Stochastic effects during the establishment of β-lactam resistant *E. coli* mutants indicate conditions for collective resistance

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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. Comprehending the stochastic factors that influence 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 antibiotic concentration in both unstructured (liquid) and structured (agar) environments. We show that concentrations well below the minimum inhibitory concentration (MIC) can substantially hamper establishment, particularly for highly resistant mutants. While the pattern of establishment suppression is comparable in both tested environments, we find greater variability in establishment probability on agar. Using a simple branching model, we investigate possible sources of this stochasticity, including environment-dependent lineage variability. Lastly, we use the single-cell establishment probability to predict each strain's MIC in the absence of social interactions. We observe substantially higher measured than predicted MIC values, particularly for highly resistant strains, which indicates cooperative effects among resistant cells at large cell numbers, such as in standard MIC assays.

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 and 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 generalized and extended by a great number of models, but none of them has been tested empirically until about a decade ago (Patwa and Wahl 2008). With advances in genomic techniques, the issue of fixation probability has more 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 once they reach a relatively high 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 *et al.* 2013), bacteria (Farrell *et al.* 2017; Giometto *et al.* 2018) and nematodes (Chelo *et al.* 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 bacterial pathogens has led to the awareness that this problem must be tackled from different angles, including a better understanding of the emergence and evolution of antibiotic resistance (Baym, Stone, & Kishony, 2016; Smith et al., 2016; Furusawa, Horinouchi, & Maeda, 2018). Resistance alleles can be acquired via horizontal gene transfer or *de novo* mutation, resulting in the resistant allele initially being present in one or a few individuals within a susceptible population. But how likely is it that this single genotype can establish in the population and what factors influence this process? Using time-lapse microscopy, Coates et

50 al. (2018) followed the growth of single *Escherichia coli* cells on agar, supplemented with
51 different antibiotics. They found that the establishment of a single cell, determined by its
52 growth into a visible macrocolony, was a highly stochastic process driven by fluctuations in
53 cell death and birth, leading to the extinction of microcolonies at various time points. Similar
54 results were found for two *Pseudomonas aeruginosa* strains, resistant to streptomycin or
55 meropenem (Alexander and MacLean 2020). By seeding one to a few cells of a resistant strain
56 in liquid medium with increasing antibiotic concentrations, it was found that at concentrations
57 as low as 1/8 of the minimum inhibitory concentration (MIC) of this strain, its establishment
58 probability was only 5% and that establishment of one cell was independent of the presence
59 of other cells. The latter finding, however, may differ in systems where the antibiotic is broken
60 down in the environment, leading to positive social interactions (Medaney *et al.* 2016).

61 A common resistance mechanism potentially allowing for social effects involves β-lactamase
62 enzymes. β-lactamases hydrolyze 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 is the ancestor of a large family of extended-spectrum β-lactamases, that have acquired
69 mutations causing enhanced activity against more recently introduced penicillins and
70 cephalosporins, including CTX (Salverda *et al.* 2010; Schenk *et al.* 2012; Van Dijk *et al.* 2017).
71 As β-lactamases are expressed in the periplasmic space of gram-negative pathogens like *E.*
72 *coli*, this system has been recognized as potentially cooperative, since the enzymatic
73 breakdown in the periplasm reduces the antibiotic concentration in the environment (Brown

74 *et al.* 2009), thereby cross-protecting susceptible bacteria nearby (Medaney *et al.* 2016). Such
75 a cooperative behavior can be expected to have more pronounced consequences on agar due
76 to the local breakdown of the antibiotic, creating microenvironments that may allow for the
77 co-existence of strains with different levels of antibiotic resistance (Nicoloff & Andersson,
78 2016; Frost et al., 2018; Geyrhofer & Brenner, 2020).

79 Here, we examined the single-cell establishment probability of antibiotic-degrading bacteria
80 under a range of antibiotic concentrations in both a structured (agar) and an unstructured
81 (liquid) environment. We used four *E. coli* strains expressing β-lactamase alleles with different
82 activities toward CTX, and hence different levels of resistance. Each strain was tested
83 separately at low cell densities in both liquid and on agar medium with increasing CTX
84 concentrations. To determine the single-cell establishment probability from the data, we
85 developed a simple branching model, followed by a more detailed investigation of the cause
86 of differences in stochasticity between the two environments. We show that environmental
87 structure and cooperative behavior play at most a minor role in the establishment of resistant
88 cells at low initial densities. Based on our results, we further introduce a new measure for
89 resistance level that takes inoculum size and stochasticity into account. Application of the new
90 measure to our data indicates substantial cooperative effects at higher cell densities, as
91 typically used in standard MIC assays.

