Single-cell phenotypic characteristics of phenotypic resistance under recurring antibiotic exposure in *Escherichia coli*.

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Short title: Phenotypic characteristics of phenotypic antibiotic resistant cells

Abstract:

Despite increasing interest, non-heritable, phenotypic drug resistance, such as tolerance and persistence towards antibiotics, remains less characterized compared to genetic resistance. Non-heritable drug resistance challenges antibiotic treatment and have implications towards heritable resistance evolution. Phenotypically resistant cells have commonly been characterized as growth arrested cells prior and during antibiotic application that quickly resume growth post-application. Here, we add novel combinations of characteristics of phenotypic resistant *E. coli* single cells—that

are of particular interest towards genetically fixed resistance—, and contrast those to susceptible cells of the isoclonal initial population by exposure to different levels of recurrent antibiotic. We find that phenotypic resistant cells reduced their growth rate by about 50% compared to growth rates prior to antibiotic exposure, but cells do not go into near growth arrest. The growth reduction is induced by antibiotic exposure and not caused by a stochastic switch or predetermined state as frequently described. Cells exhibiting constant intermediate growth survived best under antibiotic exposure and, contrary to expectations, selection did not primarily act on fast growing cells. Our findings support diverse modes of phenotypic resistance, and we revealed resistant cell characteristics that supports acclaims of an underappreciated role of phenotypic resistant cells towards resistance evolution.

Introduction

Resistance to antibiotics, in combination with slow progress in discovering novel antibiotics, poses a major challenge to modern medicine (1). Antibiotic resistant cells sustain bactericidal concentrations of antibiotics through heritable, often well-known and explored, genetically fixed mutations. In contrast, non-heritable phenotypic resistant cells, also often referred to as antibiotic persistent or tolerant cells are in a non-heritable drug resistant phenotypic state. This state allows them to survive periods of bactericidal concentration of antibiotics and is classically characterized by non- or very slow growth prior and during exposure to the drug (2–5). More recently 'specialized' persister cells have been described that show less growth dependent persistence (6–12). Identifying persister cells is usually done through a characteristic biphasic killing curve that is observed at the population level and caused by differential selection of persister cells of a susceptible population during antibiotic exposure (2,13), in contrast tolerant cells do not show a biphasic killing curve as they are assumed to be a population wide response and not a subpopulation one (2–5,9). However, definitions on persistence and tolerance are not consistent as persistence is also referred to as a special case of tolerance, or is defined as hetero-tolerance (4). The persister cell stage can be environmentally induced or stochastically switched to, hence, cells can dynamically alter states, and progeny of persiter cells are as susceptible as the parental population from which they derived (4,14). Quantitatively, below 0.1% of cells in wild-type susceptible populations are persister cells, though variation in persister fractions exists among experiments, strains, growth media, and environmental conditions (15). Uncertainty in accurately quantifying persister cell fractions based on killing curves comes from highly sensitive determination of slopes and intercepts of killing curves (10).

Tolerance is a population level response and leads to a slowed killing curve, tolerant cells are therefore much more common than persister cells (2,9,16). Tolerant cells tolerate antibiotic exposures by mainly non- or very slow growing under antibiotic exposure, characteristics they share with persister cells (2). Though compared to persister cells, tolerant cells can only sustain shorter

59 durations of antibiotic exposure (4). Under antibiotic application, tolerant cell populations show
60 prolonged minimum duration of killing (MDK) without a change in the minimum inhibitory
61 concentration (MIC), that is, they die slowly under antibiotic application even when exposed to very
62 high concentrations.

63 A third class of cells that can sustain bactericidal concentration of antibiotics by phenotypic
64 resistance are viable but non-culturable cells (VBNC/ deep dormant cells / sleeper cells) (Dong et al
65 2020). In contrast to persister and tolerant cells, VBNC cells do not regain growth 3-4 hours post-
66 antibiotic exposure, but instead remain at low metabolic activity in a deeper dormancy state and do
67 not easily resuscitate, potentially due to a lack of intracellular ATP (17). Persister and tolerant cells
68 are therefore more susceptible to recurring exposure to antibiotics compared to VBNC cells (17).
69 VBNC cells are further phenotypically characterized by being small (2.3 ± 0.2µm for E. *coli*) and less
70 elongated compared to persister cells (18).

71 Differentiating among these different phenotypic resistant cell types can be difficult, in particular
72 when single-cell data is not available (3,4,7,19). A growing number of studies on persistence work at
73 the single-cell level and primarily aim at a mechanistic understanding of phenotypic resistance
74 (8,18,20–24), yet in depth analyses of single-cell phenotypic characteristics of phenotypic resistant
75 cells are still rare (8,10,25). Many of these single-cell studies use persister mutants, and start with
76 growth-arrested, stationary phase cells, as these conditions increase the fraction of persister cells.
77 Some recent studies suggest that persister cells arising of exponential growing populations are
78 predominantly not growth arrested prior to antibiotic exposure (10). Despite evidence for diverse
79 modes of phenotypic resistance, overall, these single-cell level studies highlight that that persister
80 cells show limited growth when exposed to β-lactam or other antibiotics that inhibit cell wall
81 formation or cell division (6,7,26), and that switching to persister stages can be irreversible or a
82 reversible dynamic stochastic process (6,10). Earlier studies reported that the stochastic switch to
83 the rare persister state occurred for growth-limited cells within exponentially growing *E. coli*

84 populations, with ~80% of persisters arising from the non-growing sub-population prior to
85 treatment, while the remaining persister cells originated from cells that were growing prior to
86 treatment (11,27). Other high throughput experiments, not working on persister mutant strains,
87 suggest that large fractions of persister cells arise from non-dormant cells when selected of an
88 exponentially growing population (10). Quantitative high-quality single-cell data on phenotypic
89 characteristics of phenotypic resistant cells are limited, as persister cells are so rare and selecting for
90 them remains challenging (10). However, characterizing single-cell growth, division and mortality of
91 phenotypically resistant cells would provide deeper understanding about the evolutionary
92 importance of non-heritable resistant cells for the evolution of heritable (genetic) resistance and
93 efficacy of antibiotic treatment, as high frequencies of non-heritable tolerant cells provide
94 evolutionary reservoirs and increase the probability of genetically fixed resistance mutations to
95 occur (28–37).

96 Here, we use a demographic and phenomenological approach to quantify the fraction of cells in a
97 phenotypic resistant stage among susceptible stage cells, we investigate how phenotypic resistant
98 cells differ in their single-cell characteristics compared to susceptible cells, and we study what
99 triggers changes in their phenotypes. To increase the number of cells that sustained antibiotic
100 exposure, we reduce exposure periods to recurring 1.5 h periods compared to a typical single 3-5h
101 exposure period. Whether the resulting selected cells should be referred to as hetero-tolerant cells,
102 or persiter cells depends on definitions, we follow with the term persister. We started with a
103 susceptible clonal exponentially growing population of cells that we expose to recurrent antibiotic
104 treatment at sub- to supra level antibiotic concentrations, we track each cell for their growth,
105 division, and survival in a microfluidic device called a mother machine (38–40). By exposing cells to a
106 β-lactam, ampicillin, that corrupts cell wall synthesis, we expected quickly-growing cells to be heavily
107 selected against, leaving primarily slow-growing phenotypic resistant cells that also arose from the
108 slowest-growing population of cells prior to antibiotic exposure (3). Phenotypic resistant cells,
109 compared to genetically resistant cells, should not be sensitive to antibiotic concentrations beyond

110 the MIC of the susceptible cell population (3), and are expected to regain normal growth rates within
111 3-4 hours post-antibiotic treatment, as long as they are not VBNC cells. We expected few cells to
112 stochastically switch to tolerant resistance stages or switch by environmental induction, as we
113 expected phenotypic resistant stages to be mainly predetermined prior to antibiotic exposure. To
114 evaluate how our findings at the single-cell level might scale to cell culture dynamics, we exposed
115 cell cultures to similar recurring exposures of antibiotics and counted colony forming units (CFU)
116 before and after each exposure to antibiotics. In addition, we performed an experiment by
117 measuring the optical density dynamics of constantly exposed cell cultures to the same levels of
118 antibiotics. We discuss why scaling and quantitative comparison between single-cell characteristics
119 and experiments, and cell culture level exploration can be challenging.

