Stochastic bacterial population dynamics prevent the emergence of antibiotic resistance within the mutant selection window

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Abstract:
Understanding how antibiotic exposure impacts the emergence and spread of antibiotic resistant strains is key to preventing resistance in pathogenic bacteria. The conventional approach to this problem is to measure the range of antibiotic concentrations over which resistance is favoured – the 'mutant selection window'. Here we investigate how antibiotic concentration impacts the *de novo* establishment of antibiotic resistance from a single cell, specifically of a *Pseudomonas aeruginosa* strain that is resistant to streptomycin. The key finding of our study is that the range of antibiotic concentrations over which resistance is likely to emerge *de novo* is much smaller than the range of concentrations over which antibiotic resistance is selectively favoured. Strikingly, we observe that a single resistant cell has less than a 5% chance of establishing a large surviving population under streptomycin concentrations that are well below (1/8x) the minimum inhibitory concentration of the resistant strain (MIC_R). Sub-MIC_R doses of streptomycin extend the lag-time and increase the mortality rate of the resistant strain, corresponding to a dramatic reduction in the likelihood of successful outgrowth from a resistant cell. Our results show that the emergence of rare resistant strains is dominated by stochastic population dynamics, with frequent loss of lineages due to the detrimental effects of sub-MIC_R antibiotic exposure on resistant cells. Importantly, these effects dramatically restrict the range of antibiotic concentrations over which resistance is likely to emerge *de novo* relative to the range over which established resistant strains are favoured.

Introduction

The emergence of antibiotic resistance is a key obstacle to successful treatment of bacterial infections, with resistant infections associated with poorer clinical outcomes for patients [1]. A better understanding of how antibiotic dosing drives resistance evolution could aid the design of better treatment strategies.

A crucial question is how selection for resistance depends on antibiotic concentration. A major step towards in answering this question was the formulation of the mutant selection window (MSW) hypothesis, which proposes that resistance arises within a predictable range of antibiotic concentrations [2] [3]. Specifically, the lower boundary of this window is the minimum inhibitory concentration (MIC) of the sensitive strain, i.e. the concentration that is sufficient to prevent its growth. The upper boundary, the so-called "mutant prevention

concentration", was originally defined as the minimal concentration at which no colonies are formed when plating a culture of at least 10^{10} cells [2] [3], and gradually became equated with the MIC of the most resistant strain available [4] [5]. The MSW hypothesis has become an important paradigm in the field and a framework for suggesting dosing strategies to avoid emergence of resistance [4] [5] [6].

In recent years, it has been recognized both on theoretical grounds and with accumulating empirical evidence that resistance can also be selectively favoured below the MIC of the sensitive strain [7] [8] [9] [10] [6]. This occurs because sub-MIC concentrations of antibiotic can already be sufficiently detrimental to render the sensitive strain less fit than the resistant strain. Indeed, the "minimal selective concentration" (MSC) favouring resistance can be as low as a few percent of the sensitive strain's MIC [8]. Thus, the window of antibiotic concentrations over which resistance can emerge is potentially very wide.

Selection is expected to operate efficiently when both sensitive and resistant cell populations are reasonably large, resulting in an increase in frequency of the fitter strain (i.e. of the resistant strain at concentrations beyond the MSC). However, a crucial distinction must be drawn between situations in which resistance is already present at appreciable frequency, and cases in which it emerges *de novo* [11]. If a resistant strain is not already present, it must first arise (e.g. by mutation or horizontal gene transfer), and then increase in frequency from a single cell to a sizeable population. Although the <u>spread</u> of an existing resistant strain is a more or less deterministic process driven by selective differences, the <u>establishment</u> of an initially rare resistant strain is highly stochastic [11]. That is, due to chance events on the individual cell level (division and death), a resistance mutation present in only a few cells may be lost even when it is selectively favoured. Indeed, predicting the probability that beneficial mutations escape stochastic loss when rare is a classical problem in population genetics [12].

While a large body of work has addressed the question of antibiotic concentrations at which resistance is selectively favoured, and thus expected to spread once present in appreciable numbers, it is largely unknown how antibiotic concentration impacts the stochastic establishment phase of *de novo* resistant mutants. Theory predicts that the probability of establishment gradually decreases as cell division rate decreases or death rate increases, even while the expected population net growth rate remains positive, albeit reduced [12]. Thus, if antibiotics have detrimental effects on resistant cells below the resistant strain's MIC, it is possible that small initial populations of cells are stochastically lost and thus establishment fails, despite being within the MSW.

We set out to test this idea in the bacterium *Pseudomonas aeruginosa* challenged with the aminoglycoside antibiotic streptomycin. We used a strain carrying a plasmid that confers streptomycin resistance through production of an enzyme that deactivates the antibiotic. To study stochastic establishment, we tested growth in hundreds of cultures inoculated with resistant cells at extremely low density. Consistent with an important role for stochasticity, under identical culture conditions some replicate cultures grew and others did not. By fitting a mathematical model to these data, we were able to quantify the probability that a single resistant cell establishes a large surviving population across a range of antibiotic concentrations. Consistent with antibiotics exerting detrimental effects on resistant cells,

this establishment probability began to decrease at concentrations below the resistant strain's MIC (MIC_R). This decline occurred at surprisingly low antibiotic concentrations: significant effects were seen as low as 1/16 x MIC_R, and we estimate that establishment from single cells succeeds less than 5% of the time at 1/8 x MIC_R. We show that these results are consistent with a simple model of how inoculum size affects population growth, whereby each individual cell has a small, independent chance of establishment, but cumulatively, many cells virtually ensure growth from in standard MIC assays using larger inoculum sizes. Our results imply that *de novo* emergence of resistance may be unlikely throughout a substantial portion of the mutant selection window.

Materials & Methods

Bacterial strains, media and culture conditions

Bacterial strains: We use a set of *Pseudomonas aeruginosa* PA01 strains described previously [13]. These include both streptomycin-sensitive and -resistant strains, which are isogenic except that the resistant strains carry the clinically derived, non-conjugative plasmid Rms149 [14]. Streptomycin resistance is conferred by the *aadA5* gene, which is located within a class 1 integron, and codes for an enzyme that adenylates streptomycin [15]. For both plasmid carriers (resistant) and non-carriers (sensitive), we have strains with a chromosomal YFP label, a DsRed label, or unlabelled. The competition experiment was conducted with the DsRed-labelled sensitive and YFP-labelled resistant strains. The live-dead staining experiment was conducted with the unlabelled resistant strain. All other experiments were conducted with the YFP-labelled resistant strain. The minimum inhibitory concentrations of streptomycin in LB broth were previously determined to be MIC_S = 16μg/ml for the sensitive strain and MIC_R = 2048μg/ml for the resistant strain [13].