92

93 **Results and Discussion**

94 In this study, we aimed at quantifying how the establishment of single bacterial cells with
95 different levels of antibiotic resistance is affected by: (i) the antibiotic concentration, (ii) the
96 environmental structure, and (iii) positive social interactions via the breakdown of the
97 antibiotic in the environment. To determine single cell establishment probabilities, we

98 performed one experiment in liquid (unstructured environment) and one on agar medium
99 (structured environment). In each experiment, four *E. coli* strains (referred to as Ancestor,
100 Single mutant, Double mutant, Triple mutant; cf. Table 1) with increasing levels of CTX
101 resistance conferred by different antibiotic-hydrolyzing β-lactamase enzymes, were tested
102 separately under exposure to a range of CTX concentrations. The tested concentrations
103 differed between strains and were chosen to display establishment probabilities between 0
104 and 1 (see Material and Methods, and Supplement S1 - Table S1). We use the notation $p_e(x)$
105 to denote the probability that a single cell establishes a macroscopic population (i.e. turbidity
106 in liquid or colony formation on agar, visible to the naked eye) at CTX concentration x. When
107 the antibiotic concentration is zero, a population should establish with near certainty.
108 Likewise, when the concentration is above the strains' minimum inhibitory concentration
109 (MIC), establishment is expected to be prevented. Stochastic outcomes can arise only at
110 intermediate concentrations.

111 <u>Establishment probability declines well below the MIC in liquid</u>

112 In the liquid medium experiment, we grew bacteria in multi-well plates and observed their
113 establishment as a function of CTX concentration. Per strain, inoculum size (about 1 and 3 cells
114 on average) and antibiotic concentration, a total of 285 replicate wells were tested, spread
115 across three plates, including one medium control well per plate. Estimates of the viable cell
116 numbers present in the inocula for evaluating the probabilities were obtained by applying cell
117 culture aliquots on agar, taken from the culture dilutions that were used for inoculating the
118 well plates. Using a bootstrapping algorithm and assuming a Poisson distribution of
119 independently acting cells in each inoculum (details in Supplement S2), we estimated the
120 single-cell establishment probability $p_e(x)$ for all strains and conditions. The results in Figure

121 1 show that the establishment probability nears zero well below each strain's MIC and that
122 this effect is more pronounced for strains with higher resistance levels.

123 The observation that the establishment probability of a single cell nears zero at antibiotic
124 concentrations well below the strain's MIC has been shown before in *E. coli* for various
125 bactericidal antibiotics (Coates *et al.* 2018) and in *P. aeruginosa* for streptomycin and
126 meropenem (Alexander and MacLean 2020). These studies showed that the underlying causes
127 for this are rooted in stochastic effects in population dynamics due to among-cell variability in
128 death rate (Coates *et al.* 2018; Alexander and MacLean 2020) and lag-phase (Alexander and
129 MacLean 2020), which become more pronounced with increasing antibiotic concentration.
130 Therefore, the establishment probability is higher with increasing initial cell numbers, simply
131 because more cells can survive and outgrow into a population (Coates *et al.* 2018; Alexander
132 and MacLean 2020). This explains the decreased establishment probability of single cells at
133 lower antibiotic concentrations compared to each strain's MIC, as the latter is measured for
134 inocula containing hundreds of thousands of cells per mL. While we confirm the results of this
135 general pattern in our study, we extend the findings by using four *E. coli* strains with the same
136 genetic background but different sensitivities toward the β-lactam antibiotic CTX. As these
137 differences in sensitivity are based on differences in the level of β-lactamase activity (i.e.
138 enzymatic capacity in breaking down CTX), our system provides the opportunity to study the
139 effect of positive social interactions during establishment. Indeed, the increasingly
140 pronounced discrepancy between the single-cell establishment probability and standard MIC
141 estimates that is observed with increasing resistance level is, at least partially, attributed to
142 an increased collective breakdown at high cell densities (see section "MIC and the inoculum
143 effect" for details).

