Stochastic bacterial population dynamics prevent the emergence of antibiotic resistance from single cells

Helen K. Alexander[1,2,*], R. Craig MacLean[1]

[1] Department of Zoology, University of Oxford, 11a Mansfield Rd, Oxford, OX1 3PS, United Kingdom

[2] Current address: Institute of Evolutionary Biology, University of Edinburgh, Charlotte Auerbach Rd, Edinburgh, EH9 3FL, United Kingdom

* Corresponding author: helen.alexander@ed.ac.uk

Abstract:

A better understanding of how antibiotic exposure impacts the evolution of resistance is crucial for designing more sustainable treatment strategies. The conventional approach to relating antibiotic dose to resistance evolution within a bacterial population is to measure the range of concentrations over which resistant strain(s) are selectively favoured over a sensitive strain – the "mutant selection window". Here, we instead investigate how antibiotic concentration impacts the initial establishment of resistance from single cells, mimicking the clonal expansion of a resistant lineage following mutation or horizontal gene transfer. Using two *Pseudomonas aeruginosa* strains carrying distinct resistance plasmids, we show that single resistant cells have <5% probability of outgrowth at antibiotic concentrations as low as 1/8th of the resistant strain's minimum inhibitory concentration. This low probability of establishment is due to detrimental effects of antibiotics on resistant cells, coupled with the inherently stochastic nature of cell division and death on the single-cell level, which leads to loss of many nascent resistant lineages. Our findings suggest that moderate doses of antibiotics, within the traditional mutant selection window, may be more effective at preventing *de novo* emergence of resistance than predicted by deterministic approaches.

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

Significance statement:

The emergence of antibiotic resistance poses a critical threat to the efficacy of antibiotic treatments. A resistant bacterial population must originally arise from a single cell that mutates or acquires a resistance gene. This single cell may, by chance, fail to successfully reproduce before it dies, leading to loss of the nascent resistant lineage. Here we show that antibiotic concentrations that selectively favour resistance are nonetheless sufficient to reduce the chance of outgrowth from a single cell to a very low probability. Our findings suggest that lower antibiotic concentrations than previously thought may be sufficient to prevent, with high probability, emergence of resistance from single cells.

41 # Introduction

42

43 Antibiotics have had a huge impact on human health by reducing the burden associated with
44 bacterial infections, and the use of antibiotics now underpins many areas of medicine.
45 Unfortunately, antibiotic treatment is also associated with the evolution of resistance [1], resulting
46 in poorer patient outcomes [2]. A better understanding of how antibiotic dosing affects resistance
47 evolution could aid the design of more effective treatment strategies that suppress pathogenic
48 bacteria without driving the emergence of resistance.

49

50 To date, *in vitro* work addressing how antibiotics affect evolution of resistance has focused on
51 identifying the range of antibiotic concentrations at which resistant mutants are selectively favoured
52 by antibiotic treatment, known as the "mutant selection window" (MSW) [3, 4, 5, 6]. Here we will
53 refer to any strain with reduced susceptibility relative to a wild-type ("sensitive") strain simply as
54 "resistant", as is common in evolutionary microbiology literature (e.g. [7, 8, 9]), as opposed to
55 defining resistance with respect to clinical breakpoints. Originally, the lower boundary of the MSW
56 was approximated by the minimum inhibitory concentration of the sensitive strain (MIC_S) [3, 4, 5, 6],
57 i.e. the lowest antibiotic concentration that abolishes its growth in a standardized assay (such as
58 [10]). However, more recent work has emphasized that a resistant strain can be selectively favoured
59 down to a minimal selective concentration (MSC) that is often well below MIC_S [11, 12, 13, 7, 8]. The
60 upper boundary of the MSW is conventionally defined by the lowest antibiotic concentration that
61 prevents growth of all mutant subpopulations [3, 4]. This upper bound is often equated with the
62 minimum inhibitory concentration of the most resistant single-point mutant [5] or of a specific
63 resistant strain under study [13, 8], which we denote MIC_R. The MSW therefore ranges between
64 antibiotic concentrations that are so low that they are unlikely to have any clinical benefit (below
65 MIC_S) and very high concentrations (up to MIC_R) that may be difficult to achieve in practice because
66 of physiological constraints on the accumulation of antibiotics in tissues (pharmacokinetics) and
67 toxic side-effects of antibiotics [14, 15].

68

69 Selection operates efficiently when both sensitive and resistant populations are large, resulting in an
70 increase in frequency of the fitter strain in an antibiotic dose-dependent manner. Correspondingly,
71 the mutant selection window is typically measured by direct competition between large numbers of
72 cells (typically $>10^4$ colony-forming units, CFU) of both resistant and sensitive strains across a
73 gradient of antibiotic concentrations (e.g. [13]). This provides a powerful approach to measure the
74 impact of antibiotic dose on selection for established resistant strains, i.e. those that are already

reasonably prevalent. However, *de novo* emergence of resistant strains, when not initially present, should be subject to stochastic processes [16] that are not reflected by the MSW, nor captured by this experimental design. First, resistance must stochastically arise in a sensitive cell by mutation, or by acquisition of a resistance gene through horizontal gene transfer. Next, the single resistant cell thus generated must survive and successfully divide to produce daughter cells that likewise survive, and so on to generate a large number of resistant descendant cells. The latter process, which we will refer to throughout as "establishment" of resistance [16], will be our focus here. Importantly, due to the stochastic nature of cell divisions and deaths on the individual level, establishment is not guaranteed, even under conditions in which the resistant strain is selectively favoured [17]. Moreover, if antibiotics even partially inhibit the resistant strain below its MIC_R, the chance that a resistant cell dies or fails to divide, and thus the risk that a resistant lineage is stochastically lost, should increase in the presence of antibiotics at concentrations within the MSW. Despite the substantial body of work addressing the selection of resistance, very little work has addressed the stochastic establishment phase (see however [18, 19, 20]).

We set out to quantify stochastic establishment using *in vitro* experiments with *Pseudomonas aeruginosa*, an important opportunistic pathogen that evolves resistance at an exceptionally high rate during infections [1, 21]. To isolate the establishment phase, we inoculated hundreds of cultures, each with a very small number of resistant cells (on average, approx. 1-3), and assessed culture growth. We tested two strains carrying non-conjugative plasmids (Rms149 and PAMBL2) that confer resistance to streptomycin and meropenem, respectively, across a gradient of the corresponding antibiotic concentrations within the MSW. By fitting mathematical models to these data, we estimated the probability of establishment, i.e. of detectable culture growth due to clonal expansion from a single resistant cell, as a function of antibiotic concentration. Our key finding is that the establishment probability of resistant cells drastically declines at concentrations well below the MIC of the resistant strain, reaching $\lesssim$5% at $1/8^{th}$ of MIC_R in both systems. These concentrations lie well above the corresponding MIC values of the sensitive strain and within the conventional mutant selection window. Our results highlight that antibiotic selection pressure is not a sufficient condition for *de novo* emergence of resistance starting from single cells. Accounting for the demographic stochasticity inherent to the outgrowth of mutant lineages substantially narrows the window of concentrations at which resistant mutants are likely to establish, suggesting that moderate antibiotic dosing may be an effective strategy to prevent the emergence of resistance.

Results

Establishment of resistance is inhibited by sub-MIC$_R$ antibiotic concentrations

To elucidate the direct impact of antibiotics on resistant cells, we first investigated establishment of a resistant strain in the absence of a sensitive strain. We first focused on the streptomycin-resistant PA01:Rms149 strain. To estimate its probability of establishment, defined as outgrowth of a detectable (i.e. large) population from a single cell, we conducted large-scale "seeding" experiments (see also [20]). In this assay (**Fig. 1**), a highly diluted overnight culture of the resistant strain is inoculated into fresh media in a large number of replicate cultures. The high dilution factors yield average inoculum sizes of <1 to ~3 cells per culture. Importantly, however, the actual number of cells inoculated into each replicate culture is random, and can be described by a Poisson distribution (**SI Appendix, Fig. S1**). One implication of this protocol is that many cultures are not inoculated with any cells, while others receive more than one cell; our modelling approach will account for this variation statistically. We inoculated parallel replicate cultures in streptomycin-free media and at a range of streptomycin concentrations below the MIC of the resistant strain, denoted MIC$_R$, as measured using standard protocols [10] (**SI Appendix, Table S1**). We then scored the number of replicate cultures showing growth based on reaching a threshold optical density (OD) of 0.1 within 3 days post-inoculation.

A culture could fail to grow either because the inoculum did not contain any cells, or because every cell in the inoculum failed to give rise to a surviving lineage. To infer the probability that a single cell yields detectable population growth (i.e. the per-cell establishment probability), we fit a mathematical model, accounting for both the random inoculum size and demographic stochasticity, to the observed number of replicate cultures showing growth (**Materials and Methods**). All probabilities are normalized by the result in streptomycin-free media, which corresponds to scaling inoculum size by the mean number of cells that establish in benign conditions (which we call the "effective" inoculum size). Thus, relative establishment probability $\tilde{p}_c$ equals one by definition in streptomycin-free conditions, while we expect $\tilde{p}_c \leq 1$ with streptomycin treatment; however, values larger than one can arise due to sampling error.

Our seeding experiments revealed that the probability of establishment of a single resistant cell declines with increasing streptomycin concentration (**Fig. 2** and **SI Appendix, Table S2**). While exposure to the lowest tested concentrations of streptomycin (up to 1/32 x MIC$_R$) had no detectable

impact on establishment, 1/16 x MIC_R was already sufficient for significant declines, to $\tilde{p}_c$ of 55-73% (maximum likelihood estimates in two independent experiments). At 1/8 x MIC_R, $\tilde{p}_c$ dropped to just 3-5%. These results suggest that a *de novo* resistant mutant would only rarely establish at antibiotic concentrations that are well below its MIC_R, i.e. within the MSW.