Media and antibiotics: For culture media we used LB broth containing 5g/L NaCl (Sigma-Aldrich, product no. L3022). For plating to assess colony-forming units we used LB Agar, Vegitone, containing 5g/L NaCl and 15g/L agar (Sigma-Aldrich product no. 19344). Streptomycin was prepared as stock from streptomycin sulfate salt (Sigma-Aldrich product no. S6501), stored according to supplier directions, and added to media on the day of experiments. Bacterial cultures were diluted in phosphate buffered saline (PBS) prepared from tablets (Sigma-Aldrich product no. P4417). Note that treatment cultures were generally set up with 90% media plus 10% inoculating culture by volume; thus, the final concentrations of LB and streptomycin in the treatments are 90% of the prepared media values denoted on plots.

Culture conditions: All cultures were incubated at 37°C, shaking at 225rpm. Overnight cultures were inoculated directly from freezer stocks into 2ml of LB and then incubated for approximately 16h. Overnight cultures were then diluted in PBS and used to inoculate treatment plates. All experimental treatments were conducted in 200μl cultures on 96-well plates (Thermo Scientific Nunc™ flat-bottom 96-well microplate with lid, product no. 260860) unless otherwise noted.

Competition experiment

Competitions were carried out between the YFP-labelled resistant strain and the DsRed-labelled sensitive strain, in streptomycin-free media and streptomycin at 1/16 to 16 x MIC_S in 2-fold concentration steps. Treatment cultures were inoculated with a 1:1 mixture of overnight cultures of the two strains, with expected total inoculum size around 10^5 CFU. We tested six replicate mixed cultures along with two of each pure culture as controls. After 24h, treatment cultures were diluted 500-fold and 66µl of each diluted culture was sampled by flow cytometry (BD Accuri C6 Flow Cytometer, fast fluidics, discarding events with forward scatter FSC-H < 10,000 or side scatter SSC-H < 8000). By gating based on fluorescence and correcting for the overlap of each strain into the opposing gate using the pure culture controls (see **Suppl. Text** and **Suppl. Fig. 2**), we estimated the final frequency of each strain.

Seeding experiment

Experimental protocol: An overnight culture was serially diluted up to 10^6-fold, then in three further independent steps, diluted to 4 x 10^7-, 8 x 10^7, and 1.6 x 10^8-fold. Each of these diluted cultures was used to inoculate an entire 96-well test plate for each tested streptomycin concentration, at 20µl per well. Test plates were incubated and scored for growth after approximately 1, 2, and 3d.

Scoring culture growth: We evaluated culture growth by measuring optical density (OD_{595}) using a BioTek Synergy 2 plate reader. Lids on 96-well plates were briefly removed for the reading; comparison to controls mock-inoculated with PBS indicated that contamination was rare. We set a threshold OD of 0.1 to score as growth, as background OD in controls was typically <0.05. OD showed a clearly bimodal distribution after a sufficiently long growth period; thus, the precise choice of threshold is unimportant.

Estimating establishment probability: From the number of replicate cultures showing growth by 3d at each condition, we obtained a likelihood-based point estimate and confidence intervals for the single-cell establishment probability, relative to streptomycin-free controls, by fitting a mathematical model to the data (see section "Mathematical model of establishment" below).

Generalized linear model: To evaluate the effect of streptomycin on establishment, culture growth data was additionally fit with a generalized linear model using the built-in R function 'glm'. Number of replicate cultures showing growth was treated as binomial data, with the dilution factor applied for inoculation and streptomycin concentration taken as explanatory variables, using the complementary log-log link function (see Suppl. Text for details).

MIC assay with varying inoculation density

Test cultures were inoculated with 20µl of overnight culture diluted 10^3-, 10^4-, 10^5-, or 10^6-fold. These dilution factors were chosen such that the highest inoculation density was expected to be similar to a standard MIC test (5 x 10^5 CFU/ml; EUCAST); actual density was estimated by plating out diluted overnight culture on LB-agar and counting colony-forming

units. We tested six replicates per inoculation density and per streptomycin concentration, ranging from 1/16 x MIC_R to 2 x MIC_R in 2-fold steps, plus streptomycin-free controls. Culture growth was evaluated by OD at approximately 20h, 2d, and 3d, according to the same procedure as for the seeding experiments. In rare cases, MIC within a replicate is ambiguous due to growth failing in a culture at lower streptomycin concentration but succeeding at the next highest step before being definitively abolished. In these cases, we take MIC to be the higher concentration, at and beyond which no further growth was observed.

Testing the null model of the inoculum size effect

To test the relationship between inoculum size and probability of population growth, we evaluated growth at selected streptomycin concentrations using many different dilution factors for inoculation: five in streptomycin-free conditions and 6-10 in each streptomycin concentration. These dilution factors were chosen for each streptomycin concentration based on the results of previous experiments, with the aim of capturing the range at which the proportion of replicate cultures showing growth increases from near 0 to near 1. In each case, 54 replicate cultures were evaluated per inoculum size. Culture growth was evaluated daily by OD, according to the same procedure as for the seeding experiments, up to 5d. The final number of replicate cultures showing growth was used for model fitting (see section "Mathematical model of establishment" below).

Fraction of dead cells by live-dead staining

For this experiment, we used the resistant strain with no fluorescent label, to avoid interfering with the signal from the live-dead stain. We inoculated treatment cultures (six replicates per streptomycin concentration) with 10^3-fold diluted overnight culture, as in the MIC assay at standard inoculation density. After 7h of treatment, we diluted test cultures a total of 100-fold and stained with thiazole orange and propidium iodide (BD Cell Viability Kit, product no. 349483). In parallel, we diluted and stained media and heat-killed cultures as controls. We sampled 50μl per diluted culture using flow cytometry (with the same settings as in the competition experiment). In order to better discriminate cells from background, we first gated on events according to forward and side scatter before defining clusters of dead (membrane-compromised) and intact cells based on fluorescence; see **Suppl. Text** and **Suppl. Fig. 7** for details.

Viable cell density dynamics

Here we tracked the number of viable cells over time in streptomycin-free media and at 1/32, 1/16, 1/8, and 1/4 x MIC_R streptomycin. An independent test plate was used for sampling at each time point. Six replicate cultures per concentration, per test plate (twelve replicates for streptomycin-free) were inoculated with 20μl of 5 x 10^5-fold diluted overnight culture. Streptomycin treatments were split across two sets of plates, which were sampled at generally different time points. At each sampling time, undiluted cultures were plated in arrays of 4μl spots, repeated across 5 square LB-agar plates for a total sampled volume of 20μl per culture. LB-agar plates were incubated at 37°C for the rest of the day of the

experiment, moved to the bench overnight to prevent overgrowth, then incubated again the next day at 37°C until colonies were visible, but still separated, to count CFUs.

Mathematical model of establishment

Model: We used a simple stochastic model to describe population (i.e. bacterial culture) growth as a function of inoculum size and streptomycin concentration. We denote by p_w the probability that a population inoculated with a small number of cells grows to a large size (detected by OD as described above). Among a set of n independent replicate populations (inoculated at the same density and cultured in the same conditions), the number of populations showing growth is thus described by a Binomial(n,p_w) distribution. Under the assumptions that (i) the number of cells in the inoculum that establish surviving lineages is Poisson-distributed with some mean α (an assumption supported by the observed distribution of colony-forming units counted in low-density diluted cultures; **Suppl. Fig. 1**), and (ii) population growth is observed provided at least one cell establishes, we have the relationship:

$$p_w = 1 - e^{-\alpha} \qquad \textbf{(Eqn. 1)}$$

Relative per-cell establishment probability $\tilde{p}_c(x)$ is generally calculated as the ratio of the mean number of established cells at any given streptomycin concentration x to that in the absence of streptomycin: $\tilde{p}_c(x) = \alpha(x)/\alpha(0)$.