144 <u>Cells do not interact in liquid medium during establishment</u>

145 One complication in estimating the single-cell establishment probability in liquid medium is
146 that multiple cells may be present initially in some wells, and these cells may in principle
147 interact, for example through competition for resources or the breakdown of antibiotic
148 molecules. In that case, the establishment probabilities of individual cells would no longer be
149 independent, biasing our estimates of $p_e(x)$. To test whether cells interact within initially small
150 inocula, we conducted the liquid experiment with two different mean inoculum sizes (with
151 approximately 1 and 3 cells, respectively) and estimated $p_e(x)$ for both cases under the
152 assumption that there is no interaction (details in Supplement S2). If interactions occurred,
153 the inferred values should disagree. However, Figure 1 shows that they agree closely,
154 indicating that interactions between cells are insignificant at these low cell densities in
155 unstructured environments.

156 The absence of social interactions in liquid medium at low cell density was also found in the
157 study with *P. aeruginosa*, exposed to streptomycin or meropenem under similar conditions
158 regarding culture volume and inoculum size (Alexander and MacLean 2020). In that system,
159 positive social interactions are less likely to occur, because streptomycin resistance does not
160 involve antibiotic-degrading enzymes. Our *E. coli* β-lactamase system on the contrary involves
161 the potential for cooperative effects due to an active breakdown of the antibiotic in the
162 environment. The absence of an effect in our data confirms that cell-to-cell interactions (both
163 positive and negative) play an insignificant role during the establishment of single cells at such
164 low densities in liquid medium and further shows that this observation is independent of the
165 resistance mechanism.

