Stochastic bacterial population dynamics prevent the emergence of antibiotic resistance

Helen K. Alexander*, R. Craig MacLean

Department of Zoology, University of Oxford, Oxford, OX1 3PS, United Kingdom

* Corresponding author: helen.alexander@zoo.ox.ac.uk

Keywords: antimicrobial resistance, *Pseudomonas aeruginosa*, streptomycin, minimum inhibitory concentration (MIC), inoculum effect, mathematical model, extinction probability, demographic stochasticity, evolutionary rescue, experimental evolution

Abstract:

Understanding how antibiotic exposure impacts the evolution of resistance is key to optimizing antibiotic treatment strategies. The conventional approach to this problem is to measure the range of antibiotic concentrations over which resistance is selectively favoured – the "mutant selection window". Here we take an alternative approach by investigating how antibiotic concentration impacts *de novo* establishment of resistance from single cells of a streptomycin-resistant *Pseudomonas aeruginosa* strain. We show that demographic stochasticity prevents outgrowth of resistant cells with >95% probability across ~90% of the range of streptomycin concentrations where resistance is selectively favoured. This effect occurs because exposure to streptomycin extends lag time and increases the mortality rate of streptomycin-resistant cells. Our study highlights fundamental differences between the processes that drive emergence versus spread of resistance. It suggests that moderate doses of antibiotics, within the traditional mutant selection window, may effectively prevent emergence of resistance in cases where transmission of resistant strains is negligible, for instance when using new antibiotics.

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 more effective treatment strategies.

A crucial question is how evolution of resistance depends on antibiotic concentration. A major step towards 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 higher resistance can also be selectively favoured below the MIC of a sensitive or less-resistant strain [7] [8] [9] [10]. This selection 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 [9]. 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 spread of a common resistant strain is a more or less deterministic process driven by selective differences, the establishment 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, 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 (aadA5) that deactivates the antibiotic intracellularly. To study stochastic establishment, we tested growth in hundreds of cultures inoculated with resistant cells at extremely low density (around a single cell per culture). 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. This establishment probability began to decrease at concentrations below the resistant strain's MIC (MIC_R), suggesting that antibiotics are exerting detrimental effects on resistant cells. 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. Collectively, our results imply that *de novo* emergence of resistance may be unlikely throughout a substantial portion of the mutant selection window.

Results

Resistance is selectively favoured over a wide range of streptomycin concentrations

We measured the impact of antibiotic exposure on selection for resistance by directly competing fluorescently labelled sensitive and resistant strains across a gradient of streptomycin concentrations, ranging from 1/16 to 16 times the MIC of the sensitive strain (MIC_S = 16 μg/ml; [13]),

corresponding to 1/2048 to 1/8 times the MIC of the resistant strain (MIC_R = 2048 μg/ml; [13]). Strains were inoculated at approximately 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. 1**). 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**, **Suppl. Fig. 2** and **Suppl. Table 1**). Strikingly, resistance was still strongly selected as low as 1/4 x MIC_S (two-sided t-test: p = 5e-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 an appreciable population size, is expected to spread by outcompeting the sensitive strain over a wide range of streptomycin concentrations, ranging from well below (1/4x or less) to well above (at least 16x) the MIC of the sensitive strain (MIC_S).