MIC depends on inoculum size

The frequent failure of the resistant strain to grow in our seeding experiments at concentrations well below its MIC is, at face value, surprising. We hypothesized that these results could be explained by the difference in inoculum size between these assays. Specifically, standard MIC values are assessed from an inoculation density of $5x10^5$ CFU/mL [10], which corresponds to an inoculum size of 10^5 CFU per 200µl culture on our microtitre plates. In contrast, our seeding experiments used an inoculum size on the order of 1 CFU per culture. MIC for many antibiotics has been observed to increase with higher-than-standard inoculation densities (CFU/ml) [22, 23, 24] which corresponds to higher absolute inoculum size (CFU) for a fixed culture volume. Although less well-explored, it has also occasionally been noted that MIC can decrease when lower absolute inoculum sizes are used [25, 26].

To test the hypothesis that inoculum size influences MIC in the present system, we conducted a modified MIC assay using the PA01:Rms149 strain with inoculum sizes ranging over three orders of magnitude, from approximately 10^2 to 10^5 CFU per culture (corresponding to inoculation densities of $5x10^2$ up to the standard $5x10^5$ CFU/ml). We found that MIC indeed increases with inoculum size (**Fig. 3a**). This pattern arises regardless of whether growth is scored at 20h, as per the standard MIC assay protocol [10], or up to 3d post-inoculation, as in our seeding experiments, although the number of cultures showing detectable growth, and thus the measured MIC, tends to increase over time (**SI Appendix, Fig. S2**).

Since all cultures contained the same volume in the above experiment, this pattern could be due to changes either in absolute inoculum size (i.e. CFU) or in inoculation 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 interactions among cells (e.g. 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. In contrast, inoculation density per unit volume does not have a significant effect within the range that we tested, after controlling for absolute cell numbers (**SI Appendix, Fig. S3**).

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

Taken together, our seeding experiments and MIC assays reveal that the absolute number of cells in the inoculum has a strong effect on whether the culture eventually shows detectable growth. The simplest explanation for this result is that population growth can be attributed to the stochastic outgrowth of one or more lineages, each initiated by a single cell in the inoculum, acting independently. This independence assumption yields a "null model" that mathematically describes the effect of inoculum size on the probability of outgrowth of a detectable population (**Materials and Methods**, **Eqn. 1**). Here the probability of establishment of each cell in the inoculum ($\tilde{p}_c$) is a scaling parameter, which does not depend on inoculum size. Note that this null model would not hold if interactions among cells substantially influenced their chances of successful replication. For example, if cells secrete an enzyme that breaks down an antibiotic extracellularly, then the establishment probability of each cell could increase with inoculum size. On the other hand, if cells compete for limiting resources or secrete toxins, the per-cell establishment probability could decrease with inoculum size.

To formally test the null model, we again conducted seeding experiments with the PA01:Rms149 strain, but now using many different inoculum sizes, spanning approximately three orders of magnitude. We tested two streptomycin concentrations (1/16 and 1/8 x MIC_R) for which growth often failed from a single cell, but succeeded from standard inoculum size in MIC assays. In parallel, we tested growth in streptomycin-free media in order to estimate the effective mean inoculum size (**SI Appendix, Fig. S4**). This left one free parameter, the per-cell relative establishment probability ($\tilde{p}_c$), to fit at each streptomycin concentration.

We found good agreement between the null model and our experimental data at all tested streptomycin concentrations, consistent with the hypothesis that cells establish independently (main experiment, **Fig. 3b**, and repeat experiments, **SI Appendix, Fig. S5**). More precisely, the null model did not show significant deviance from the observed proportion of populations that grew

211 (according to the likelihood ratio test), and thus we accept it as a parsimonious explanation for the
212 data. Furthermore, we obtain estimates of relative establishment probability, $\tilde{p}_c$, at 1/16 and 1/8 x
213 MIC_R similar to those from the previous seeding experiments (**SI Appendix, Table S2**).
214
215 To summarize, the probability of culture growth at any given streptomycin concentration depends
216 on inoculum size, according to a simple quantitative relationship. Our experimental data are
217 consistent with a simple model of cells behaving independently, such that a fixed per-cell
218 establishment probability can explain our growth data across inoculum sizes. That is, cells are not
219 "more susceptible" to streptomycin at lower inoculum sizes, but rather, culture growth is less likely
220 to be observed simply because fewer cells are available to establish, and not all cells succeed. In
221 turn, the minimal concentration of streptomycin required to prevent growth in some proportion of
222 replicate cultures (i.e. the observed MIC) increases with inoculum size.
223
224
225 ***Sub-MIC_R streptomycin concentrations induce resistant cell death and extend lag phase***
226
227 We hypothesized that resistant cells sometimes failed to establish in our seeding experiments
228 because exposure to streptomycin compromised cell division rate and/or viability. As a simple test
229 of this idea, we measured the relative abundance of dead cells in cultures of the resistant strain
230 grown at sub-MIC_R concentrations of streptomycin. We found that the fraction of dead cells after 7h
231 of treatment, as determined by propidium iodide staining, increased from an average of 3-4% in
232 streptomycin-free conditions to >20% at 1/8 x MIC_R streptomycin (**Fig. 4a** and **SI Appendix, Fig. S6**
233 and **Table S3**). Note that this is a conservative measure of cell death, because this assay only detects
234 cells that have compromised membrane permeability, and not, for example, cells that have already
235 lysed. Furthermore, this assay provides only a snapshot in time.
236
237 To gain further insight into how sub-MIC_R streptomycin impacts the population dynamics of the
238 resistant strain, we quantified viable cell density over the first few hours after inoculation into
239 streptomycin-containing media. Cultures were inoculated with approximately 100 cells in this
240 experiment, to ensure that cell numbers were low enough for demographic stochasticity to be
241 relevant, yet large enough to be detectable using conventional plating methods.
242
243 We found that streptomycin treatment has a significant effect on the growth of resistant cultures
244 (ANOVA, main effect: p < 2e-16), and this effect varies over time (ANOVA, interaction term: p < 2e-

245 16; **Fig. 4b** and **SI Appendix, Fig. S7**). Following inoculation, cultures exhibited a lag phase of
246 approximately 2 hours. Control cultures in streptomycin-free media then began to grow
247 exponentially. The lowest tested concentration of streptomycin (1/32 x MIC_R) had no significant
248 effect on these dynamics (Dunnett's test: p=0.87); however, 1/16 x MIC_R was already sufficient to
249 slow growth (p=4e-4). Nonetheless, all replicate cultures (n=48 per concentration) eventually grew,
250 as detected by OD. Meanwhile, higher doses of streptomycin (1/8 x or 1/4 x MIC_R) had dramatic
251 effects on growth dynamics (p < 1e-4), with cultures exhibiting an extended lag phase of at least 7-8
252 hours, in which viable cell density initially declined. After further incubation (up to 3 days), 25% of
253 cultures (15/60) exposed to 1/8 x MIC_R eventually showed growth, while the remaining 75% (45/60)
254 failed to reach detectable OD. At 1/4 x MIC_R, no viable cells were detected in most cultures from 4h
255 on, and only 1/60 cultures reached detectable OD within 3 days.
256
257 In summary, sub-MIC_R streptomycin treatment has the effect of extending the lag phase, before
258 cultures eventually either grow to saturation or die out. Failure to grow can be explained by
259 significantly elevated cell death rates beginning at 1/16 x MIC_R, which can lead to stochastic loss of
260 initially small populations.
261
262
263 ***Stochastic establishment is recapitulated for a clinically relevant antibiotic and resistance plasmid***
264
265 If the frequent failure of resistant cells to establish surviving populations at antibiotic doses well
266 below their MIC is a general phenomenon, it would have important implications for understanding
267 the emergence of resistance during antibiotic treatment. To confirm that our result was not driven
268 by the specific choice of antibiotic or resistance mechanism, we repeated the key seeding
269 experiment using a *P. aeruginosa* PA01 strain carrying a recently isolated multi-drug resistance
270 plasmid, PAMBL2 [27, 28], that confers resistance to meropenem through the $bla_{\text{vim-1}}$
271 carbapenemase. Carbapenems are an important treatment option for serious infections caused by
272 gram-negative bacterial pathogens, and resistance is of current clinical concern [29, 30];
273 carbapenem-resistant *P. aeruginosa* has been identified as a "critical priority" for new antibiotic
274 development by the WHO [31]. In agreement with our previous findings, the establishment
275 probability of PA01:PAMBL2 cells declined at concentrations of meropenem well below this strain's
276 MIC_R (**SI Appendix, Table S1**), reaching ~5% at 1/8 x MIC_R, while no establishment was observed at
277 1/4 x MIC_R (**Fig. 5** and **SI Appendix, Table S4**). This result highlights that the stochastic loss of
278 resistant cells is not unique to our primary model system of PA01:Rms149 in streptomycin.

279
280
281 ***The sensitive population modulates probability of establishment of resistant cells***
282
283 So far, we focused on the direct effects of antibiotics on resistant cells by conducting experiments
284 with monocultures of resistant strains. However, *de novo* resistance will actually arise within a
285 sensitive population, by mutation or transfer of a mobile genetic element into a sensitive cell.
286 Moreover, antibiotic treatment will only begin in clinical settings once the total pathogen population
287 is large enough to cause symptoms. We therefore asked whether the presence of a large sensitive
288 population affects the establishment of initially rare resistant cells during antibiotic treatment,
289 returning to the interaction between PA01 (sensitive) and PA01:Rms149 (streptomycin-resistant) as
290 a model system.
291
292 We expect the sensitive population and the antibiotic to have interacting effects on establishment of
293 resistance. In particular, at sufficiently low antibiotic concentrations, a sensitive strain is generally
294 expected to outcompete a resistant strain due to the fitness cost associated with resistance [13, 7,
295 8]. We confirmed this expectation in our experimental system using a standard competition assay,
296 where both strains start from reasonably large inoculum sizes (**SI Appendix, Fig. S8-S10** and **Table**
297 **S5**). We found that the sensitive strain is favoured up to a minimum selective concentration (MSC)
298 between 1-2 μg/ml streptomycin (equivalent to 1/32 – 1/16 x MIC_S, or 1/2048 – 1/1024 x MIC_R), in
299 agreement with previous results for these strains [32]. We hypothesized that competition from the
300 sensitive strain would prevent establishment of resistance at streptomycin concentrations below the
301 MSC.
302
303 As a simple test of this idea, we modified the seeding experiment to inoculate very few resistant
304 cells into a large sensitive population. Since bacterial densities in clinical infections can vary widely
305 [9, 33], we inoculated the sensitive strain at two different densities: approximately 5 x 10^5 CFU/ml
306 (as in a standard MIC assay; labelled "low") and 5 x 10^7 CFU/ml (labelled "high"). The resistant strain
307 was seeded, with mean inoculum size on the order of one cell per culture, immediately thereafter.
308
309 As hypothesized, we found that the presence of the sensitive population (at either density)
310 abolished establishment of resistant cells in the absence of streptomycin (**Fig. 6** and **SI Appendix,**
311 **Fig. S11** and **Table S6**). Meanwhile, at streptomycin concentrations above the MSC (1/256 to 1/8 x
312 MIC_R, or 1/4 to 8 x MIC_S), adding the sensitive population at low density had a negligible effect on