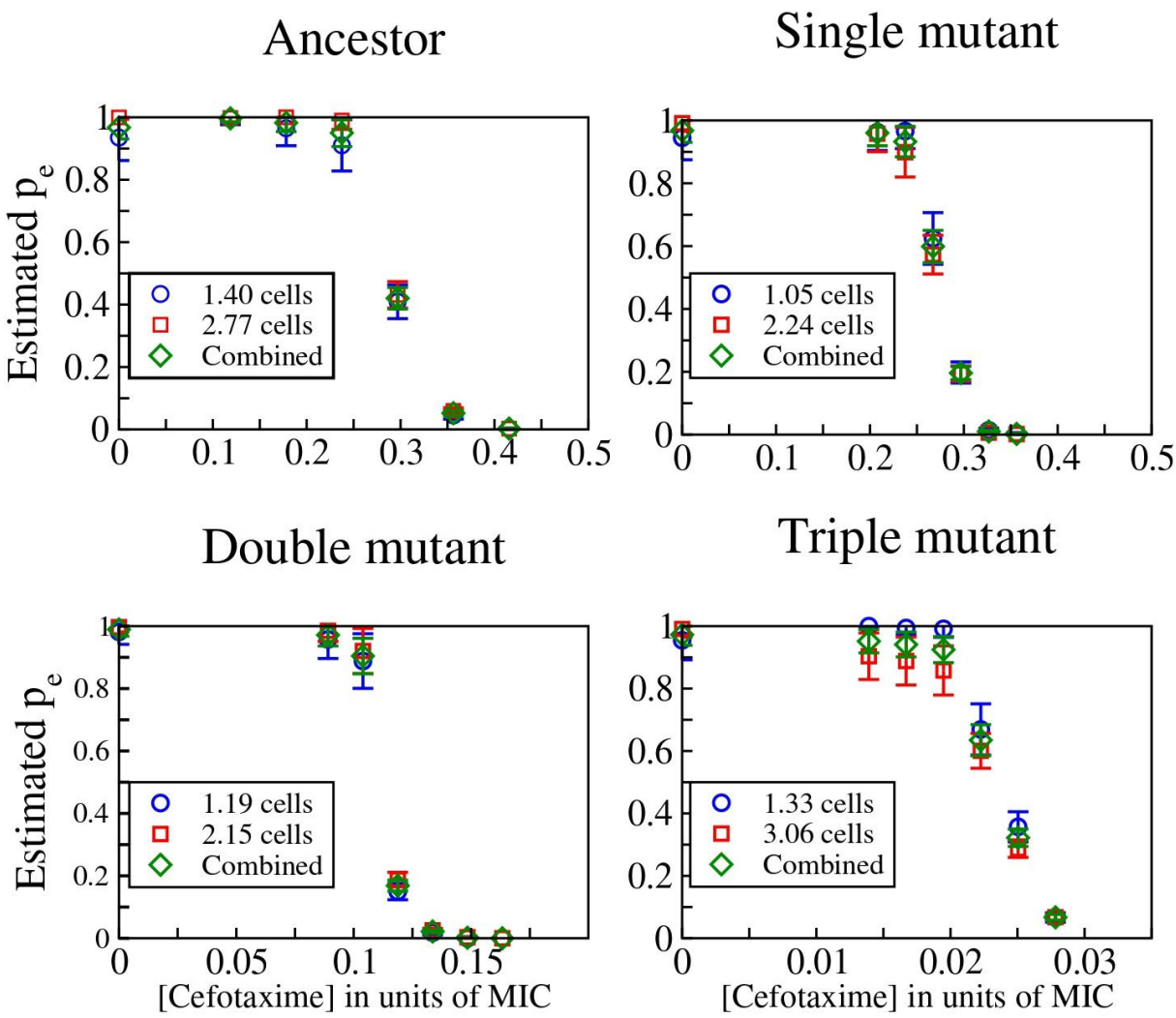

Figure 1. Single-cell establishment probabilities, p_e, in liquid medium, estimated for the four tested strains as a function of antibiotic concentration, shown as a fraction of each strain's measured MIC (cf. Table 1). For each strain, two inocula were used, one of approximately 1 cell (blue circles) and another of 2-3 cells (red squares); the average inoculum size estimated from separate CFU counts is shown. The inferred single-cell establishment probabilities are nearly identical for the two cases, implying that the establishment probability of a cell is not influenced by the presence of other cells at these low initial cell densities. The green diamonds are weighted averages over the two inoculum sizes (see Supplement S2 for details on the method used to estimate p_e).

<u>Establishment probability shows moderately higher stochasticity on agar</u>

In the previous section, we found that the establishment probability of one cell was not influenced by the presence of other cells in liquid medium at very low densities. But how is this on agar? Previous studies have shown that evolutionary outcomes can largely differ depending on the environmental structure in the absence of migration (Perfeito *et al.* 2008;

181 Lavrentovich *et al.* 2016; Santos-Lopez *et al.* 2019). For instance, while conditions in
182 unstructured (liquid) environments commonly select for one strain to dominate the
183 population, structured (agar) environments can maintain various strains due to the creation
184 of microenvironments (Rainey and Travisano 1998; Kerr *et al.* 2002; Habets *et al.* 2007). One
185 condition that can be affected locally on agar is the concentration of the antibiotic due to
186 enzymatic breakdown. While it has been shown that this can result in cooperative effects,
187 where a resistant strain protects more sensitive ones (Medaney *et al.* 2016; Rojo-Molinero *et*
188 *al.* 2019) and such cooperation is more prominent in structured environments (Geyrhofer and
189 Brenner 2020), it is, to the best of our knowledge, unknown whether such social interactions
190 can influence the establishment of single antibiotic-resistant cells. To determine the effect of
191 environmental structure on the establishment of β-lactamase-expressing cells, we plated
192 about 200 cells of each *E. coli* strain on CTX-containing agar (eight plates per condition),
193 followed by counts of colony-forming units (CFUs) after incubation. As for the experiment in
194 liquid medium, the tested CTX concentration range depended on the strain to display
195 establishment probabilities between 0 and 1 (see Material and Methods, and Supplement S1
196 - Table S1). The establishment probability was estimated as the CFU count relative to that at
197 no CTX. The patterns of p_e as a function of CTX concentration are broadly similar to those in
198 liquid medium (Figure 2), indicating a minor role of environmental structure being involved in
199 mutant establishment at low cell densities.

200 Although we found a general agreement of the establishment probability pattern between
201 the liquid and agar experimental data, a closer comparison of the estimates of both
202 environments shows that the decline of p_e with CTX concentration is less steep on agar than
203 in liquid (Figure 3a). This implies a higher degree of stochasticity on agar, since p_e remains
204 between 0 or 1 over a larger range of concentrations. We quantified this effect by calculating

205 the Shannon entropy for the establishment probabilities in both environments and all four
206 strains, finding that the entropy is higher on agar (Figure 3b).

207 Next, using a modelling approach we explored possible underlying mechanisms for
208 stochasticity in the system. The simplest model is a branching process, where every cell divides
209 with a fixed probability p_d, or dies otherwise (see Supplement S3 for details), allowing us to
210 estimate the division probability p_d using the measured p_e values. Figure 2 shows that under
211 the simple branching model, the division probability appears to approach the value ½
212 asymptotically as opposed to the natural expectation of approaching zero asymptotically (see
213 Figure S1 for an illustration of the possible shapes of the p_e and p_d curves in the simple
214 branching model). This is a clear indication that the simple model does not fully capture the
215 establishment process. One way to modify the model is to introduce variability in p_d among
216 cell lineages, and this fits the data reasonably well (see Supplement S4). Within this
217 framework, the higher establishment stochasticity on agar could be due to small differences
218 in p_d of different cells on the agar surface (see Supplement S4 and Figure S3 for details).
219 However, other possibilities remain; for instance, the cell division probability may change with
220 time due to accumulating damages in cell wall structure under antibiotic action, changing
221 rates of antibiotic and enzyme flux or changing CTX concentration over time at the location of
222 a growing colony. Distinguishing conclusively between these alternatives is not possible based
223 on our data, and we do not explore this issue any further here.

