Supporting Information

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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: η_{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 Ω 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 ω in Ω, 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 ω 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 $\eta(\omega,.)$ 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

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

Large groups of individuals in the population may possess the same response to stress. We therefore describe the population Ω 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 ω in Ω, there is an index $i \in I$ for which under any stress, ω behaves like ω_i: $\forall x = (x_1, \ldots, x_n) \in \Re^{+n}$, $\eta(\omega, x) = \eta(\omega_i, x)$. We define in this case $\eta_i(.) \equiv \eta(\omega_i,.)$. We then associate to any index $i \in I$ the proportion $p(i)$ of individuals in Ω, which behave like ω_i. We naturally have $\int_{i \in I} p(i) di = 1$. The equation for the frequency of the trait then becomes

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

The quantity p describes the population Ω in terms of its response to each stress alone, while the quantities η_i 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 η_i 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 (η_i 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 Ω 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 Ω 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 Ω 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., $p_X(x) = \int_{y>0} p_{XY}(x, y) dy$ and $p_Y(y) = \int_{x>0} p_{XY}(x, y) dx$. 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

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

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 $p_{XY}^{\text{Indep}}(x, y) \equiv p_X(x) p_Y(y)$. 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}^{Correl} 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: $p_{XY} = \xi p_{XY}^{\text{Correl}} + (1 - \xi) p_{XY}^{\text{Indep}}$. The parameter ξ 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 $F(z) = \int_{u<z} p(u)du$. 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_{XY}^{Correl} is the density of that Fréchet–Hoeffding upper-bound. The family $p_{XY} = \xi p_{XY}^{\text{Correl}} + (1 - \xi) p_{XY}^{\text{Indep}}$ 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 p_{XY} 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}^{Correl} is the density of the Fréchet–Hoeffding upper bound if and only if in any two-by-two submatrix of p_{XY}^{Correl}, at least one of the two nondiagonal elements is null:

$$\forall (i < j,\ k < l), p_{XY}^{Correl}(i,\ l) \cdot p_{XY}^{Correl}(j,\ k) = 0. \qquad \textbf{[S4]}$$

From this characterization, we obtain a simple algorithm (6) that gives p_{XY}^{Correl}. 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}^{Correl}.

Algorithm. M is the input stochastic matrix.

- For all row indexes $i<j$, column indexes $k<1$:
  - 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 $N = M$, and $N(i, l) = M(i, l) - m$, $N(j, k) = M(j, k) - \text{m}$, $N(i, k) = M(i, k) + m$,$N(j, l) = M(j, l) + m$. 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: $F_{XY}(C_X, C_Y) = F_X(C_X)F_Y(C_Y)$. 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 η_{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: $\eta_{XY}(C_X, C_Y) = 1$ when $F_{XY}(C_X, C_Y) = 1$, and zero elsewhere. Since $F_{XY}(C_X, C_Y) = F_X(C_X)F_Y(C_Y)$, 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 $\varepsilon = \infty$. 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**:

$$F_{XY}(C_X, C_Y) = \iint_{x,y>0} \eta\left(\frac{C_X}{x}\right)\eta\left(\frac{C_Y}{y}\right)p_x(x)p_Y(y)dxdy$$

and

$$F_{XY}(C_X, C_Y) = \iint_{x,y>0} \eta_{XY}\left(\frac{C_X}{x}, \frac{C_Y}{y}\right)p_X(x)p_Y(y)dxdy.$$

Then, defining

$$p_{XY} \equiv p_X p_Y : F_{XY}(C_X, C_Y) = \iint_{x,y>0} \eta_{XY}\left(\frac{C_X}{x}, \frac{C_Y}{y}\right)p_{XY}(x,y)dxdy.$$

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 $p_{XY} = p_X p_Y$, 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.