120 MATERIAL METHODS

121 We conducted four set of experiments, three of them with three recurring antibiotic (ampicillin)
122 exposure periods of 90 minutes; exposure periods started 60 min, 330 min and 1080 min past onset
123 of the experiments. Two of these sets collected single-cell data using a mother machine microfluidic
124 device and time-lapse microscopy (Movie SI) (38,39,41) and one set collected cell culture level data,
125 i.e., CFU counts (details SI). Cells were derived of an exponentially growing susceptible isoclonal *E.*
126 *coli* strain K12-MG1655, ATCC® 700926TM, population. Exposure was at one of eight different
127 concentrations ranging from sub-MIC to supra-MIC levels (0-128μg/ml; details SI). Cell death in the
128 microfluidic device was determined using propidium iodide. The fourth experiment, which was at
129 the cell culture level, continuously exposed cells to antibiotics and served as a control experiment.
130 All experiments, single-celled and cell culture experiments, were done in supplemented minimal
131 media (M9), and all cultures were derived from the same *E. coli* strain, that have a replication time
132 of ~25-30 minutes in M9 medium at 37°C (details SI). We chose this strain as it is well adapted and
133 explored and has been used in previous work on persister cells (11,18,26,42). More details on
134 bacterial cell culturing, microchip casting and mounting, microchip loading, life cell imaging, image

135 analysis, experimental treatment are provided in the SI. We remain brief in our description here as
136 most methods follow previously published protocols (39,43).

137 The resulting data was subsequently analysed in program R (44) using general linear, generalized
138 linear, and nonlinear (Generalized Additive Models, GAM) models. Models were compared based on
139 information criteria (AIC)(45). We considered an ΔAIC >2 as better support between competing
140 models. Survival analyses (Kaplan-Meier Fig. 1a) are computed with R package survival (survfit
141 function) and compared amongst them with a Cox proportional hazard model. Probability of death
142 curves (Fig. 1b, Fig. 3b), division rate curves (Fig. 2a), and size curves (Fig. 2b) were plotted with
143 ggplot2 package and a loess smoothing (geometric_smooth function; package ggplot2), growth
144 curves were fitted with loess smoothed GAM models (Fig. 2c), competing GAM models (Table 1) are
145 fitted with the restricted maximum likelihood method (REML). Details on the statistical testing,
146 exclusion of extreme values, and model specifics are given in the SI, which also include details on
147 comparisons of growth differences before death (GLM's; post-hoc Tukey test using glht function of
148 mutlcomp package), and testing for the robustness of growth rates (Fig. 4; GAM).

149 For the cell culture experiments, to collect the number of colony forming unit (CFU) data and the cell
150 culture density data (OD_{600}), we initially seeded these experiments using cells from an exponential
151 growing culture. Each experiment had 8 replicates, the experiments were done in 96 well-plates, and
152 cells grew at 37°C and vigorous shaking in their respective well (Supplementary File 2). Recurrent
153 exposure to different antibiotic concentrations was achieved through serial dilution, centrifuging,
154 removing super natant, resuspending and culturing cells, followed by serial dilution and plating on
155 agar plates and CFU were counted after 16h of incubation, more details are provided in the SI.

156

157 RESULTS

158 **Mortality and selection for tolerance**

159 As expected, the probability of cell death, i.e., the force of mortality, increased after the first
160 exposure of the susceptible parental population to the antibiotic, when exposed to MIC or higher
161 concentrations (Fig. 1b, Movie SI). Selection for phenotypic resistant cells was strong with about two
162 thirds to three quarter of cells dying before the second exposure period, when exposed to MIC or
163 higher concentrations (Fig. 1a, Movie SI). The survivorship patterns (Fig. 1a) somewhat resemble a
164 biphasic killing curve as frequently observed at the cell culture level of persister cells, though we
165 highlight that direct comparison among cell culture level killing curves and single-cell survival can
166 only be made in a crude way. Mortality peaked 30 to 60 minutes after the first 90-minute antibiotic
167 exposure period, which suggests that the force of selection peaked with a lag time of 2 to 2.5 hours
168 after the start of the first exposure. Survival patterns were similar at, or above, the MIC of 4 µg/ml
169 (Fig. 1a, Cox proportional hazard model Likelihood ratio test 477.1, $p<0.0001$, $n= 3496$; for post hoc
170 testing see SI Table S1). The subsequent 90-minute exposure period triggered a less pronounced
171 mortality force compared to the first exposure period with a similar lag time of about 30 to 90
172 minutes after the second antibiotic exposure. Note, cell numbers became small (~20-30 cells per
173 concentration) and estimates of mortality rates less reliable towards the third exposure period. For
174 the control, 0 µg/ml, cells died, as expected, at a low rate and in agreement with experiments aiming
175 at senescence patterns of aging single cells (40).

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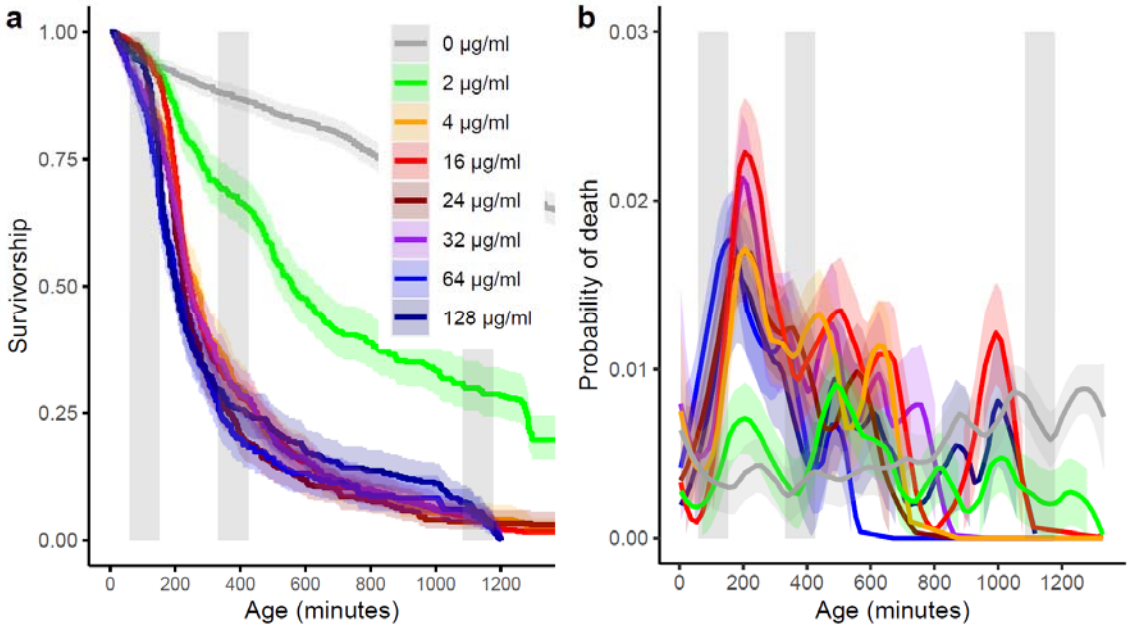

Fig. 1: Survivorship curves (a) and probability of death, i.e., force of mortality (b) of cells exposed to different levels of antibiotics across the duration of the experiment. The grey curves are control group cells that were not exposed to any antibiotics. The grey vertical bars mark the exposure period to the antibiotic. Kaplan-Meier survivorship curves (a) are shown with 95% CI. Probability of death curves (b) are loess smoothed with 95% CI. Note, the two panels (a & b) are built from the same single-cell mortality data.

Division rate, size, and growth responses

In contrast to the control group of no antibiotic exposure, cells that had been exposed to MIC or higher antibiotic concentrations reduced their division rate after the first exposure period, changing from ~2 to ~1 division per hour (Fig. 2a, Table 1). Most of this reduction in reproductive rate was reached ~150-200 minutes after the onset of the first exposure (300-360 minutes after onset of experiment). The division rates tended to only slowly decline after the second exposure, patterns that might also arise due to senescence and not only antibiotic exposure (41).

