A different type of tolerance: selection for and phenotypic characteristics of antibiotic tolerance evaluated at the single cell level under recurring antibiotic exposure in *Escherichia coli*.

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Abstract:

Tolerance and persistence are states that allow non-genetically resistant bacterial cells to survive periods of bactericidal concentrations. Compared to resistance, tolerance and persistence appear to be more diverse in their mechanisms but are less studied. Here we report, using a high throughput microfluidic single-cell microfluidic device, selection for and phenomenological characteristics of cells exposed to recurring exposure to antibiotics. We find a high fraction of tolerant cells, these cells reduce their growth rate but do not go into slow growth or growth arrest, a characteristic previously reported on. Here, tolerance is induced by antibiotic exposure and not caused by a stochastic switch or predetermined state. The tolerance state only weakly depends on the applied concentration of the antibiotic and post-antibiotic exposure there is no fast resumption of growth contrasting again with previous studies. Tolerant cells remain susceptible to antibiotic exposure suggesting recurrent selection for tolerance. Selection also did not act primarily on cells showing relatively high growth rates, as often assumed under exposure to a β-lactam antibiotic. The cells with relatively high growth rates, but still reduced growth compared to prior antibiotic exposure conditions, showed equal mortality risk than cells that were in growth arrest or shrank, slowly growing cells showed the lowest mortality risk. Relatively fast-growing cells and non-growing or shrinking cells exhibited less robust growth patterns compared to slowly, but constantly, growing cells. Our findings suggest a type of tolerance that differs from previously described tolerance and persister characteristics. The high frequency of such tolerant cells, in combination with the fact that they do not arise from growth arrested cells and do not go into growth arrest under antibiotic exposure, suggest that they might play an important role for the evolution to resistance and for antibiotic treatment failure of recurring infections.

Resistance to antibiotics, in combination with the slow progress in discovering novel antibiotics, poses a major challenge to modern medicine around the globe (Ventola 2015). Antibiotic resistant cells sustain bactericidal concentrations of antibiotics through genetically fixed alterations and therefore their resistance is heritable. By contrast to resistant cells, persister cells do not possess heritable genetic resistance, they are in a phenotypic state that allows them to sustain bactericidal concentration of antibiotics. These persistent states are either environmentally induced or caused by stochastic switching, and, due to this dynamic in switching states, their progeny is as susceptible as the parental population the subpopulation of persister cells was collected from (Lewis 2007; Balaban et al. 2019). Experimentally, increased transition rates to the persister state can be induced by starvation or other environmental stresses and transitions to persistence occur frequently during early stationary phase (Sulaiman and Lam 2021). Quantitatively, persister cells build a small subpopulation—typically below 0.1% for wild-type strains—in an otherwise susceptible population. Persister cells are phenotypically characterized as non- or very slow growing under antibiotic exposure and arise from a non- or very slowly growing subpopulation of a susceptible population prior to antibiotic exposure (Brauner et al. 2016, 2017; Balaban et al. 2019). Another type of cells are tolerant cells, they are more common than persister cells, and like persister cells they can tolerate antibiotic treatment, even at high antibiotic concentrations. Though compared to persister cells, for tolerant cell the duration of antibiotic exposure needs to be more limited (Balaban et al. 2019). As for persister cells, the progeny of tolerant cells is as susceptible to antibiotics as the parental (susceptible) population it was collected from, and like persister cells tolerant cells are non- or very slowly growing under antibiotic exposure. Tolerant cell populations are characterized by a lower mortality rate under antibiotic exposure and show prolonged minimum duration of killing (MDK), without a change in the minimum inhibitory concentration (MIC). The similarities between persister cells and tolerant cells sometimes define persister cells a special and rare subgroup of tolerant cells (Windels et al. 2020).

A third class of cells that can sustain bactericidal concentration of antibiotics are viable but non-culturable cells (VBNC). These cells are different from persister and tolerant cells in not regaining growth post-antibiotic exposure. VBNC cells remain at low metabolic activity, while persister and tolerant cells typically regain growth within 3-4 hours post-antibiotic treatment. Persister cells are considered therefore easier to resuscitate, while VBNC cells are in a deeper dormancy state (Pu et al. 2019). Persister and tolerant cells utilize high amounts of sugars post-antibiotic exposure, which might make them more susceptible to recurring exposure to antibiotics compared to VBNC cells. VBNC cells might not have enough intracellular ATP available for resuscitation (Pu et al. 2019). VBNC cells are further phenotypically characterized by being small (2.3 ± 0.2µm for E. *coli*) and less elongated compared to persister cells (Bamford et al. 2017).

These phenotypically, but non-genetically heritable, resistant cell types—persister, tolerant, and VBNC cells— elevate the chance of resistance evolution by serving as evolutionary steppingstones towards resistance through the provision of evolutionary reservoirs, and by increasing the mutant supply due to their increased mutation rate (Levin-Reisman et al. 2017, 2019; Bakkeren et al. 2019; Barrett et al. 2019; Liu et al. 2020; Sulaiman and Lam 2021). The distinction and definition among different antibiotic sustaining cell types and states is not always consistent, and—dependent on experimental setup—it can be difficult to distinguish among them empirically (Brauner et al. 2017; Balaban et al. 2019).