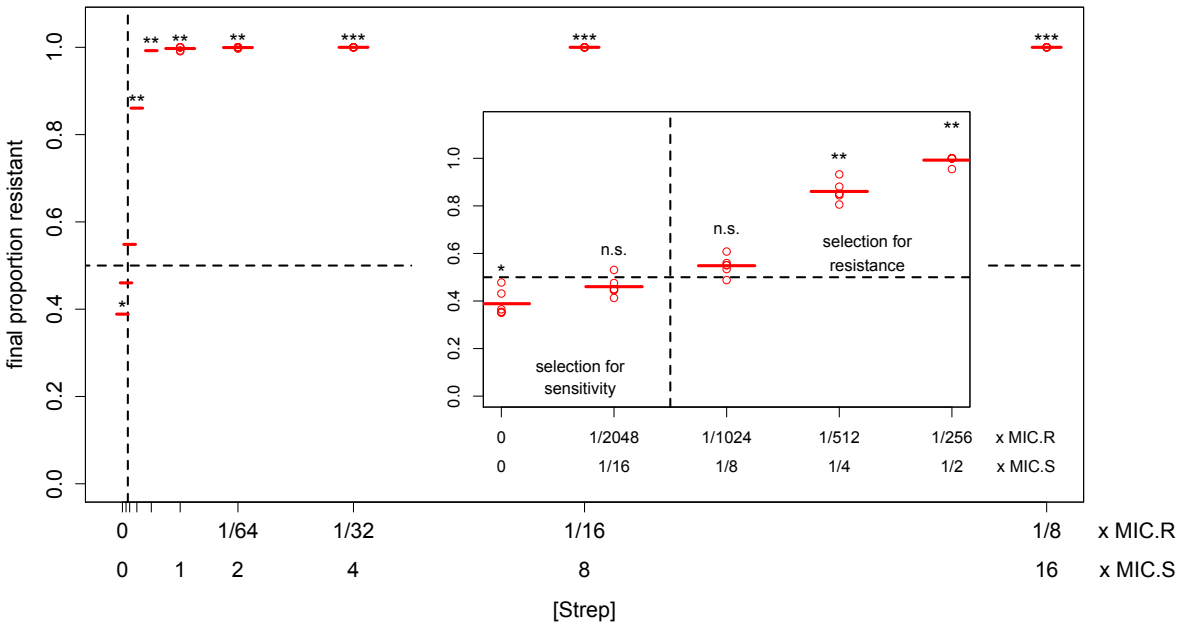

Figure 1: Evaluating selection for resistance by competition assays across streptomycin concentrations. Concentration on the x-axis is scaled by the previously measured MIC values of these strains (sensitive: MIC_S = 16 μg/ml; resistant: MIC_R = 2048 μg/ml; [13]). Cultures were inoculated with a 1:1 volumetric mixture of overnight cultures of the sensitive (DsRed) and resistant (YFP) strains, 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). The inset shows a zoomed-in version at low streptomycin concentration, on a log scale. Points (shown only in the inset for lower concentrations) represent six biologically independent replicates at each concentration, with line segments indicating their mean (see also **Suppl. Table 1**). 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.: $p > 0.05/7$; *: $p = 4e\text{-}3$; **: $p \leq 5e\text{-}6$; ***: lack of variation among replicates precludes a t-test).

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 [14]). 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, the number of cells inoculated into each culture is random, and can be described by a Poisson distribution (**Suppl. Fig. 3**). 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 absolute establishment probability because this quantity cannot be separated from the effect of inoculum size: inoculating on average

two cells, each with 50% chance of establishment, will give equivalent results to one cell with 100% chance of establishment. Therefore, all results are expressed in terms of relative per-cell establishment probability, denoted $\tilde{p}_c$, which is normalized by the result in streptomycin-free culture medium. Thus, $\tilde{p}_c$=1 by definition in streptomycin-free conditions, while we expect streptomycin treatment to yield $\tilde{p}_c \leq 1$; however, values larger than one can arise due to sampling error. Since the distribution of inoculum size is the same in streptomycin-treated and streptomycin-free cultures, any significant 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 2**). 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**).