1. Nelsen RB (1999) *An Introduction to Copulas* (Springer, New York).
2. Embrechts P, Lindskog F, McNeil A (2003) in *Handbook of Heavy Tailed Distributions in Finance*, ed Rachev T (Elsevier, Amsterdam), pp 329–284.
3. Sklar A (1959) On n-dimensional distributions and their margins (Fonctions de répartition à n dimensions et leurs marges). *Statist Univ Paris* 8:229–231.
4. Fréchet M (1951) On correlation matrixes with fixed margins (Sur les tableaux de corrélation dont les marges sont données). *Ann Univ Lyon A* 9:53–77.
5. Hoeffding W (1940) Scale-invariant correlation theory. *Schr Math Inst Univ Berlin* 5:181–233.

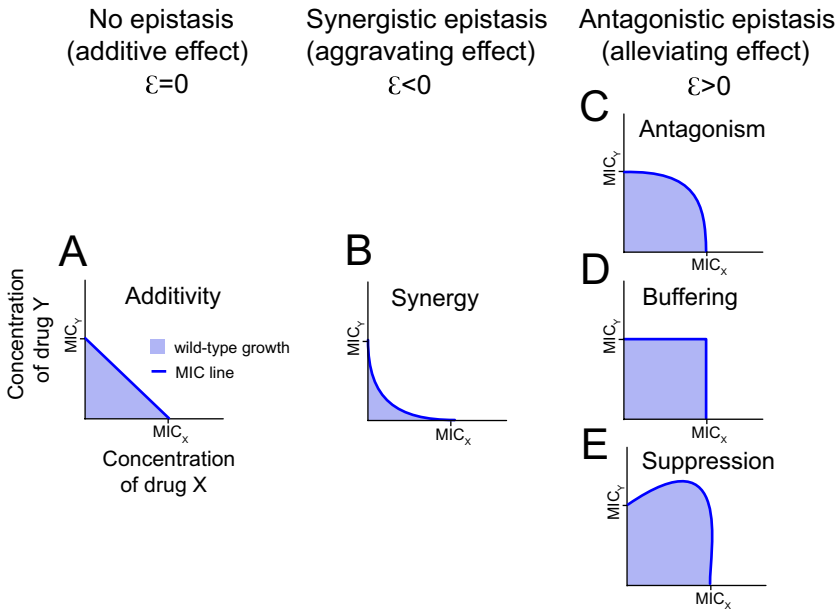

Fig. S1. Drug epistasis with respect to growth inhibition. Epistasis between drugs is marked by an inhibition of wild-type growth that deviates from a null expectation based on the effect of each drug alone. The shape of the MIC line, which bounds the region of wild-type growth, reveals epistatic interactions. (*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*).