Compared to antibiotic resistance, interest in antibiotic persistence and tolerance has only recently risen (Balaban et al. 2019). Despite this recent rise in interest, already a rich variety of mechanisms have been revealed. The overarching communalities of these molecular mechanisms suggest lower protein synthesis, reduced metabolism, or complete dormancy as mechanisms, and we refer here to various reviews on such molecular mechanisms (Lewis 2007; Lee and Lee 2010; Vega et al. 2012; Maisonneuve and Gerdes 2014; Van Acker et al. 2014; Olsen 2015; Harms et al. 2016; Michiels et al. 2016; Page and Peti 2016; Wilmaerts et al. 2019; Diez et al. 2020). Compared to resistance that shows several clearly defined mechanisms, the diversity of molecular mechanisms that allow for non-genetic

tolerance seems to be large. Mechanisms seem to be more specific and vary how closely they are linked to different types of antibiotics, still an ongoing debate lingers whether there is a general mechanism or various mechanisms behind persistence and tolerance (Balaban et al. 2019; Sulaiman and Lam 2021).

Most studies on resistance, tolerance, and persistence, have been done on cell cultures, rather than at the single-cell level, which can make it challenging to differentiate among persister, tolerant and VBNC cells (Goormaghtigh and Van Melderen 2019). Single-cell studies revealed how antibiotic-induced ATP switching can be a fast, all-or-nothing, and irreversible event that is correlated with immediate growth arrest of the cell (Maglica et al. 2015). Other studies at the single-cell level revealed that persister cells do not go into complete growth arrest when exposed to β-lactam antibiotics or other antibiotics, that inhibit cell wall formation or cell division, but they show very limited growth (Balaban et al. 2004; Wakamoto et al. 2013; Goormaghtigh and Van Melderen 2019). In addition to an irreversible all-or-nothing switch, persistence has also been shown as a dynamic process where stochastic antibiotic activating pulses increase mortality, hence persistence highlights the importance of balancing division and mortality for sustaining antibiotics at high concentrations and uncoupling persistence from single-cell growth rates (Wakamoto et al. 2013). The stochastic switch to a persister state has been observed for growth-limited cells within exponentially growing populations, with ~80% of persisters arising from the non-growing subpopulation in *E. coli*, while the remaining persister cells originated from cells that were growing prior to treatment (Roostalu et al. 2008; Orman and Brynildsen 2013). Even though this type of high-quality single-cell phenomenological data allows for a quantitative investigation of heterogeneity among cells—including fractional killing and responses in cell growth and division—such high-quality single-cell phenomenological data remains scarce and the potential to gain deeper understanding about the evolutionary importance of persister and tolerant cells for the evolution of resistance and efficacy of antibiotic treatment has not yet been deeply exploited.

Here, we follow a quantitative demographic and phenomenological approach, to ask how tolerant cells differ in their characteristics and what triggers changes in their phenotypes. Starting with a susceptible clonal population of cells that we expose to recurrent antibiotic treatment at different concentrations, we track each cell for their growth, division, and survival over nearly one day in a microfluidic device, a mother machine (Wang et al. 2010; Jouvet et al. 2018; Steiner et al. 2019). We chose antibiotic concentrations similar to previously explored ranges, that spans from below MIC to >30 times MIC, and exposed cells to antibiotics for periods that would cover about three to four divisions under non-antibiotic conditions, Post-exposure times were also chosen on previous results reporting on regained growth of persister cells within 3-4 hours post-antibiotic treatment (Balaban et al. 2004; Wakamoto et al. 2013; Goormaghtigh and Van Melderen 2019). By exposing cells to a β-lactam, ampicillin, that corrupts cell wall synthesis, we expected fast growing cells to be heavily selected against. Persister and tolerant cells should both be little sensitive to increasing levels of antibiotic concentration beyond the MIC (Brauner et al. 2017). Most cells that can withstand limited duration of antibiotics should be tolerant cells, as they are expected to be more common than persister cells (Brauner et al. 2017). Tolerant and persister cells should regain normal growth rates within hours post antibiotic treatment. Comparing growth dynamics, size, division dynamics, and sensitivity on mortality in response to recurring exposure to antibiotics allows us to differentiate among tolerant and VBNC cells, and in comparing lag times, we should be able to differentiate antibiotic induced and stochastic switching to tolerance.

We show that the susceptible clonal parental population, that was derived of a commonly used K-12 *E. coli* lab strain (Mg 1655), consists of phenotypically heterogenous cells, or at least of cells that have different capacities to sustain exposure to antibiotics. Despite observing a growth reduction of tolerant cells under antibiotic exposure, these tolerant cells did not go into growth arrest and reduced growth by about fifty percent. Post-antibiotic treatment, tolerant cells did not resume normal growth rates but stayed for more than 11 h in a reduced, but not very low, growth state. Surprisingly, cells

that showed high growth rates showed similar high mortality than cells that did not grow or shrink. Cells with intermediate growth showed the lowest mortality, but significant growth dynamics remained among cells. The combined characteristics of these tolerant cells suggest a new type of tolerance that has not previously be described (e.g. no growth arrest, no quick resumption of growth), and that might have different implications for the evolution of resistance and failure of antibiotic treatment.

