Stochastic effects on the establishment of mutants resistant to β-lactam antibiotics

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Abstract

For antibiotic resistance to arise, new resistant mutants must establish in a bacterial population before they can spread via natural selection. Understanding the stochastic factors influencing mutant establishment is crucial for a quantitative understanding of antibiotic resistance emergence. Here, we quantify the single-cell establishment probability of four *Escherichia coli* strains expressing β-lactamase alleles with different activity against the antibiotic cefotaxime, as a function of concentration. Using a simple branching model, we show that concentrations well below the minimal inhibitory concentration (MIC) can substantially hamper establishment, particularly for highly resistant mutants, suggesting cooperative effects due to antibiotic breakdown at high cell densities. We further show that variation among cell lineages may explain the more gradual influence of increased antibiotic concentrations on agar. Finally, we use the single-cell establishment probability to predict a strain's MIC in the absence of social interactions, as a general reference to detect cooperative resistance effects.

Introduction

A classical question in population genetics addresses the fate of a new adaptive allele in a population. Any new allele is initially present at a low frequency in the population and thus prone to extinction by genetic drift, even if the allele is beneficial (Patwa & Wahl, 2008). In 1927, Haldane stated that a novel beneficial allele has to overcome drift loss and *establish* in the population before it can be picked up by selection; the probability of which he estimated to be roughly two times its selective benefit (Haldane, 1927). Since then, Haldane's approximation has been generalised and extended by a great number of models, but none of them have been tested empirically until about a decade ago (Patwa & Wahl, 2008). With advances in genomic techniques, the issue of fixation probability has recently been studied in experimental populations of microorganisms (e.g. Lang, Botstein, & Desai, 2011; Frenkel, Good, & Desai, 2014; Good, McDonald, Barrick, Lenski, & Desai, 2017). These studies looked at the fate of beneficial alleles that are already established at a certain frequency in the population, finding that their fixation strongly depends on the competition with further beneficial alleles that appear via mutation over time. Only a small number of empirical studies has investigated the stochastic process of establishment of an initially rare beneficial allele arisen by *de novo* mutation in fungi (Gifford, De Visser, & Wahl, 2013), bacteria (Farrell, Gralka, Hallatschek, & Waclaw, 2017; Giometto, Nelson, & Murray, 2018) and nematodes (Chelo, Nédli, Gordo, & Teotónio, 2013). Overall, these studies found that genetic drift dominates the establishment process, and the probability for a beneficial allele to establish in a population depends on the alleles' initial frequency, its selective benefit, and the size of the population.

Whether a beneficial allele establishes successfully in a population is of particular relevance in cases of unwanted evolution, such as the evolution of antibiotic resistance. The worldwide growing concern about failing treatments of infections due to the emergence of resistant

40 bacterial pathogens has led to the awareness that this problem has to be tackled from
41 different angles, one of which is the better understanding of the emergence and evolution of
42 antibiotic resistance (Baym, Stone, & Kishony, 2016; Smith et al., 2016; Furusawa, Horinouchi,
43 & Maeda, 2018). Resistance alleles can be acquired via horizontal gene transfer or *de novo*
44 mutation. In the latter case, the resistant allele is initially present in a single individual within
45 a susceptible population. But how likely is it that this single genotype can establish in the
46 population and what factors influence this process? Using time-lapse microscopy, Coates et
47 al. (2018) followed the growth of single *Escherichia coli* cells on agar, supplemented with
48 different antibiotics. They found that the establishment of a single cell, i.e. the growth into a
49 visible macrocolony, was a highly stochastic process driven by fluctuations in cell death and
50 birth, leading to the extinction of microcolonies at various time points. This stochasticity was
51 more pronounced with increasing antibiotic concentration, resulting in higher extinction
52 rates. Similar results were found for streptomycin resistance in *Pseudomonas aeruginosa*
53 (Alexander & MacLean, 2020). By seeding one to a few cells of a resistant strain in liquid
54 medium with increasing antibiotic concentrations, it was found that at concentrations as low
55 as 1/8 of the minimum inhibitory concentration (MIC) of this strain, its establishment
56 probability was only 5% and that establishment of one cell was independent of the presence
57 of other cells in the inoculum. The authors speculated that the lack of social effects on mutant
58 establishment may depend on the mechanism of antibiotic resistance, and could be modified
59 when positive social interactions play a role, e.g. due to the expression of enzymes that break
60 down the antibiotic (Alexander & MacLean, 2020).