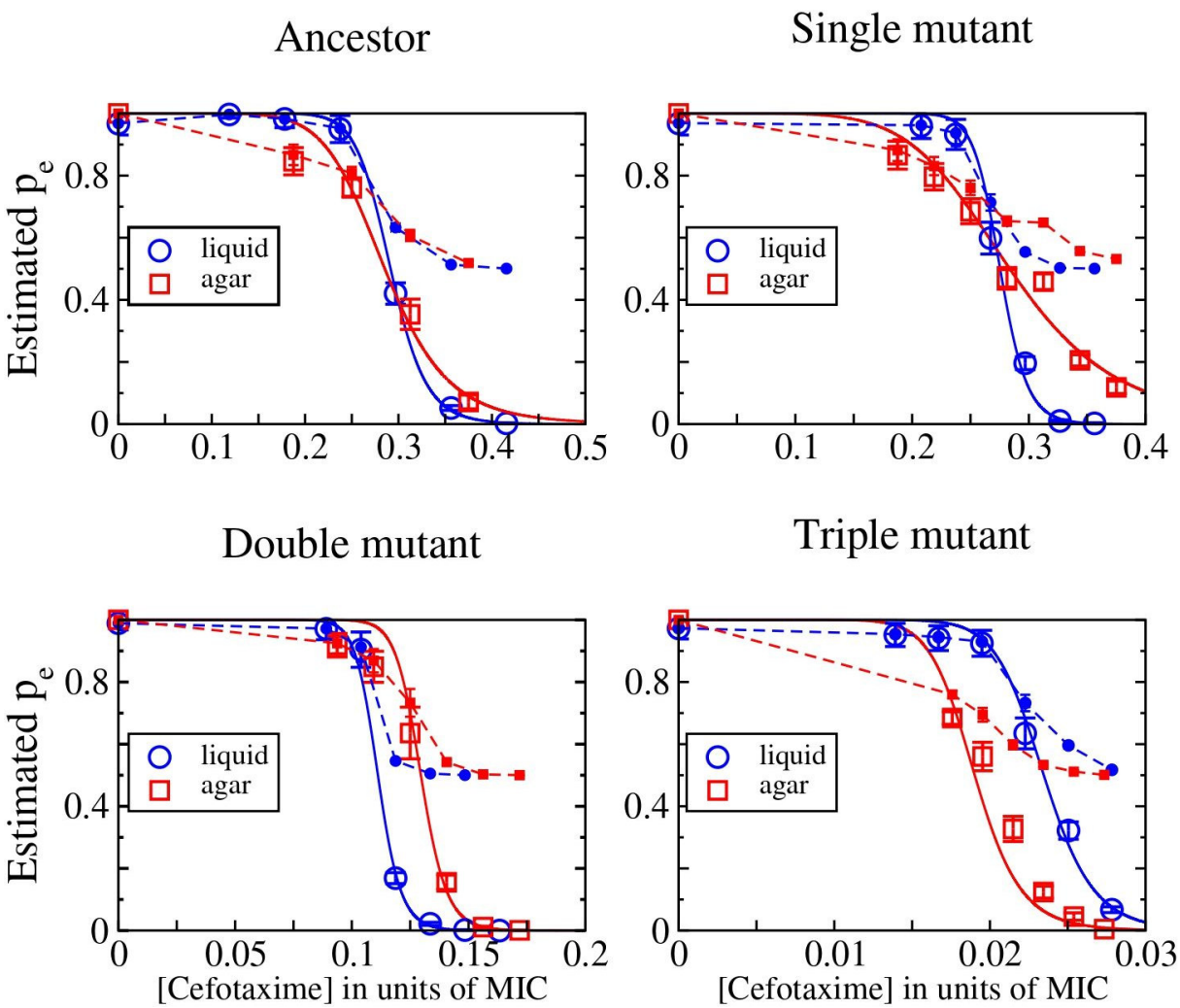

Figure 2. The single-cell establishment probability, p_e, is shown as open symbols for the four tested strains in liquid (blue) and agar environments (red) for different CTX concentrations as the fraction of each strain's measured MIC. For liquid medium, the combined results from both tested inoculum sizes (cf. Figure 1) are shown. The solid lines are fits performed with the Hill function $p_e(x) = (1 + (x/x_0)^n)^{-1}$ with x_0 and n as parameters, where x_0 is the concentration at which p_e drops to $\frac{1}{2}$, and n indicates the steepness of the curve. The values of the inferred parameters are given in Supplement S5, Table S2. The filled symbols connected by dashed lines show the cell division probability p_d inferred from a simple branching model (see main text).