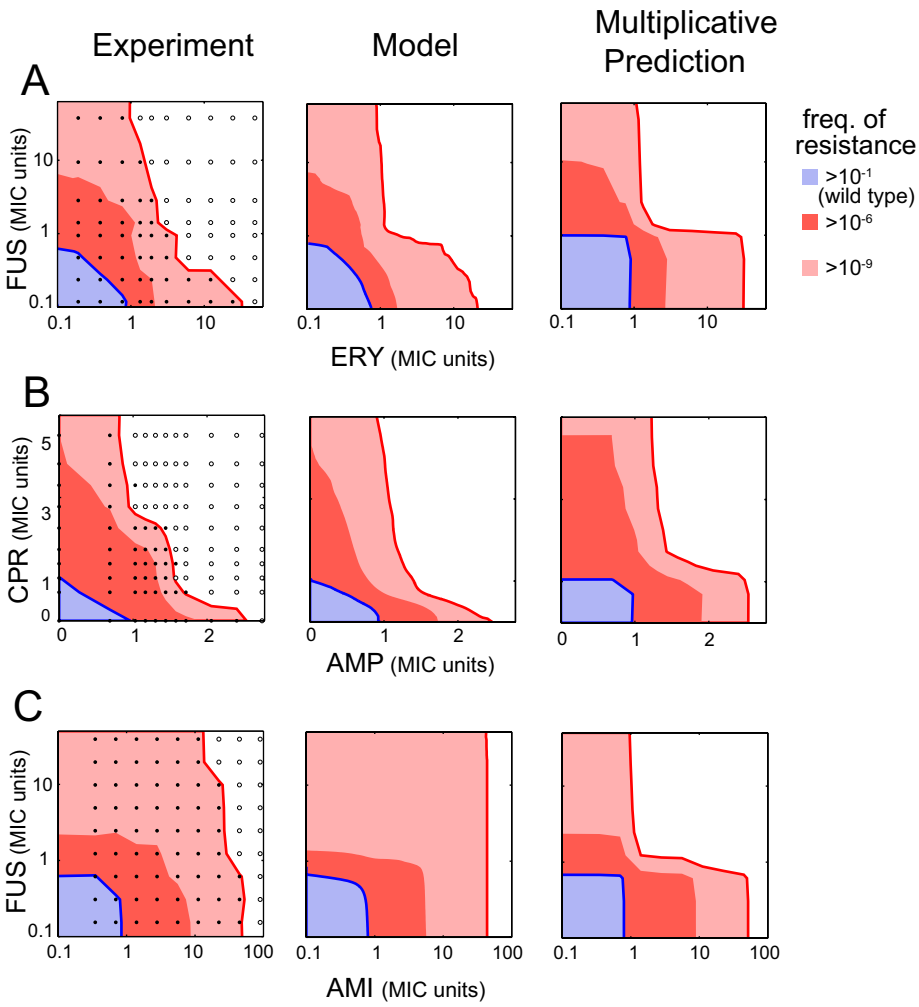

Fig. S2. The data do not support the multiplicative model. We represent in blue or shades of red the regions where experimental data shows wild-type growth or resistance, respectively. We represent the same regions as predicted by our model (second column) after the cross-resistance parameter was fitted to the data. In the third column we show the predictions of the multiplicative model $F_{XY} = F_X F_Y$. 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.