61 A common resistance mechanism potentially involving social effects involves β-lactamase
62 enzymes. β-lactamases hydrolyse the lactam ring of β-lactam antibiotics, which target
63 penicillin-binding proteins (PBPs) that are involved in cell wall synthesis of Gram-negative

64 bacteria, thereby leading to loss of cell wall integrity and eventual cell lysis (Samaha-Kfoury &
65 Araj, 2003; Bush, 2010). A well-studied and clinically relevant example is TEM-1 β-lactamase,
66 which has high activity toward first-generation penicillins, but low activity toward third-
67 generation β-lactams, including the cephalosporin cefotaxime (CTX). However, the TEM-1
68 allele can acquire mutations, rendering it more active to CTX (Salverda, de Visser, & Barlow,
69 2010; Schenk, Szendro, Krug, & de Visser, 2012; Van Dijk, Hwang, Krug, De Visser, & Zwart,
70 2017). As β-lactamases are expressed in the periplasmic space of gram-negative pathogens
71 like *E. coli*, this system has been recognized as potentially cooperative, since the enzymatic
72 breakdown in the periplasm reduces the antibiotic concentration in the environment (Brown,
73 West, Diggle, & Griffin, 2009), thereby cross-protecting susceptible strains (Medaney,
74 Dimitriu, Ellis, & Raymond, 2016). Such a cooperative behaviour can be expected to be more
75 pronounced on agar due to the local breakdown of the antibiotic, creating microenvironments
76 that allow for co-existence of strains with different levels of antibiotic resistance (Nicoloff &
77 Andersson, 2016; Frost et al., 2018; Geyrhofer & Brenner, 2020).

78 Here, we examined the single-cell establishment probability of antibiotic-degrading bacteria
79 under a range of antibiotic concentrations in a structured and an unstructured environment.
80 We used four *E. coli* strains expressing β-lactamase alleles with different activity, and hence
81 different levels of resistance, toward CTX. Each strain was tested separately at low cell
82 densities in both liquid and on agar medium with increasing CTX concentrations. To determine
83 the single-cell establishment probability from the data, we developed a simple branching
84 model, followed by a more detailed investigation of the cause of differences in stochasticity
85 between the two environments. We show that environmental structure and cooperative
86 behaviour play at most a minor role in the establishment of resistant cells at low initial

87 densities. Based on our results, we further introduce a new measure for resistance level that
88 takes inoculum size and stochasticity into account, in contrast to the classical MIC assay.

89

90 **Results and Discussion**

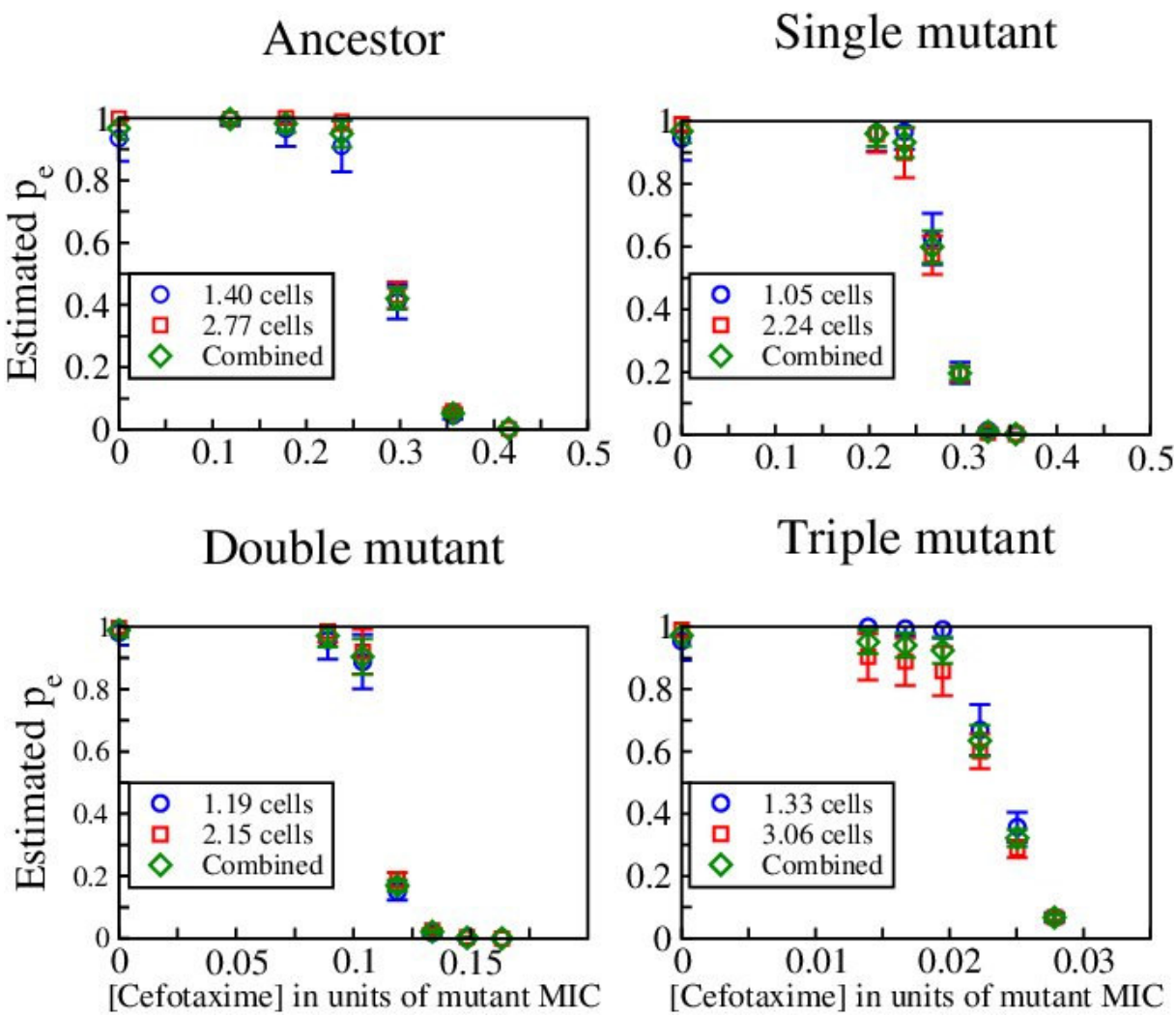
91 Single-cell establishment probability