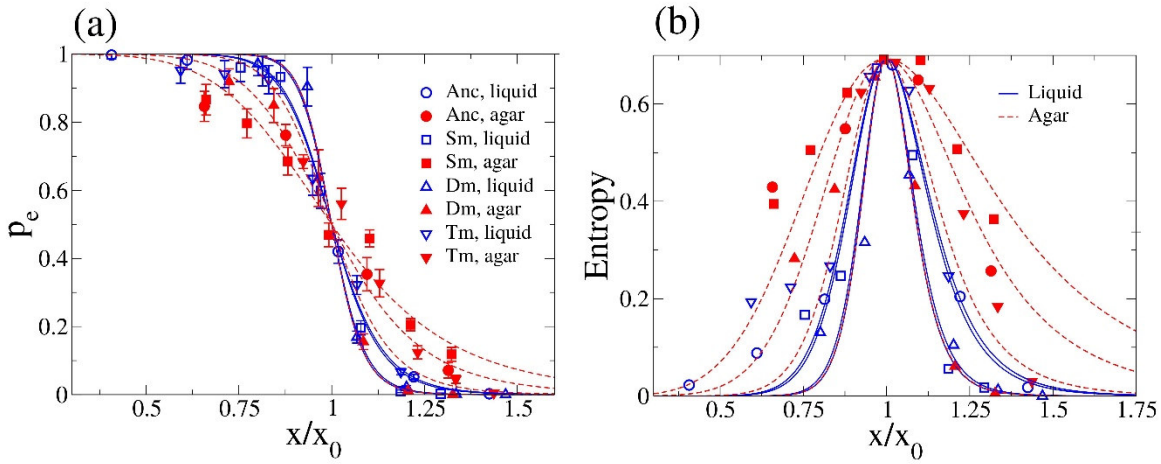

Figure 3. (a) Establishment probabilities for all the strains in both liquid and agar environments as a function of CTX concentration (Anc: Ancestor, Sm: Single mutant, Dm: Double mutant, Tm: Triple mutant), scaled by the corresponding estimate x_0 (i.e. the concentration at which p_e drops to $\frac{1}{2}$). The curves for agar medium (red dotted lines) are less steep than those for liquid medium (blue solid lines). **(b)** The Shannon entropy is calculated and plotted for the p_e values, using the standard expression $S = -p_e \ln p_e - (1 - p_e)\ln(1 - p_e)$. It is seen to be higher for agar (red lines and symbols) compared to liquid (blue lines and symbols), as expected from the shallower slopes of the $p_e(x)$ curves for agar in panel (a).

<u>MIC and the inoculum effect</u>

Results from our experiments in liquid medium have shown a discrepancy between the CTX concentration where the single-cell establishment probability, p_e, nears zero and the standard MIC value. As mentioned above, the reasons for this lie in the fact that standard MIC values are measured from large inocula of 5 x 10^5 cells/mL, while establishment is a highly stochastic process at the cellular level. The stochastic nature of MIC estimates can be seen from the limited reproducibility of MIC assays, despite its coarse scale (Coates *et al.* 2018).

A conceptually cleaner approach to determine the MIC of a strain would therefore be to focus on a quantity that considers the role of stochasticity and initial inoculum size in the establishment of a population. For this purpose, we define the measure $\mathrm{MIC}_q(N)$ as the minimum concentration at which an inoculum of initially N cells has a probability q of not being established (see Supplement S6). We suggest a null-model for this quantity, which assumes that the fates of the lineages arising from the N initial cells are independent, and