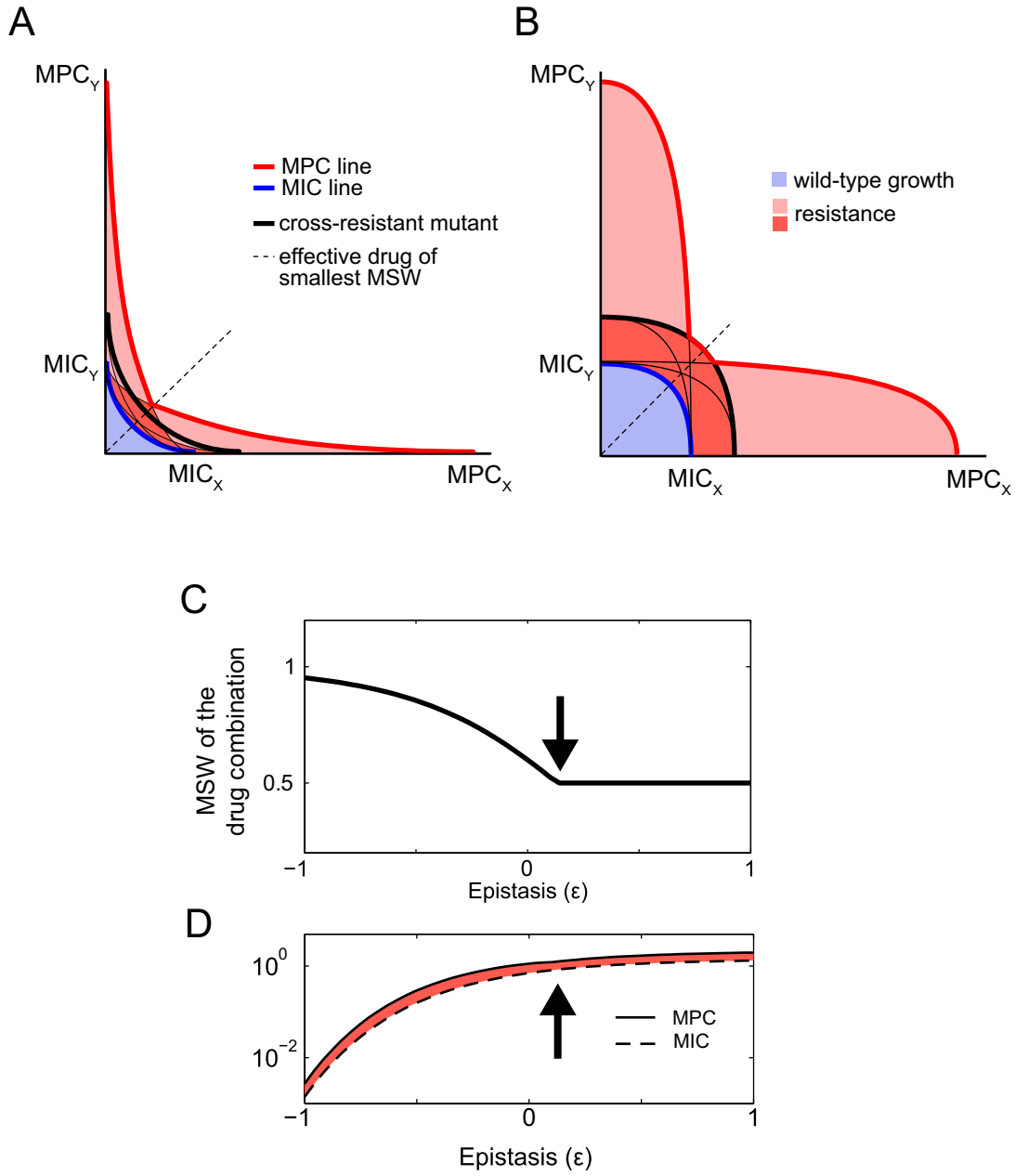

Fig. S3. Impact of epistasis on the MSW of a drug combination in the presence of cross-resistance. Five mutants exist in this bacterial populations: two are increasingly resistant to *X*, two are increasingly resistant to *Y*, and the last mutant is resistant to both *X* and *Y*. (*A* and *B*) The MIC 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.

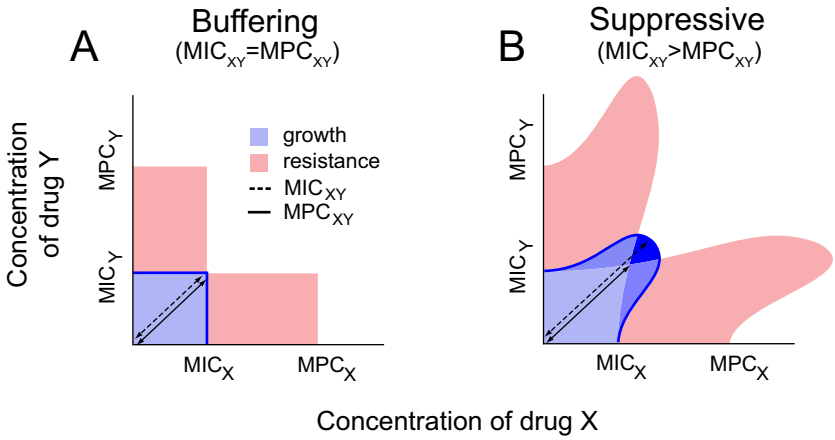

Fig. S4. Suppressive drug combinations close the mutant selection window (MSW). We consider again the simple case where only two mutants exist, one conferring resistance to drug *X* and the other to drug *Y*, where cross-resistance is absent and no double mutant exists due to low mutation rates. We show 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 ($\varepsilon = \infty$), 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.