RESULTS

After a one-hour lasting acclimation phase of the cells in the microfluidic device, and upon the initial exposure of the susceptible parental population, survival dropped, and a cell's probability of death increased, i.e. the force of mortality increased due to the antibiotic (Fig. 1). Mortality peaked, 30 – 60 minutes post the initial 90-minute lasting exposure period, which suggests that the force of selection (probability of death) peaked with a lag time of 2-2.5 hours after initiation of the first exposure period. Survival pattern were similar among different concentrations of antibiotics, if it was at, or above, the MIC of 4 µg/ml (Fig. 1a, Cox proportional hazard model Likelihood ratio test 477.1, $p<0.0001$, $n= 3496$; for post hoc testing see SI Table S1). At 2 µg/ml, mortality was higher compared to the control group without any antibiotic exposure but lower than for concentrations at or above MIC, but the lag time in response to the antibiotic exposure of mortality was similar for all concentrations of antibiotics (Fig. 1b). Subsequent exposure periods did not trigger such a pronounced force of mortality. This suggests a heterogeneous initial population on which the antibiotic differentially selects, leaving mainly tolerant cells after the first period of exposure, of which a small fraction should be persister cells. After the initial rise in mortality triggered by the antibiotic exposure, probability of death declined, and increased again towards the end of the second exposure period, though in a less synchronized way among cells exposed to different antibiotic concentrations, and the rise in mortality (force of selection) was less pronounced compared to the first mortality peak. The (second) peak force of mortality being reached about 30-90 min post second antibiotic exposure shows that the lag time of 2-3 h after onset of exposure was similar to the lag time after the first exposure. The sensitivity in mortality to the second exposure period suggests that the cells that survived the first mortality peak are not resistant cells, but rather tolerant cells that remained to some degree susceptible to antibiotics or these cells switched to such a susceptible state before the second exposure. Mortality rates continued to decline after this second peak of force of selection, and fluctuated for the remainder of the experiment, including the third period of exposure. Note, cell numbers became small and estimates of mortality rates less and less reliable towards the end of the experiment.

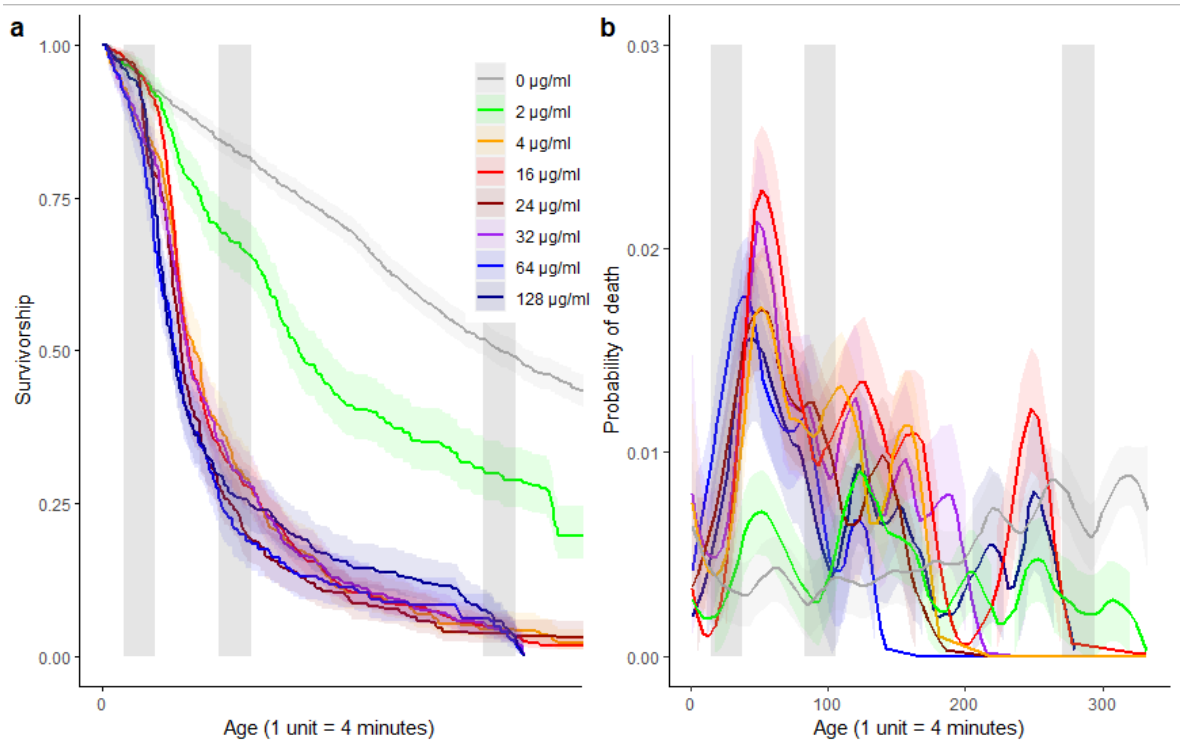

Fig. 1: Survivorship curves (a) and probability of death, i.e. force of mortality (b) of cells exposed to different levels of antibiotics across the duration of the experiment. The grey curves are control group cells that did not get exposed to antibiotics. The grey vertical bars mark the exposure period to the antibiotic. Kaplan-Meier survivorship curves (a) are computed with the survival package (survfit function) and shown with 95% CI. Probability of death curves (b) are plotted with a loess smoothing function with 95% CI (geometric_smooth function; package ggplot2).

Cell division rates decreased during and after the initial exposure period—for cells exposed to MIC or higher concentrations—from initially ~2 division per hour to ~1 division per hour (Fig. 2a, Table 1). This level of reduced division rates (reproductive rates) was reached around 5.5 h after onset of the first exposure, i.e. towards the end of the second exposure period. Division rates remained almost constant after the second exposure for another 6-7 hours, and then dropped off towards the end of the experiment. Control group cells and cells exposed to below MIC concentrations (2 µg/ml) had constant division rates throughout most of the experiment and did not show an initial decline in division rate. Towards the end of the experiment, cells exposed to 2 µg/ml declined in their division rate, while control group cells did not alter in their division rate.