92 To determine single cell establishment probabilities, we performed each one experiment in
93 liquid medium and on agar plates. In each experiment, four *E. coli* strains with increasing levels
94 of CTX resistance (referred to as Ancestor, Single mutant, Double mutant, Triple mutant; cf.
95 Table 1) were tested separately under exposure to a range of CTX concentrations. We use the
96 notation $p_e(x)$ to denote the probability that a single cell establishes a macroscopic
97 population (i.e. growth in liquid or colony formation on agar visible with the naked eye) at CTX
98 concentration x. When the antibiotic concentration is zero, a population should establish with
99 near certainty. Likewise, when the concentration is above the strains' minimum inhibitory
100 concentration (MIC), establishment is completely prevented. Stochastic outcomes can arise
101 only at intermediate concentrations.

102 In the liquid medium experiment, we grew bacteria in multi-well plates starting from a small
103 number of cells (1-3 on average) in each well and observed their establishment as a function
104 of CTX concentration. Applying aliquots of the cell cultures used for inoculating the well-plates
105 on agar, provided estimates of the viable cell numbers needed to evaluate the probabilities.
106 Using a bootstrapping algorithm and assuming a Poisson distribution of independently acting
107 cells in each inoculum (details in Supplement S4), we estimated the single-cell establishment
108 probability $p_e(x)$ for all strains and conditions, which are shown in Figure 1. As expected, the
109 mutants with higher resistance require higher antibiotic concentration to inhibit

110 establishment, and all strains show intermediate values of p_e for a range of subMIC CTX

111 concentrations.



112

113 **Figure 1.** Single-cell establishment probabilities, p_e, in liquid medium, estimated for the four
114 tested strains as a function of antibiotic concentration. For each strain, two inocula were used,
115 one of approximately 1 cell (blue circles) and another of 2-3 cells (red squares); average
116 inoculum size estimated from separate CFU counts are shown. The inferred single-cell
117 establishment probabilities are nearly identical for the two cases, implying that the
118 establishment probability of a lineage is not influenced by the presence of other lineages at
119 these low initial cell densities. The green diamonds are weighted averages over the two
120 inoculum sizes (see Supplement S3 for details on the method used to estimate p_e).

121

122 One complication in estimating the single-cell establishment probability is that multiple cells

123 may be present initially in some wells, and the lineages arising out of them may in principle

124 interact, for example through competition for resources or breakdown of antibiotic molecules.

125 In that case, the establishment probabilities of multiple lineages arising from different cells
126 are no longer independent, biasing our estimates of $p_e(x)$. To test whether lineages interact
127 from initially small inocula, we conducted the liquid experiment with two different mean
128 inoculum sizes and estimated $p_e(x)$ for both cases under the assumption that there is no
129 interaction (cf. Supplement S4). If interaction occurred, the inferred values should disagree.
130 However, Figure 1 shows that they agree closely, indicating that interaction between lineages
131 is insignificant at these low cell densities.

132 The absence of social interactions in liquid medium was also found in the study with *P.*
133 *aeruginosa* and streptomycin under similar conditions regarding culture volume and inoculum
134 size (Alexander & MacLean, 2020). In that study, social interactions were less expected
135 because streptomycin resistance does not involve degrading enzymes with the potential for
136 such interactions. Possibly social interactions play a role during the establishment of new β-
137 lactamase mutants in structured environments, where antibiotic breakdown may locally affect
138 establishment probability.

139 To determine the effect of environmental structure on establishment, we plated about 200
140 cells of each *E. coli* strain on CTX-containing agar, followed by counts of colony-forming units
141 (CFUs) after incubation. The establishment probability was estimated as the CFU count
142 relative to that at zero concentration (Figure 2). The patterns of p_e as a function of CTX
143 concentration are broadly similar to those in liquid medium. Overall, these results show that
144 the establishment process of single *E. coli* cells in our system can be described by simple
145 branching processes, assuming a uniform cell death probability within each lineage (see
146 Supplement S2), in both liquid and agar environments. However, when comparing the
147 estimates for liquid and agar medium more closely, one notices that the decline of p_e with
148 CTX concentration is less steep on agar than in liquid, even though the average establishment

probabilities are not systematically different. In the following section, we analyse the origin and nature of this difference by modelling the function $p_e(x)$ and fitting it to our data.