In the full model (statistically speaking), p_w, or equivalently α, is taken to be distinct for each inoculum size and each streptomycin concentration. Simpler nested models make additional assumptions (see Suppl. Text for details). In particular, the null model of the inoculum size effect supposes that cells behave independently of one another, such that the true per-cell probability of establishment, p_c, depends only on streptomycin concentration. Then the mean number of established cells, α, is directly proportional to the mean inoculum size, $\bar{N}$ (where the actual inoculum size is Poisson-distributed about this mean). In this null model, the probability of population growth can be expressed as:

$$\begin{aligned} p_w &= 1 - e^{-\bar{N}p_c} \\ &= 1 - e^{-\bar{N}_{eff}\tilde{p}_c} \end{aligned} \qquad \textbf{(Eqn. 2)}$$

where $\bar{N}_{eff} = \alpha(0) = \bar{N}p_c(0)$ denotes the "effective mean inoculum size", defined as the mean number of established cells in the absence of streptomycin; and the relative establishment probability at streptomycin concentration x can simply be expressed as $\tilde{p}_c = p_c(x)/p_c(0)$. Note that by scaling up $\bar{N}_{eff}$ by the dilution factor(s) applied to the inoculating culture, we have an estimate of bacterial density in this culture, determined equivalently to the historical "most probable number" method [16] [17]. This definition of effective inoculum size based on cells that grow in benign conditions is no different in principle to the usual quantification of "viable" cells according to successful formation of a colony; we simply assess growth in liquid rather than on solid media. We also note that if cells (or possibly clumps of cells acting as individual units) are heterogeneous, p_c should be interpreted as the mean establishment probability (see **Suppl. Text**).

Likelihood-based model fitting and comparisons: These stochastic models were fit to experimental population growth data using likelihood-based methods. Specifically, we obtained a maximum likelihood estimate and a 95% confidence interval (determined by the range of parameter values that would not be rejected by a likelihood ratio test at 5% significance level) on the parameter p_w, which can be transformed to an estimate for α. In the case of relative establishment probability, $\tilde{p}_c(x) = \alpha(x)/\alpha(0)$, the (profile likelihood) confidence interval takes into account the uncertainty in both numerator (i.e. results at streptomycin concentration x) and denominator (i.e. results in streptomycin-free conditions). The fit of nested models is compared using the likelihood ratio test (LRT) at 5% significance level, i.e. a χ^2 test on model deviance with degrees of freedom equal to the difference in number of fitted parameters between the two models.

To test the null model of the inoculum size effect, we assume that mean inoculum size $\overline{N}$ is precisely inversely proportional to the dilution factor applied to the inoculating culture, i.e. we neglect any experimental error in the dilution steps relative to one another. Effective mean inoculum size, $\overline{N}_{eff}$, is estimated by fitting Eqn. 2 to population growth data in streptomycin-free media. Per-cell relative establishment probability $\tilde{p}_c$ then remains as the single free parameter to fit at each tested streptomycin concentration. The goodness of fit of the null model (**Eqn. 2**) is assessed for each test concentration separately. Here, the relevant comparison in the LRT is to the full model (**Eqn. 1**), where a distinct parameter p_w (or equivalently α) is estimated for each inoculum size, without any assumed relationship among them.

All model fitting was implemented in R, version 3.3.1 (The R Foundation for Statistical Computing, 2016).

Results

Resistance is selectively favoured over a wide range of streptomycin concentrations

The mutant selection window (MSW) hypothesis posits that antibiotic resistance will be selected between the minimum inhibitory concentrations (MIC) of the sensitive and resistant strains [3] [2] [5]. More recently, it has become widely recognized that fitness of the resistant strain generally exceeds that of the sensitive strain beginning at a minimal selective concentration (MSC) that is below the MIC of the sensitive strain, sometimes as little as a few percent of it [7] [8] [9] [10]. Thus, a potentially very broad range of antibiotic concentrations favours the spread of resistance.

We quantified this selective window in our system by competing fluorescently labelled sensitive and resistant strains at various concentrations of streptomycin, ranging in 2-fold steps from 1/16 to 16 x the previously measured MIC of the sensitive strain (MIC_S = 16 μg/ml; [13]), corresponding to 1/2048 to 1/8x the MIC of the resistant strain (MIC_R = 2048 μg/ml; Frost et al. 2018). Strains were inoculated at equal starting frequencies, with a total density around 5 x 10^5 CFU/ml, and their final frequencies were evaluated by flow cytometry after 24h (**Methods** and **Suppl. Fig. 2**). An increase in resistant frequency to significantly above 50% thus indicates selection for resistance. We were unable to

quantitatively estimate the selection coefficient of the resistant strain, because virtually no sensitive cells were recovered after culturing with streptomycin above MIC_S, leading to 100% final resistance frequency (**Fig. 1** and **Suppl. Table 2**). Strikingly, resistance was still strongly selected as low as 1/4 x MIC_S (two-sided t-test: p = 4e-6), while sensitivity was only significantly favoured in the complete absence of streptomycin (p = 4e-3). Based on the point at which final resistance frequency crosses the 50% mark, we estimate that the MSC of streptomycin lies between 1/16 and 1/8 x MIC_S, in agreement with previous results for these strains [13].

These results demonstrate that the resistant strain, once present at appreciable size, is expected to spread by outcompeting the sensitive strain over a wide range of streptomycin concentrations, ranging from well below (1/8-1/4x) to well above (at least 16x) the MIC of the sensitive strain (MIC_S).