313 the probability of establishment of resistant cells. At high density, the sensitive population also had
314 negligible effects on establishment of resistance at streptomycin concentrations up to 1/16 x MIC_R (4
315 x MIC_S). However, at 1/8 x MIC_R (8 x MIC_S), the presence of a high-density sensitive population
316 increased the establishment probability from near zero to 65%. To confirm and further probe the
317 extent of this apparent protective effect, we repeated the experiment over a higher range of
318 streptomycin concentrations. The boost in establishment probability was repeatable and highly
319 significant at 1/8 x MIC_R (Wilcoxon rank-sum test, high- vs. zero or low-density sensitive: $p < 5e\text{-}8$ in
320 both experiments). However, at 1/4 x MIC_R (16 x MIC_S), an apparent slight boost in establishment
321 probability was non-significant, and by 1/2 x MIC_R (32 x MIC_S) the effect was abolished. Thus, a
322 sufficiently dense sensitive population can extend the range of streptomycin concentrations at
323 which the resistant strain is likely to emerge, but does not change the qualitative pattern of
324 stochastic establishment.
325
326

327 Discussion

328
329 In order for resistance to emerge *de novo*, not only must a resistance gene arise in a bacterial
330 population by mutation or horizontal gene transfer; this first resistant cell must also successfully
331 expand to form a large population. Since any individual cell may fail to replicate, particularly in
332 challenging environmental conditions, the expansion of newly arisen resistant strains is not
333 guaranteed. Our key finding is that demographic stochasticity imposes a significant barrier to the
334 emergence of resistance in the presence of antibiotics at concentrations within the mutant selection
335 window.
336
337 We empirically demonstrated the importance of stochasticity with a simple "seeding experiment"
338 mimicking the growth of clonal resistant lineages founded by a single cell. First, to assess the direct
339 impact of antibiotics, we inoculated fresh antibiotic-containing media with approximately one
340 resistant cell per replicate culture and quantified the per-cell probability of establishing a detectable
341 population. Strikingly, this establishment probability dropped off at concentrations well below the
342 MIC of the corresponding resistant strain (MIC_R). For example, the establishment probability of
343 PA01:Rms149 was significantly reduced by streptomycin concentrations as low as 1/16 x MIC_R, and
344 dropped to <5% at 1/8 x MIC_R (**Fig. 2**). Resistant cells failed to establish viable populations because of
345 the toxic effects of exposure to sub-MIC_R concentrations of antibiotics (**Fig. 4**) coupled with the
346 inherently stochastic nature of individual cell death and division. Importantly, we were able to

347 replicate our key finding of frequent stochastic loss using a different, meropenem-resistant strain
348 (PA01:PAMBL2; **Fig. 5**). This demonstrated that our results are not limited to a particular model
349 system, but are also relevant to bacterial pathogens of clinical concern: carbapenem-resistant *P.*
350 *aeruginosa* is considered by the WHO to be of "critical priority" for antibiotic development [31].
351
352 In clinical settings, antibiotic treatment will typically begin only when the total bacterial population is
353 large enough to cause symptoms. Assuming resistance has not been transmitted, this population will
354 be predominantly antibiotic-sensitive, and *de novo* mutation or acquisition of a mobile element
355 conferring resistance will occur within a sensitive cell. We therefore next asked how the presence of
356 a large sensitive population would combine with the above effects of antibiotics to shape the
357 emergence of resistance from this first cell, again in the streptomycin model system. As predicted
358 from standard competition assays, emergence of resistance was abolished in the absence of
359 antibiotics (**Fig. 6**), presumably due to competitive suppression by the sensitive strain [8]. More
360 interestingly, a sufficiently dense sensitive population (inoculated at ~5 x 10^7 CFU/ml here) was able
361 to shift the range of concentrations at which resistance established upwards by approximately two-
362 fold. We speculate that this apparent protection is due to sensitive cells absorbing antibiotics, thus
363 lowering their concentration in the media [23, 34], despite these concentrations being high enough
364 to cause decline of the sensitive population (i.e. > MIC_S). *A priori*, one may not expect resistant cells
365 to "need" protection at sub-MIC_R antibiotic concentrations. However, in the stochastic regime of
366 establishment, any increase in the probability of individual cells surviving and dividing can make a
367 qualitative difference to the fate of a rare resistant lineage. This protective effect depends on
368 bacterial density at the time of treatment, within a realistic range for some bacterial infections [9,
369 33]: when the sensitive population was inoculated at 100-fold lower density (~5 x 10^5 CFU/ml),
370 protection was not apparent. We emphasize that although these experiments provide an initial
371 proof of concept, a complete investigation of the interacting effects of sensitive population density,
372 antibiotic dose and timing remains an important direction for future work. Importantly, however,
373 our main message continues to hold even in the more realistic context of resistance arising within a
374 sensitive population: stochastic loss of resistant cells is frequent at antibiotic concentrations within
375 the MSW.
376
377 The failure of resistant cells to establish successful lineages at concentrations well below the MIC_R
378 shows a clear disconnect between antibiotic susceptibility of individual cells and populations. To
379 explain this effect rigorously, we quantified the probability of outgrowth of a detectable population
380 at a fixed streptomycin concentration, starting from inoculum sizes spanning three orders of

magnitude. We fit these data to a mathematical model relating inoculum size to probability of population growth, under the hypothesis that each cell in the inoculum behaves independently (**Eqn. 1**). This simple stochastic model, with a constant per-cell probability of establishment ($\tilde{p}_c$), provides a good explanation for inoculum size-dependent population growth in PA01:Rms149 (**Fig. 3b**). In this model, individual cells are not "more susceptible" to antibiotic in smaller populations. Instead, the cumulative effect of many cells, each with a small chance of establishment (e.g. <5% at 1/8 x MIC_R in this system), virtually guarantees population growth from a sufficiently large inoculum size, reconciling our results with the standard definition of the MIC. We thus emphasize that MIC is not an innate property of a cell or strain, but rather an emergent property of a population of cells. We also note that the inoculum size effect on MIC that we found here – a purely stochastic phenomenon arising at low absolute numbers (CFU) – is distinct from the inoculum size effect already widely recognized in the literature, which is seen at high cell density (CFU/ml) and attributed to various density-dependent mechanisms, such as titration or enzymatic inactivation of antibiotics [35, 36, 24, 37, 32, 23, 38]. Although there are hints of the former absolute-number effect in earlier studies [25, 26], to our knowledge we are the first to provide a rigorous explanation in terms of stochastic population dynamics.

The good fit of the null model, in which every cell has an equal probability of establishment, at first seems to be at odds with the growing recognition that bacteria exhibit phenotypic heterogeneity, which could affect individual cells' susceptibility to antibiotics [39, 40]. Indeed, it is entirely possible that the resistant cells that successfully established in our experiments were those with a particular metabolic state or gene expression level. However, this variability among cells would have no effect on our experimental outcomes: under our mathematical model (**Eqn. 1**), the probability of observing growth in a given number of replicate cultures is the same for any degree of cell-to-cell variation around a fixed mean, assuming the susceptibilities of cells within an inoculum are independent from one another (**Suppl. Text**, section 10.1). Thus, the establishment probability that we infer empirically should more accurately be interpreted as a mean among cells.

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 [17]. Our study joins a small handful of others that have now experimentally quantified establishment probability from single cells [41, 18, 42, 19], including two [18, 19] investigating establishment of bacterial cells in the presence of antibiotics, using different methods to ours (see **Suppl. Text**, section 10.2, for a more detailed comparison). Bacterial evolution of resistance to

415 antibiotic treatment is also a prime example of the more general phenomenon of evolutionary
416 rescue, whereby adaptation prevents extinction of populations facing severe environmental change
417 [43]. Experiments quantifying the probability of rescue in initially large, but declining, populations
418 were pioneered around a decade ago in yeast exposed to high salt concentrations [44]. More recent
419 work clarified the quantitative relationship between initial population size and probability of rescue
420 [45], coinciding with our **Eqn. 1** (see **Suppl. Text**, section 10.2, for further discussion). More broadly,
421 the concepts and statistical methods developed here are applicable to a variety of situations where
422 growth depends on success of rare cells and is thus highly stochastic, for instance the establishment
423 of productive infection in a host following pathogen transmission [46], the onset of invasive bacterial
424 infections [47], the outgrowth of bacteria in food products from small initial contaminants [48], or
425 the establishment of metastases from cancerous tumours [49].
426
427 In summary, our study highlights the stochastic nature of *de novo* emergence of antibiotic
428 resistance. In a practical sense, this stochasticity implies that to accurately assess the risk of
429 resistance emerging, we must evaluate not only mutation rates, but also the probability that
430 resistant mutants escape extinction when rare [9], which will depend on the antibiotic dosing
431 regimen. Our results caution against the naïve use of the mutant selection window (MSW) for this
432 purpose. While a positive selection coefficient is a necessary condition for resistance to outcompete
433 an initially prevalent sensitive strain, it does not guarantee emergence when rare; indeed, we
434 showed that single resistant cells are frequently lost at antibiotic concentrations well within the
435 MSW. Thus, our findings suggest that moderate antibiotic doses may be more effective than
436 previously thought at preventing *de novo* emergence of resistance, especially in infections where
437 total pathogen density is relatively low. For antibiotics that have mutagenic effects, the chance of a
438 resistant lineage arising in the first place might also be reduced at lower doses ([50]; see however
439 [51]). Furthermore, use of lower antibiotic doses could reduce both adverse effects on patients and
440 release of antibiotics into the environment [52].
441
442