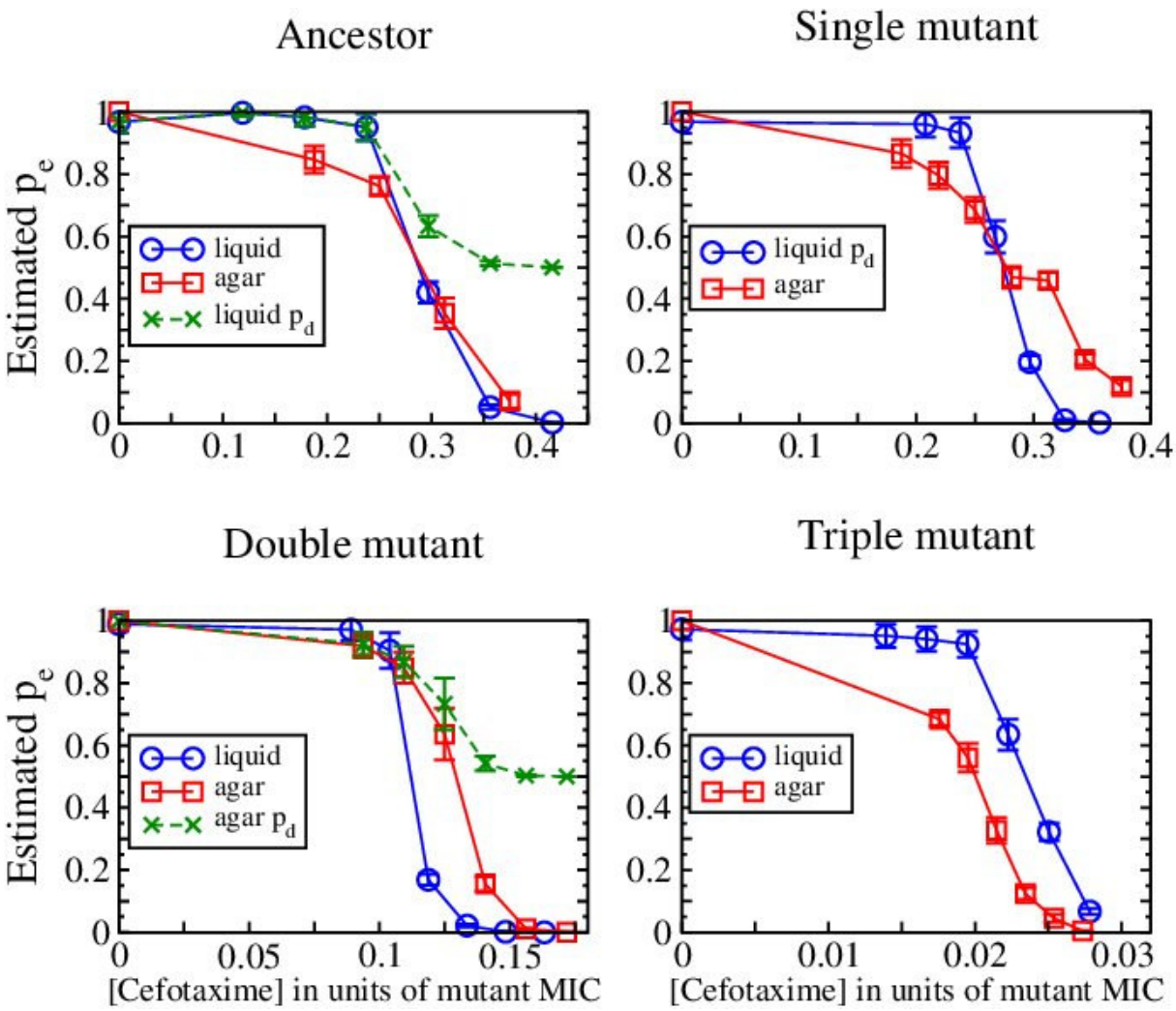


Figure 2. Estimated single-cell establishment probabilities for the four strains as a function of CTX concentration in liquid and on agar. For liquid, the values are obtained as weighted averages over the two inoculum sizes (as shown in Figure 1). For agar, the inoculum size is effectively 1, since each colony usually grows from a single cell. Green dots (with dashed lines) show the per-cell division probability, p_d, (estimated using the branching model without lineage variability), for two particular strains, one each for liquid and agar. The shape of the p_d curve is similar for the other cases (not shown).