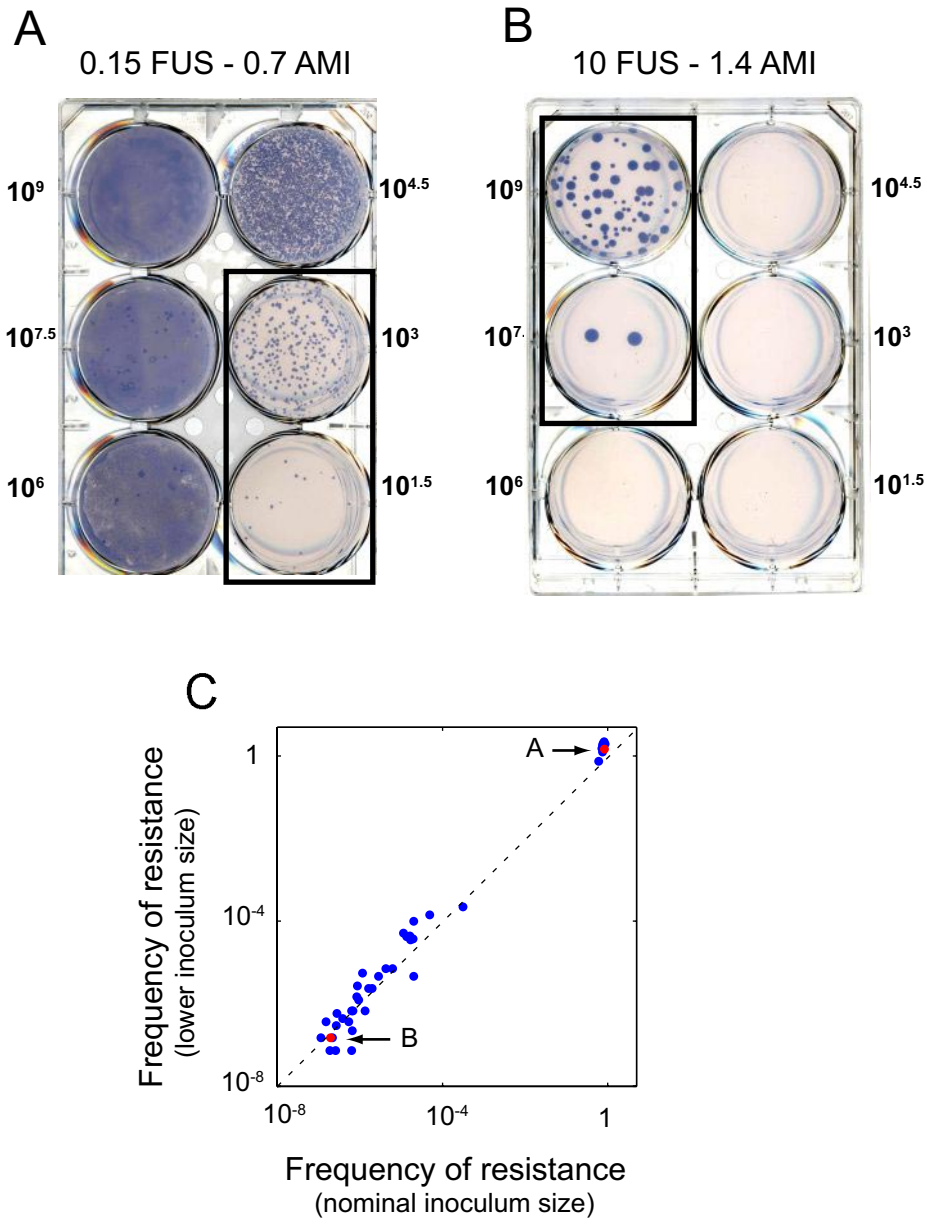

Fig. S5. The resolution of the colony count spans nine orders of magnitude. For each two-drug concentration, we used one six-well plate, with each well containing 7 ml of agar supplemented with the same concentration of drugs (*A* and *B*). We inoculated six different number of cells in each well ($10^{1.5}$, 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].