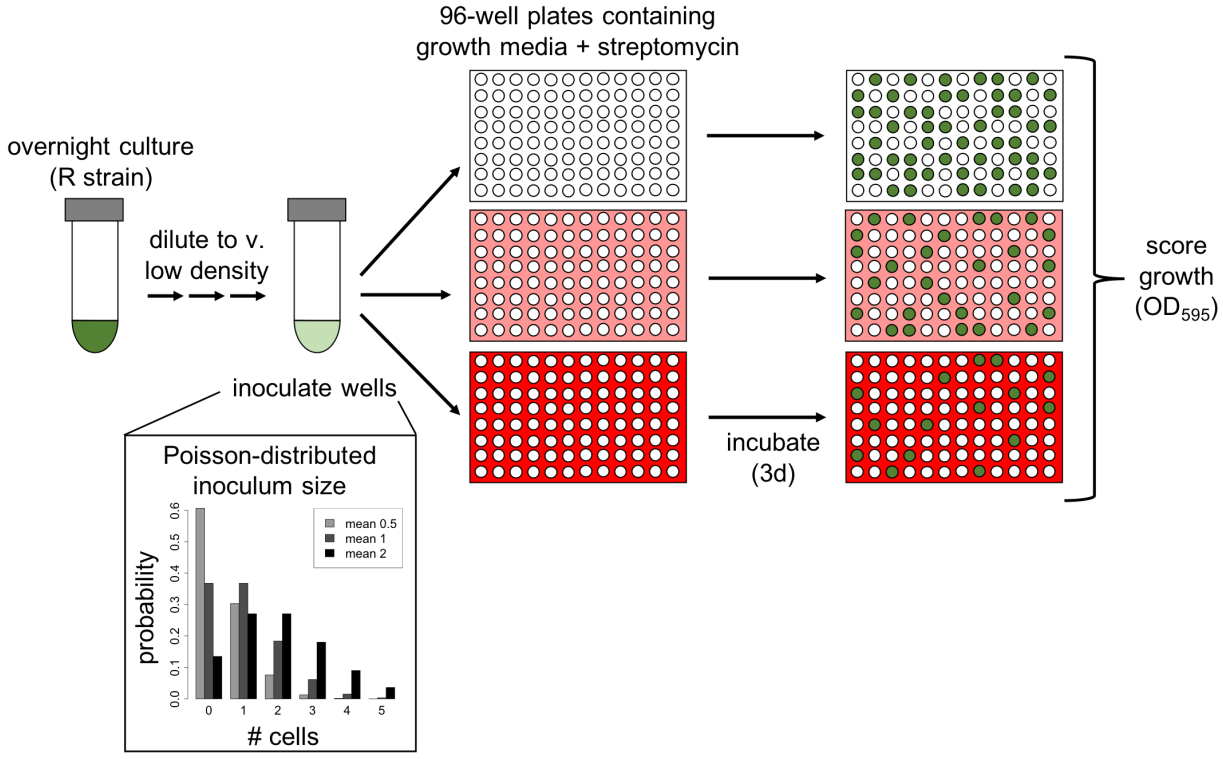

Figure 2: Design of seeding experiment to estimate establishment probability. An overnight culture of the resistant strain is highly diluted and used to inoculate 96-well plates containing growth media (LB) with streptomycin at various concentrations (higher concentration indicated by darker red). The 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**).