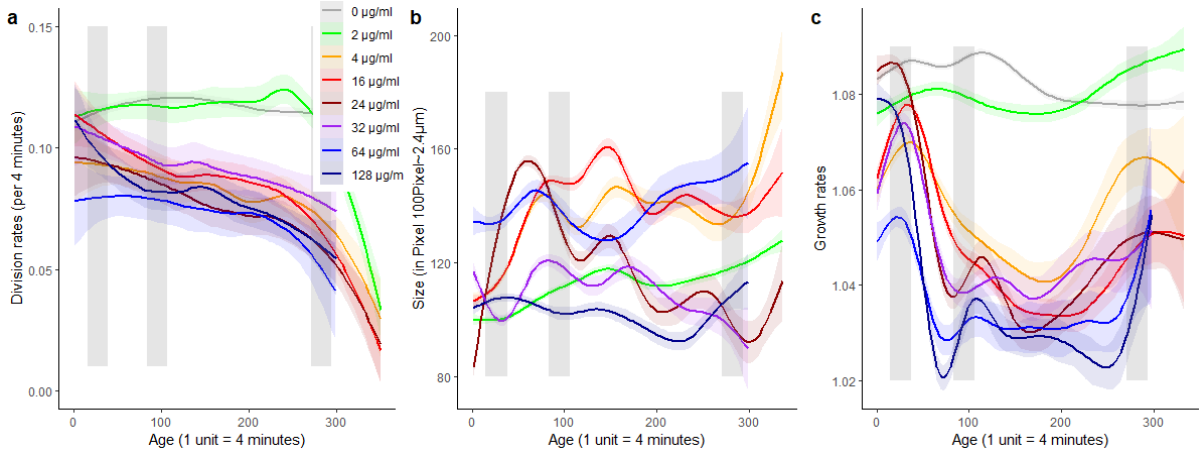

Fig. 2: Cell division rates (a), cell size (in pixel with 100 pixels~2.4µm) (b), and growth rates (c) of cells exposed to different levels of antibiotics across the duration of the experiment. The grey vertical bars mark the exposure period to the antibiotics. Division rate curves (a) are plotted with a loess smoothing function with 95% CI (geometric_smooth; package ggplot2), size and growth rate curves (b & c) are fitted with a GAM with 95% CI. Note, size data (b) for the control group (0µg/ml) was not available.

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

One set of competing models for Division rates, Size, and Growth rates are listed respectively. The best supported model for each set is highlighted in bold (one set for each response variable, Division rate, Size, and Growth rate). Competing GAM models are fitted with the restricted maximum likelihood method (REML), a smoothing parameter for age with a shrinkage version of a cubic regression spline as smoothing parameter (bs=cs) and/or the concentration. Division rates (Fig.2a) are fitted using a binomial error structure with a logit link function, size data (Fig. 2b) is fitted with a Gaussian error structure and an identity link function, and the Growth rates (Fig. 2c) are fitted with a Gamma error structure and an inverse link function.

Cell size remained approximately constant for the average cell exposed to the same level of antibiotic concentration, but we found a surprising variation in average size among cells exposed to different levels of antibiotics (Fig. 2b, Table 1). No relationship to increasing or decreasing levels of antibiotics was revealed; even cells exposed to below MIC (2 µg/ml) did not differ in their size from those exposed to higher doses of antibiotics. Note, smoothing functions are highly sensitive at the extremes as partly illustrated by the diverse initial average cell sizes of cells that came from the same parental population and that had not yet been exposed to antibiotics during the 60-minute lasting acclimation phase. These patterns in cell size suggest that selection has not been favoring small cells over the course of the experiment, as one could expect if VBNC cells would have been selected for. If the rate of filamentous cells would have increased over the course of the experiment, we should have seen an increase in average cell size, but we did not find such pattern, hence filamentation rate likely did not increase. Finally, the variation in cell size did not increase substantially across the course of the experiment, as one would expect if simultaneously higher fractions of small cells (VBNC) and filamentous cells would emerge.

Growth rates responded as expected in declining during and after first antibiotic exposure for cells exposed to MIC or above (≥4 µg/ml), but growth rates only approximately halved, and cells did not go into growth arrest (Fig. 2c, Table 1). Control group cells and cells exposed to concentrations below the MIC (2 µg/ml) did not show any change in cell growth. The induced growth reduction for cells exposed to MIC or above shows a lag time of about 30 minutes after the onset of antibiotic exposure. This lag time corresponds to approximately the doubling rate (division time) of the parental population. The lowered growth rates stabilized about 4.5 hours after onset of the initial exposure period, suggesting substantial variance and prolonged lag time in growth response among cells. This lag time suggests an

antibiotic induced switch to a tolerant state and contrasts with predictions of instantaneous switching or a predisposed tolerance state prior to antibiotic exposure, similar induced switching has been found for fluoroquinolone induced persistence (Dörr et al. 2009). Growth rates after exposure depended partly on antibiotic concentrations with higher levels of antibiotics leading to lower growth rates, but even under the highest level of antibiotics, growth rates did not drop below 1.02-1.03, i.e. 2-3% cell elongation within 4 minutes. Further, post second antibiotic exposure, growth did not resume in the 11-hour recovery phase. Previous findings on persister and tolerant cells regained normal growth after 3-4 hours post antibiotic exposure (Brauner et al. 2017; Balaban et al. 2019).