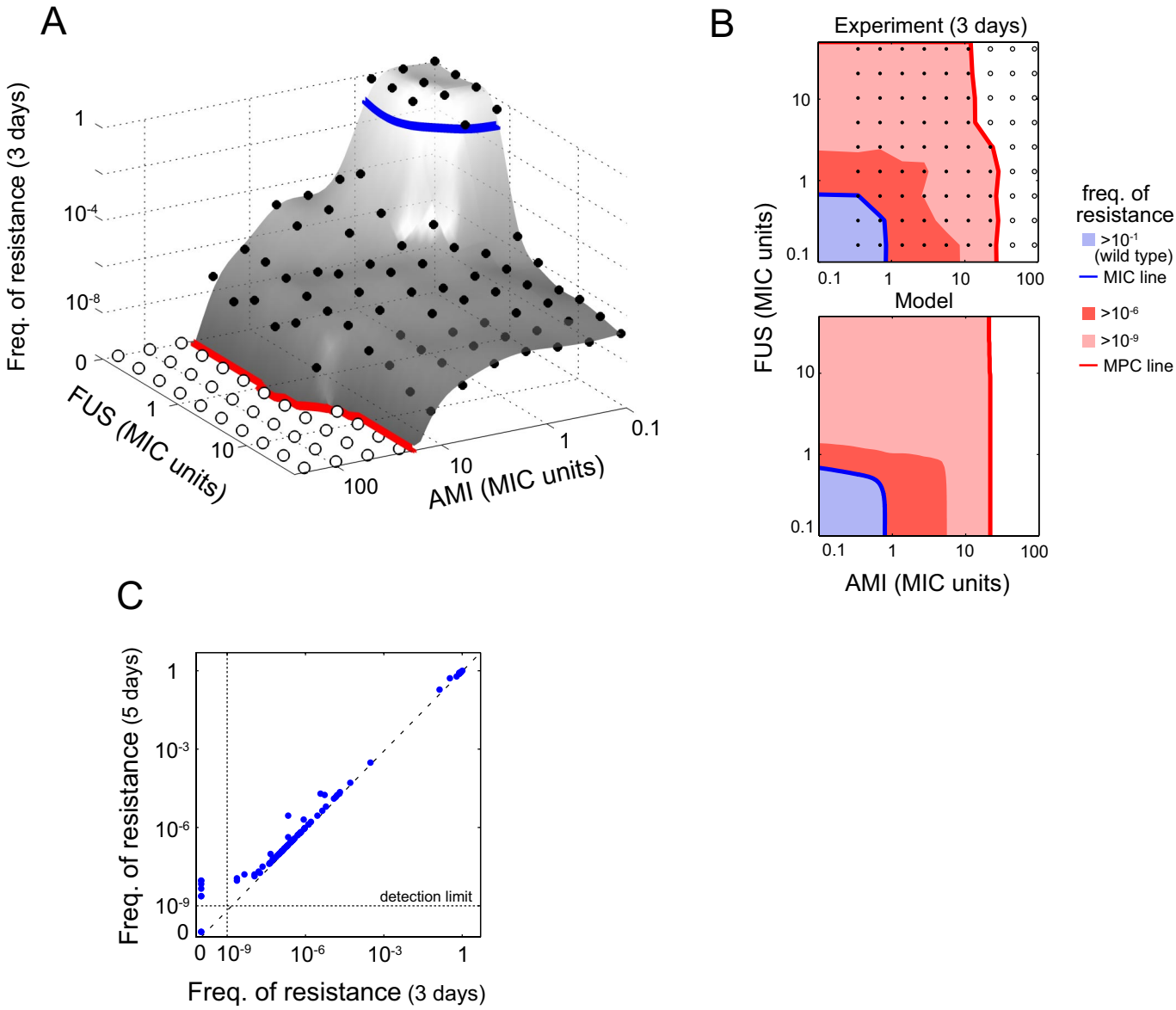

Fig. S6. Analysis of FUS-AMI after 3 days of incubation time. Using our automated image analysis platform, we have counted the colonies resistant to combinations of FUS and AMI that were detectable after 3 days of growth in a controlled environment. (*A*) We plot the experimental points together with 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).