<u>Sources of stochasticity and the branching model</u>

When the establishment probability is different from 0 or 1, it means that the colony growth process arising from genetically identical cells in the same environment can have different

outcomes. There can be two different sources of stochasticity in the system. One source we call *inherent noise*, which arises from random thermal fluctuations in chemical reactions. Two nascent cells that are identical in structure and composition may still have different establishment fates due to inherent noise, potentially leading to different outcomes like cell division for one and cell lysis for another. The other source we call *lineage variability*, which we define as the systematic variation between founder cells of colonies, that can arise from phenotypic differences already present between cells in the inoculum or can be caused by small variations across the micro-environments in which the colonies grow. We aim to differentiate between the two sources of stochastic effects in our data and understand the relative contribution of each in the two tested environments with a different structure.

To do so, we model population growth as a branching process, where each cell has a cell division probability, p_d, and consequently a cell death probability, $1 - p_d$. Inherent noise is present whenever p_d is different from 0 and 1 and we assume all cells in a lineage to have the same p_d. In a branching process, the simplest definition of establishment is that the population continues to be of non-zero size indefinitely. For our branching model,

$$p_e = 2 - \frac{1}{p_d} \tag{1}$$

when $p_d \geq \frac{1}{2}$, and $p_e = 0$ when $p_d \leq \frac{1}{2}$ (see Supplement S2 for details). Considering p_d to be identical for all cell lineages rules out lineage variation, and cell fate is purely a result of inherent noise. To see if this is sufficient to explain the data, we estimate p_d from the observed values of p_e, which are shown in Figure 2. The way p_d approaches $\frac{1}{2}$ asymptotically with increasing concentrations is counter to the expectation that $p_d \to 0$ at very high concentration. This indicates either that our definition of establishment is not biologically realistic, or that inherent noise is inadequate for explaining the intermediate values of p_e. We can modify the definition of establishment to make it more realistic by requiring that the process reaches a

188 certain threshold cell number in the time window of the experiment (Schenk et al., 2012). The
189 results (shown in Supplement S2) indicate that this is not sufficient to explain the data. We,
190 therefore, need to include lineage variability into our model.
191
192 Model for $p_e(x)$
193 To arrive at a model that incorporates both sources of stochasticity, inherent noise and lineage
194 variability, we start by modelling p_d as a Hill function of the antibiotic concentration, x, and
195 the antibiotic concentration where p_d is ½, x_0:

196 $$p_d(x, x_0) = \frac{1}{1+\left(\frac{x}{x_0}\right)^n}. \quad (2)$$

197 A mechanistic basis for this functional form is as follows: due to inactivation of penicillin-
198 binding proteins (PBPs, which are the main target of CTX; Rossolini, Arena, & Giani, 2017),
199 there is a shortage of PBP molecules, leading to the formation of cell wall pores, some of which
200 can eventually lead to bulging of the cell wall and lysis (Yao, Kahne, & Kishony, 2012). Let the
201 number of pores be Poisson distributed with mean λ, and let the probability that a pore does
202 not bulge be q. Then p_d is the probability that there are no bulging pores, which can be shown
203 to be equal to $e^{-\lambda(1-q)}$. The dependence of λ on the concentration is not known, but the
204 choice of a simple empirical form for λ produces the function in (2) (see Supplement S5 for
205 details). We emphasize that the choice of the Hill function is not unique; any inverted
206 sigmoidal function would suffice for the purpose. Note that in (2), x_0 is the concentration at
207 which p_d drops to $\frac{1}{2}$, and consequently p_e drops to 0; in other words, x_0 is the single-cell MIC
208 (Artemova, Gerardin, Dudley, Vega, & Gore, 2015). The value of x_0 is determined in part by the
209 level of β-lactamase activity, as a higher antibiotic degradation rate would produce a higher
210 x_0. The quantity $\frac{x}{x_0}$ can be interpreted as the effective (dimensionless) intracellular antibiotic
211 concentration. The Hill exponent n serves as a measure of the inherent noise. In the limit $n \rightarrow$

212 $\infty, p_d(x)$ behaves like a step function that drops sharply from 1 to 0 at $x = x_0$, implying no
213 stochasticity since all cells behave identically (they all divide or all die). Thus high values of n
214 correspond to low stochasticity. For low n, the curve of $p_d(x)$ decays slowly, and therefore has
215 a larger window in which values of p_e are substantially different from 0 or 1, implying higher
216 stochasticity.

217 Lineage variability, on the other hand, is introduced via among-lineage variation in the
218 parameter x_0. Specifically, we model x_0 as a random variable drawn independently for each
219 lineage from the gamma distribution:

220 $$P(x_0) = \frac{1}{\Gamma(k)a^{k-1}} x_0^{k-1} e^{\frac{-x_0}{a}}.$$

221 The mean of x_0 is ka, and the variance is ka^2. The parameter a provides a scaling factor of
222 x_0. The coefficient of variation of x_0 is a measure of lineage variability, which in this case is
223 $k^{-\frac{1}{2}}$; k is therefore inversely related to the degree of lineage variability. The single-cell
224 establishment probability inferred from a large number of observations is then simply the
225 establishment probability (1) averaged over the distribution of x_0. The final model for
226 establishment probability is then:

227 $$p_e(x) = \int_x^\infty dx_0\, P(x_0) \left(1 - \left(\frac{x}{x_0}\right)^n\right). \quad (3)$$

228 The parameter n for intrinsic noise is the same for all mutants, but may, in general, differ
229 between liquid and agar environments. The parameters for the distribution of x_0 can be
230 different for the tested strains and can depend on the environment as well. We performed
231 collective fits using the same value of n for both media, shown in Figure 3. It was estimated
232 that $n = 7.18 \pm 0.60$ and the coefficient of variation on agar was higher than in liquid for
233 most strains, consistent with the less steep curve for agar. In Figure 3, the x-values for each
234 strain have been scaled by the corresponding estimate for mean x_0. The curves for different

strains largely collapse, indicating small (but non-zero) lineage variability in x_0. The coefficients of variation of x_0, which contain the effect of lineage variability, are larger for the agar than for the liquid environment in most cases, as expected, but the difference is small.

We also performed model fits with separate estimates of n for liquid and agar. In this case, the lower mean slope on agar was explained chiefly by a smaller value of n, as shown in the Supplement (S3: Figure S3). The lineage variability was, in this case, similar for both media except in the case of the Single mutant. The fit was equally good (see Supplement S6 for details), making it hard to distinguish between the contribution of both sources of stochasticity based on curve-fitting alone. However, the assumption of a universal value for n is more parsimonious, and higher lineage variability on agar compared to liquid may also be expected from its supposedly greater environmental heterogeneity. It thus seems likely that a higher lineage variability is the main contributor for the lower slope of $p_e(x)$ on agar, but a contribution from inherent noise cannot be ruled out.

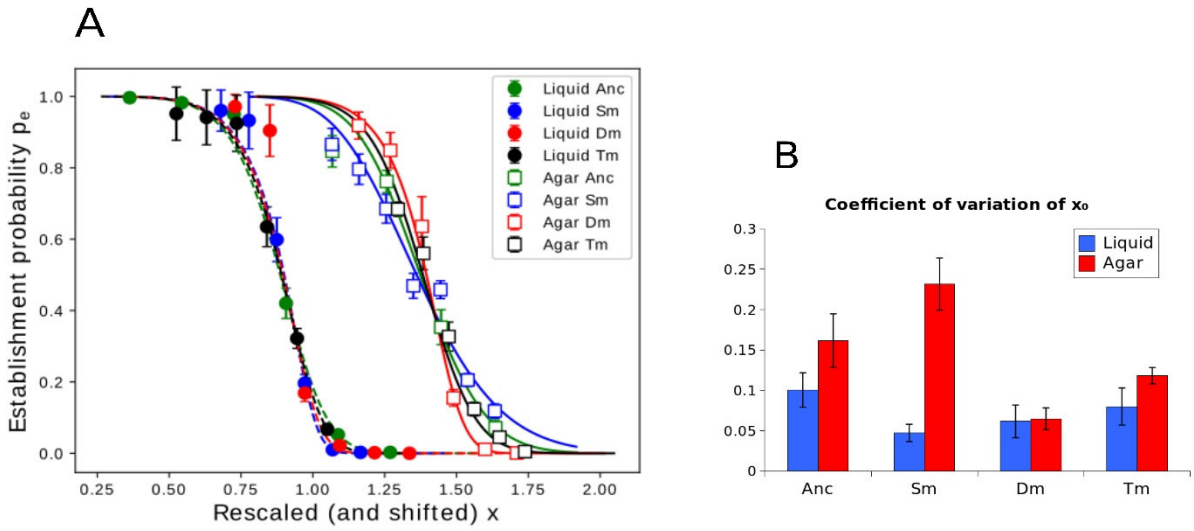


Figure 3. Effect from intrinsic noise and lineage variability in x_0 on single-cell establishment probability in liquid compared to agar. A joint fit of equation (3) with the same value for n for both environments was used. **A**: $p_e(x)$ obtained from curve-fitting. The x-values for each strain (Anc: TEM-1 Ancestor, Sm: Single mutant, Dm: Double mutant, Tm: Triple mutant) have been scaled by their respective mean x_0, and the values for agar have been shifted along the x-axis to the right by 0.5 to improve visibility only. **B**: The coefficient of variation of x_0 (mean ± S.D.), indicating higher lineage variability on agar than in liquid for most strains.