The growth rates as discussed above (Fig. 2c) are average growth rates at a given point in time, and even though variance in growth rates does not increase substantially during the experiment (beyond the expected increase due to decreasing numbers of cells towards the end of the experiment), selection might still act preferentially against cells with high growth as expected from a β-lactam antibiotic and as previously described for persister and tolerant cells (Lewis 2007). To evaluate such differential selection based on cell growth we combined all cells exposed to MIC or above (≥4 µg/ml), but then separated them out into three growth categories, cells that shrank or did not grow (growth rates<1.0), cells that grew at low to intermediate rates (growth rates 1.0-1.1), and cells that grew fast (growth rates >1.1) (Fig. 3a). Note, that cells can switch dynamically among these categories throughout the experiment and that mortality and survival is associated only with the current growth rate of a cell, not a cell's growth rate averaged across time. In contrast with our expectations, probability of death of non-growing/shrinking and fast-growing cells increased substantially upon exposure to antibiotics, whereas slow/intermediate growing cells did not change in their probability of death (Fig. 3b, see also SI Table S2 and Table S3). We would have expected to see a graded mortality pattern of cells with high growth rates showing high mortality rates and non-growing/shrinking cells showing low mortality rates upon exposure to antibiotics.

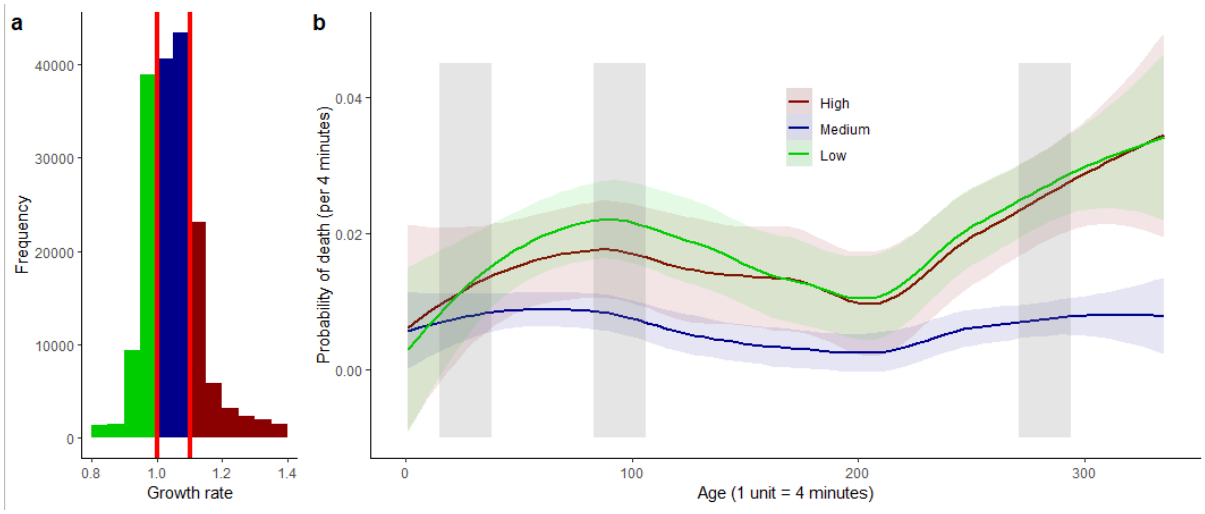

Fig. 3: Cell growth rate distribution divided into three categories: shrinking or non-growing (green), intermediate growth (blue), and fast growth (red) (a), and probability of death for these three groups of shrinking/non-growing, intermediate, and fast-growing cells across the duration of the experiment (b). The grey vertical bars in panel (b) mark the exposure period to the antibiotics. Probability of death curves are plotted with a loess smoothing function with 95% CI (geometric_smooth; package ggplot2). 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 High and Low growth groups did not differ from each other in age at death, but the medium growth group did.

To verify the robustness of the finding on selective mortality dependent on growth rates and to broaden our understanding of how growth influences beyond the current growth rate (Fig. 3b), 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, and conducted similar comparisons between cells dying during or shortly after the first exposure period (cut off at peak of first mortality) to those surviving past the first peak of mortality, and for cells that died between the first peak of mortality and the second peak of mortality to those that survived past the second peak of mortality (Table 2). Before first exposure, cells that died had lower average growth rates, compared to those that survived past the onset of first exposure, showing that without selection of antibiotics slower growing cells are preferentially selected against. Cells that died during the first exposure or shortly after, i.e. during times of strong selection, had slightly higher growth rates than those that survived past this first increased selection period; this pattern of growth dependent selection remained for the next period of increased selection after the second antibiotic exposure. These findings support the initial expectation that faster growing cells would be preferentially selected against under antibiotic exposure but note that the effect sizes (growth differences) among surviving and dying cells are fairly small, even though they are significantly different. One can interpret these findings as selection by antibiotic exposure not primarily acting on fast growing cells.

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

GROWTH RATE	**0 – 60 MIN**	**60 – 328 MIN**	**328 – 600 MIN**	**+600 MIN**
DEATH DURING (±STDEV)	1.047 (±0.039)	1.067 (±0.032)	1.057 (±0.030)	1.045 (±0.027)
DEATH AFTER	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	

Model comparison among a null model (intercept only model) and a model with the two groups of cells dying during a particular period or surviving past that period as explanatory variable. The mean growth rate of each cell was used as the response variable and three sets of models were fit, one for each time period [0 min – 60 min; 60 min – 328 min; 328 min – 600 min]. A gaussian error structure was used to fit these models.