(a) **(b)**

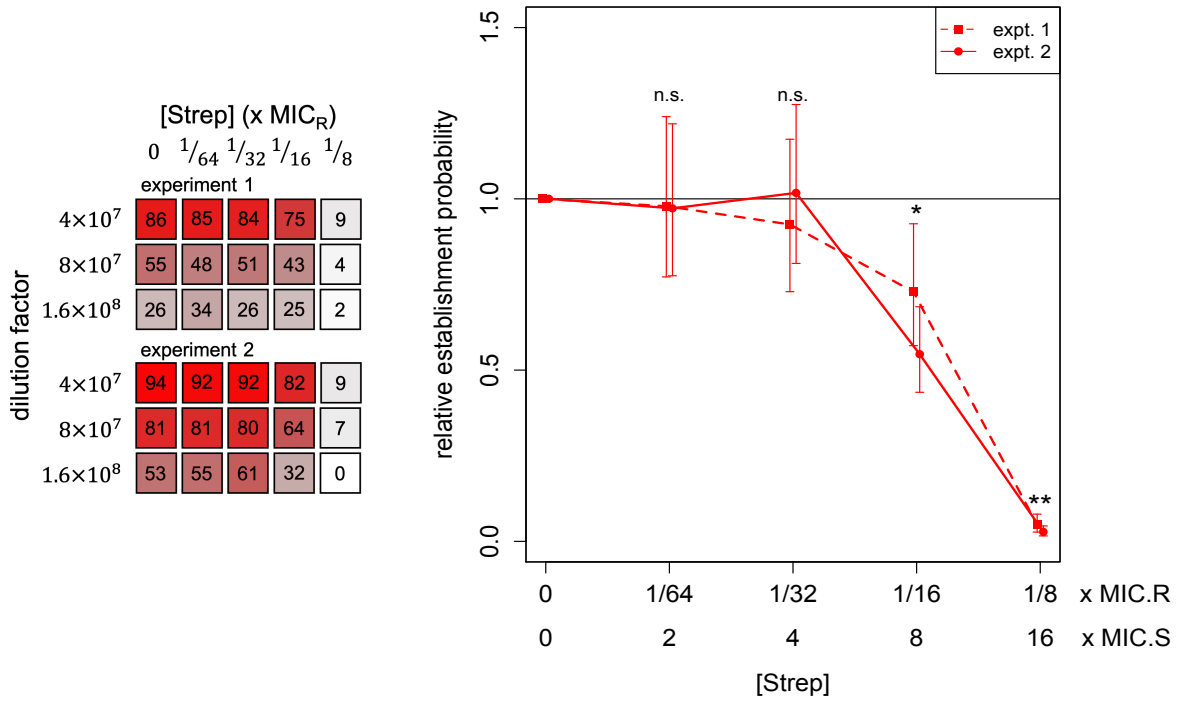

Figure 3: Establishment probability of single resistant cells, estimated from seeding experiments. (a) A visual representation of the growth data, indicating the number of replicate cultures (out of 96) that grew in each test condition. **(b)** The estimated relative per-cell establishment probability $\tilde{p}_c$ of the resistant strain, scaled by the probability in streptomycin-free medium, plotted as a function of streptomycin concentration, scaled by the MICs 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; ** $p < 2e\text{-}16$ 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 [15], 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 [16] [17] [18]. Although less well-explored, it has also occasionally been noted that MIC can decrease when lower inoculum sizes are used [19] [20].

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 increased with inoculum size (**Fig. 4**). This pattern is apparent regardless of whether growth is scored at 20h, as per the standard MIC assay protocol [15], 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. 4**).

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 experiment 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. 5**).

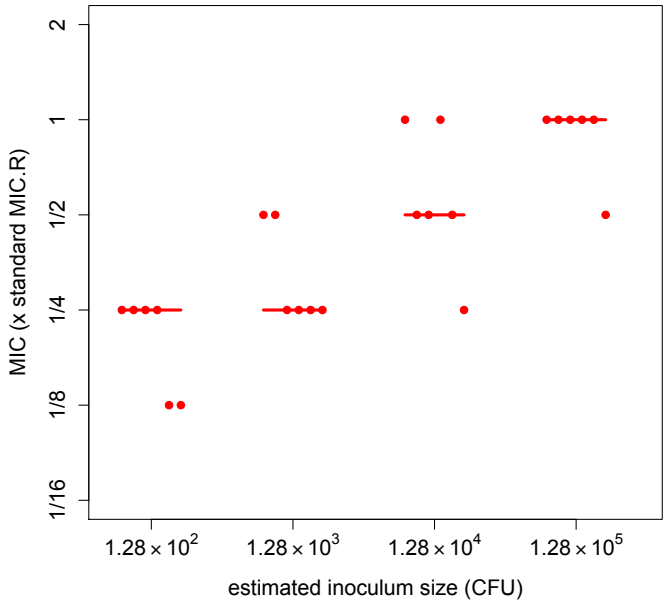

Figure 4: MIC as a function of inoculum size. Test cultures at two-fold concentration steps of streptomycin were inoculated at four different densities. MIC was evaluated as the minimal tested concentration that prevented detectable growth up to 3d post-inoculation; a qualitatively similar pattern arose if growth was evaluated at 20h (**Suppl. Fig. 4**). 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 null 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. 6**).