257 therefore the extinction probability of the entire population equals $(1-p_e)^N$. Predictions
258 based on this null-model can be used to test for the presence of interactions in the
259 establishment process. For example, in a traditional MIC assay with an inoculum of 10^5
260 cells/200µL, the null model predicts that $\mathrm{MIC}_{\frac{1}{2}}(10^5)$ has the values of 0.053 (±3.5%), 0.31
261 (±4.7%), 2.0 (±3.6%), and 4.5 (±6.6%) µg/mL for the Ancestor, Single mutant, Double mutant
262 and Triple mutant, respectively (see Supplement S6 for further details). The differences
263 between these predicted values and the experimentally determined MICs (cf. Table 1) become
264 increasingly pronounced with the level of resistance of the mutants. A choice of q other than
265 ½ produces only modest changes in the predicted values (unless q is very close to the limits 0
266 or 1; see Supplement S6), and given the limited resolution of the MIC assay with two-fold
267 increases in concentration, the precise choice of q does not affect the conclusion. The pattern
268 of differences between the null-model $\mathrm{MIC}_{\frac{1}{2}}$ prediction and the measured MICs prompted us
269 to calculate the likelihood, L, based on the null-model, that the inoculum establishes at the
270 highest concentration at which growth is observed in the MIC assay (i.e. at half the reported
271 MIC, due to the two-fold dilution steps). The results are shown in Table 1. While L is high for
272 the Ancestor, it is low for all the mutants (L<2%), indicating that the measured MIC for the
273 mutants is significantly above what we predict in the absence of social interactions (Table 1).
274 This suggests that positive interactions among cells present at high density substantially
275 enhance the collective establishment of mutant cell populations.

276 The effect of inoculum size on the MIC through social interactions has been recognized before
277 (Brook 1989; Tan *et al.* 2012) and causes have been suggested to include the binding of the
278 antibiotic to cellular components (Queenan *et al.* 2004) or the expression of proteins that
279 inactivate the antibiotic (Lenhard and Bulman 2019). The four *E. coli* strains used here are
280 genetically identical apart from the point mutations in the TEM allele, meaning that the

281 number of penicillin-binding-proteins (PBPs), which are the main binding target of CTX (Piras
282 *et al.* 1990; Cho *et al.* 2014), are comparable between them. Thus, binding of CTX molecules,
283 although possibly contributing to the discrepancy between measured and predicted MICs,
284 cannot explain the increasingly pronounced differences between the two resistance measures
285 with increasing resistance levels of the mutant strains. As the level of resistance is conferred
286 by increased enzyme activity against CTX with each additional point mutation (Knies *et al.*
287 2017), the collective breakdown of the antibiotic in the classical MIC assay is a likely
288 explanation for the observed effect. Overall, our results on the discrepancy between the
289 standard MIC value and the single-cell establishment probability are in line with the findings
290 of other studies that criticized the MIC assay for dosage determination for the treatment of
291 antibiotic-resistant infections (Gould & MacKenzie, 1997; Artemova et al., 2015; Alexander &
292 MacLean, 2020).

293 **Table 1:** Overview of the used strains with their respective measured minimum inhibitory
294 concentration (MIC) and null-model likelihood of the corresponding predicted MICs (see main
295 text).

Wildtype strain (without TEM allele)	**TEM amino acid substitution**	**Strain name (as used here)**	**MIC for Cefotaxime* (µg/mL)**	**Null-model likelihood L****
MG1655 *galK*::SYFP2-CAT	none (TEM-1)	Ancestor	0.08	>99.99%
	G238S	Single mutant	0.64	**1.1%**
	E104K/G238S	Double mutant	10.24	**<0.01%**
	E104K/M182T/G238S	Triple mutant	81.92	**<0.01%**

296 *As determined prior to the study by standard MIC assays (see Material and Methods). Note that the
297 true MIC lies between this value and half of this concentration (due to the 2-fold dilution during the
298 setup).
299 **Assuming that the inoculum establishes at the highest concentration at which growth is observed in
300 the MIC assay (see the last section of Results and Discussion); values that show significant differences
301 between the measured and predicted MICs are depicted in bold.
302

303 **Conclusion**

304 This study advances our understanding of the stochastic nature of the establishment of
305 initially rare adaptive mutants (e.g. due to *de novo* mutation), a topic that has been under

306 much theoretical scrutiny, but has rarely been investigated empirically. We find that the
307 establishment of *E. coli* single mutant cells is negatively affected by CTX concentrations well
308 below each of the four tested strain's MIC, consistent with other recent findings (Alexander
309 and MacLean 2020).

310 One aim of our study was to test if cooperative behavior via collective antibiotic breakdown
311 among cells of the same strain, would affect the establishment probability and whether this
312 would differ between unstructured and structured environments. Despite higher stochasticity
313 on agar, our data show a reasonable agreement between the establishment in liquid and agar
314 environments, indicating that environmental structure plays only a modest role, and that
315 cooperative behavior has a negligible effect on the establishment probability of single
316 resistant mutant cells in isolation. However, the process of establishment of antibiotic
317 resistant mutants is particularly relevant for *de novo* mutants arising in populations of
318 relatively susceptible bacteria, and in recent work, we demonstrated positive net effects on
319 mutant establishment from susceptible cells in the same experimental system (Saebelfeld et
320 al. 2021).