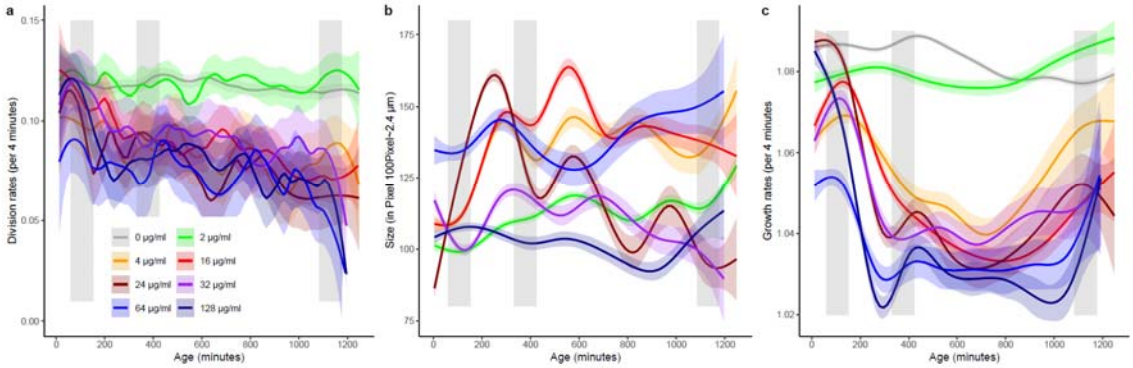

Fig. 2: Cell division rates (a), cell size (in pixels with 100 pixels~2.4µm) (b), and growth rates (c) of cells exposed to different levels of antibiotics across the duration of the experiment. The grey vertical bars mark the exposure period to the antibiotics. Division rate (a) size (b) and growth rate (c) curves are with 95% CI. Note, size data (b) for the control group (0µg/ml) was not available.

198 Table 1: Model comparison among competing GAM models.

		DF	AIC
Division rate	**S(Age) + Concentr.**	**15.8**	**455232**
	S(Age)	8.4	487667
	Concentr.	8.0	455263
Size	**S(Age) + Concentr.**	**16.8**	**2455427**
	S(Age)	10.8	2460359
	Concentr.	8.0	2458502
Growth rate	**S(Age) + Concentr.**	**17.7**	**-1091065**
	S(Age)	10.5	-1073598
	Concentr.	9.0	-1087172

For each of the three response variables (Division rates, Size, and Growth rates), we compared three competing models that had two explanatory variables (Age, i.e. time since onset of experiment, and antibiotic concentration). The best supported of these three models is highlighted in bold.

199

200 Average cell size remained approximately constant within a given antibiotic concentration. Although
201 we found a surprising amount of variation in size among cells exposed to different levels of
202 antibiotics (Fig. 2b, Table 1), there was no relationship between size and the levels of antibiotics. Cell
203 growth rates responded as predicted by declining during and after the first antibiotic exposure for
204 cells exposed to the MIC or above. We highlight here that growth rates only approximately halved
205 (Fig. 2c, Table 1), which contrasts our predictions based on previous findings where persister cells
206 were growth arrested or near growth arrested (4). The induced growth reduction started about 30
207 minutes after the onset of antibiotic exposure, which is a lag time that corresponds to approximately

208 the doubling rate (division time) of the parental population. The lowered growth rates stabilized
209 about 4-5 hours after onset of the initial exposure period, suggesting substantial variance in lag time
210 among individual cells' growth response. Induced growth reductions depended partly on antibiotic
211 concentrations with higher levels of antibiotics leading to lower growth rates. However, even with
212 the highest antibiotic concentration, the growth rates did not drop below 1.02-1.03/4 minutes, i.e.,
213 2-3% cell elongation within 4 minutes. Further, growth did not resume to prior exposure levels rates
214 within 3-4 hours after release of exposure.

215 **Growth specific selection**

216 The growth rates as discussed above (Fig. 2c) are average growth rates at a given point in time, and
217 even though variance in growth rates (see CI Fig. 2c) does not increase substantially during the
218 experiment (beyond the expected increase due to decreasing number of cells towards the end of the
219 experiment), selection might still act preferentially against cells with high growth as expected from a
220 β-lactam antibiotic and as previously described for phenotypic resistant cells (14). To evaluate the
221 possibility of such differential selection based on cell growth we analysed together all cells exposed
222 to MIC or above, and then separated them out into three growth categories: cells that did not grow
223 or shrank (growth arrested cells with growth rates <1.0), cells that grew at low to intermediate rates
224 (growth rates 1.0-1.1), and cells that grew quickly (growth rates >1.1) (Fig. 3a). Note that cells can
225 switch dynamically among these categories throughout the experiment and that mortality and
226 survival is associated only with the current growth rate of a cell and not a cell's growth rate averaged
227 across time. As expected, quickly growing cells showed increased probabilities of death upon
228 exposure to antibiotics, but contrasting to our expectations, the probability of death of growth
229 arrested cells increased substantially upon exposure to antibiotics, whereas the probability of death
230 of slow-intermediate growing cells did not change (Fig. 3b, see also SI Table S2 and Supplementary
231 file 1C).

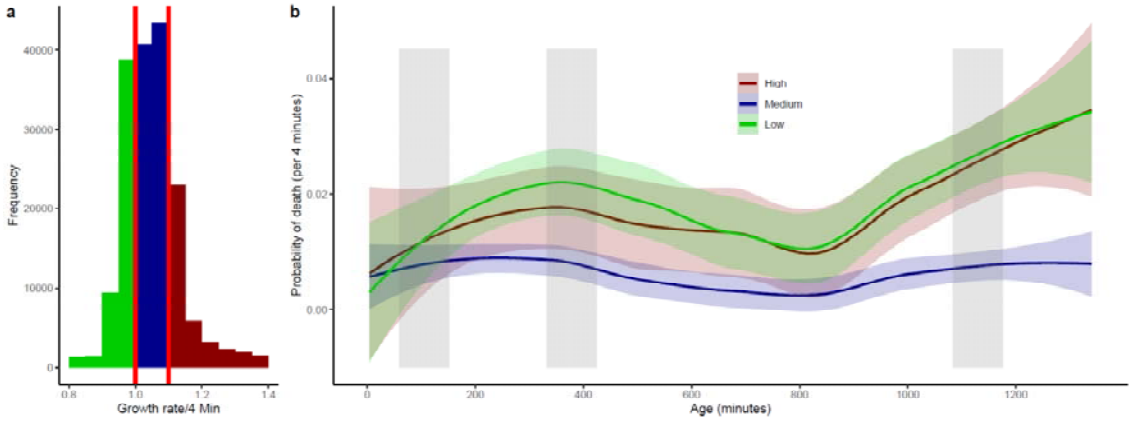

Fig. 3: Cell growth rate distribution divided into three categories: growth arrested (shrinking or non-growing; green), intermediate growth (blue), and fast growth (red) (a), and probability of death for these three groups of growth arrested, intermediate, and quickly-growing cells across the duration of the experiment (b). Confidence intervals (95% CI) are shown in shading in panel (b). The grey vertical bars in panel (b) mark the exposure period to the antibiotics. Note that cells can switch among growth categories throughout the experiment, that is cells are categorized according to their current cell growth rate. For this analysis, we have combined cells across levels of antibiotic exposure ≥4 µg/ml and excluded all cells exposed to 2 µg/ml. A statistical model comparison showed that mortality differed substantially among the three growth category groups: ΔAIC 25.8 comparing a growth category model and a null model, see also Supplemental Information. Classical p-value post-hoc analyses showed that quickly growing and growth arrested groups did not differ from each other in age at death, but the intermediate group did.

To verify the robustness of our finding that selective mortality is dependent on growth rates, and to broaden our understanding of how growth influences mortality beyond the current growth rate (Fig. 3b), we did three comparisons between average growth rates of cells dying during specific periods in time. First, we compared the average growth rate (averaged across time) of cells that survived the first exposure period to those that died before the first exposure period. Second, we compared the average growth rate of cells dying during or shortly after the first exposure period (cut off at peak of

252 first mortality) to those surviving past the first peak of mortality, and third we compared similarly for
253 cells that died between the first peak of mortality and the second peak of mortality to those that
254 survived past the second peak of mortality (Table 2). Before the first exposure, cells that died had
255 lower average growth rates compared to those that survived past the first exposure, showing that
256 without selection of antibiotics slower growing cells are preferentially selected against. Cells that
257 died during the first exposure or shortly after, i.e., during times of strong selection, had slightly
258 higher growth rates than those that survived past this first increased selection period. This pattern of
259 growth dependent selection remained for the next period of increased selection after the second
260 antibiotic exposure. These findings support our initial prediction that faster growing cells would be
261 preferentially selected against under antibiotic exposure. However, note that the effect sizes
262 (growth differences) among surviving and dying cells are small, even though they are significantly
263 different. Combined with the findings shown in Fig. 3, we interpret our findings on growth specific
264 selection under antibiotic exposure as not primarily acting on fast-growing cells.