256 Minimum inhibitory concentration

257 One of the main findings in the *P. aeruginosa* study mentioned above (Alexander & MacLean,
258 2020), was that the used mutant strain, when seeded alone at a few cells in a similar setup as
259 ours, showed a <5% establishment probability at a streptomycin concentration of 1/8 of the
260 strain's MIC. At the same MIC fraction, the establishment probabilities for our strains are
261 about 100, 100, 8 and 0% for the Ancestor, Single mutant, Double mutant and Triple mutant,
262 respectively. These results show that there is no general relationship between the MIC and
263 the single-cell establishment probability, neither across systems nor among genotypes of the
264 same species within the same system. The cause for this is likely rooted in how the MIC is
265 measured. In a standard MIC assay, 10^5 cells are inoculated into 200 µl medium in a series of
266 wells with 2-fold differences in antibiotic concentration. Thus, the "real" MIC value lies
267 between the smallest concentration where no growth is observed (defined as MIC) and half
268 this concentration. The concentration range of where the real MIC lies, is then smaller or
269 larger depending on the MIC of the respective strain. Another issue with the MIC is that even
270 the growth of an inoculum as large as 10^5 cells is a stochastic process under sufficiently high
271 antibiotic concentrations (Coates et al., 2018), which generally results in a reduced
272 reproducibility of MIC assays.

273 A conceptually cleaner approach to determine the MIC would be to recognise this
274 stochasticity. Here, we introduce a new measure, $MIC_q(N)$, defined as the concentration at
275 which an inoculum of size N has a probability q of not becoming established. In the absence
276 of interactions between lineages, $MIC_q(N)$ can be predicted from $p_e(x)$ (see Supplement S7
277 for details). Setting $q = \frac{1}{2}$ and $N = 10^5$, we obtain $MIC_q(N)$ values of 0.038, 0.23, 1.6, and
278 2.9 µg/ml CTX for the Ancestor, Single mutant, Double mutant and Triple mutant, respectively.
279 The values are smaller than the previously determined MICs (Table 1), and the differences

280 become increasingly pronounced with the level of resistance of the mutants. This suggests
281 that positive interactions among cell lineages enhance their collective establishment due to
282 the enzymatic breakdown of the antibiotic in the medium, which is particularly pronounced
283 at high cell densities and for cells expressing enzymes with high activity against CTX.

284 The effect of inoculum size on the MIC through social interactions has been recognised before
285 (Brook, 1989; Tan et al., 2012). Causes for such social effects have been suggested to include
286 binding of the antibiotic to cellular components (Queenan, Foleno, Gownley, Wira, & Bush,
287 2004) or the expression of proteins that inactivate the antibiotic (Lenhard & Bulman, 2019).
288 Our new measure includes a purely statistical inoculum effect, which leads to a logarithmic
289 increase of $MIC_q(N)$ for large N (see Supplement S7 for details). Our results on the
290 discrepancy between the MIC and the single-cell establishment probability are in line with the
291 findings of other studies that criticised the MIC assay as not suitable for dosage determination
292 for the treatment of antibiotic-resistant infections (Gould & MacKenzie, 1997; Artemova et
293 al., 2015).

294

295 **Conclusion**

296 Our study contributes to the understanding of the establishment of adaptive mutants, a topic
297 which has been under much theoretical scrutiny but is rarely investigated empirically. We
298 show that establishment of populations starting from a single cell in the presence of an
299 antibiotic can be well described by a simple branching model both in liquid and agar
300 environments, where the cell division probability is dependent on the antibiotic
301 concentration. We find that the establishment of single mutant cells is negatively affected by
302 CTX concentrations well below each tested strains' MIC, consistent with previous
303 findings(Alexander & MacLean, 2020).

304 One aim of our study was to test if cooperative behaviour (i.e. collective antibiotic breakdown)
305 among cells of the same strain would affect establishment probability and whether such an
306 effect differed between unstructured and structured environments. Our data show a
307 reasonable agreement between establishment in liquid and agar media, indicating that
308 environmental structure plays only a modest role, and that cooperative behaviour has a
309 negligible effect on the establishment probability of single resistant mutant cells. However,
310 the $p_e(x)$ curves for agar are slightly shallower than for the liquid environment, indicating
311 higher stochasticity. By evaluating the effect of two potential sources of stochasticity, random
312 intrinsic noise and systematic (e.g. heritable) variability among cell lineages, we found that
313 the simplest explanation for this observation is an enhanced lineage variability on agar,
314 possibly arising from small differences in local agar conditions.

315 Lastly, we show that social interaction effects on the establishment of antibiotic-resistant
316 mutants can be strong at high cell densities, making conventional MIC values highly sensitive
317 to the assay conditions. We suggest a more robust measure of resistance which includes both
318 inoculum size and probabilistic effects, $MIC_q(N)$, and can be compared easily with p_e to test
319 for the presence of interactions.