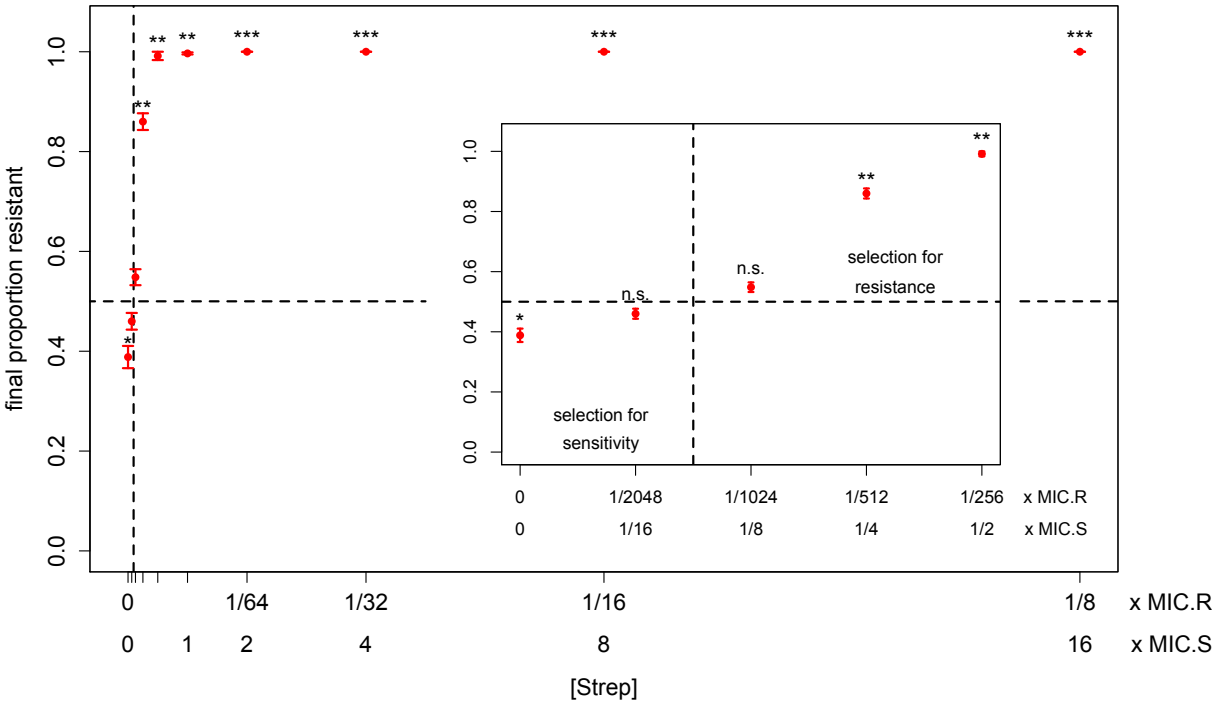

Figure 1: Evaluating selection for resistance by competition assays across streptomycin concentrations. Concentration on the x-axis is scaled by the previously measured MIC values of these strains (sensitive: MIC_S = 16 μg/ml; resistant: MIC_R = 2048 μg/ml; Frost et al 2018). Cultures were inoculated with a 1:1 mix of sensitive (DsRed) and resistant (YFP) strains from overnight cultures, at total density similar to a standard MIC test. After 24h, cultures were sampled by flow cytometry to estimate the proportion of each strain (**Methods**). The horizontal dashed line at 0.5 indicates the starting proportion, which would be maintained if resistance were selectively neutral. Final proportions falling below this line indicate selection favouring the sensitive strain, while those above this line indicate selection favouring the resistant strain. The vertical dashed line indicates the approximate position of the minimum selective concentration (MSC). Each data point represents the mean of six biologically independent replicates, with error bars indicating the standard error of the mean (see also **Suppl. Table 2**). Asterisks indicate that the mean final proportion of the resistant strain significantly differs from 0.5 in a two-sided t-test at each streptomycin concentration, with a Bonferroni correction for multiple testing (n.s.: $p > 0.005$; *: $p < 0.005$; **: p < 5e-6; ***: lack of variation among replicates precludes a t-test). The inset shows a zoomed-in version at low streptomycin concentration, on a log scale.

Outgrowth of resistance from single cells is significantly inhibited within the MSW

Although a wide range of streptomycin concentrations select for already-established resistant strains, it does not follow that streptomycin resistance will emerge *de novo* at these concentrations. To test how antibiotic exposure affects establishment of the resistant strain, which we define as the growth of a large population starting from a single cell, we conducted a large-scale "seeding experiment" (see also [18]). In this experiment (**Fig. 2**), we inoculated a large number of cultures at very low densities, averaging <1 to around 3 cells per culture. Importantly, cells are randomly distributed between cultures, and the number of cells inoculated into each culture therefore follows a Poisson distribution (**Suppl. Fig. 1**). One implication of this protocol is that many cultures are not inoculated with any cells. Our approach is to account for this variation statistically. We conducted seeding experiments at three different inoculation densities and across a range of streptomycin concentrations (up to 1/8 x MIC_R). We then scored the number of cultures showing growth based on reaching a threshold optical density up to 3 days post-inoculation.

Cell division and death in individual bacterial cells are probabilistic events, with the consequence that a single cell will not necessarily give rise to a successful lineage of cells. This chance of stochastic loss applies even under benign culture conditions, but is expected to be exacerbated by stressors such as antibiotic exposure that reduce the probability of successful cell division compared to cell death. In the context of our experiment, a culture could fail to grow either because the inoculum did not contain any cells, or because cells in the inoculum failed to give rise to a surviving lineage. To discriminate between these possibilities, we fit a stochastic model to infer the per-cell probability of establishment (i.e. probability that a single cell produces a large number of surviving descendants leading to detectable growth) from the number of replicate cultures showing growth, across streptomycin concentrations (Methods). We cannot estimate an <u>absolute</u> establishment probability because this quantity cannot be separated from the effect of inoculum size: inoculating a mean of two cells, each with 50% chance of establishment, will give equivalent results to one cell with 100% chance of establishment. Thus, all results are expressed in terms of <u>relative</u> per-cell establishment probability, denoted $\tilde{p}_c$, which is normalized by the result in streptomycin-free culture medium. Since mean inoculum size is the same in streptomycin-treated and streptomycin-free cultures, any differences in estimated $\tilde{p}_c$ can be attributed to effects of streptomycin on the dynamics of resistant cells.

Exposure to very low concentrations of streptomycin (up to 1/32 x MIC_R) had no detectable impact on the probability of establishment of a single resistant cell, but this probability declined sharply as concentration increased (**Fig. 3** and **Suppl. Table 1**). Strikingly, $\tilde{p}_c$ is already significantly reduced by 1/16 x MIC_R streptomycin and is only marginally above zero (3-5%) at 1/8 x MIC_R. Thus, the chance of *de novo* establishment of the resistant strain is nearly abolished under antibiotic concentrations well below its MIC, where resistance is strongly favoured in competition with the sensitive strain (compare **Fig. 3** to **Fig. 1**).