265 Table 2: Differences in average growth rates of cells surviving past or dying during or after specific
266 points in time.

PERIOD	0 – 60 MIN	60 – 328 MIN	328 – 600 MIN	+600 MIN
GOWTH RATE OF CELLS DYING DURING PERIOD (±STDEV)	1.047 (±0.039)	1.067 (±0.032)	1.057 (±0.030)	1.045 (±0.027)
GROWTH RATE OF CELLS SURVIVING PERIOD (±STDEV)	1.072 (0.0412)	1.060 (±0.035)	1.050 (±0.028)	
AIC GROWTH DURING/AFTER	**-8020.8**	**-8816.3**	**-3170.8**	
AIC NULL MODEL	-7985.4	-8772.0	-3161.4	
ΔAIC	35.4	44.3	9.4	

267 *Model comparison among a null model (intercept only model) and a model with the two groups of*
268 *cells dying during a particular period or surviving that period as explanatory variable and the mean*
269 *growth rate (per 4 min) as response variable. Three sets of models were fit, one for each period [0*

min – 60 min; 60 min – 328 min; 328 min – 600 min]. A Gaussian error structure was used to fit these models.

Robustness of growth rates within cells

In Fig. 2c, we investigated the average growth rate at a given time point. As cells can dynamically alter growth rates between the current and a future point in time, we correlated a cell's current growth rate to its growth rate in the future, which provides a measure of robustness of growth. Overall correlation between an individual's cell growth rate at a given time t and its growth rate in the immediate future (time *t*+1 [4 minutes later]) or future time points (time *t*+3 [12 min later]; time *t*+5 [20 minutes later]) was weak (Fig.4, Table 3), that is, growth rates are little robust and a cell's current growth rate does not well predict its future growth rate. However, there was the exception for cells with intermediate growth rates (1.0-1.1). Cells growing in that range (Fig. 4, x-axis range 1.0-1.1) showed a strong correlation between current and future growth, illustrating robust constant growth for these cells. Growth arrested cells (growth rate <1 at time t) tended to increase in their growth rates at future points in time, while quickly-growing cells (growth rates >1.1 at time t) tended to decrease their growth rates (Fig. 4). Overall, we observed substantial temporal dynamics in growth rates with less robust growing cells (growth arrested and quickly-growing cells) being more affected by the concentration of antibiotics, which suggests that the growth rate responses to different concentrations of antibiotics shown in Fig. 2c are mainly influenced by the fastest growing and growth arrested cells.

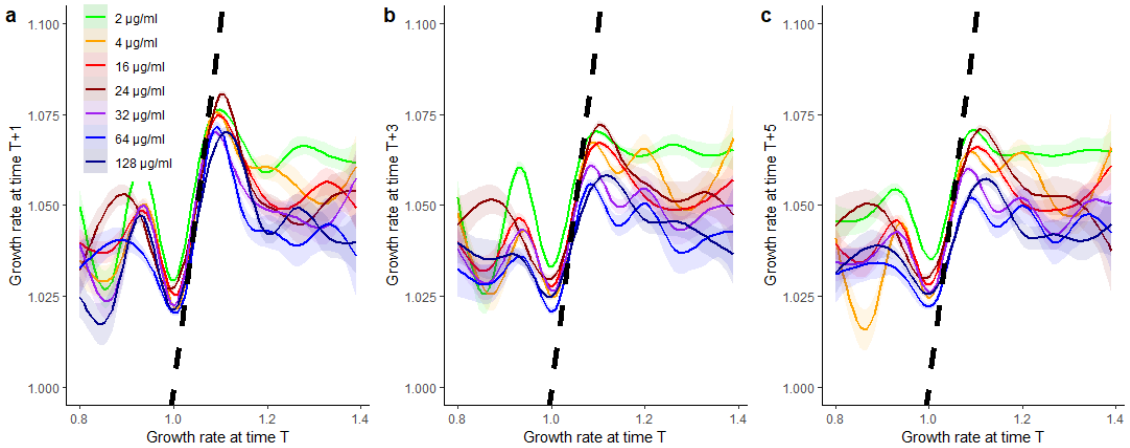

Fig. 4: Correlation in cell growth rates at a given time t, compared to growth rates at time t+1 (4 minutes later) (a), t+3 (12 minutes later) (b), and t+5 (20 minutes later) separated out into populations recurrently exposed to different levels of antibiotics (as described in the main experiment). All growth rate correlations are fitted with a GAM with 95% CI. The dashed black line illustrates the direct correlation among time points.

Table 3: Model comparison among competing GAM models, for correlation of current growth rates to growth rates at future points in time.

	MODEL	DF	AIC
GROWTH T+1	**Concentr. + S(Growth by Concentr)**	**70.3**	**-441151**
	Concentr. + S(Growth)	17.0	-440468
	S(Growth)	11.0	-438626
	Concentr.	8.0	-425891
GROWTH T+3	**Concentr. + S(Growth by Concentr)**	**69.2**	**-421883**
	Concentr. + S(Growth)	17.0	-421503
	S(Growth)	11.0	-420235
	Concentr.	8.0	-407188
GROWTH T+5	**Concentr. + S(Growth by Concentr)**	**69.0**	**-412563**

	Concentr. + S(Growth)	17.0	-412180
	S(Growth)	11.0	-410925
	Concentr.	8.0	-398709

297

298 *Best supported model for each response variable (Growth t+1, t+3, t+5 respectively) is highlighted in*
299 *bold. GAM models are fitted with the restricted maximum likelihood method, a smoothing parameter*
300 *for Growth rate at time t with a shrinkage version of a cubic regression spline as smoothing*
301 *parameter (bs=cs) and/or the concentration, including a model that accounts for the interaction of*
302 *the two explanatory variables, growth rate and concentration of antibiotics. Growth rates are fitted*
303 *with a Gaussian error structure and an inverse link function.*

304

305 **Population level responses**

306 Much research investigating phenotypic resistance has been done in cell cultures, i.e. at the
307 population level, which makes it challenging to partition mortality, reproduction and growth
308 responses, characteristics that need single-cell level evaluation. Further, density dependence and
309 resource depletion need consideration at the cell culture level but are controlled for by the
310 microfluidics setup at the single-cell level. Scaling and comparing single-cell characteristics to
311 population level patterns can therefore be challenging. In the following section we report on
312 'control' experiments done in cell cultures. For these cell culture experiments we took two
313 approaches: CFUs were counted before and after each exposure period to antibiotics in order to
314 mimic the recurrent application of antibiotics as in the single-cell experiments, or we evaluated cell
315 densities (OD) under constant antibiotic concentrations to understand longer exposure to
316 antibiotics. Cell cultures exposed up to the MIC showed similar qualitative dynamics in terms of
317 numbers of CFUs when recurrently exposed to antibiotics (Fig. 5a). The higher the antibiotic
318 concentration the stronger the reduction in CFUs, and the stronger the increase after relieving

319 exposure to antibiotics, with dampened responses to the second and third exposure and subsequent
320 releases. These patterns align with predictions of selection for phenotypic resistant cells as revealed
321 for the single-cell findings. During the long recovery time after the second exposure, most cultures,
322 except those previously exposed to 64 and 128 µg/ml, reached densities that likely imposed growth
323 limitation due to resource limitation (i.e., the cultures approached stationary phase). The decline in
324 CFU during the recurrent exposure shows the continued susceptibility of the cells after series of
325 selection due to antibiotic exposure. When populations were permanently exposure to antibiotics
326 (Fig. 5b), they, as expected, initial increased in density before densities were reduced to very low
327 levels for all cultures exposed to MIC or supra-MIC concentrations, illustrating the susceptibility of
328 the initial population and no occurrence of resistance mutations during the experiment.