443 Materials and Methods

444
445 Further details of experimental protocols, data processing, mathematical models and statistical
446 methods are provided in the **SI Appendix, Suppl. Text**.
447
448 ***Bacterial strains, media, and culture conditions***

449

450 Bacterial strains: The majority of our experiments, in streptomycin, were conducted with a set of
451 *Pseudomonas aeruginosa* PA01 strains studied previously [32]. The streptomycin-sensitive and -
452 resistant strains are chromosomally isogenic, while resistant strains additionally carry the clinically
453 derived, non-conjugative plasmid Rms149 [53], which is stably maintained in PA01 at approximately
454 two copies per cell [54]. Streptomycin resistance is conferred by the *aadA5* gene on Rms149, which
455 codes for an enzyme that adenylates streptomycin [55]. Both plasmid carriers (resistant) and non-
456 carriers (sensitive) are available with either YFP or DsRed chromosomal fluorescent markers or with
457 no marker [32]. The live-dead staining experiment was conducted with the unlabelled resistant
458 strain. All other experiments reported in the main text were conducted with the YFP-labelled
459 resistant strain and, where applicable, the DsRed-labelled sensitive strain. We chose this pairing
460 because YFP provides a stronger signal, facilitating detection of the resistant strain in mixed cultures.
461 Previous work with these strains suggests that the two fluorescent labels have similar fitness effects
462 [32], and we confirmed that the label had no substantive effect on the MIC values of the sensitive
463 strain (**Suppl. Text**, section 2.1). For the seeding experiment in meropenem, we transformed the
464 plasmid PAMBL2 into the same PA01-YFP background (**Suppl. Text**, section 1). This plasmid, isolated
465 in 2007 from a patient in a Spanish hospital [27], confers meropenem resistance through three
466 copies of the bla_{VIM-1} gene, which codes for a metallo-beta-lactamase [27, 28]. It is non-conjugative
467 [28] and stably maintained in PA01 at an average of 2-3 copies/cell [54]. MIC values of all relevant
468 strain-antibiotic pairs are reported in **Table S1**.

469

470 Media and antibiotics: We cultured bacteria in LB broth containing 5g/L NaCl (Sigma-Aldrich, product
471 no. L3022). To assess colony-forming units, we plated on LB Agar, Vegitone, containing 5g/L NaCl
472 and 15g/L agar (Sigma-Aldrich, product no. 19344). Streptomycin was prepared from streptomycin
473 sulfate salt (Sigma-Aldrich, product no. S6501) and meropenem was prepared from meropenem
474 trihydrate (Santa Cruz Biotechnology, Inc., product no. SC-485799). Stocks prepared in water were
475 stored according to supplier directions and added to media on the day of experiments. When high
476 antibiotic concentrations were required, stocks were instead prepared directly in LB on the day of
477 experiments to avoid excessive dilution of the media with water. Bacterial cultures were diluted in
478 phosphate buffered saline (PBS) prepared from tablets (Sigma-Aldrich product no. P4417).
479 Treatment cultures were set up with 90% media plus 10% inoculating culture by volume; thus, the
480 final concentrations of LB and antibiotics in the treatments are 90% of the prepared media values
481 denoted on plots.

482

483 Culture conditions: All cultures were incubated at 37°C, shaking at 225rpm. Overnight cultures were
484 inoculated directly from freezer stocks into 2ml of LB in 14ml culture tubes and incubated for
485 approximately 16h. Overnight cultures were then diluted in PBS and used to inoculate treatment
486 plates. Unless otherwise noted, experimental treatments were conducted in 200μl cultures in flat-
487 bottom 96-well microtitre plates.
488
489 Scoring culture growth: In all experiments, we evaluated culture growth by measuring optical
490 density (OD_{595}) using a BioTek Synergy 2 plate reader, at room temperature. Lids on microtitre plates
491 were briefly removed in a non-sterile environment for the reading; comparison to controls mock-
492 inoculated with PBS indicated that contamination was rare (see **Suppl. Text** for detailed
493 quantification in each experiment). We set a threshold of $OD_{595} > 0.1$ to score as growth, whereas
494 background OD in media-only controls was typically below 0.05. Final readings at 3d post-inoculation
495 were used for data analysis unless otherwise noted. By this time, growth had typically stabilized,
496 with OD much higher than the threshold.
497
498 ***MIC assays***
499
500 Standard MIC values for all applicable strain-antibiotic pairs (i.e. resistant Rms149-carrier against
501 streptomycin; resistant PAMBL2-carrier against meropenem; sensitive non-carrier against both
502 antibiotics) were determined under our culture conditions using the broth microdilution method.
503 Overnight cultures were diluted 10^3-fold and inoculated into antibiotic-containing media at
504 20μl/well on 96-well test plates. This dilution factor consistently yielded an inoculation density close
505 to 5 x 10^5 CFU/ml, in accordance with standard protocol [10]; actual density was estimated by
506 plating. Test plates were incubated and scored for growth at approximately 20h (as per standard
507 protocol [10]), 2d, and 3d post-inoculation. For consistency with growth scoring in seeding
508 experiments, the standard MIC values (MIC_S and MIC_R) used to scale antibiotic concentrations on
509 plot axes are based on results at 3d. Consensus MIC values of all tested strain-antibiotic pairs, at
510 both 20h and 3d, are reported in **Table S1**, with results of individual replicates reported in the **Suppl.**
511 **Text**, section 2.1. For the YFP-labelled Rms149-carrying resistant strain, an additional MIC assay in
512 streptomycin was conducted varying inoculum size (**Fig. 3a**). Here, inoculations were conducted with
513 overnight culture diluted 10^3-, 10^4-, 10^5-, and 10^6-fold (see **Suppl. Text**, section 2.2 for details).
514
515 ***Seeding experiments: resistant strains in isolation***
516

517 Experimental protocol: A highly diluted overnight culture of the YFP-labelled resistant strain
518 (Rms149- or PAMBL2-carrier) was inoculated at 20μl/well into antibiotic-containing media on 96-
519 well test plates. For experiments with PA01:Rms149 screening across many streptomycin
520 concentrations (**Fig. 2**), we used three dilution factors (4×10^7-, 8×10^7, and 1.6×10^8-fold), each to
521 inoculate 96 replicate wells at each concentration. To test the null model of the inoculum size effect
522 (**Fig. 4b**), we screened fewer streptomycin concentrations across a larger number of dilution factors
523 (five in streptomycin-free conditions and six to ten in each streptomycin concentration), each with
524 54 replicates. These dilution factors were chosen differently for each streptomycin concentration to
525 capture the range over which the proportion of replicate cultures showing growth increased from
526 near 0 to near 1. For the experiment with PA01:PAMBL2 in meropenem (**Fig. 5**), we used two
527 dilution factors (5×10^7- and 2×10^8-fold), each with 96 replicates per concentration. In all cases, test
528 plates were incubated and scored for growth after approximately 1, 2, and 3d; for the null model
529 test, incubation and readings were continued up to 5d to confirm stabilization of growth. See **Suppl.**
530 **Text**, sections 4-5, for further details.
531
532 Model fitting: The number of replicate cultures showing growth by 3d (or, additionally, by 5d for the
533 null model test), at each inoculating dilution factor and antibiotic concentration, was used for
534 subsequent model fitting. To estimate single-cell establishment probability and evaluate the null
535 model of the inoculum size effect, likelihood-based methods were used to fit a stochastic model of
536 population growth to these data (see ***Mathematical model of establishment*** below). In addition, to
537 evaluate the effect of antibiotic concentration on establishment, generalized linear models were fit
538 to data from the seeding experiments screening across streptomycin (**Fig. 2**) or meropenem (**Fig. 5**)
539 concentrations. Using the built-in R function 'glm', growth data were treated as binomial, with
540 inoculating dilution factor and antibiotic concentration taken as explanatory variables, applying the
541 complementary log-log link function (**Suppl. Text**, section 12).
542
543 ***Seeding experiments: resistant strain in presence of sensitive population***
544
545 Overnight culture of the DsRed-labelled PA01 sensitive strain was diluted 5-fold to obtain the "high
546 density" inoculating culture, and (in the first experiment only) further to 500-fold to obtain the "low
547 density" inoculating culture. Overnight culture of the YFP-labelled PA01:Rms149 resistant strain was
548 diluted up to 5×10^7-fold and 2×10^8-fold. These cultures were inoculated as follows into media at
549 various streptomycin concentrations on 96-well plates. Pure sensitive control cultures (24 replicates
550 per test condition) were inoculated with 10μl/well of the appropriate diluted culture plus 10μl/well

551 PBS. "Blank" wells to serve as background fluorescence controls were inoculated with 20μl/well PBS.
552 Seeding test plates were first inoculated with 10μl/well of either PBS (for pure resistant control
553 cultures), low-density or high-density sensitive culture. The resistant strain was inoculated at
554 10μl/well immediately thereafter (all sensitive and resistant culture inoculations were completed
555 within an hour). Seeding was conducted with 30-60 replicates per test condition and resistant
556 dilution factor (see **Suppl. Text**, section 6, for details). All test plates were then incubated as before,
557 with optical density (OD_{595}) and fluorescence (excitation: 500+/-27 nm; emission: 540+/-25 nm)
558 measured at approximately 1, 2, and 3d post-inoculation. Among wells showing growth (OD>0.1),
559 we considered the YFP-labelled resistant strain to have established if fluorescence exceeded 5 x 10^5
560 units, chosen by comparison to pure cultures. In each test condition, the number of replicates in
561 which resistance established was taken as data for model fitting, as in the previous seeding
562 experiments.
563
564 ***Fraction of dead cells by live-dead staining***
565
566 This experiment used the PA01:Rms149 resistant strain with no fluorescent label, to avoid
567 interfering with the signal from the stains. We inoculated streptomycin treatment cultures (six
568 replicates per concentration) with 10^3-fold diluted overnight culture, as in the standard MIC assay.
569 After 7h of treatment, we diluted test cultures 100-fold and stained with thiazole orange and
570 propidium iodide (BD Cell Viability Kit, product no. 349483). In parallel, we diluted and stained
571 media and heat-killed cultures as controls. We sampled 50μl per diluted culture using flow
572 cytometry (BD Accuri C6 Flow Cytometer with fast fluidics, discarding events with forward scatter
573 FSC-H < 10000 or side scatter SSC-H < 8000). The staining and flow cytometry steps were carried out
574 in groups containing one replicate per concentration plus controls, to avoid potentially toxic effects
575 of stain exposure over prolonged times (**Suppl. Text**, section 7). To better discriminate cells from
576 background in the flow cytometry data, we first gated on events according to forward and side
577 scatter before defining clusters of dead (membrane-compromised) and intact cells based on
578 fluorescence; see **Suppl. Text**, section 7, and **Suppl. Fig. 6** for details.
579
580 ***Viable cell density dynamics***
581
582 Using the YFP-labelled PA01:Rms149 strain, we tracked the number of viable cells over time in
583 streptomycin-free media (twelve replicates per time point) and at 1/32, 1/16, 1/8, and 1/4 x MIC_R
584 streptomycin (six replicates per time point). An independent test plate was used for sampling at