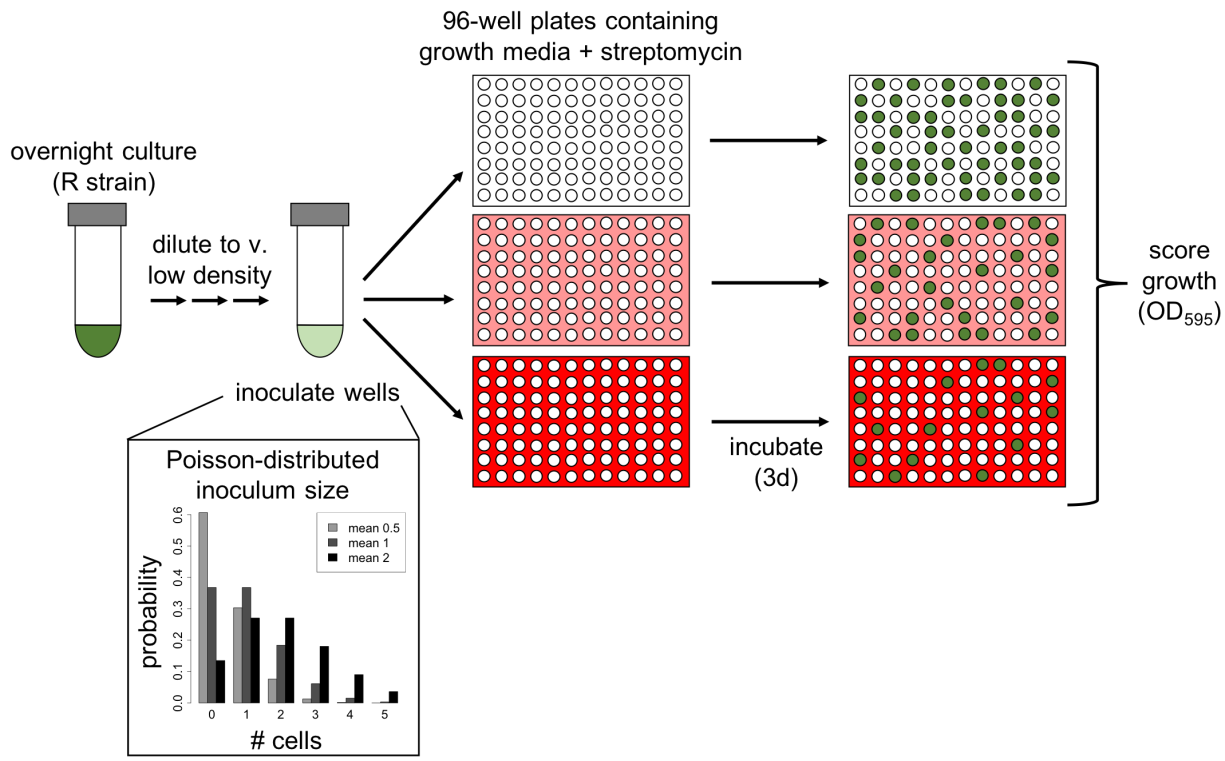

Figure 2: Design of seeding experiment to estimate establishment probability. An overnight culture of the resistant strain is highly diluted and used to inoculate 96-well plates containing growth media (LB) with streptomycin at various concentrations (higher concentration indicated by darker red). The number of cells inoculated per well follows a Poisson distribution (examples illustrated in the inset plot for mean inoculum size of 0.5, 1, or 2 cells per well). Plates are incubated up to 3d and optical density is measured to score growth in wells (OD_{595} > 0.1; indicated by dark green colouring). The final number of replicate cultures showing growth at each inoculum size and streptomycin concentration is used to estimate the per-cell establishment probability by fitting a mathematical model (Methods).

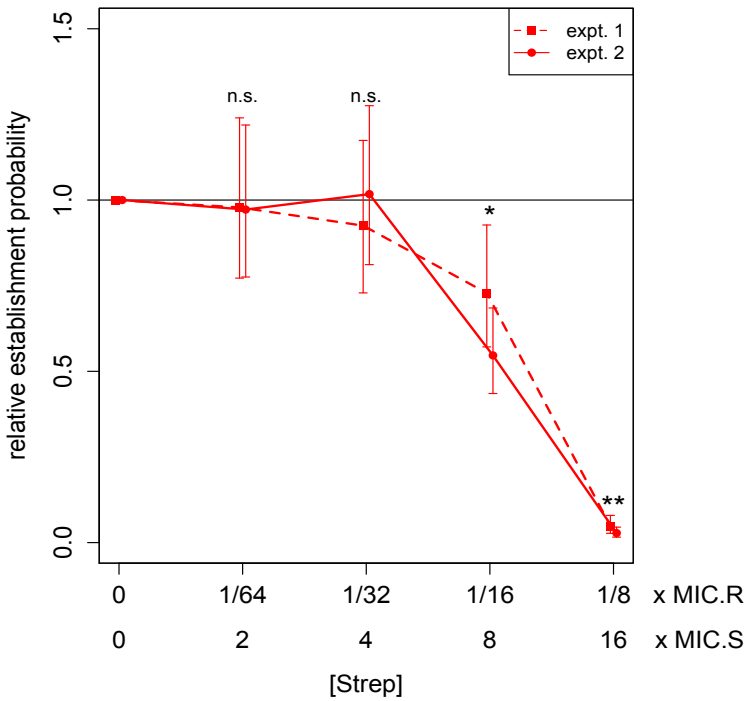

Figure 3: Establishment probability of single resistant cells, estimated from seeding experiments. The relative per-cell establishment probability $\tilde{p}_c$ of the resistant strain, scaled by the probability in streptomycin-free medium, is plotted as a function of streptomycin concentration, scaled by the previously measured MIC values of the resistant and sensitive strains (MIC_R and MIC_S, respectively). Results are shown for two separate experiments. Plotted points indicate the maximum likelihood estimate of $\tilde{p}_c$ and error bars indicate the 95% confidence interval, using the fitted model selected by the likelihood ratio test (Model B' in experiment 1 and Model C' in experiment 2, both of which pool data across three inoculation densities; see **Suppl. Text**). Significance of the streptomycin effect is determined by fitting a generalized linear model to the population growth data (n.s.: not significant, p >0.05; * p = 0.01 in expt. 1 and 2e-7 in expt. 2; ** p < 2e-16 in both experiments).

MIC depends on inoculum size

We hypothesized that the resistant strain's low probability of establishment even far below its MIC could be explained by an inoculum size effect. Specifically, MIC is standardly measured with an inoculation density of $5x10^5$ CFU/mL [19], which corresponds to 10^5 CFU inoculated per well in our experimental set-up. In contrast, in the seeding experiments above, we inoculated on the order of 1 CFU per well. MIC for many antibiotics is known to increase with inoculation densities higher than standard [20] [21] [22]. Although less well-explored, it has also occasionally been noted that MIC can decrease when lower inoculum sizes are used [23] [24].

To test the hypothesis that MIC is inoculum size-dependent, we conducted MIC assays with inoculation densities ranging over three orders of magnitude, from approximately $5x10^2$ CFU/ml up to the standard $5x10^5$ CFU/ml (i.e. 10^2-10^5 CFU/well). We found that MIC indeed increases with inoculum size (**Fig. 4**). This pattern is apparent regardless of whether growth is scored at 20h, as per the standard MIC assay protocol [19], or up to 3d post-inoculation, as in our seeding experiments, although the number of cultures showing detectable growth and thus the observed MIC tends to increase over time (**Suppl. Fig. 3**).

The observed increase in MIC with inoculum size could have been due either to the increase in absolute number of individuals in the inoculum (i.e. CFU), or in their density (i.e. CFU per unit volume). These two possibilities are not typically distinguished in the literature; however, they lead to distinct interpretations. If demographic stochasticity is the dominant force, we expect absolute numbers to matter, whereas if ecological effects (competition or cooperation) affect establishment, cell density per unit volume could be more important. To disentangle these two factors, we repeated the MIC assay co-varying inoculation density and culture volume. This confirmed that absolute inoculum size has a strong effect on MIC, but revealed that when controlling for absolute size, inoculation density per unit volume does not have a significant effect on the MIC, within the range that we tested (**Suppl. Fig. 4**).