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. 7**). More precisely, in no case did the null model show significant deviance from the observed proportion of populations that grew (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 2**).

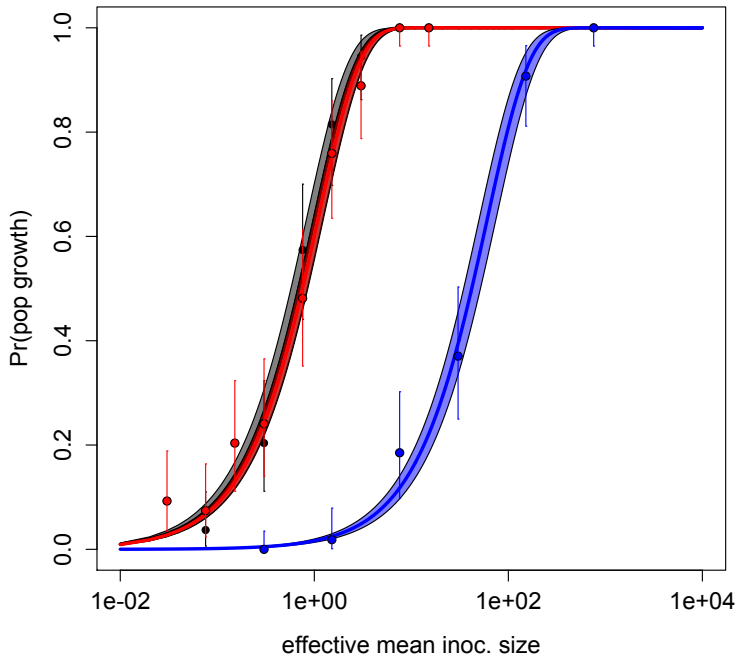

Figure 5: Null model of the inoculum size effect fit to population growth data. Probability of population growth of the resistant strain is plotted as a function of effective mean inoculum size ($\overline{N}_{eff}$, calibrated by the results in streptomycin-free media; see **Suppl. Fig. 6**). Black: 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 found 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. 8** 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-16), and this effect varies over time (ANOVA, interaction term: p < 2e-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-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-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 3 days), 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 3 days.

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 sub-MIC_R concentrations can be explained by a significant fraction of cells dying rather than successfully dividing, which can lead to stochastic loss of initially small populations.