each time point. Lower (Set A) and higher (Set B) streptomycin concentrations were split across separate plates and sampled at different times. Cultures were inoculated with 20µl of 5 x 10^5-fold diluted overnight culture. At each sampling time, we plated 5 x 4µl spots of undiluted cultures (10% sampling by volume). The number of viable cells was estimated from total colony count following incubation. Comparison of streptomycin-free controls from both sets (A and B) indicated that the plate set effect was non-significant (ANOVA: p = 0.10); thus, controls were pooled for further analysis of the streptomycin effect (see **Suppl. Text**, section 8, for further details).

Mathematical model of establishment

Model: We denote by p_w the probability that a small number of inoculated cells grows into a large population, i.e. that the culture reaches detectable OD as described above. Among a set of n independent replicates, the number of cultures showing growth is thus described by a Binomial(n,p_w) distribution.

In the "null" model, similar to previous work [46, 45], a simple expression for p_w is derived under the assumptions that: (i) the number of cells in the inoculum is Poisson-distributed with mean $\overline{N}$; (ii) each cell, *independently*, establishes a surviving lineage with probability p_c, which depends only on antibiotic concentration x; and (iii) culture growth is observed provided at least one cell establishes a surviving lineage. Then the probability of observing culture growth, as a function of mean inoculum size and antibiotic concentration, can be written as follows (**Suppl. Text**, section 10):

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

In the second line, we have rewritten the expression in terms of the "effective mean inoculum size", $\overline{N}_{eff} = \overline{N} p_c(0)$, which is the mean number of established lineages in the absence of antibiotics; and the "relative establishment probability", $\tilde{p}_c(x) = p_c(x)/p_c(0)$. Although we expect that $p_c(0)$ is close to 1, $\overline{N}$ and $p_c(0)$ play indistinguishable roles in this model, so that in practice we can only estimate their product. This definition of effective inoculum size based on cells that grow in benign conditions is similar 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. Scaling up $\overline{N}_{eff}$ by the dilution factor applied to the inoculating culture, we have an estimate of bacterial density in this culture, equivalent to the historical "most probable number" method [56, 57]. If cells are

phenotypically heterogeneous (i.e. vary in their propensity to establish), or if the individual units in the inoculum are actually clumps of cells, then p_c should be interpreted as the mean establishment probability among individuals (**Suppl. Text**, section 10.1).

More generally, we need not assume that cells establish independently. If we suppose simply that the number of established lineages is Poisson-distributed with some mean α (which is supported empirically by the distribution of colony-forming units counted in highly diluted cultures; **Suppl. Fig. 1**), we have the relationship

$$p_w(\bar{N}, x) = 1 - e^{-\alpha(\bar{N},x)} \qquad \textbf{(Eqn. 2)}$$

where α, and hence p_w, have an arbitrary dependence on mean inoculum size and antibiotic concentration. In the statistical "full model", we estimate a distinct p_w (or equivalently α, by the one-to-one mapping in **Eqn. 2**) in each test condition. Relative establishment probability is then generally defined by $\tilde{p}_c(\bar{N}, x) = \alpha(\bar{N}, x)/\alpha(\bar{N}, 0)$. Nested models, including the null model above, make additional assumptions about the form of α (see **Suppl. Text**, section 10, for details).

<u>Likelihood-based model fitting and comparisons</u>: These stochastic models are fit to experimental population growth data using likelihood-based methods (**Suppl. Text**, section 11). Specifically, under each model we obtain 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, $\tilde{p}_c(x) = \alpha(x)/\alpha(0)$, we use a profile likelihood confidence interval accounting for the uncertainty in both numerator (i.e. results at antibiotic concentration x) and denominator (i.e. results in antibiotic-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 neglect any experimental error in preparing overnight culture dilutions, and assume that mean inoculum size $\bar{N}$ is inversely proportional to the applicable dilution factor. Effective mean inoculum size, $\bar{N}_{eff}$, is estimated by fitting **Eqn. 1** to population growth data in antibiotic-free media. Per-cell relative establishment probability $\tilde{p}_c$ then remains as the single free parameter to fit at each tested antibiotic concentration. The goodness of

fit of the null model (**Eqn. 1**) is assessed for each test concentration separately, using the LRT to compare it to the fit of the full model (**Eqn. 2**).

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. We thank Isabel Frost, Natalia Kapel, Lois Ogunlana, Andrei Papkou, and Célia Souque for advice and assistance on experimental protocols.

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 mathematical model, wrote the code and carried out model fitting. H.K.A. and R.C.M. wrote the manuscript.

Data and code availability: Data generated in this study, as well as custom R scripts for likelihood-based model fitting and comparisons, will be made available upon manuscript acceptance.

References

[1] D. N. Fish, S. C. Piscitelli and L. H. Danziger, “Development of resistance during antimicrobial therapy: a review of antibiotic classes and patient characteristics in 173 studies,” *Pharmacotherapy,* vol. 15, no. 3, pp. 279-291, 1995.

[2] World Health Organization, “Antimicrobial Resistance: Global Report on Surveillance,” World Health Organization, Geneva, 2014.

[3] X. Zhao and K. Drlica, “Restricting the selection of antibiotic-resistant mutants: a general strategy derived from fluoroquinolone studies,” *Clin. Infect. Dis.,* vol. 33 (Suppl 3), pp. S147-S156, 2001.

[4] X. Zhao and K. Drlica, “Restricting the selection of antibiotic-resistant mutant bacteria: measurement and potential use of the mutant selection window,” *J. Infect. Dis.,* vol. 185, pp. 561-565, 2002.

[5] K. Drlica and X. Zhao, “Mutant selection window hypothesis updated,” *Clin. Infect. Dis.,* vol. 44, pp. 681-688, 2007.

[6] R. Canton and M.-I. Morosini, “Emergence and spread of antibiotic resistance following exposure to antibiotics,” *FEMS Microbiol. Rev.,* vol. 35, pp. 977-991, 2011.

[7] D. I. Andersson and D. Hughes, “Evolution of antibiotic resistance at non-lethal drug concentrations,” *Drug Resistance Updates,* vol. 15, pp. 162-172, 2012.

[8] T. Day, S. Huijben and A. F. Read, “Is selection relevant in the evolutionary emergence of drug resistance?,” *Trends Microbiol.,* vol. 23, pp. 126-133, 2015.

[9] D. I. Andersson, “Improving predictions of the risk of resistance development against new and old antibiotics,” *Clin. Microbiol. Infect.,* vol. 21, pp. 894-898, 2015.

[10] European Committee for Antimicrobial Susceptibility Testing (EUCAST), “Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution,” *Clin. Microbiol. Infect.,* vol. 9, pp. 1-7, 2003.

[11] M.-C. Negri, M. Lipsitch, J. Blázquez, B. R. Levin and F. Baquero, “Concentration-dependent selection of small phenotypic differences in TEM beta-lactamase-mediated antibiotic resistance,” *Antimicrob. Agents Chemother.,* vol. 44, pp. 2485-2491, 2000.

[12] A. Liu, A. Fong, E. Becket, J. Yuan, C. Tamae, L. Medrano, M. Maiz, C. Wahba, C. Lee, K. Lee, K. P. Tran, H. Yang, R. M. Hoffman, A. Salih and J. H. Miller, “Selective advantage of resistant strains at trace levels of antibiotics: a simple and ultrasensitive color test for detection of antibiotics and genotoxic agents,” *Antimicrob. Agents Chemother.,* vol. 55, no. 3, pp. 1204-1210, 2011.

[13] E. Gullberg, S. Cao, O. G. Berg, C. Ilbäck, L. Sandegren, D. Hughes and D. I. Andersson, “Selection of resistant bacteria at very low antibiotic concentrations,” *PLoS Pathogens,* vol. 7, p. e1002158, 2011.

[14] S. Kalghatgi, C. S. Spina, J. C. Costello, M. Liesa, J. R. Morones-Ramirez, S. Slomovic, A. Molina, O. S. Shirihai and J. J. Collins, “Bactericidal antibiotics induce mitochondrial dysfunction and oxidative damage in mammalian cells,” *Sci. Transl. Med.,* vol. 5, p. 192ra85, 2013.

[15] A. Prayle, A. Watson, H. Fortnum and A. Smyth, “Side effects of aminoglycosides on the kidney, ear and balance in cystic fibrosis,” *Thorax,* vol. 65, pp. 654-658, 2010.

[16] P. Abel zur Wiesch, R. Kouyos, J. Engelstädter, R. R. Regoes and S. Bonhoeffer, “Population biological principles of drug-resistance evolution in infectious diseases,” *Lancet Infect. Dis.,* vol. 11, pp. 236-247, 2011.

[17] Z. Patwa and L. M. Wahl, “The fixation probability of beneficial mutations,” *J. R. Soc. Interface,* vol. 5, pp. 1279-1289, 2008.

[18] M. F. Schenk, I. G. Szendro, J. Krug and J. A. G. M. de Visser, “Quantifying the adaptive potential of an antibiotic resistance enzyme,” *PLoS Genet.,* vol. 8, p. e1002783, 2012.

[19] J. Coates, B. R. Park, D. Le, E. Simsek, W. Chaudhry and M. Kim, “Antibiotic-induced population fluctuations and stochastic clearance of bacteria,” *eLife,* vol. 7, p. e32976, 2018.