321 Lastly, we point out limitations of the traditional MIC measurements that make MIC values
322 highly sensitive to the assay conditions. We suggest a more robust measure of resistance,
323 $\mathrm{MIC}_q(N)$, which includes both inoculum size and probabilistic effects, for which we
324 constructed a null model based on the single-cell establishment probability, p_e, by assuming
325 homogeneous cell populations and no social interactions across lineages. The null-model
326 prediction disagrees with the measured MIC in systematic ways, indicating strong cooperative
327 effects at high cell densities (Saebelfeld *et al.* 2021). We propose that this measure may be
328 used as a reference for detecting conditions of collective resistance for other bacterial strains
329 and antibiotics.

330

331 **Material and Methods**

332 Strains and culture conditions

333 Four strains were used for the experiments, derived from *Escherichia coli* strain MG1655
334 *galK*::SYFP2-CAT (a kind gift from the lab of Dan Andersson via Peter Lind). This strain
335 (DA28100) had previously been modified with a YFP (yellow fluorescent protein) marker
336 cassette, containing a resistance gene for chloramphenicol (Gullberg *et al.* 2014). For the
337 current study, the chloramphenicol resistance was removed, and each one of four TEM
338 variants was inserted into the *galK* locus, together with the pTac promoter from plasmid
339 pACTEM (Barlow and Hall 2002): the ancestral TEM-1 allele (referred to as "Ancestor"), and
340 three mutant alleles with either 1, 2 or 3 point mutations in the TEM allele (referred to as
341 "Single mutant", "Double mutant" and "Triple mutant", respectively). While the ancestral
342 TEM-1 allele confers only low activity towards the β-lactam antibiotic cefotaxime (CTX), the
343 three mutants show increasing CTX resistance with each additional substitution (Table 1). All
344 TEM loci are under the control of the LacI repressor and are expressed by adding 50 µM
345 Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the growth medium. The minimum inhibitory
346 concentration (MIC) of each strain for CTX had previously been determined in duplicates
347 (Table 1), using 2-fold increases in CTX concentration in microtiter plates filled with 200 µL M9
348 minimal medium (containing 0.4% glucose, 0.2% casaminoacids, 2 µg/mL uracil and 1 µg/mL
349 thiamine) and 50 µM IPTG, inoculated with 10^5 cells and incubated for 24h at 37°C.

350 For culturing, all strains were first recovered from glycerol stocks by streaking them out on LB
351 (Luria-Bertani) agar plates and incubated overnight at 37°C. From those plates, one colony
352 was picked and introduced into 1 mL M9 medium (as above) and incubated overnight at 37°C,
353 250 rpm. The cultures were then serially diluted with phosphate-buffered saline (PBS) to the

354 density needed for the particular experiment (see below), assuming an initial density of
355 $2.5x10^9$ cells/mL.

356 Liquid experiment

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

362 Per strain and CTX concentration, on average either about 1 or 2-3 cells were seeded into 285
363 wells across three 96-well microtiter plates. For this, 190 µL M9 medium with the respective
364 cefotaxime concentration and 50 µM IPTG were pipetted into all wells. Overnight cultures of
365 the strains were diluted to 200 and 100 cells/mL with PBS. 10 µL of the dilutions were pipetted
366 into the wells. One well per plate served as medium control, mock-inoculated with 10 µL PBS.
367 The plates were incubated for about 40 h at 37°C (static). After incubation, OD at 600 nm of
368 all wells was measured in a plate reader (Victor3™, PerkinElmer) without the plate lid. All
369 medium controls across the experiment showed no sign of growth; the OD ranged from 0.033
370 to 0.038. Thus, a threshold of >0.05 was applied to determine whether a population
371 successfully established.

372 To determine the mean number of seeded cells in culture dilutions, aliquots of the dilutions
373 were dropped on LB agar plates (120 x 120 mm). Per dilution and strain, a total of 3x32 10 µL
374 drops were applied to the plates, let dry for about 10 to 20 minutes, incubated at 37°C until
375 leaving, and then moved to 30°C overnight to prevent overgrowth, followed by counting the
376 number of colonies per droplet.

377 Agar experiment

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

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