320

321 **Material and Methods**

322 Strains and culture conditions

323 Four strains were used for the experiments, derived from the *Escherichia coli* strain MG1655
324 DA28100. This strain had previously been modified with a YFP (yellow fluorescent protein)
325 marker cassette, containing a resistance gene for chloramphenicol (Gullberg, Albrecht,
326 Karlsson, Sandegren, & Andersson, 2014). For the current study, the chloramphenicol
327 resistance was removed, and each one of four TEM variants was inserted into the *galK* locus:

328 the ancestral allele TEM-1 (referred to as "Ancestor" strain), and three mutant alleles with
329 either 1, 2 or 3 point mutations in the TEM allele (referred to as "Single mutant", "Double
330 mutant" and "Triple mutant", respectively). While the TEM-1 ancestral allele confers only low
331 activity towards the β-lactam antibiotic cefotaxime (CTX), the three mutants show increasing
332 CTX resistance with each additional substitution (Table 1). All TEM loci are under the control
333 of the *lacI* repressor and are expressed by adding 50 µM Isopropyl β-D-1-
334 thiogalactopyranoside (IPTG) to the growth medium. The minimum inhibitory concentration
335 (MIC) of each strain for CTX had previously been determined in duplicates (Table 1), using 2-
336 fold increases in CTX concentration in microtiter plates filled with 200 µl M9 minimal medium
337 (containing 0.4% glucose, 0.2% casaminoacids, 2 µg/ml uracil and 1 µg/ml thiamine) and 50
338 µM IPTG, inoculated with 10^5 cells and incubated for 24h at 37°C.

339 For culturing, all strains were first recovered from glycerol stocks by streaking them out on
340 each one LB (Luria-Bertani) agar plate and incubated overnight at 37°C. From those plates,
341 each one colony was picked into 1 ml M9 medium (as above) and incubated overnight at 37°C,
342 250 rpm. The cultures were then serially diluted with phosphate-buffered saline (PBS) to the
343 density needed for the particular experiment (see below), assuming an initial density of
344 2.5×10^9 cells/ml.

345

346 **Table 1:** Overview and minimum inhibitory concentration (MIC) of the used strains.

Wildtype strain (without TEM allele)	**TEM amino acid substitution**	**MIC for Cefotaxime (µg/ml)**		**Strain name (as used here)**
		MIC*	$\boldsymbol{MIC_q(N)}$**	
MG1655 DA28100 galK::YFPΔCAT-	TEM-1	0.08	0.038	Ancestor
	G238S	0.64	0.23	Single mutant
	E104K/G238S	10.24	1.6	Double mutant
	E104K/M182T/G238S	81.92	2.9	Triple mutant

347 *As determined prior to the study by standard MIC assays (see Material and Methods).

348 **As predicted from $p_e(x)$ in the current study (see the last section of Results and Discussion, and
349 Supplement S7).
350

351 Liquid experiment

352 Each of the four strains was tested separately in the absence of CTX and six CTX concentrations
353 (Supplement S1: Table S1), where the particular tested concentrations depended on the
354 strains' resistance level. The range of concentrations was chosen to display establishment
355 probabilities between 0 and 1, based on pilot experiments to find the right conditions (data
356 not shown).

357 Per strain and CTX concentration, on average either about 1 or 2 cells were seeded into 285
358 wells across three 96-well microtiter plates. For this, 190 µl M9 medium with the respective
359 cefotaxime concentration and 50 µM IPTG were pipetted into all wells. Overnight cultures of
360 the strains were diluted to 200 and 100 cells/ml with PBS. 10 µl of the dilutions were pipetted
361 into the wells. One well per plate served a medium control, mock-inoculated with 10 µl PBS.
362 The plates were incubated about 40 h at 37°C (static). After incubation, OD at 600 nm of all
363 wells was measured in a plate reader (Victor3™, PerkinElmer) without the plate lid. All medium
364 controls across the experiment showed no sign of growth; the OD ranged from 0.033 to 0.038.
365 Thus, a threshold of >0.05 was applied to determine whether a well successfully established
366 (i.e. showed growth).

367 To test for correct culture dilutions and Poisson distribution of seeded cell numbers, the same
368 culture dilutions used for seeding the well plates were dropped on LB agar plates (120 x 120
369 mm). Per dilution and strain, a total of 3x32 10 µl drops were applied to the plates, let dry for
370 about 10 to 20 minutes, incubated at 37°C until leaving, and then moved to 30°C overnight to
371 prevent overgrowth, followed by counting the number of colonies per droplet.
372

373 Agar experiment

374 As in the liquid experiment, all strains were tested separately and the CTX concentration range
375 depended on the strain. Five to eight concentrations were tested per strain (Supplement S1:
376 Table S1). The overnight cultures were diluted to 4000 cells/ml. 50 µl (~200 cells) of the
377 dilutions were spread onto 92 mm agar plates containing M9 medium (as above) with 1.5 %
378 agar, 50 µM IPTG and the respective cefotaxime concentration, using a bacterial spreader. Per
379 strain and cefotaxime treatment, eight replicate plates were used. The plates were incubated
380 at 37°C until colonies were big enough to count them unambiguously (20 to 48 hours). Each
381 colony is regarded as a successfully established single cell.

382

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386

387 **Author contribution**

388 All authors conceived the study. MS and AH conducted the experiments. SGD developed the
389 models with help from JK. MS and SGD wrote the manuscript with help from JK and JAGMdV.
390 All authors read the manuscript and approved the final version.

391

392 **Competing Interests Statement**

393 All authors declare that they have no significant competing financial, professional, or personal
394 interests that might have influenced the performance or presentation of the work described
395 in this manuscript.

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