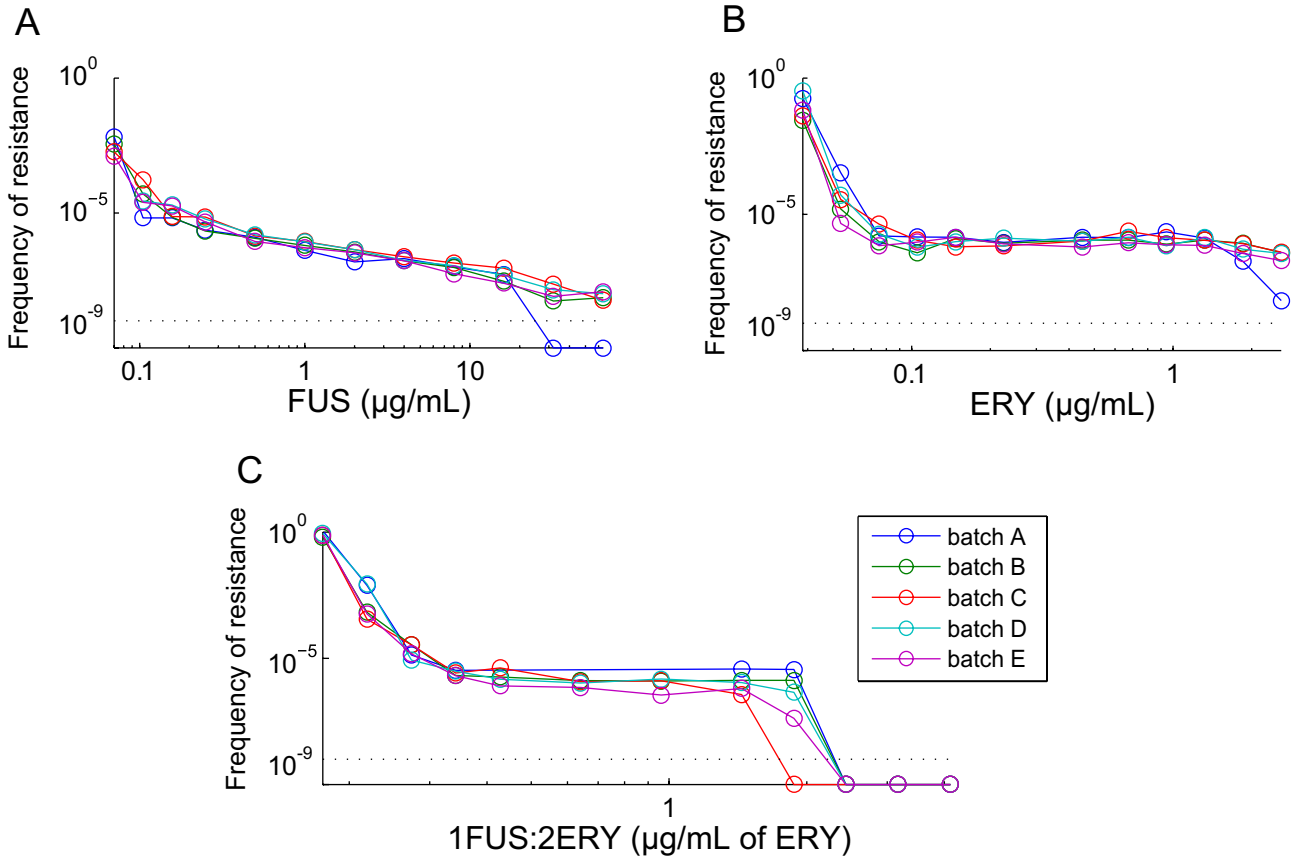

Fig. S7. Limited Luria–Delbrück fluctuations in the measurement of the frequencies of resistance. Five bacterial cultures were grown in five separate overnight flasks, each inoculated with five different frozen aliquot issued from the same bacterial culture (initially prepared from one single colony). 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 S1. Antibiotics, mode of action, and MIC

Antibiotic	Abbr.	MIC, μg/ml	Mode of action
Fusidic acid	FUS	0.7	Protein synthesis (EFG)
Erythromycin	ERY	150	Protein synthesis (50S)
Amikacin	AMI	4	Protein synthesis (30S)
Ciprofloxacin	CPR	0.1	DNA gyrase
Ampicillin	AMP	0.3	Cell wall formation