[20] I. Levin-Reisman, I. Ronin, O. Gefen, I. Braniss, N. Shoresh and N. Q. Balaban, “Antibiotic tolerance facilitates the evolution of resistance,” *Science,* vol. 355, pp. 826-830, 2017.

[21] D. M. Livermore, “Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare?,” *Clin Infect Dis,* vol. 34, pp. 634-640, 2002.

[22] I. Brook, “Inoculum effect,” *Rev. Infect. Dis.,* vol. 11, pp. 361-368, 1989.

[23] K. I. Udekwu, N. Parrish, P. Ankomah, F. Baquero and B. R. Levin, “Functional relationship between bacterial cell density and the efficacy of antibiotics,” *J. Antimicrob. Chemother.,* vol. 63, pp. 745-757, 2009.

[24] T. Artemova, Y. Gerardin, C. Dudley, N. M. Vega and J. Gore, “Isolated cell behavior drives the evolution of antibiotic resistance,” *Molec. Syst. Biol.,* vol. 11, p. 822, 2015.

[25] K. C. Haltalin and J. D. Nelson, “In vitro susceptibility of Shigellae to sodium sulfadiazine and to eight antibiotics,” *JAMA,* vol. 193, pp. 705-710, 1965.

[26] S. E. McLinn, J. D. Nelson and K. C. Haltalin, “Antimicrobial susceptibility of Hemophilus influenzae,” *Pediatrics,* vol. 45, pp. 827-838, 1970.

[27] M. Tato, T. M. Coque, F. Baquero and R. Cantón, “Dispersal of carbapenemase blaVIM-1 gene associated with different Tn402 variants, mercury transposons, and conjugative plasmids in Enterobacteriaceae and Pseudomonas aeruginosa,” *Antimicrob. Agents Chemother.,* vol. 54, pp. 320-327, 2010.

[28] A. San Millan, M. Toll-Riera, J. A. Escudero, R. Cantón, T. M. Coque and R. C. MacLean, “Sequencing of plasmids pAMBL1 and pAMBL2 from Pseudomonas aeruginosa reveals a blaVIM-1 amplification causing high-level carbapenem resistance,” *J. Antimicrob. Chemother.,* vol. 70, pp. 3000-3003, 2015.

[29] M. Fritzenwanker, C. Imirzalioglu, S. Herold, F. M. Wagenlehner, K.-P. Zimmer and T. Chakraborty, “Treatment options for carbapenem-resistant gram-negative infections,” *Dtsch. Arztebl. Int.,* vol. 115, pp. 345-352, 2018.

[30] M. S. Walters, J. E. Grass, S. N. Bulens, E. B. Hancock, E. C. Phipps, D. Muleta, J. Mounsey, M. A. Kainer, C. Concannon, G. Dumyati, C. Bower, J. Jacob, P. M. Cassidy, Z. Beldavs, K. Culbreath, W. E. Phillips Jr., D. J. Hardy, R. L. Vargas, M. Oethinger, U. Ansari, R. Stanton, V. Albrecht, A. L. Halpin, M. Karlsson, J. K. Rasheed and A. Kallen, “Carbapenem-resistant Pseudomonas aeruginosa at US emerging infections program sites, 2015,” *Emerg. Infect. Dis.,* vol. 25, pp. 1281-1288, 2019.

[31] World Health Organization, “Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis,” World Health Organization, Geneva, 2017.

[32] I. Frost, W. P. J. Smith, S. Mitri, A. San Millan, Y. Davit, J. M. Osborne, J. M. Pitt-Francis, R. C. MacLean and K. R. Foster, “Cooperation, competition and antibiotic resistance in bacterial colonies,” *ISME J.,* vol. 12, pp. 1582-1593, 2018.

[33] M. A. Brockhurst, F. Harrison, J.-W. Veening, E. Harrison, G. Blackwell, Z. Iqbal and C. Maclean, “Assessing evolutionary risks of resistance for new antimicrobial therapies,” *Nature Ecol. Evol.,* vol. 3, pp. 515-517, 2019.

[34] P. Abel zur Wiesch, S. Abel, S. Gkotzis, P. Ocampo, J. Engelstädter, T. Hinkley, C. Magnus, M. K. Waldor, K. Udekwu and T. Cohen, “Classic reaction kinetics can explain complex patterns of antibiotic action,” *Sci. Transl. Med.,* vol. 7, p. 287ra73, 2015.

[35] E. A. Yurtsev, H. X. Chao, M. S. Datta, T. Artemova and J. Gore, “Bacterial cheating drives the population dynamics of cooperative antibiotic resistance plasmids,” *Molec. Syst. Biol.,* vol. 9, p. 683, 2013.

[36] N. M. Vega and J. Gore, “Collective antibiotic resistance: mechanisms and implications,” *Curr. Opin. Microbiol.,* vol. 21, pp. 28-34, 2014.

[37] R. A. Sorg, L. Lin, G. S. van Doorn, M. Sorg, J. Olson, V. Nizet and J.-W. Veening, “Collective resistance in microbial communities by intracellular antibiotic deactivation,” *PLoS Biol.,* vol. 14, p. e2000631, 2016.

[38] H. R. Meredith, J. K. Srimani, A. J. Lee, A. J. Lopatkin and L. You, “Collective antibiotic tolerance: mechanisms, dynamics and intervention,” *Nat. Chem. Biol.,* vol. 11, pp. 182-188, 2015.

[39] I. El Meouche, Y. Siu and M. J. Dunlop, “Stochastic expression of a multiple antibiotic resistance activator confers transient resistance in single cells,” *Sci. Rep.,* vol. 6, p. 19538, 2016.

[40] T. Bergmiller, A. M. C. Andersson, K. Tomasek, E. Balleza, D. J. Kiviet, R. Hauschild, G. Tkačik and C. C. Guet, “Biased partitioning of the multidrug efflux pump AcrAB-TolC underlies long-lived phenotypic heterogeneity,” *Science,* vol. 356, pp. 311-315, 2017.

[41] D. R. Gifford, J. A. G. M. de Visser and L. M. Wahl, “Model and test in a fungus of the probability that beneficial mutations survive drift,” *Biol. Lett.,* vol. 9, p. 20120310, 2012.

[42] D. R. Gifford and R. C. MacLean, “Evolutionary reversals of antibiotic resistance in experimental populations of Pseudomonas aeruginosa,” *Evolution,* vol. 67, pp. 2973-2981, 2013.

[43] H. K. Alexander, G. Martin, O. Y. Martin and S. Bonhoeffer, “Evolutionary rescue: linking theory for conservation and medicine,” *Evol. Appl.,* vol. 7, pp. 1161-1179, 2014.

[44] G. Bell and A. Gonzalez, “Evolutionary rescue can prevent extinction following environmental change,” *Ecol. Lett.,* vol. 12, pp. 942-948, 2009.

[45] G. Martin, R. Aguilée, J. Ramsayer, O. Kaltz and O. Ronce, “The probability of evolutionary rescue: towards a quantitative comparison between theory and evolution experiments,” *Phil. Trans. R. Soc. B,* vol. 368, p. 20120088, 2013.

[46] H. A. Druett, “Bacterial invasion,” *Nature,* vol. 170, p. 288, 1952.

[47] R. E. Moxon and P. A. Murphy, “Haemophilus influenzae bacteremia and meningitis resulting from survival of a single organism,” *PNAS,* vol. 75, no. 3, pp. 1534-1536, 1978.

[48] K. Koutsoumanis, “A study on the variability in the growth limits of individual cells and its effect on the behavior of microbial populations,” *Int. J. Food Microbiol.,* vol. 128, pp. 116-121, 2008.

[49] L. A. Liotta, G. M. Saidel and J. Kleinerman, “Stochastic model of metastases formation,” *Biometrics,* vol. 32, no. 3, pp. 535-550, 1976.

[50] M. A. Kohanski, M. A. DePristo and J. J. Collins, “Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis,” *Molec. Cell,* vol. 37, pp. 311-320, 2010.

[51] A. Frenoy and S. Bonhoeffer, “Death and population dynamics affect mutation rate estimates and evolvability under stress in bacteria,” *PLoS Biol.,* vol. 16, p. e2005056, 2018.

[52] S. J. Dancer, “How antibiotics can make us sick: the less obvious adverse effects of antimicrobial chemotherapy,” *Lancet Infect. Dis.,* vol. 4, pp. 611-619, 2004.

[53] A. S. Haines, K. Jones, M. Cheung and C. M. Thomas, “The IncP-6 plasmid Rms149 consists of a small mobilizable backbone with multiple large insertions,” *J. Bacteriol.,* vol. 187, pp. 4728-4738, 2005.

[54] A. San Millan, K. Heilbron and R. C. MacLean, “Positive epistasis between co-infecting plasmids promotes plasmid survival in bacterial populations,” *ISME J.,* vol. 8, pp. 601-612, 2014.

[55] D. Sandvang, “Novel streptomycin and spectinomycin resistance gene as a gene cassette within a class 1 integron isolated from Escherichia coli,” *Antimicrob. Agents Chemother.,* vol. 43, pp. 3036-3038, 1999.

[56] W. G. Cochran, “Estimation of bacterial densities by means of the "most probable number",” *Biometrics,* vol. 6, pp. 105-116, 1950.

[57] M. A. Hurley and M. E. Roscoe, “Automated statistical analysis of microbial enumeration by dilution series,” *J. Appl. Bacteriol.,* vol. 55, pp. 159-164, 1983.