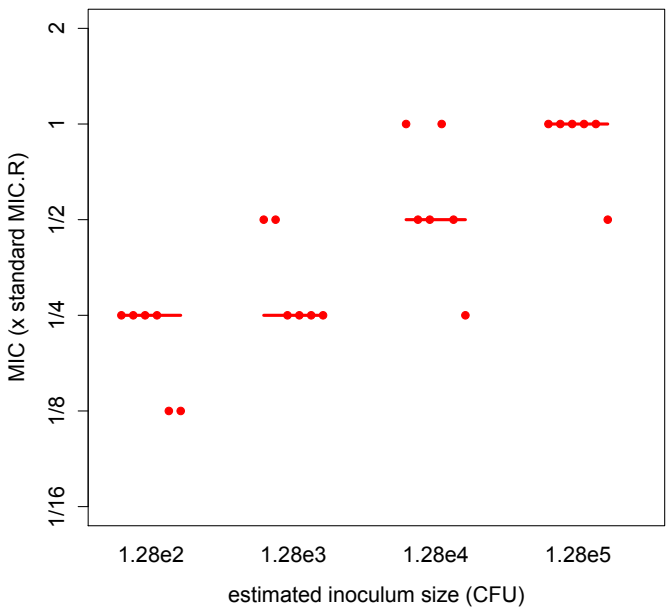

Figure 4: MIC as a function of inoculum size. Test cultures at 2-fold concentration steps of streptomycin were inoculated at four different densities. MIC was evaluated as the minimal tested concentration that prevented detectable growth up to 3d post-inoculation; a qualitatively similar pattern arose if growth was evaluated at 20h (see **Suppl. Fig. 3**). The y-axis is scaled by the previously measured MIC of the resistant strain at standard inoculation density (MIC_R), as before. The points represent six biologically independent replicates at each inoculum size, with the line segments indicating their median.

Population growth can be explained by an independent chance of each cell to establish

The above MIC tests revealed that the absolute number of cells in the inoculum has a strong effect on whether the population eventually shows detectable growth at streptomycin concentrations within the MSW. The simplest explanation for this effect is that at a given streptomycin concentration, each inoculated cell has a certain probability of establishing a surviving lineage (leading to detectable population growth), independently of all other cells in the inoculum. This independence assumption leads to a simple stochastic model capturing the effect of inoculum size on the probability of population growth (**Methods**, **Eqn. 2**). Note that this independent model of cellular replication need not be true; for example, if cells secrete an enzyme that breaks down an antibiotic extracellularly, then the chance that an individual cell establishes a successful lineage could increase with initial cell

density due to cooperative resistance. On the other hand, if cells strongly compete for limiting resources or secrete toxins, the per-cell chance of establishment could decrease with initial cell density.

To formally test the hypothesis that each inoculated cell has an independent probability of establishment, we again conducted high-replicate population growth experiments. These were similar to the seeding experiments, but used multiple inoculum sizes spanning approximately three orders of magnitude, in order to capture the full range of response (i.e. 0-100% of cultures showing growth) at a given streptomycin concentration. We tested two concentrations (1/16 and 1/8 x MIC_R) for which growth often failed in the seeding experiment, but succeeded at standard inoculation density in the MIC tests. In parallel, we tested growth in streptomycin-free media in order to calibrate the "effective" mean inoculum size (**Methods** and **Suppl. Fig. 5**).

We found good agreement between the null model and our experimental data at all tested streptomycin concentrations, as we would expect if establishment were independent (main experiment, **Fig. 5**, and repeat experiments, **Suppl. Fig. 6**). More precisely, in no case did the null model show significant deviance (according to the likelihood ratio test; see **Suppl. Text** for full results), and thus we accept it as the most parsimonious explanation for the data. At 1/16 and 1/8 x MIC_R we obtain estimates of relative establishment probability, $\tilde{p}_c$, comparable to those from the seeding experiments (**Suppl. Table 1**).

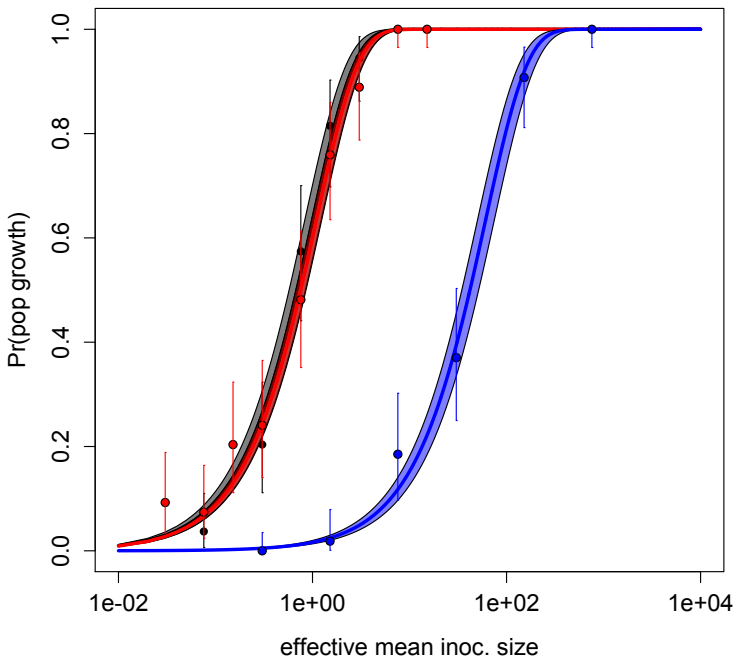

Figure 5: Null model of the inoculum size effect fit to population growth data. Probability of population growth of the resistant strain is plotted as a function of effective mean inoculum size ($\overline{N}_{eff}$, calibrated by the results in streptomycin-free media; see **Suppl. Fig. 5**). Black: streptomycin-free; red: streptomycin at 1/16 x MIC_R; blue: 1/8 x MIC_R. The solid line shows the best fit of the null model (using the maximum likelihood estimate [MLE] of $\tilde{p}_c$) and the shaded area corresponds to the 95% confidence interval (CI). Points and error bars indicate the MLEs and 95% CIs in the full model, i.e. treating each inoculum size separately (here MLE simply equals the proportion of experimental replicate cultures showing growth). According to the likelihood ratio test, the null model deviance from the full model is not significant at any streptomycin concentration (streptomycin-free: p=0.55; 1/16 x MIC_R: p=0.28; 1/8 x MIC_R: p=0.71).

Sub-MIC streptomycin concentrations extend lag phase and induce cell death

To explain the reduced establishment probability of the resistant strain when exposed to sub-MIC_R concentrations of streptomycin, we hypothesized that even these low doses must compromise cell division rate and/or viability. As a simple test of this idea, we measured the relative abundance of dead cells in cultures of the resistant strain grown under various concentrations of streptomycin. We find that the fraction of dead cells after 7h of treatment, as determined by propidium iodide staining, increases from an average of 3-4% in streptomycin-free conditions to >20% at 1/8 x MIC_R streptomycin (**Fig. 6a**, **Suppl. Fig. 7** and **Suppl. Table 3**). Note that this is a conservative measure of cell death, because this assay only detects cells that have compromised membrane permeability, and does not detect, for example, lytic cell death.

