth, reveals epistatic interactions. (A) The null expectation is that drugs do not interact, which results in a straight MIC line. (B) Synergistic epistasis signifies that wild-type inhibition is stronger than expected: the drugs aggravate each other’s effects. (C–E) Conversely, antagonistic epistasis signifies that the drugs alleviate each other’s effects, so that inhibition is weaker than expected. Antagonism between drugs can be so pronounced as to suppress each other’s effects. Adding a small concentration of drug Y to a concentration of drug X that normally inhibits growth can suppress the inhibition: the wild type is recovered (E).
Fig. S2. The data do not support the multiplicative model. We represent in blue or shades of red the regions where experimental data shows wild-type growth or resistance, respectively. We represent the same regions as predicted by our model (second column) after the cross-resistance parameter was fitted to the data. In the third column we show the predictions of the multiplicative model $F_{XY}/F_{H11005}$. This model produces surfaces whose shapes do not match the experimental results. The multiplicative model implicitly postulates strong antagonism and no cross-resistance, contrasting with our model which accounts for any type of epistasis and degree of cross-resistance.
Fig. S3. Impact of epistasis on the MSW of a drug combination in the presence of cross-resistance. Five mutants exist in this bacterial populations: two are increasingly resistant to X, two are increasingly resistant to Y, and the last mutant is resistant to both X and Y. (A and B) The MIC line of this cross-resistant mutant is shown in thick black; in blue and red are the MIC and MPC lines of the drug combinations. Depending on the degree of epistasis, the cross-resistant mutant can contribute to the MPC line of the combination (B). In this example, once this is the case the MSW of the drug combination no longer depends on the epistatic interactions. (C) The MSW of the drug combination decreases as epistasis goes from synergy to antagonism, but reaches a plateau (arrow). This plateau marks the point after which the cross-resistant mutant defines the MPC of the effective drug with smallest MSW. The position of this point can be geometrically derived and is a function of the cross-resistance, the single-drug frequencies of resistance, and the single-drug MSW. (D) After this point, the MPC increases at the same pace as the MIC. Note that the MSW plateaus because the cross-resistant mutant resists to equal amounts of the drugs X and Y: in general, the MSW will keep decreasing as epistasis increases, at a slower pace in the presence of cross-resistance.
Fig. S4. Suppressive drug combinations close the mutant selection window (MSW). We consider again the simple case where only two mutants exist, one conferring resistance to drug X and the other to drug Y, where cross-resistance is absent and no double mutant exists due to low mutation rates. We show in light blue the regions of wild-type growth, bounded by the MIC line (blue), and in red regions where resistant mutants grow. The smallest MSW is achieved by combining X and Y in equal proportions: the size of this minimal MSW depends on epistatic interactions. (A) When the two drugs buffer each other (\(x = \infty\)), the MIC (dashed) and the MPC (solid) of this combination of drugs are equal: drug concentrations where resistant mutants or the wild type grow are the same. Therefore, there is no drug concentration that could selectively enrich resistant mutants. When the two drugs buffer each other, the size of the MSW is zero: the MSW is closed. (B) If the antagonism between X and Y is so pronounced as to be suppressive, the MSW is again null, and there even exist regions where the wild type can grow but not the resistant mutants [Chait R, Craney A, Kishony R (2007) Nature 446:668–671]. These regions of drug space (dark blue) select against resistance: the susceptible strain is selectively enriched.
Fig. S5. The resolution of the colony count spans nine orders of magnitude. For each two-drug concentration, we used one six-well plate, with each well containing 7 ml of agar supplemented with the same concentration of drugs (A and B). We inoculated six different numbers of cells in each well (10^{1.5}, 10^{3}, 10^{4.5}, 10^{6}, 10^{7.5}, and 10^9 cells) and let them grow for 5 days in a controlled environment (see Materials and Methods). This setup ensures that at least one of the six plates will contain a countable number of resistant colonies as long as the frequency of resistance is >10^{-9}. (A) The frequency of resistance to a combination of 0.15 FUS–0.7 AMI is on the order of 0.3. Almost all cells can grow: the plates where >10^3 cells were inoculated show a lawn of bacteria. Distinct colonies can be counted in the two plates with the lowest initial number of cells. (B) The frequency of resistance to 10 FUS–1.4 AMI is low, on the order of 10^{-7}. Only the plates with the two highest initial numbers of cells exhibit bacterial growth. (C) We compare the estimates of the resistance frequencies obtained with different inoculum sizes, when available (42 plates). Overall, there is no significant difference between the frequency estimates obtained with the most diluted plates or least diluted plates (one-way ANOVA: 0.17). We do not observe a significant effect of the inoculum size on the estimates of resistance frequencies [Amsterdam D (2005) in Antibiotics in Laboratory Medicine, ed Lorian V (Lippincott Williams & Wilkins, Philadelphia), pp 61–143].
**Fig. S6.** Analysis of FUS-AMI after 3 days of incubation time. Using our automated image analysis platform, we have counted the colonies resistant to combinations of FUS and AMI that were detectable after 3 days of growth in a controlled environment. (A) We plot the experimental points together with a standard interpolated surface. (B) The model (Lower) is again in good agreement with the data (Upper). (C) We compare the frequencies of resistance estimated at 3 and 5 days. The estimates stay within the same order of magnitude. Some slow-growing colonies are detectable only after 5 days of growth and not after 3 days. This can affect the precise determination of the MPC and MPC line, and is the reason why we generally conducted the analysis after 5 days rather than after 3 days. This does not, however, affect the goodness of the fit or the estimated cross-resistance parameter (0.3 in both cases; not shown graphically).
Fig. S7. Limited Luria–Delbrück fluctuations in the measurement of the frequencies of resistance. Five bacterial cultures were grown in five separate overnight flasks, each inoculated with five different frozen aliquots issued from the same bacterial culture (initially prepared from one single colony). The five populations obtained came from the same initial isogenic colony but may differ in the frequency of the different mutants (Luria–Delbrück fluctuations). We measured on agar the frequencies of resistance of each population to increasing concentrations of FUS, ERY, and a constant combination of the two drugs, 1FUS:2ERY (see Materials and Methods). We plot with the same color the frequencies of resistance for each population (batch A in blue, batch B in green, etc.). Fluctuations are limited but can impact significantly the determination of the MPC.
<table>
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<tr>
<th>Antibiotic</th>
<th>Abbr.</th>
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<td>DNA gyrase</td>
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<td>Ampicillin</td>
<td>AMP</td>
<td>0.3</td>
<td>Cell wall formation</td>
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