Recall, that 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 at subsequent times. Overall correlation between an individual's cell growth rates at a given time t and its growth rate at the next time point (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), except for cells with low to intermediate growth rates (1.0-1.1). Such slow to intermediate growing cells showed a strong correlation (Fig. 4), illustrating robust constant growth of such slow to intermediate growing cells. Shrinking cells (growth rate at time t<1) tended to have higher growth rates in the next time steps, while fast growing cells (growth rates at time t >1.1) tended to have lower growth rates in the next time steps. Overall, slow to intermediate growing cells showed robust and stable growth rates, while shrinking and fast-growing cells tended to fall back towards average growth rates in the next time steps. Across cells we observed substantial temporal dynamics in growth rates. Non-growing/shrinking and fast-growing cells showed larger differences in growth rates at different levels of antibiotics compared to slow and intermediate growing cells, which suggests that growth rate differences among cells exposed to different concentrations of antibiotics (Fig.2c) are mainly influenced by the fastest growing and non-growing/shrinking cells.

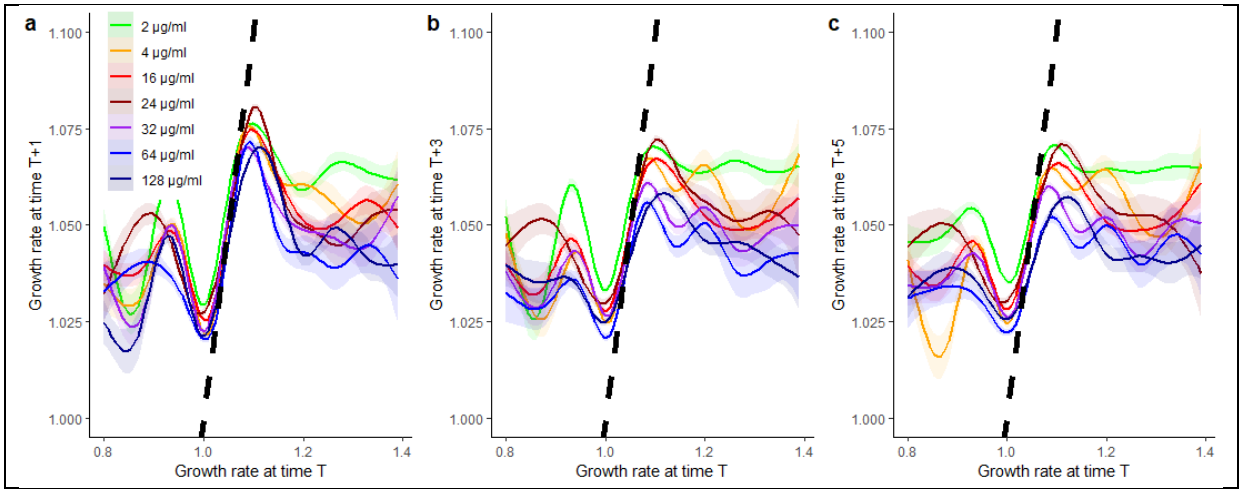

Fig. 4: Correlation in cell growth rates at a given time t, compared to growth rates at time t+1 (4 minutes later) (a), t+3 (12 minutes later) (b), and t+5 (20 minutes later) separated out into populations recurrently exposed to different levels of antibiotics (as described in the main experiment). All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

Table 3: Model comparison among competing GAM models, one set of competing models for Correlation of current growth rates to growth rates at next time step, growth at time t+3, and growth at time t+5 rates respectively.

	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

Best supported model for each of the three sets highlighted in bold (one set for each response variable, Growth at time T+1, Growth at time T+3, and Growth at time T+5). GAM models are fitted with the restricted maximum likelihood method, a smoothing parameter for Growth rate at time T with a shrinkage version of a cubic regression spline as smoothing parameter (bs=cs) and/or the concentration, including a model that accounts for the interaction of the two explanatory variables. Growth rates are fitted with a Gaussian error structure and an inverse link function.

Discussion

We report a different, previously not described, combination of phenological and demographic cell responses in *E. coli* to recurring exposure to antibiotics. Like previously described types of tolerant cells, a substantial fraction (>50%) of cells survived antibiotic exposure independent of the level of antibiotics, a fraction much larger than described for persister cells (Fig. 1a) (Brauner et al. 2016, 2017; Balaban et al. 2019). Below MIC concentrations, survival was higher compared to exposure at or above MIC, illustrating that the initial population has been susceptible and not resistant (Fig. 1). Growth and

division rates of our cells, like previously described types of tolerant cells, are reduced under antibiotic exposure, but growth reduction in our cells was only halved, and cells did not go into growth arrest as previously described for tolerant and persister cells (Balaban et al. 2004, 2019; Brauner et al. 2016). Other studies showed severe growth reduction in persisters that did not go into growth arrest (Balaban et al. n.d.; Goormaghtigh and Van Melderen 2019). Contrasting previously defined characteristics of tolerant and persister cells, a fast growth resumption within 2-4 hours was not observed for our cells. Our cells did not resume to growth rate levels observed prior to antibiotic exposure when released from antibiotics. Such lacking growth resumption is characteristic of some type II persister cells and VBNC cells, however VBNC are in complete growth arrest and are characterized by small cell size, and persister II cells are not environmentally induced but predetermined (Balaban et al. 2004; Bamford et al. 2017), features not found in our study.