676
677

Figures

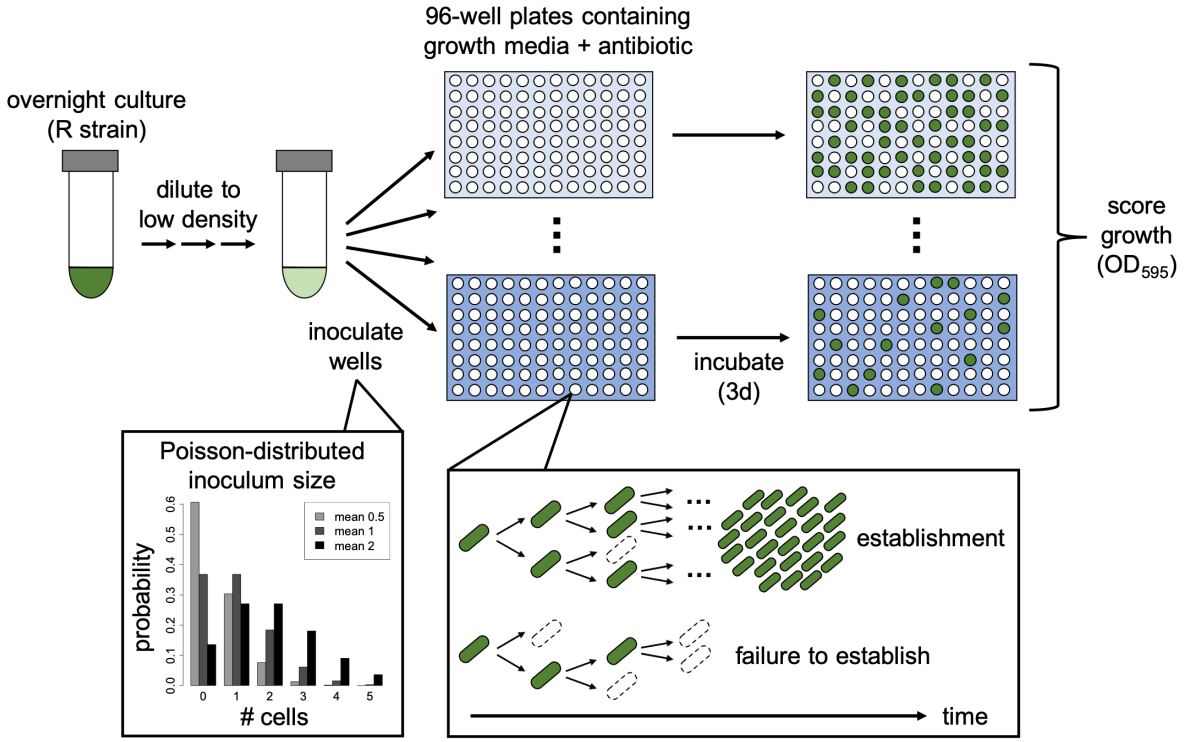
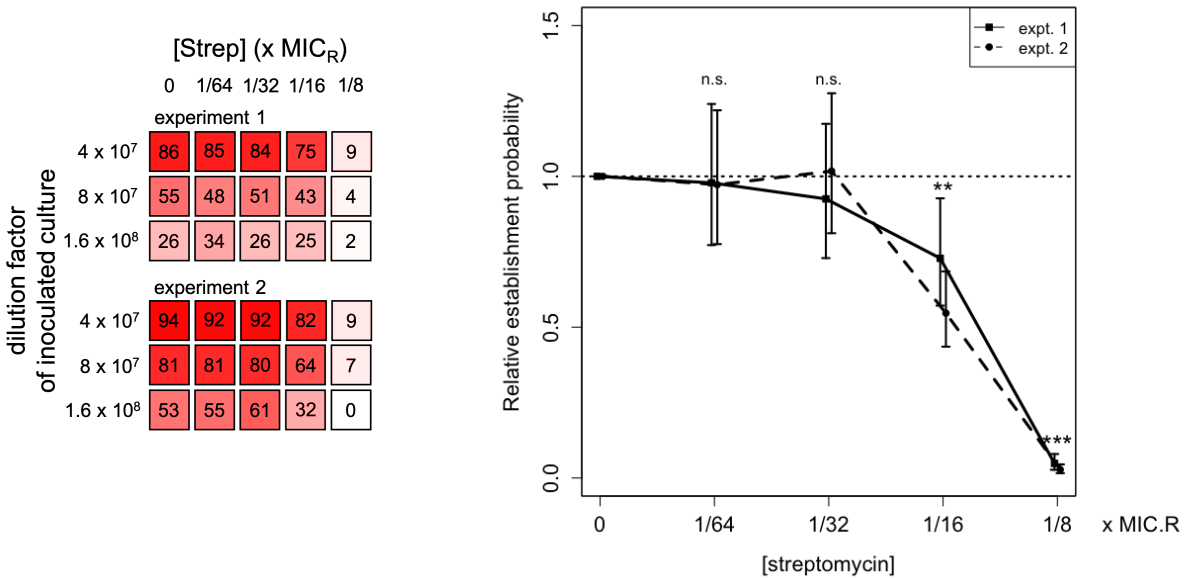


Figure 1: Design of seeding experiments to estimate establishment probability. An overnight culture of the resistant strain is highly diluted and used to inoculate 96-well plates containing growth media (LB broth) with antibiotic at various concentrations (shades of blue). The number of cells inoculated per well follows a Poisson distribution (examples plotted for mean inoculum size of 0.5, 1, or 2 cells per well). Within these culture wells, stochastic population dynamics imply that each inoculated cell may either produce a large number of descendants (*establishment*) or produce no/few descendants that ultimately die out (*failure to establish*). Plates are incubated for 3 days and optical density is measured to score growth in wells (OD_{595} > 0.1; dark green). The number of replicate cultures showing growth is used to estimate the per-cell establishment probability at each antibiotic concentration by fitting a mathematical model.

693 **(a)** **(b)**

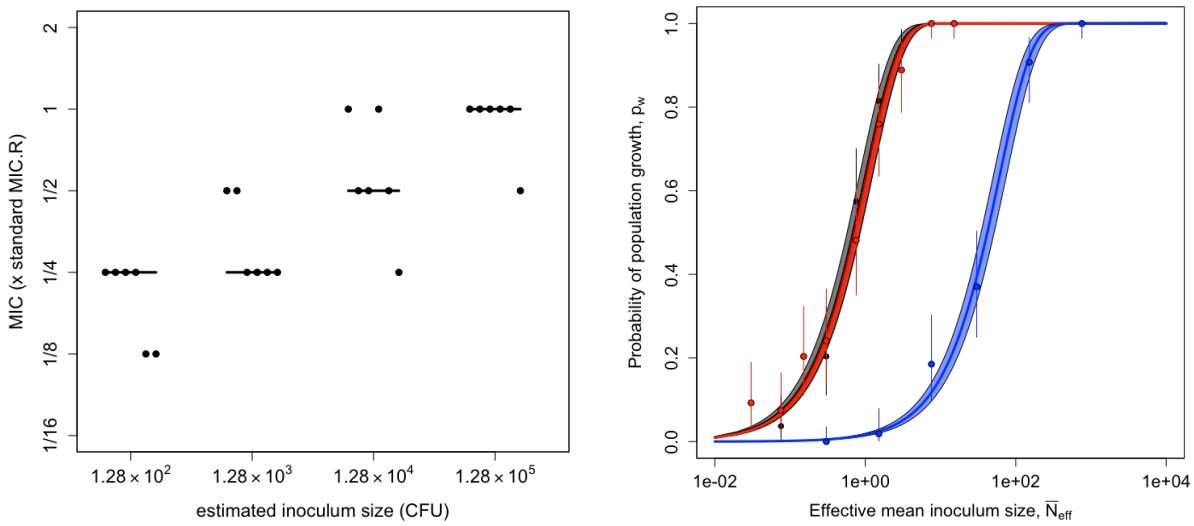


694

695 **Figure 2: Establishment probability of single PA01:Rms149 streptomycin-resistant cells, estimated**
696 **from seeding experiments. (a) Visual representation of the growth data**, indicating the number of
697 replicate cultures (out of 96) that grew in each test condition up to 3d post-inoculation. **(b)**
698 **Estimated relative per-cell establishment probability ($\tilde{p}_c$)**, scaled by the probability in
699 streptomycin-free medium, as a function of streptomycin concentration, scaled by the standard MIC
700 value of the resistant strain (MIC_R = 2048μg/ml; **Table S1**). Results are shown for two separate
701 experiments. Plotted points indicate the maximum likelihood estimate of $\tilde{p}_c$ and error bars indicate
702 the 95% confidence interval, using the fitted model selected by the likelihood ratio test (experiment
703 1: Model B', fixed environmental effect; experiment 2: Model C', the null model [**Eqn. 1**]. Both of
704 these models pool data across three inoculation densities; see **Suppl. Text**, section 10, for details).
705 Significance of the streptomycin effect is determined by fitting a generalized linear model to the
706 population growth data (n.s.: not significant, $p > 0.05$; ** p=0.01 in expt. 1, p=2e-7 in expt. 2, and
707 p=2e-8 pooling both experiments; *** $p < 2e-16$ in both experiments; see **Suppl. Text**, section 14.1**,**
708 for full results).