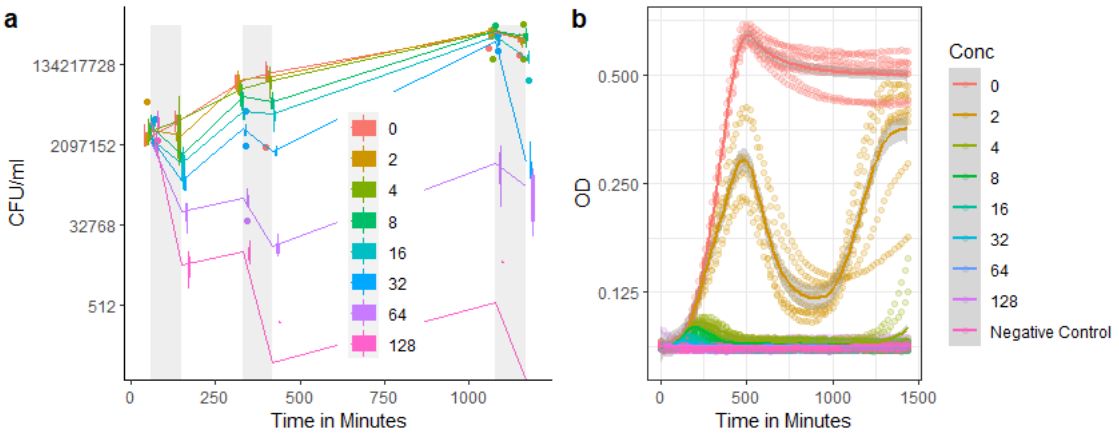

Fig. 5: Colony forming units (CFU) (a), and optical density, OD_{600} (b) of cell cultures exposed to different levels of antibiotics across the duration of the experiment (a) or constant exposure to antibiotics throughout the experiment (b). The grey vertical bars (a) mark the exposure period to the antibiotics. CFU (a) are plotted as boxplots with line connecting mean number of CFU per time when CFU were counted. Single data points are outliers. Note, a jitter is added (a) for better visibility. For the OD curves (b) the dotted lines show the different replicates. the negative control (b) is no bacteria added to medium. CFU (a) and OD (b) are plotted on a log scale.

329

330 DISCUSSION

331 We report on phenological characteristics of single-cells in *E. coli* in response to recurring exposure
332 to a β-lactam antibiotic. Our findings suggest additional diversity of modes of phenotypic resistance,
333 given that we show previously undescribed combination of characteristics. Compared to previous
334 persister studies that quantify fractions of <0.1% persisters (2–4,8,10,25), we reveal a substantial
335 larger fraction (~25%) of cells that survived 1.5 hours of antibiotic exposure, independent of the level
336 of antibiotics (Fig. 1a, see also findings at cell culture for <64 µg/ml; Fig. 5a). This considerably larger
337 fraction we find is best explained by the shorter exposure period to antibiotics we used to select for
338 phenotypically resistant cells compared to the commonly used 3-5h exposure periods to select for
339 persisters. We choose the exposure period as 1.5 hours exceed pharmacokinetics actions of
340 antibiotics (46), illustrated for instance by the time to kill a cell by antibiotics often being comparable
341 with bacterial generation time (16). For our conditions division time is much shorter than the 1.5 h
342 exposure period and other studies report on substantially reduced mortality rates after 2h of
343 exposure (16). Our single-cell survival curves (Fig. 1a) resemble those of a biphasic culture level
344 killing curve, as is characteristic for persister cells. However the biphasic pattern cannot be directly
345 compared, as the second phase of our findings concerns a higher fraction of cells and this phase
346 might also be influenced by the release of the antibiotics after 1.5 h (as killing curves in other
347 persister studies might be influenced by release time). Fractions of persisters varied among previous
348 persister studies as selection conditions varied with respect to drug concentration, growth stage
349 before selection (stationary exponential), preselection (priming of lineages, mutations), or growth
350 media, to name a few. All of these conditions have been shown to alter persister fractions
351 (2,3,15,29). In line with previous findings and as expected by selecting on persisters and against
352 susceptible cells, the MIC marked a survival threshold, illustrating that the initial population was
353 susceptible and not genetically resistant (Fig. 1), this susceptibility was also confirmed when
354 evaluated at the cell culture level (Fig. 5). Our results show that the susceptible clonal parental
355 population consists of phenotypically heterogeneous cells, whereby the cells have different

356 capacities to sustain exposure to antibiotics, leaving predominantly phenotypic resistant cells after
357 the first period of exposure.

358 We found reduced cell growth and division rates under antibiotic exposure, but this reduction was
359 quantitatively different compared to most previously described persister cells. Growth reduction in
360 our cells was about halved when exposed to antibiotics, while many other studies have reported
361 growth arrest or near growth arrest of persister cells during antibiotic exposure (2,4,7,26). Single
362 studies highlight that fast growth phenotypes with high efflux activity can avoid accumulation of
363 antibiotics, findings that do not align well with the fact that antibiotics of exponentially growing cells
364 accumulate within minutes in exposed cells (8,21). In contrast to previously described characteristics
365 of persiter cells, after release from antibiotics, we did not observe resumption of normal growth
366 within 2-4 hours (3,4). Our findings hence support studies that explored persisters derived from
367 exponentially growing cells that showed longer lag times to regain growth, note these cells had been
368 growth arrest during drug exposure, or highlighting substantial variation in lag times after antibiotic
369 exposure release (10,25,47). A lack of growth resumption, as we found, is also a characteristic of
370 persister type II cells and VBNC cells, however VBNC are found in complete growth arrest and are
371 characterized by a small cell size, and persister type II cells are not environmentally induced but
372 predetermined (18,26), features not found in our study and other studies (10). If classified, our
373 phenotypic resistant cells fall best under specialized antibiotically induced persister cell definitions
374 (9). Quantitative comparison to understand the resumed population growth at the cell culture level
375 (Fig. 5) in light of the single cell growth reduction (Fig. 2) are challenging, as it remains opaque
376 whether population growth dynamics are driven by altered cell division rates, altered cell growth, or
377 altered mortality rates (48). In addition, with respect to heterogeneous selection and cell size, the
378 average cell size remained constant over the course of the experiment (Fig. 2b) suggesting that
379 selection did not favour small cells like VBNC cells or small birth sizes as described for persister cells
380 (18,49), alternatively increased cell size should have been observed if increased frequencies in
381 filamentation of cells occurred; here we only observed rare filamentation. Finally, variation in cell

382 size did not increase substantially across the course of the experiment, which would have been
383 predicted had higher proportions of small cells (VBNC) and filamentous cells simultaneously
384 emerged, we also did not find other extreme morphological changes as reported for non-dormant
385 persisters (10).

386 Our observations differ from genetically fixed resistant cells in various ways. First, at the single-cell
387 level, unlike resistant cells, we did not find graded survival to different levels of antibiotics (Fig. 1 &
388 2a), which supports studies that show insensitivity of fractions of persisters up to ~100 μg/ml (15),
389 although at the cell-culture level we observed graded responses in CFUs (Fig. 5). Second, our cells
390 approximately halved their growth rates (~50% reduction in cell elongation rates) under drug
391 exposure (Fig. 2c), whereas a 20% reduction in population growth (evaluated in cell cultures) is
392 already considered to be an extreme growth cost for genetically fixed resistant cells (50). Third, in
393 contrast to genetic resistant cells, our cells showed continued susceptibility to repeated exposure to
394 antibiotics (Fig. 1, Fig. 5a). Finally, resistance is not expected for our single-cell findings, as our
395 population of cells was directly derived from a susceptible, standard *E. coli* lab strain K-12 MG1655
396 (see also Fig. 5b). A heteroresistant population, i.e., one composed of multiple sub-populations that
397 differ in their genetically fixed resistance to antibiotics (exemplified by different MIC), should have
398 left, post exposure, a resistant population with graded responses to increased levels of antibiotic
399 concentrations; we found none of these survival, division or growth rate patterns typical for
400 resistant cells (Fig. 1&2) (50). Despite the isogeneic origin, our initial population was heterotolerant
401 with a high fraction of cells (~25%) surviving the first exposure and continued showing dampened
402 sensitivities with respect to mortality for following exposure periods (Fig. 2a). Such continued, but
403 dampened, sensitivity could be explained by stochastic switching of smaller fractions of cells out of
404 the tolerant state, which might be related to stochastic gene expression prior, during, and after
405 exposure to antibiotics (51).