Our cells also differed from genetically fixed resistant cells in various ways. First, our cells did not show graded survival or division response to different levels of antibiotics as resistant cells do. Second, our cells halved their growth rates (50% reduction) under exposure, whereas a reduction of growth by 20% is already considered to be an extreme growth cost for resistant cells (Melnyk et al. 2015). Third, other than resistant cells, our cells showed continued susceptibility to repeated exposure to antibiotics. Finally, resistance is not expected for our cells, as our population of cells was directly derived from a susceptible, standard *E. coli* lab strain K-12 MG1655. Evolution towards a heteroresistant population, being composed of multiple subpopulations that differ in their resistance to antibiotics (exemplified by different MIC), would not be expected in the time prior to the first exposure when no selective force towards resistance was applied; and a heteroresistant population should have left, after exposure, a resistant population with graded responses to increased levels of antibiotic concentrations. Such a graded response should not only be visible with respect to survival, but also with regards to division and growth rates (Melnyk et al. 2015). Still, the direct derivation of the MG1655 lab strain did not prevent our initial population to show heterotolerance, as illustrated by the differential selection for tolerant cells after first antibiotic exposure. The high fraction of cells, that survived the first exposure to antibiotics (75% still alive at the end of the first exposure period), suggests that these cells are not rare persister cells (Balaban et al. 2019), but tolerant cells. We therefore argue that we describe new characteristics of tolerance, that might be another type of tolerance, for which resistance, VBNC or persistence, play no significant role, but this new tolerance has important characteristics relevant for antibiotic treatment and the evolution of antibiotic resistance.

Our tolerant cells did not, as other tolerant cells, rise out of stationary phase cells, they were also not growth-arrested or very slowly growing prior to exposure (Balaban et al. 2004, 2019; Lewis 2007; Brauner et al. 2016). These growth characteristics have important implications of tolerant cells for the evolution of resistance. “Classical” tolerant and persister cells, that are growth arrested and that emerge predominantly out of stationary phase cells, only contribute to resistance development through repeated rounds of population expansion by their offspring. Such expansions and repeated selection opportunities towards resistance are best achieved through multiple rounds of antibiotic exposure, resumed growth post-exposure, and going into stationary phase, whereas our tolerant cells contribute before, during, and after antibiotic exposure to population expansion, and therefore provide continued opportunities for the accumulation of mutations towards resistance (Barrett et al. 2019). Note, that our tolerant cells cannot be, *a priori*, distinguished from the normal non-tolerant cells by their reduced growth rate (Balaban et al. 2004). Other studies found persisters that show somewhat similar characteristics to our cells, in not arising from stationary phase cells, being not easily identifiable prior to exposure, showing slow growth resumption post-exposure, but these cells are still very rare and go into almost growth arrest under antibiotic exposure (Goormaghtigh and Van Melderen 2019). The tolerance we observe is not linked to cell growth rates under antibiotic exposure, slow to intermediate growing cells had higher survival rates compared to non-growing, shrinking, and fast-growing cells. Further, intermediate growing cells showed more robust growth rates compared

to high dynamics in growth rates found for non-growing, shrinking, and fast-growing cells, suggesting that tolerant cells with high survival might have more balanced and robust intrinsic metabolism and less dynamic biochemical changes. Our findings might therefore contrast with findings at the molecular level that suggest that tolerance might be the result of a perturbed biological network, that does not mean that the tolerance observed here falls into the reductionist perspective frequently applied to resistance (Sulaiman and Lam 2021). To us it was surprising that growth arrested or shrinking cells were not the cells that were the least affected cells by β-lactam exposure, given that β-lactam acts by corrupting enzymes involved in cell wall biogenesis.

The growth response of the tolerant cells we observe also suggests that the tolerant state is an induced state and not a pre-existing state as previously described for exponential growing cells that have been observed to switch very rarely, and in a stochastic manner, to a persistence or tolerant state (Balaban et al. 2004). Our findings however, support other findings on persister cells, and increased tolerance and persistence induced by antimicrobial peptide exposure (Goormaghtigh and Van Melderen 2019; Rodríguez-Rojas et al. 2021). The suggested antibiotic induced switching to the tolerant state we observe, takes about 2-3 hours, a lag time that spans over a minimum of 2-3 divisions. During that time about 4-8 times the initial cell's biomass is produced, and it is interesting that such a substantial increase in biomass was independent of the level of antibiotics, as one could imagine that the range of antibiotic concentrations we explored would have resulted in a graded response in survival, division, size, or growth. The emergence of our tolerant cells out of an exponential growing population and descending from a strain that has not evolved under growth limiting conditions, also suggests that the evolution, or at least the maintenance, of tolerance is not conditional on nutrient limiting condition as being suggested to be the favorable evolutionary route when antibiotic treatment occurs in an environment that does not promote growth (Windels et al. 2020).

Our finding that cells cannot be easily identified prior to antibiotic exposure by their reduced growth rates also supports findings that there are various evolutionary routes to tolerance (Balaban and Liu 2019), which contrasts our knowledge of evolution to genetically fixed resistance, that tends to arise from mutations in a few defined genes that relate directly to the mechanism the antibiotic acts by (Sulaiman and Lam 2021). Experimental evolution studies that evolved high tolerance by repeated exposure to ampicillin showed that tolerance was achieved through increased lag time (Fridman et al. 2014), while other experimental evolution studies, exposing stationary phase cells, evolved tolerance without prolonged lag times (Van Den Bergh et al. 2016), and still other experimental evolution studies found again other mutations, which illustrates the diversity of evolutionary paths to tolerance (Sulaiman and Lam 2020*a*, 2021). As our cells did not go into growth arrest, certain underlying mechanism might be excluded and others might be more likely, but as our study did not aim at molecular mechanism we could only speculate. It remains interesting to see the diversity of molecular and evolutionary path to tolerance, that seems to result in a diversity of phenomenological characteristics of tolerance, which our findings add to.