709

710 **(a)** **(b)**

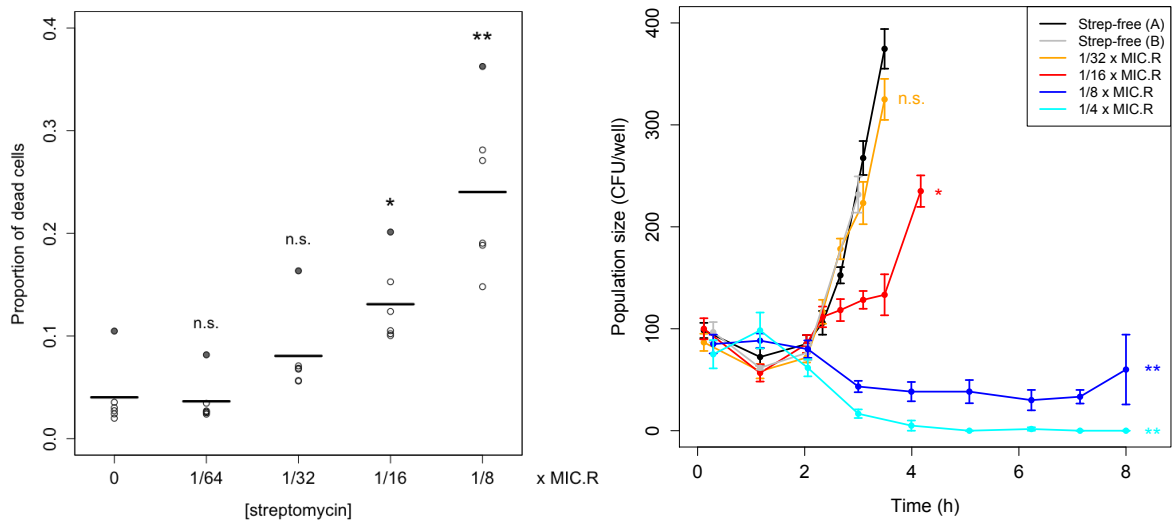


711

712 **Figure 3: Inoculum size effects on MIC and probability of population growth of the resistant**
713 **PA01:Rms149 strain in streptomycin. (a) MIC as a function of inoculum size.** Cultures were
714 inoculated with PA01:Rms149 at four different inoculum sizes. MIC was evaluated as the minimal
715 tested streptomycin concentration that prevented detectable growth up to 3d post-inoculation; a
716 qualitatively similar pattern arose if growth was evaluated at 20h (**Fig. S2**). The y-axis is scaled by the
717 MIC of this strain at standard inoculation density (MIC_R). The points represent six replicates at each
718 inoculum size, with the line segments indicating their median. **(b) Null model of the inoculum size**
719 **effect (Eqn. 1) fit to culture growth data.** Probability of population growth (p_w) is plotted as a
720 function of effective mean inoculum size ($\overline{N}_{eff}$, calibrated by the results in streptomycin-free media;
721 see **Fig. S4**). Black: streptomycin-free; red: streptomycin at 1/16 x MIC_R; blue: 1/8 x MIC_R. These
722 results are based on growth in streptomycin up to 5d post-inoculation; see **Suppl. Text**, section 15,
723 for results at 3d post-inoculation. Points indicate the proportion of replicate cultures showing
724 growth, i.e. the maximum likelihood estimate (MLE) of p_w in the full model, with error bars indicating
725 the 95% confidence interval (CI). The solid line shows the best fit of the null model (i.e. **Eqn. 1**
726 parameterized with the MLE of $\tilde{p}_c$) and the shaded area corresponds to the 95% CI. According to the
727 likelihood ratio test, the null model deviance from the full model is not significant at any
728 streptomycin concentration (streptomycin-free: p=0.55; 1/16 x MIC_R: p=0.28; 1/8 x MIC_R: p=0.71; see
729 **Suppl. Text**, section 15, for full results).

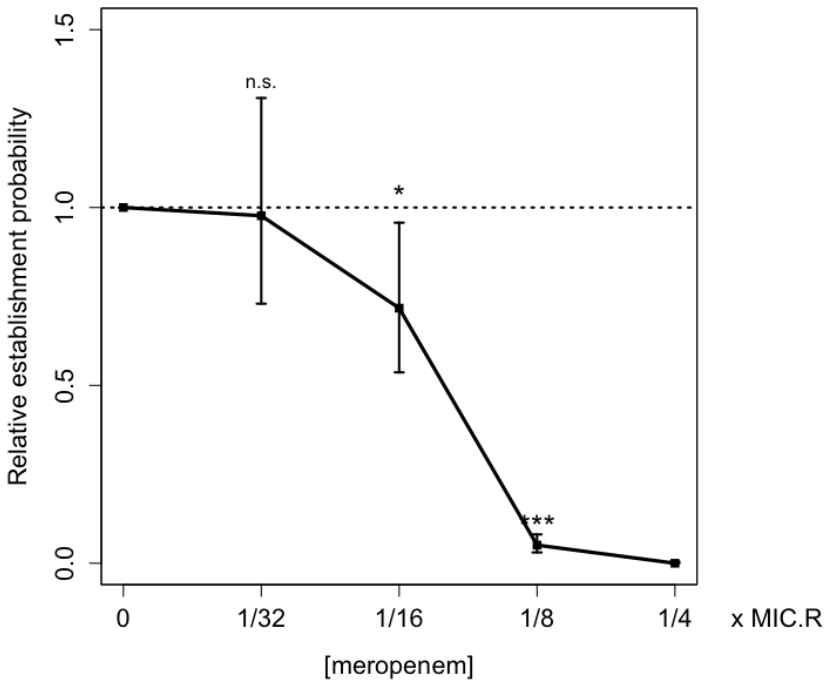
730

731 **(a)** **(b)**



732

733 **Figure 4: Effects of sub-MIC$_R$ streptomycin treatment on PA01:Rms149 resistant cell dynamics. (a)**
734 **Proportion of dead cells after 7h in sub-MIC$_R$ streptomycin.** The proportion of dead cells in
735 streptomycin-treated cultures was estimated using live-dead staining and flow cytometry. Points
736 represent six independent treatment replicates at each concentration and line segments indicate
737 their mean. Differences from the streptomycin-free control cultures were assessed using a one-way
738 ANOVA followed by a post-hoc Dunnett's test (n.s.: not significant, $p > 0.05$; *: p = 9e-3, **: p < 1e-
739 4). Effects identified as significant do not change if we exclude an outlier replicate (shaded-in points)
740 showing consistently elevated dead cell fractions (**Table S3**). **(b) Viable cell population dynamics in**
741 **sub-MIC$_R$ streptomycin.** Points with connecting lines indicate the mean number of viable cells across
742 six replicate cultures per streptomycin concentration, per sampling time point (or twelve replicates
743 for streptomycin-free controls); the error bars indicate standard error. **Fig. S7** shows all individual
744 replicates. Viable cell numbers were estimated by plating undiluted culture samples; plots are
745 truncated when colonies became too dense to count. Significance of each streptomycin
746 concentration compared to the streptomycin-free control was assessed by a post-hoc Dunnett's test
747 (n.s.: not significant, p=0.87; * p=4e-4; ** p<1e-4).
748



749

750 **Figure 5: Estimated relative per-cell establishment probability of the PA01:PAMBL2 meropenem-**
751 **resistant strain as a function of meropenem concentration.** Concentration is scaled by the standard
752 MIC of this strain in meropenem (MIC_R = 512 μg/ml; **Table S1**). Plotted points indicate the maximum
753 likelihood estimate of $\tilde{p}_c$ and error bars indicate the 95% confidence interval, using the fitted model
754 selected by the likelihood ratio test (Model C', the null model [**Eqn. 1**], which pools data across two
755 tested inoculation densities). Significance of the meropenem effect is determined by fitting a
756 generalized linear model (GLM) to population growth data (n.s.: not significant, $p > 0.05$; * $p = 0.02$;
757 *** $p < 2e\text{-}16$; see **Suppl. Text**, section 14.2, for full results). 1/4 x MIC_R meropenem was excluded
758 from the GLM because zero replicates established.

759

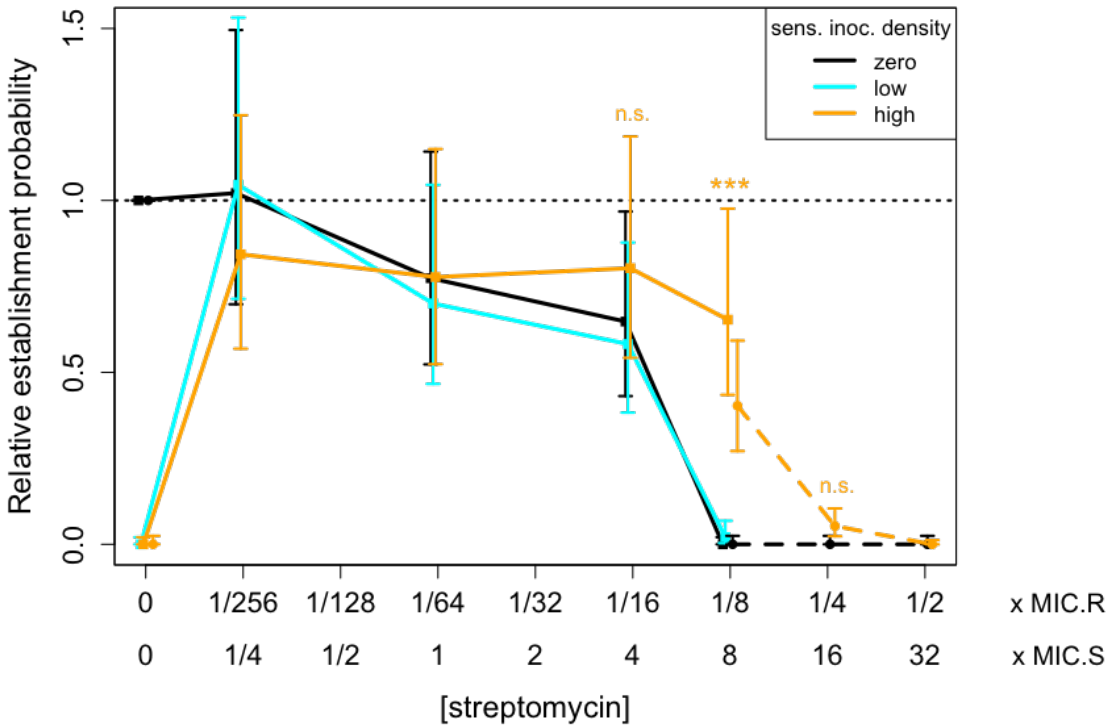


Figure 6: Impact of a large sensitive population on the establishment probability of a resistant cell. The PA01:Rms149 resistant strain was seeded either alone (black) or into a low-density (cyan) or high-density (orange) sensitive PA01 population, across a range of streptomycin concentrations. Results are shown from two separate experiments, testing different subsets of conditions (experiment 1 – data points in squares with solid line; experiment 2 – data points in circles with dashed line). Within each experiment, the estimated relative establishment probability per resistant cell ($\tilde{p}_c$) in each condition is normalized by the result for the resistant strain alone in streptomycin-free media. 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 C', the null model [**Eqn. 1**] for both experiments). At streptomycin concentrations of particular interest, the number of replicates in which the resistant strain established in the presence of no or low-density sensitive (pooled where applicable) vs. high-density sensitive was compared using a two-sided Wilcoxon rank-sum test, with significance annotated on the plot (1/16x MIC_R: experiment 1, p=0.17; 1/8x MIC_R: experiment 1, p < 2.2e-16 and experiment 2, p=4.8e-8; 1/4x MIC_R: experiment 2, p=0.042, not significant after Bonferroni correction); see **Suppl. Text**, section 16, for further details.