To gain further insight into how the toxic effects of streptomycin impact the population dynamics of the resistant strain, we quantified viable cell density (colony-forming units in plated samples) over the first few hours of sub-MIC_R streptomycin treatment. In this experiment, cultures were inoculated with approximately 100 CFU/well, such that populations are small enough for demographic stochasticity to be relevant, yet large enough to be detectable by plating samples.

We found that streptomycin treatment has a significant effect on population growth (ANOVA, main effect: $p < 2e\text{-}16$), and this effect varies over time (ANOVA, interaction term: $p < 2e\text{-}16$). Following inoculation into fresh medium, the resistant strain experienced a lag phase of approximately 2 hours. Control cultures that were exposed to streptomycin-free medium then exited lag phase and began to grow exponentially. The lowest tested dose of streptomycin (1/32 x MIC_R) had no significant effect on these dynamics (Dunnett's test: $p = 0.87$); however, 1/16 x MIC_R was already sufficient to slow population growth ($p = 4e\text{-}4$). Nonetheless, all replicate populations (48/48 at each concentration, pooled across all sampled treatment plates) eventually grew, as detected by OD, at streptomycin concentrations up to 1/16 x MIC_R. Meanwhile, higher doses of streptomycin had dramatic effects (1/8 x or 1/4 x MIC_R vs. control: $p < 1e\text{-}4$), with cultures exhibiting an extended lag phase of at least 7-8 hours, in which viable cell density initially declined. After further incubation (up to 3d), 25% of cultures (15/60) exposed to 1/8 x MIC_R eventually showed growth to saturation, while the remaining 75% of cultures (45/60) failed to reach detectable OD. At 1/4 x MIC_R, no viable cells were sampled in most replicates from 4h on, and only 1/60 replicates reached detectable OD within 3d.

In summary, these population dynamics experiments reveal that sub-MIC streptomycin treatment has the effect of extending the lag phase, before populations eventually either grow to saturation or die out. The failure of many populations to establish at concentrations $\leq$ 1/8 x MIC_R can be explained by a significant fraction of cells dying rather than successfully dividing, which can lead to stochastic loss of initially small populations.

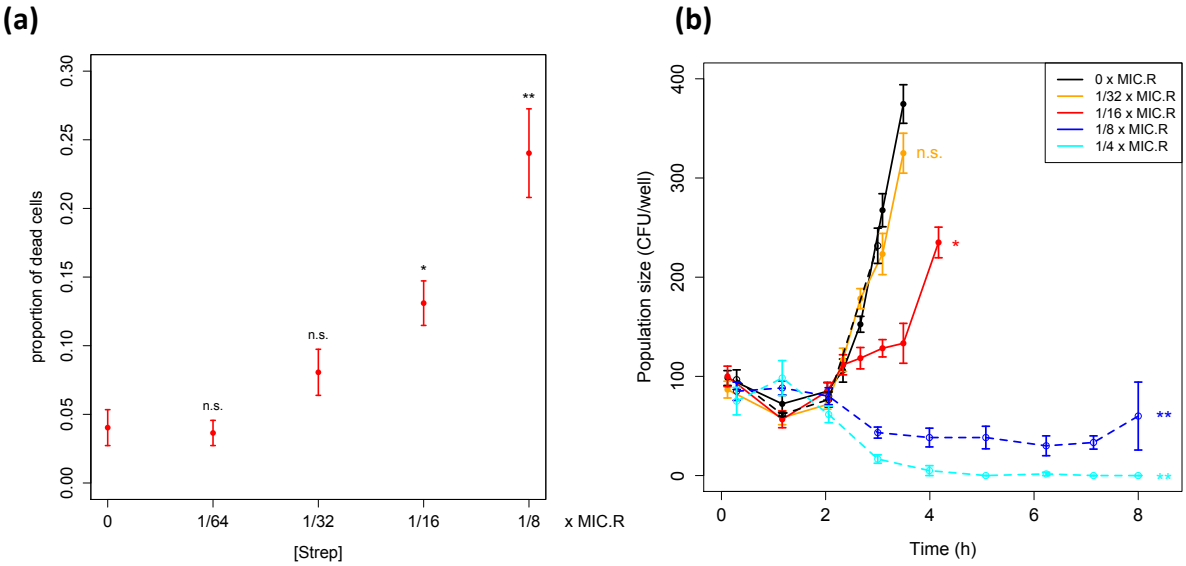

Figure 6: Sub-MIC$_R$ streptomycin treatment has profound effects on resistant cell dynamics. (a) Proportion of dead cells after 7h treatment in sub-MIC$_R$ streptomycin. The proportion of dead cells was estimated using live-dead staining and flow cytometry (Methods). Note that this is a minimal estimate, since staining only detects cells with compromised membranes. The points represent the mean and the error bars represent the standard error of the mean among six independent treatment replicates. Differences from the streptomycin-free control were assessed with a two-sample, two-sided t-test with Bonferroni correction for multiple testing (n.s.: not significant; *: p = 1.6e-3, **: p = 8.7e-4). If one outlier replicate with a consistently elevated fraction of dead cells across all streptomycin concentrations is excluded, means slightly decrease and the result at 1/32 x MIC$_R$ also becomes significant (see **Suppl. Table 3**). **(b) Early post-inoculation population dynamics in sub-MIC$_R$ streptomycin.** The plotted points (with connecting lines) indicate mean estimated population size of six independent replicate cultures per streptomycin concentration (or twelve replicates for each of the streptomycin-free controls), at each sampling time point; the error bars indicate standard error of the mean. Population size in each replicate was estimated by scaling up the number of colony forming units in a total plated volume of 20µl of undiluted culture (i.e. 10% of culture volume). Plotted population size is truncated where plated colonies became too dense to count. Treatments were split over two sets of plates, each including a streptomycin-free control, with the two lower streptomycin concentrations in Set A (solid lines and filled points) and the two higher in Set B (dashed lines and open points). Comparing controls indicates that the plate set effect is non-significant (ANOVA: p = 0.103); thus, controls were pooled for further analysis of the streptomycin effect. Significance of each streptomycin concentration compared to the control in a post-hoc Dunnett's test is indicated (n.s.: not significant, p=0.87; * p = 0.0004; ** p < 0.0001).

Discussion

Understanding how antibiotic concentration affects selection for resistance is key to designing more effective antibiotic dosing strategies. To this end, the mutant selection window (MSW) hypothesis has been a valuable and influential paradigm in the field. More recent work has highlighted that resistance is selectively favoured not only between the MICs of the sensitive and resistant strains, as originally posited [2] [3] [4], but even below the MIC of the sensitive strain [7] [8] [9] [10]. Thus, resistance is expected to spread over a wide window of antibiotic concentrations. Indeed, when bacteria are inoculated at sizeable densities, we confirmed that the resistant strain outcompetes the sensitive strain in our experimental system beginning at streptomycin concentrations of ≤1/4 x MIC$_S$ (**Fig. 1**), and the resistant strain can continue to grow until reaching its standard MIC, 1 x MIC$_R$ = 128 x MIC$_S$ (**Fig. 4**).