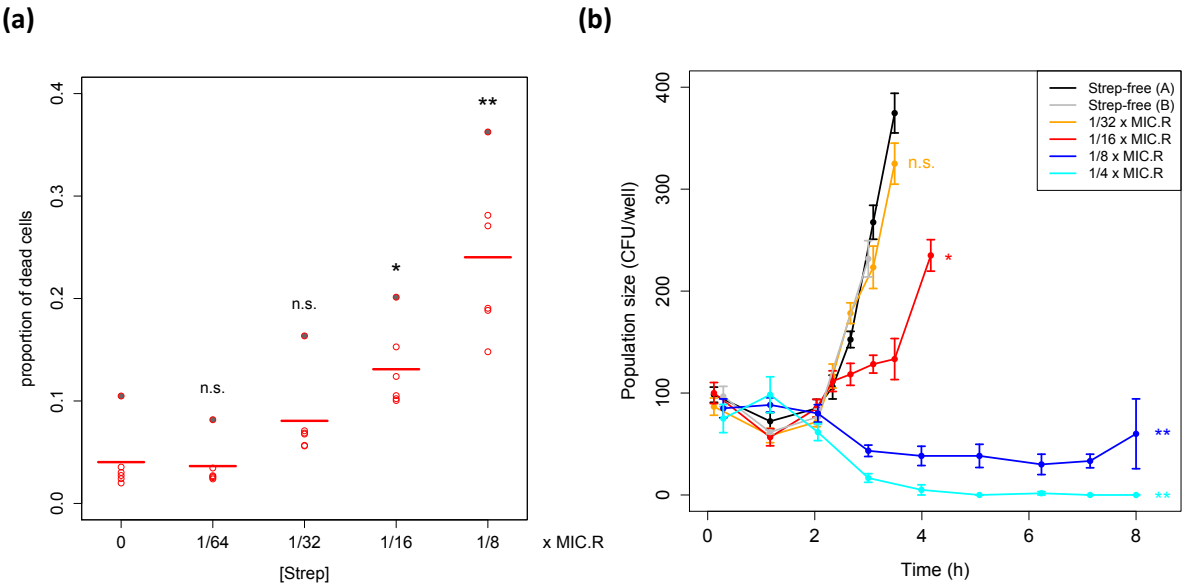

Figure 6: Sub-MIC_R streptomycin treatment has profound effects on resistant cell dynamics. (a) Proportion of dead cells after 7h treatment in sub-MIC_R streptomycin. The proportion of dead cells was estimated using live-dead staining and flow cytometry (**Methods**). Note that this is a minimal estimate of the proportion of dead cells, since propidium iodide staining only detects cells with compromised membranes. The points represent six treatment replicates at each concentration and the line segments indicate their mean. Differences from the streptomycin-free control cultures were assessed using a one-way ANOVA followed by a post-hoc Dunnett's test (n.s.: not significant, $p > 0.05$; *: p = 9e-3, **: $p <$ 1e-4). The significant effects do not change if we exclude an outlier replicate (shaded-in points) showing consistently elevated dead fractions (**Suppl. Table 3**). **(b) Viable cell population dynamics in sub-MIC_R streptomycin.** The plotted points with connecting lines indicate mean population size of six independent replicate cultures per streptomycin concentration and per sampling time point (or twelve replicates for each streptomycin-free control; see **Methods**); the error bars indicate standard error. See **Suppl. Fig. 9** for a version showing all individual replicates. Population size was estimated by plating and truncated when plated colonies became too dense to count. Significance of each streptomycin concentration compared to the streptomycin-free control was assessed by a post-hoc Dunnett's test (n.s.: not significant, p=0.87; * p = 4e-4; ** $p <$ 1e-4).

Discussion

In order for a novel resistant strain to emerge, it must successfully expand from a single cell to form a large population of cells. At the level of individual cells, division and death occur as probabilistic events, and demographic stochasticity should therefore be critical to the fate of newly arisen resistant strains. Our key finding is that these stochastic effects impose a significant barrier to the emergence of antibiotic resistance. 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). One way to quantify the importance of stochasticity is to measure the difference between the range of antibiotic concentrations at which resistance is selectively favoured and the range at which resistance is likely to establish when rare. In this case, we estimate that resistance is selectively favoured, and will on average grow, between 1/512 x and 1 x MIC_R, or 1/4 x and 128 x MIC_S (**Fig. 1** and **Fig. 4**); in contrast, resistance is very unlikely to emerge from a single cell at concentrations $\geq$ 1/8 x MIC_R (**Fig. 3**). Therefore, stochastic effects impose a major constraint on the emergence of resistance across nearly 90% of the mutant selection window.

It is possible that survival of resistant lineages, while appearing stochastic at the macro-scale, is governed by intrinsic differences among cells in their phenotypic level of resistance [21] [22]. Our model is still valid in case of such phenotypic heterogeneity, but the per-cell establishment probability must then be interpreted as an average among cells (**Suppl. Text**). Further investigation of the cell-level factors that influence the fate of lineages represents an interesting avenue for future work.