406 The growth characteristics of cells we describe in arising from exponential growing cells and not
407 going into growth arrest have important implications for the evolution of genetic resistance.
408 "Classical" persister cells only contribute to resistance development through repeated rounds of
409 population expansion by their offspring, as they are growth arrested prior to and during antibiotic
410 exposure, whereas our phenotypic resistant cells contribute before, during, and after antibiotic
411 exposure to population expansion, and therefore provide continued opportunities for the
412 accumulation of mutations towards resistance (31,37). Our phenotypic resistant cells cannot, *a*
413 *priori*, be distinguished from susceptible cells by their reduced growth rate (26). Rare types of
414 persister cells, that have previously described, did not arising from stationary phase cells and
415 showed slow growth resumption post-exposure, these cells were almost growth arrested under
416 antibiotic exposure (7,10), and we did not find such growth arrest for our cells.

417 Our finding that intermediate growing cells, that exhibited robust growth rates, had higher survival
418 rates compared to growth arrested and quickly-growing cells (Fig. 3 & 4) is surprising to us, as we
419 had predicted growth arrested cells to be the least affected by β-lactam exposure (2,4,14,26). Our
420 findings might indicate that phenotypic resistant cells that exhibit high survival have more balanced
421 and robust intrinsic metabolism (homeostasis) and less dynamic biochemical changes, which
422 contrasts with molecular level findings that link phenotypic resistance to a perturbed biological
423 network (33).

424 The phenotypic resistant state that our cells are characterized by is an induced state, and not an
425 instantaneous switch, a pre-existing state (4), or a rare occasional stochastic switch to a persistence
426 or tolerant state out of exponentially growing cells(26). Similar induced switching as we find has
427 been found for different persistence (10,52) and aligns with findings where increased phenotypic
428 resistance were induced by antimicrobial peptide exposure (7,53), though our cells seem not to
429 instantaneously switch to their persistence stage, or at least require different lag times to do so. The
430 emergence of our phenotypic resistant cells out of an exponentially growing population suggests

431 that the evolution, or the maintenance, of phenotypic resistance is not conditional on nutrient
432 limiting condition as has previously been suggested to be the favorable evolutionary route (54).

433 Our finding that cells cannot be easily identified prior to antibiotic exposure by their reduced growth
434 rates also supports findings that there are various evolutionary routes to phenotypic resistance
435 (4,5,9,10,33,55). This contrasts with our knowledge of the much better understood evolution of
436 genetically fixed resistance, which tends to arise from mutations in a few defined genes that relate
437 directly to the mechanism by which the antibiotic acts (33). The diversity of evolutionary paths to
438 phenotypic resistance is illustrated by experimental evolution studies that evolved high phenotypic
439 resistance by repeated exposure to ampicillin and showed that resistance was achieved through
440 increased lag time (56); other experimental evolution studies, which exposed stationary phase cells,
441 evolved phenotypic resistance without prolonged lag times (57), and still other experimental
442 evolution studies found again other, lag time independent, mutations (33,58).

443

444 Future investigations should explore varying exposure time and varied inter exposure time periods
445 to better understand how selection and characteristics depend on exposure periods. Prolonged
446 treatment cycles can cause tolerant strains to attain resistance at higher rates (28), while multi-drug
447 treatment can prolong time to persitence evolution (59), another dimension that could easily be
448 explored in the future. Different strains should be explored to understand how specific the diverse
449 phenotypic resistance mechanisms are, but such exploration should be systematic, as part of the
450 diverse findings on phenotypic resistance might be caused by small differences in experimental
451 settings not related to the main target (33). A rigorous phenomenological exploration, as we have
452 done, can provide synergistic insights for tailored molecular exploration. Such molecular exploration
453 will be interesting as of the expected diversity of molecular and evolutionary paths to phenotypic
454 resistance. For instance, our cells did not show alterations in cell size, which suggests that our
455 persistence might not be related to known resistance mechanisms to β-lactams, where filamentation

456 rate is increased, which in turn stops ampicillin from efficiently targeting the cells (7,58,60). Our
457 approach also illustrates the challenge and potential of scaling from single cell to the population
458 level, a link that will provide a deeper understanding of the evolutionary potential towards
459 resistance, but that is still rarely explored in detail (25). Our single-cell study is a cohort study and
460 not a cross-cohort study like cell culture experiments, however cross-cohort studies can be achieved
461 at the single cell level. Other differences, such as inter cellular communication, quorum sensing,
462 nutrient limitation, or density dependence, among single-cell and cell culture explorations are more
463 challenging to circumvent but these differences can reveal important conditions that help to reveal
464 potential resistance mechanisms. The diversity and partly contrasting findings on phenotypic
465 resistance characteristics, illustrates that we are still on the verge of understanding the role and
466 mechanisms of such non-genetically determined resistance, and standardized and comparative
467 studies are needed to solidify and advance our current understanding. Nevertheless, the importance
468 of phenotypic resistance is reflected in our cell culture experiments, where population crashes were
469 prevented by fractions of resistant cells when the population was exposed recurrently to antibiotics.
470 Our results support the idea of diverse modes towards phenotypic resistance, where there is no pre-
471 determined slow growth, no growth arrest during drug exposure, and no quick resumption of
472 growth. These characteristics have implications for the evolution of resistance in providing
473 evolutionary reservoirs and increased mutant supply; this combination of characteristics might even
474 call for a wider definition of what persister cells are. As for applied implications on medical
475 treatment, we need to be cautious about interpretation, because directly relating *in vitro* to *in vivo*
476 results for antibiotic treatment remains challenging.

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484 References

485 1. Ventola CL. The antibiotic resistance crisis: causes and threats. P T J [Internet]. 2015 [cited
486 2021 Feb 24];40(4):277–83. Available from:
487 http://www.ncbi.nlm.nih.gov/pubmed/25859123%5Cnhttp://www.pubmedcentral.nih.gov/a
488 rticlerender.fcgi?artid=PMC4378521%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/25859123%
489 5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4378521

490 2. Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance
491 and persistence to antibiotic treatment. Vol. 14, Nature Reviews Microbiology. Nature
492 Publishing Group; 2016. p. 320–30.

493 3. Brauner A, Shoresh N, Fridman O, Balaban NQ. An Experimental Framework for Quantifying
494 Bacterial Tolerance. Biophys J [Internet]. 2017 Jun 20 [cited 2018 Nov 5];112(12):2664–71.
495 Available from: https://www.sciencedirect.com/science/article/pii/S0006349517305519

496 4. Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, et al. Definitions
497 and guidelines for research on antibiotic persistence. Nat Rev Microbiol. 2019 Jul
498 1;17(7):441–8.

499 5. Kaplan Y, Reich S, Oster E, Maoz S, Levin-Reisman I, Ronin I, et al. Observation of universal
500 ageing dynamics in antibiotic persistence. Nature [Internet]. 2021 Dec 9 [cited 2022 Jan
501 5];600(7888):290–4. Available from: https://doi.org/10.1038/s41586-021-04114-w

502 6. Wakamoto Y, Dhar N, Chait R, Schneider K, Signorino-Gelo F, Leibler S, et al. Dynamic
503 persistence of antibiotic-stressed mycobacteria. Science (80-) [Internet]. 2013 Jan 4 [cited
504 2021 Feb 24];339(6115):91–5. Available from: https://pubmed.ncbi.nlm.nih.gov/23288538/

505 7. Goormaghtigh F, Van Melderen L. Single-cell imaging and characterization of Escherichia coli
506 persister cells to ofloxacin in exponential cultures. Sci Adv. 2019;5(6).

507 8. Łapińska U, Voliotis M, Lee KK, Campey A, Stone RML, Tuck B, et al. Fast bacterial growth
508 reduces antibiotic accumulation and efficacy. Elife. 2022;11.