However, an important distinction must be drawn between spread of an already-established resistant strain, which is driven more or less deterministically by selective differences, and its initial emergence. Following *de novo* mutation or horizontal gene transfer, the emergence of resistance ultimately depends on the ability of a single resistant cell to establish a successful lineage. On the level of individual cells, division and death occur as probabilistic events, and demographic stochasticity is thus critical to the fate of small populations. Our key finding is that these stochastic effects can play a crucial role in shaping the emergence of antibiotic resistance, by limiting the establishment of resistant lineages within a large portion of the MSW. This result was highlighted by our seeding experiment, in which cultures were inoculated with approximately one resistant cell at streptomycin concentrations well below the MIC of the resistant strain. Strikingly, the chance of population growth was significantly reduced by streptomycin concentrations as low as 1/16 x MIC_R, and nearly abolished (<5% chance) by 1/8 x MIC_R (**Fig. 3**). This result can be explained by detrimental effects of sub-MIC antibiotic exposure, including a significant mortality rate on the individual cell level (**Fig. 6**), leading to frequent stochastic extinction of small populations even at concentrations where the expected net population growth rate is positive (albeit slowed).

The discrepancy between the antibiotic concentration required to reliably prevent growth of a larger population, as in a standard MIC test (inoculated with ~10^5 cells), and the concentration that is sufficient to drastically reduce the probability of growth of a smaller population, highlights that MIC depends on inoculum size (**Fig. 4**). To clarify this effect, we rigorously quantified the probability of population growth from inoculum sizes spanning three orders of magnitude, in the presence and absence of streptomycin. We found that a simple null model, in which each individual cell independently has some probability of establishing a surviving lineage, is sufficient to explain our data (**Fig. 5**). In other words, we need not invoke any interactions among cells (e.g. competition or cooperation). Moreover, this simple stochastic "numbers game" provides a unifying explanation for all of the preceding results. When only a single cell is inoculated, as in the seeding experiments, outgrowth is very unlikely at as little as $1/8^{th}$ of this strain's standard MIC. However, the cumulative effect of many individuals, each with a small chance of establishment, virtually guarantees population growth from a sufficiently large inoculum size, as in standard MIC assays.

The "inoculum effect" has previously been recognized primarily in reference to the reduced efficacy of antibiotic treatment (i.e. increase in MIC) as bacterial population density increases beyond that used in standard MIC assays [20] [21]. This effect can arise via various mechanisms, including titration of antibiotics out of the media [21] and enzymatic inactivation of antibiotics by resistant cells [25] [26] [22] [27] [13]. In contrast to this density-dependent, cooperative regime at high bacterial density, the inoculum effect that we found at very low bacterial population size is a stochastic phenomenon driven by cells acting effectively independently. There are hints of this low inoculum size effect in earlier literature [23] [24], but to our knowledge we are the first to provide a rigorous explanation in terms of stochastic population dynamics. These two types of inoculum effect are distinct: the cooperative growth regime arises for certain antibiotics at high bacterial <u>concentrations</u> (cells/ml), whereas we expect the stochastic growth regime to arise very generally at low

absolute numbers of cells. That is, we expect the functional relationship between probability of population growth and absolute inoculum size (**Eqn. 2**) to hold widely (with the scaling parameter $\tilde{p}_c$ depending on the bacterial strain and antibiotic); however, the relationship between MIC and inoculum size will also depend on the relationship between $\tilde{p}_c$ and antibiotic concentration. Moreover, measured MIC will become increasingly variable at low inoculum size, calling for a more precise measure of inhibition, such as the IC_{99} [28]. In these two facets of the inoculum effect, cells at the limit of low density (cells/ml) where cooperative interactions are abolished [22] are distinguished from low absolute numbers of cells, where stochastic effects become relevant. Both types of inoculum effect raise important considerations for antibiotic dosing. Cooperative growth has implications for treatment of high-density bacterial infections [26] [29] [30], whereas the stochastic effects we studied here could have implications for dosing strategies to avoid emergence of *de novo* resistant mutants.

Although the role of demographic stochasticity in the fate of *de novo* mutations has long been recognized in theoretical population genetics, until very recently it had never been addressed empirically [12]. Our study joins a small handful of others that have now experimentally quantified establishment probability from single cells [31] [28] [32] [33]. Two of these [28] [33] are particularly noteworthy in the present context, as they likewise address establishment of antibiotic-resistant bacterial cells across antibiotic concentrations. Unlike us, they used solid media and could thus directly visualize the number of established cells (α in our model notation) as colony-forming units. The ratio between CFU counts at any given concentration and CFU counts on antibiotic-free plates, sometimes called "plating efficiency" [33], is equivalent to our "relative establishment probability" ($\tilde{p}_c$). In contrast, working with liquid cultures, we visualize (by optical density) the outcome of population growth (probability p_w), and infer α or $\tilde{p}_c$ from p_w via a stochastic model. One strength of our method is that experiments can readily be conducted on large scale and are amenable to automation.

Our work is also closely connected to the topic of evolutionary rescue, the process by which a population under decline due to severe environmental change can avoid extinction through the outgrowth of rare well-adapted genetic variants. Emergence of resistance in bacterial populations during antibiotic treatment is a prime example of evolutionary rescue [34]. Generally, rescue requires two key ingredients, the *de novo* production of mutants and their establishment of surviving lineages. Here we have examined the second of these two ingredients; in fact, our results can also be interpreted as a test of the probability of rescue from pre-existing mutants. Importantly, approximations for the probability of evolutionary rescue [35] are identical in form to our Eqn. 2 (probability of population growth assuming individuals act independently), and thus the statistical model-fitting methods are transferable (see **Suppl. Text** for further discussion). More broadly, the concepts and methods developed here could be applied to a variety of situations where growth depends on establishment of rare cells and is thus highly stochastic, for instance the outgrowth of bacteria in food products from small initial contaminants [36].

In summary, our study highlights the stochastic nature of *de novo* emergence of antibiotic resistance, and sheds new light on the question of what concentrations of antibiotics drive resistance evolution. Importantly, we found that concentrations within the traditional

mutant selection window, above the MIC of the sensitive strain but still well below the MIC of the resistant strain, can be sufficient to severely limit *de novo* establishment of resistance from single cells. Taken together with evidence of selection for resistance below the MIC of the sensitive strain [7] [8] [9] [10], our work contributes to an emerging picture that resistance tends to arise at a range of concentrations shifted lower than the traditional MSW. Outside this range, at very low antibiotic concentrations, resistance is limited by competition with the sensitive strain. At higher concentrations, but potentially still well below the MIC of the resistant strain, resistance can spread if it is already established, but is unlikely to emerge *de novo*. Importantly, we studied establishment of the resistant strain in isolation; it remains to be determined how interactions with an initially dominant sensitive population shape establishment of resistance during antibiotic treatment. A key direction for future work will thus be to integrate the dual effects of competition and direct inhibition by the antibiotic, which operate with varying strengths across antibiotic concentrations [6]. By better understanding these limiting forces on the evolution of resistance, we may ultimately be able to design effective dosing strategies that also avoid emergence of resistance.

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