Our analysis provided various novel and surprising results on tolerance that contrast with previous findings on tolerance and persistence. We therefore need to ask what else than a different tolerance could explain such differences. Besides the use of different bacterial species and differences in antibiotic applied, even within the same bacteria and bacteria strains and the same antibiotic exposure, characteristics of persisters and the frequency of their occurrence is sensitive to culture conditions and growth phase (Harms et al. 2017). Studies that enriched persisters by preselecting dormant cells (Shah et al. 2006; Orman and Brynildsen 2013) are not suitable for comparison to our findings, as our cells and cells from other studies (Goormaghtigh and Van Melderen 2019) lack such clearly predefined characteristics. Working with high-frequency persister mutants, such as hip mutants, can alter characteristics compared to wild type persister cells (Balaban et al. 2004). Growth medium can influence phenotypes, for instance the filamentation rates differ among minimal medium (M9) and rich medium (LB) in *E. coli*, so does the recovery rate from filamentous states (Wang et al.

2010; Steiner et al. 2019). Exposure time to antibiotics obviously applies different selection pressure towards persister and tolerant cells, with our exposure time of 90 minutes being shorter compared to other experiments that selected cells by usually exposing them to ~5 hours (Balaban et al. 2004; Goormaghtigh and Van Melderen 2019). These differences in selection might explain the different focus on tolerant cells that sustain limited exposure time, compared to persister cells, but more exploration is needed. The core findings of by Goormaghtigh and Van Melderen (Goormaghtigh and Van Melderen 2019) rely on 23 persister cells selected out of more than 100000 cells. Such numbers of cells can, as illustrated by Goormaghtigh and Van Melderen, reveal novel insights, but are not amenable to an in-depth quantitative analysis as we carried out here. We therefore believe that our described characteristics cannot only be explained by differences in the experimental setup and growth conditions, but likely add to the diversity of tolerance mechanisms.

In our experiments, we have only evaluated treatment cycles of 30 minutes and 90 minutes. The shorter 30-minute recurrent exposure did not lead to any response, though other studies, using different antibiotics, found responses for such short time exposure (Rodríguez-Rojas et al. 2021). The 90-minute exposure treatment resulted in the results we report on here. In the future, simple experiments for varying exposure time, and varied inter exposure time periods might provide deeper understanding about the tolerance we observed and its potential role for antibiotic treatment and the evolution of resistance. Prolonged treatment cycles can cause tolerant strains to attain resistance at higher rates (Levin-Reisman et al. 2017), while multi drug treatment can prolong time to tolerance evolution (Khare and Tavazoie 2020), another dimension that could easily be explored in similar experiments we have described here in the future. Different strains can be explored, but such exploration should be systematic, as part of the diverse findings on tolerance might be caused by small differences in experimental settings not related to the main target (Sulaiman and Lam 2021). A rigorous phenomenological exploration, as we have done, can provide insights to set up more tailored experiments targeting specific molecular mechanisms. For instance, our cells did not show alterations in cell size, which suggests that our tolerance might not be related to a known tolerance to β-lactams that increases filamentation rate, which in turn circumvents ampicillin to target the cells efficiently (Goormaghtigh and Van Melderen 2019; Sulaiman and Lam 2020*a*, 2020*b*; Zahir et al. 2020). Our single cell approach also provided quantitative insights that can be scaled from the single cell to the population level and thereby can provide deeper understanding on the evolutionary potential towards resistance. The diversity of findings on persister and tolerant cells, that partly contrast each other, illustrates that we are still at the verge of understanding the role and mechanisms of tolerance, and standardized and comparative studies are needed to solidify and advance our current understanding. As for applied implications on medical treatment we need to caution interpretation, because directly relating *in vitro* to *in vivo* results for antibiotic treatment is challenging.

MATERIAL AND METHODS

We conducted two experiments, each with three antibiotic exposure periods of 90 minutes. The first exposure period was 60-150 minutes past onset of the experiments, the second exposure period was 330-420 minutes past onset of the experiments, i.e. 180 minutes past the first exposure period, and the last exposure period was 1080-1170 minutes past onset of the experiments, i.e. 660 minutes past the second exposure period. Concentrations of ampicillin during the exposure periods was set to 2, 4, 16, 24, 32, 64 or 128µg/ml. As comparison and reference, data from another experiment was added as control group, where cells were never exposed to antibiotics (Jouvet et al. 2018). We choose ampicillin as antibiotic, a β-lactamase that inhibits cell wall synthesis in *E. coli,* because this antibiotic has been used in previous persister studies (Balaban et al. 2004).

To collect individual level phenological data at a the single-cell level, each cell was tracked by time-lapse life-imaging (15 frames/hour) in a microfluidic (PDMS) chip, a mother machine (Jouvet et al. 2018), that was placed under a temperature-controlled (37°C) inverted microscope. All experiments were done in supplemented minimum media (M9). The cells loaded into the microfluidic chip came

from an exponentially growing culture that was derived from of *E. coli* strain K12-MG1655. Given that the cell culture the loaded cells originated from was in exponential growth phase, the vast majority of cells loaded should also been in an exponential growth phase (Steiner et al. 2019). The microfluidic chip the cells grew in contained main laminar flow channels (feeding channels) and dead-end side channels, in the latter the rod-shaped bacteria-cells grew. Only one focal cell, the one closest to the dead-end of the side channel, was tracked for its growth (cell elongation), size, time of each division, and death. Cell death was determined by propidium iodide (PI) that emits a strong fluorescent signal when the cell wall lysis—the moment the PI can enter the cell—and bonds to the cells' DNA. Image analysis was performed using a customized software and the resulting data was subsequently analysed in program R (R Core Team 2016) using general linear, generalized linear, and nonlinear (Generalized Additive Models, GAM) models. Models were compared based on information criteria (AIC) (Burnham and Anderson 1998). More details on the microchip production, loading of the chip, the bacteria growth, imaging, and analyses are given in the supplemental material.

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