509 9. Urbaniec J, Xu Y, Hu Y, Hingley-Wilson S, McFadden J. Phenotypic heterogeneity in persisters:
510 A novel "hunker" theory of persistence [Internet]. Vol. 46, FEMS Microbiology Reviews.
511 Oxford University Press; 2022 [cited 2023 Jan 4]. p. 1–16. Available from:
512 https://doi.org/10.1093/femsre/fuab042

513 10. Umetani M, Fujisawa M, Okura R, Nozoe T, Suenaga S, Nakaoka H, et al. Observation of non-
514 dormant persister cells reveals diverse modes of survival in antibiotic persistence. bioRxiv
515 [Internet]. 2022 May 3 [cited 2023 Jan 4];2021.10.28.466227. Available from:
516 https://doi.org/10.1101/2021.10.28.466227

517 11. Orman MA, Brynildsen MP. Dormancy is not necessary or sufficient for bacterial persistence.
518 Antimicrob Agents Chemother [Internet]. 2013 Jul [cited 2021 Mar 8];57(7):3230–9. Available
519 from: https://pubmed.ncbi.nlm.nih.gov/23629720/

520 12. Greulich P, Scott M, Evans MR, Allen RJ. Growth-dependent bacterial susceptibility to
521 ribosome-targeting antibiotics. Mol Syst Biol [Internet]. 2015 Mar 19 [cited 2023 Jan
522 5];11(3):796. Available from: https://onlinelibrary.wiley.com/doi/10.15252/msb.20145949

523 13. Vaupel JW, Yashin AI. Heterogeneity's Ruses: Some Surprising Effects of Selection on
524 Population Dynamics. Am Stat [Internet]. 1985 Aug [cited 2014 Jun 19];39(3):176. Available
525 from:

526 http://apps.webofknowledge.com/full_record.do?product=UA&search_mode=GeneralSearc
527 h&qid=3&SID=P2GPN9Uc9CCP2wpN8k4&page=1&doc=2

528 14. Lewis K. Persister cells, dormancy and infectious disease [Internet]. Vol. 5, Nature Reviews
529 Microbiology. Nature Publishing Group; 2007 [cited 2021 Feb 24]. p. 48–56. Available from:
530 www.nature.com/reviews/micro

531 15. Salcedo-Sora JE, Kell DB. A quantitative survey of bacterial persistence in the presence of
532 antibiotics: Towards antipersister antimicrobial discovery. Antibiotics [Internet]. 2020 Aug 1
533 [cited 2023 Jan 4];9(8):1–36. Available from: https://pubmed.ncbi.nlm.nih.gov/32823501/

534 16. Svenningsen MS, Svenningsen S Lo, Sørensen MA, Mitarai N. Existence of log-phase
535 Escherichia coli persisters and lasting memory of a starvation pulse. Life Sci Alliance
536 [Internet]. 2022 Feb 1 [cited 2023 Jan 4];5(2). Available from: /pmc/articles/PMC8605324/

537 17. Pu Y, Li Y, Jin X, Tian T, Ma Q, Zhao Z, et al. ATP-Dependent Dynamic Protein Aggregation
538 Regulates Bacterial Dormancy Depth Critical for Antibiotic Tolerance. Mol Cell. 2019 Jan
539 3;73(1):143-156.e4.

540 18. Bamford RA, Smith A, Metz J, Glover G, Titball RW, Pagliara S. Investigating the physiology of
541 viable but non-culturable bacteria by microfluidics and time-lapse microscopy. BMC Biol.
542 2017 Dec 21;15(1).

543 19. Kim JS, Wood TK. Persistent Persister Misperceptions. Front Microbiol [Internet]. 2016 [cited
544 2022 Jan 5];7(DEC):2134. Available from: /pmc/articles/PMC5187198/

545 20. Bergmiller T, Andersson AMC, Tomasek K, Balleza E, Kiviet DJ, Hauschild R, et al. Biased
546 partitioning of the multidrug efflux pump AcrAB-TolC underlies long-lived phenotypic
547 heterogeneity. Science (80-) [Internet]. 2017 Apr 21 [cited 2023 Jan 5];356(6335):311–5.
548 Available from: https://www.science.org/doi/10.1126/science.aaf4762

549 21. Cama J, Voliotis M, Metz J, Smith A, Iannucci J, Keyser UF, et al. Single-cell microfluidics
550 facilitates the rapid quantification of antibiotic accumulation in Gram-negative bacteria. Lab
551 Chip [Internet]. 2020 Jul 28 [cited 2023 Jan 5];20(15):2765–75. Available from:
552 https://pubs.rsc.org/en/content/articlehtml/2020/lc/d0lc00242a

553 22. Rhia M, Stone L, Łapin´ska U, Łapin´ska Ł, Pagliara S, Masi M, et al. Fluorescent macrolide
554 probes – synthesis and use in evaluation of bacterial resistance. RSC Chem Biol [Internet].
555 2020 Dec 8 [cited 2023 Jan 5];1(5):395–404. Available from:
556 https://pubs.rsc.org/en/content/articlehtml/2020/cb/d0cb00118j

557 23. Goode O, Smith A, Łapińska U, Bamford R, Kahveci Z, Glover G, et al. Heterologous Protein
558 Expression Favors the Formation of Protein Aggregates in Persister and Viable but
559 Nonculturable Bacteria. ACS Infect Dis [Internet]. 2021 Jun 11 [cited 2023 Jan 5];7(6):1848–
560 58. Available from: https://pubs.acs.org/doi/full/10.1021/acsinfecdis.1c00154

561 24. Dewachter L, Bollen C, Wilmaerts D, Louwagie E, Herpels P, Matthay P, et al. The dynamic
562 transition of persistence toward the viable but nonculturable state during stationary phase is
563 driven by protein aggregation. MBio [Internet]. 2021 Aug 1 [cited 2023 Jan 5];12(4). Available
564 from: https://journals.asm.org/doi/10.1128/mBio.00703-21

565 25. Bakshi S, Leoncini E, Baker C, Cañas-Duarte SJ, Okumus B, Paulsson J. Tracking bacterial
566 lineages in complex and dynamic environments with applications for growth control and
567 persistence. Nat Microbiol 2021 66 [Internet]. 2021 May 20 [cited 2023 Jan 6];6(6):783–91.
568 Available from: https://www.nature.com/articles/s41564-021-00900-4

569 26. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic

570 switch. Science (80-) [Internet]. 2004 Sep 10 [cited 2021 Mar 4];305(5690):1622–5. Available
571 from: www.sciencemag.org/cgi/content/full/1099390/DC1

572 27. Roostalu J, Jõers A, Luidalepp H, Kaldalu N, Tenson T. Cell division in Escherichia coli cultures
573 monitored at single cell resolution. BMC Microbiol [Internet]. 2008 Apr 23 [cited 2021 Mar
574 8];8(1):68. Available from: http://bmcmicrobiol.biomedcentral.com/articles/10.1186/1471-
575 2180-8-68

576 28. Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shoresh N, Balaban NQ. Antibiotic tolerance
577 facilitates the evolution of resistance. Science (80-). 2017 Feb 24;355(6327):826–30.

578 29. Levin-Reisman I, Brauner A, Ronin I, Balaban NQ. Epistasis between antibiotic tolerance,
579 persistence, and resistance mutations. Proc Natl Acad Sci U S A. 2019;116(29):14734–9.

580 30. Bakkeren E, Huisman JS, Fattinger SA, Hausmann A, Furter M, Egli A, et al. Salmonella
581 persisters promote the spread of antibiotic resistance plasmids in the gut. Nature [Internet].
582 2019 Sep 12 [cited 2021 Mar 11];573(7773):276–80. Available from:
583 https://doi.org/10.1038/s41586-019-1521-8

584 31. Barrett TC, Mok WWK, Murawski AM, Brynildsen MP. Enhanced antibiotic resistance
585 development from fluoroquinolone persisters after a single exposure to antibiotic. Nat
586 Commun [Internet]. 2019 Dec 1 [cited 2021 Feb 24];10(1):1–11. Available from:
587 https://doi.org/10.1038/s41467-019-09058-4

588 32. Liu J, Gefen O, Ronin I, Bar-Meir M, Balaban NQ. Effect of tolerance on the evolution of
589 antibiotic resistance under drug combinations. Science (80-). 2020 Jan 10;367(6474):200–4.