Standardized MIC assays are a widely used and crucial tool for repeatable evaluation of the susceptibility of bacterial strains to antibiotics [15]. However, the discrepancy between the antibiotic concentration required to reliably prevent growth of a larger population (as in a standard MIC assay) 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 antibiotic concentration-dependent 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) to explain the low probability of emergence of resistance when starting from small inoculum sizes. 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 [16] [17]. This effect can arise via various mechanisms, including titration of antibiotics out of the media [17] and enzymatic inactivation of antibiotics by resistant cells [23] [24] [18] [25] [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 [19] [20], 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 concentrations (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} [26]. In these two facets of the inoculum effect, cells at the limit of low density (cells/ml) where cooperative interactions are abolished [18] 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 [24] [27] [28], 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 [29] [26] [30] [31]. Two of these studies [26] [31] likewise addressed establishment of bacterial cells across antibiotic concentrations, but using different methods to ours (see **Suppl. Text** for a more detailed comparison). Bacterial evolution of resistance to antibiotic treatment is also a prime example of the more general phenomenon of evolutionary rescue, whereby adaptation prevents extinction of populations facing severe environmental change [32]. Indeed, our equation for the probability of population growth (**Eqn. 2**) is identical in form to general approximations for the probability of rescue [33] (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 onset of invasive bacterial infections [34] or the outgrowth of bacteria in food products from small initial contaminants [35].

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 (MSW), above the MIC of the sensitive strain (MIC_S) but still well below the MIC of the resistant strain (MIC_R), can be sufficient to severely limit *de novo* establishment of resistance from single cells. Thus, our findings suggest that moderate doses (between MIC_S and MIC_R) may be more effective than previously thought at preventing emergence of resistance, in cases where the chance of transmitted resistance is negligible. Taken together with our own and previous evidence of sub-MIC_S selection for resistance [7] [8] [9] [10] [13], our work contributes to an emerging picture that resistance tends to arise at a range of concentrations shifted lower than the traditional MSW. Importantly, however, 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 avoid emergence of resistance.

Methods

Bacterial strains, media and culture conditions

Bacterial strains: We used 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 [36]. Streptomycin resistance is conferred by the *aadA5* gene, which is located within a class 1 integron, and codes for an enzyme that adenylates streptomycin [37]. 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 in flat-bottom 96-well microtitre plates 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. Previous work demonstrated that these two markers have equivalent effects on bacterial fitness on LB-agar [13]. Treatment cultures were inoculated with a 1:1 volumetric 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. 1**), 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 the latter 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 interval 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 inoculating dilution factor

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; [15]); 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 two-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 was 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 took 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 over 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). The staining and flow cytometry steps were carried out in groups containing one replicate per concentration plus controls, to avoid potentially toxic effects of stain exposure over prolonged times (**Suppl. Text**). To better discriminate cells from background in the flow cytometry data, 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. 8** for details.

Viable cell density dynamics

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, each of which included a streptomycin-free control; set A additionally included the two lower streptomycin concentrations and set B included the two higher. These sets 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, for counting. Population size in each replicate culture was then estimated by scaling up total CFU count by a factor 10 (since $1/10^{th}$ of the culture volume was sampled). Comparing streptomycin-free controls from sets A and B indicated that the plate set effect was non-significant (ANOVA: $p = 0.103$); thus, controls were pooled for further analysis of the streptomycin effect.

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 highly diluted cultures; **Suppl. Fig. 3**), 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 [38] [39]. 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

medium. We also note that if cells (or possibly clumps of cells acting as individual units) are heterogeneous, then p_c should be interpreted as the mean establishment probability (**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).

Acknowledgements: H.K.A. was supported by an Early Postdoc.Mobility Fellowship (P2EZP3_165188) and an Advanced Postdoc.Mobility fellowship (P300PA_177789) from the Swiss National Science Foundation. R.C.M. was supported by Wellcome Trust Grant 106918/Z/15/Z.

Author contributions: H.K.A. and R.C.M. conceived of the study and designed experiments. H.K.A. carried out experiments and data analysis with advice from R.C.M. H.K.A. developed the

646 mathematical model, wrote the code and carried out model fitting. H.K.A. and R.C.M. wrote the
647 paper.
648
649 **Data availability:** The data generated in this study will be deposited in a public repository upon
650 manuscript acceptance.
651
652 **Code availability:** Custom R scripts for likelihood-based model fitting and comparisons will be made
653 freely available upon manuscript acceptance.
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