590 33. Sulaiman JE, Lam H. Evolution of Bacterial Tolerance Under Antibiotic Treatment and Its
591 Implications on the Development of Resistance. Front Microbiol [Internet]. 2021 Feb 26 [cited
592 2021 Mar 11];12:617412. Available from:
593 https://www.frontiersin.org/articles/10.3389/fmicb.2021.617412/full

594 34. El Meouche I, Dunlop MJ. Heterogeneity in efflux pump expression predisposes antibiotic-
595 resistant cells to mutation [Internet]. Vol. 362, Science. 2018. Available from:
596 http://science.sciencemag.org/

597 35. Van Den Bergh B, Michiels JE, Wenseleers T, Windels EM, Boer P Vanden, Kestemont D, et al.
598 Frequency of antibiotic application drives rapid evolutionary adaptation of Escherichia coli
599 persistence. Nat Microbiol 2016 15 [Internet]. 2016 Mar 7 [cited 2023 Jan 5];1(5):1–7.
600 Available from: https://www.nature.com/articles/nmicrobiol201620

601 36. Windels EM, Michiels JE, Fauvart M, Wenseleers T, Van den Bergh B, Michiels J. Bacterial
602 persistence promotes the evolution of antibiotic resistance by increasing survival and
603 mutation rates. ISME J [Internet]. 2019 May 1 [cited 2021 Mar 11];13(5):1239–51. Available
604 from: https://doi.org/10.1038/s41396-019-0344-9

605 37. Witzany C, Regoes RR, Igler C. Assessing the relative importance of bacterial resistance,
606 persistence and hyper-mutation for antibiotic treatment failure. Proc R Soc B Biol Sci
607 [Internet]. 2022 Nov 9 [cited 2023 Jan 11];289(1986). Available from:
608 https://royalsocietypublishing.org/doi/10.1098/rspb.2022.1300

609 38. Wang P, Robert L, Pelletier J, Dang WL, Taddei F, Wright A, et al. Robust growth of Escherichia
610 coli. Curr Biol [Internet]. 2010 Jun 22 [cited 2011 Jul 18];20(12):1099–103. Available from:
611 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2902570&tool=pmcentrez&ren
612 dertype=abstract

613 39. Jouvet L, Rodríguez-Rojas A, Steiner UK. Demographic variability and heterogeneity among

614 individuals within and among clonal bacteria strains. Oikos. 2018;127(5):728–37.

615 40. Steiner UK, Lenart A, Ni M, Chen P, Song X, Taddei F, et al. Two stochastic processes shape
616 diverse senescence patterns in a single-cell organism. Evolution (N Y) [Internet]. 2019 Apr 14
617 [cited 2019 Aug 20];73(4):847–57. Available from:
618 https://onlinelibrary.wiley.com/doi/abs/10.1111/evo.13708

619 41. Steiner UK, Lenart A, Ni M, Chen P, Song X, Taddei F, et al. Two stochastic processes shape
620 diverse senescence patterns in a single-cell organism. Evolution (N Y) [Internet]. 2019 Aug 14
621 [cited 2018 Aug 15];online ear:10.1111/evo.13708. Available from:
622 https://www.biorxiv.org/content/early/2018/08/14/105387

623 42. Roostalu J, Jõers A, Luidalepp H, Kaldalu N, Tenson T. Cell division in Escherichia coli cultures
624 monitored at single cell resolution. BMC Microbiol [Internet]. 2008 [cited 2021 May 7];8.
625 Available from: https://pubmed.ncbi.nlm.nih.gov/18430255/

626 43. Conde DA, Staerk J, Colchero F, da Silva R, Schöley J, Maria Baden H, et al. Data gaps and
627 opportunities for comparative and conservation biology. Proc Natl Acad Sci U S A.
628 2019;116(19).

629 44. R Core Team RA language and environment for statistical computing. R: A language and
630 environment for statistical computing. [Internet]. Team RDC, editor. R: A language and
631 environment for statistical computing. Vienna, Austria: R Foundation for Statistical
632 Computing; 2022. p. 409. (R Foundation for Statistical Computing; vol. 1). Available from:
633 http://www.r-project.org

634 45. Burnham K, Anderson DR. Model selection and multimodel inference: a practical
635 information-theoretic approach. Second. New York: Springer; 1998.

636 46. Regoes RR, Wiuff C, Zappala RM, Garner KN, Baquero F, Levin BR. Pharmacodynamic
637 functions: a multiparameter approach to the design of antibiotic treatment regimens.
638 Antimicrob Agents Chemother [Internet]. 2004 Oct [cited 2023 Feb 3];48(10):3670–6.
639 Available from: https://pubmed.ncbi.nlm.nih.gov/15388418/

640 47. Wilmaerts D, Focant C, Matthay P, Michiels J. Transcription-coupled DNA repair underlies
641 variation in persister awakening and the emergence of resistance. Cell Rep. 2022 Mar
642 1;38(9):110427.

643 48. Knudsen GM, Fromberg A, Ng Y, Gram L. Sublethal concentrations of antibiotics cause shift to
644 anaerobic metabolism in Listeria monocytogenes and induce phenotypes linked to antibiotic
645 tolerance. Front Microbiol. 2016 Jul 12;7(JUL):1091.

646 49. Hingley-Wilson SM, Ma N, Hu Y, Casey R, Bramming A, Curry RJ, et al. Loss of phenotypic
647 inheritance associated with ydcI mutation leads to increased frequency of small, slow
648 persisters in Escherichia coli. Proc Natl Acad Sci U S A [Internet]. 2020 Feb 25 [cited 2023 Jan
649 5];117(8):4152–7. Available from: /pmc/articles/PMC7049120/

650 50. Melnyk AH, Wong A, Kassen R. The fitness costs of antibiotic resistance mutations. Evol Appl
651 [Internet]. 2015 Mar 1 [cited 2021 Mar 4];8(3):273–83. Available from:
652 /pmc/articles/PMC4380921/

653 51. Rossi NA, El Meouche I, Dunlop MJ. Forecasting cell fate during antibiotic exposure using
654 stochastic gene expression. Commun Biol [Internet]. 2019 Dec 1 [cited 2021 Jun 2];2(1):1–7.
655 Available from: https://www.nature.com/articles/s42003-019-0509-0

656 52. Dörr T, Lewis K, Vulić M. SOS response induces persistence to fluoroquinolones in Escherichia
657 coli. PLoS Genet. 2009;5(12):e1000760.

53. Rodríguez-Rojas A, Baeder DY, Johnston P, Regoes RR, Rolff J. Bacteria primed by antimicrobial peptides develop tolerance and persist [Internet]. Vol. 17, PLOS Pathogens. PLoS Biol; 2021 [cited 2021 May 5]. p. e1009443. Available from: https://doi.org/10.1371/journal.ppat.1009443

54. Windels EM, Van den Bergh B, Michiels J. Bacteria under antibiotic attack: Different strategies for evolutionary adaptation. Silverman N, editor. PLOS Pathog [Internet]. 2020 May 7 [cited 2021 Mar 11];16(5):e1008431. Available from: https://dx.plos.org/10.1371/journal.ppat.1008431

55. Balaban NQ, Liu J. Evolution under antibiotic treatments: Interplay between antibiotic persistence, tolerance, and resistance. In: Persister Cells and Infectious Disease [Internet]. Springer International Publishing; 2019 [cited 2021 Apr 19]. p. 1–17. Available from: https://link.springer.com/chapter/10.1007/978-3-030-25241-0_1

56. Fridman O, Goldberg A, Ronin I, Shoresh N, Balaban NQ. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature. 2014 Sep 18;513(7518):418–21.

57. Van Den Bergh B, Michiels JE, Wenseleers T, Windels EM, Boer P Vanden, Kestemont D, et al. Frequency of antibiotic application drives rapid evolutionary adaptation of Escherichia coli persistence. Nat Microbiol [Internet]. 2016 Mar 7 [cited 2021 Apr 19];1(5):1–7. Available from: https://www.nature.com/articles/nmicrobiol201620

58. Sulaiman JE, Lam H. Proteomic Investigation of Tolerant Escherichia coli Populations from Cyclic Antibiotic Treatment. J Proteome Res [Internet]. 2020 Feb 7 [cited 2021 Apr 19];19(2):900–13. Available from: https://pubmed.ncbi.nlm.nih.gov/31920087/

59. Khare A, Tavazoie S. Extreme Antibiotic Persistence via Heterogeneity-Generating Mutations Targeting Translation. mSystems [Internet]. 2020 Jan 21 [cited 2021 Apr 19];5(1). Available from: http://msystems.asm.org/

60. Zahir T, Wilmaerts D, Franke S, Weytjens B, Camacho R, Marchal K, et al. Image-Based Dynamic Phenotyping Reveals Genetic Determinants of Filamentation-Mediated β-Lactam Tolerance. Front Microbiol [Internet]. 2020 Mar 13 [cited 2021 Mar 9];11:374. Available from: https://www.frontiersin.org/article/10.3389/fmicb.2020.00374/full