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Lineage-dependent variations in single-cell antibiotic susceptibility reveal the selective inheritance of phenotypic resistance in bacteria

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Genetically identical bacterial cells often exhibit heterogeneous responses to antibiotics - some survive, others die. Here, we show that this heterogeneity propagates across generations to give rise to phenotypic resistance. Using real-time single-cell tracking, we exposed *Escherichia coli* to the β -lactam cefsulodin at its clinical breakpoint concentration and analyzed cell fate within genealogical trees statistically. Cell survival was strongly correlated among family members, driving the selective enrichment of robust lineages within an otherwise susceptible population. Our genealogical population model identified heritable phenotypic resistance as a key factor underlying this enrichment, which was validated experimentally. Comparing enrichment dynamics between the wild-type and a *tolC* knock-out strain, deficient in multidrug efflux, uncovered nuanced changes that increased the intergenerational memory of phenotypic resistance. Our findings provide evidence for heritable phenotypic resistance and demonstrate how its propagation through cell-tocell heterogeneity enables the survival of minority cells within isogenic populations.

Antibiotics are pivotal in combating bacterial infections, but lose their efficacy when administered inappropriately or misused. This problem is further exacerbated by antibiotic-resistant bacteria, which continue to replicate in the presence of antibiotics.

Traditionally, antibiotic resistance was attributed to genetic changes, such as mutations and horizontal gene transfer¹. This mode of resistance, typically referred to as genotypic resistance, can be reliably detected by conventional antibiotic susceptibility tests². Due to its stability, it can be isolated by subsequent plating, and genetic markers responsible for resistance can be readily monitored by sequencing. Epigenetic adaptation, potentially mediated by mechanisms akin to DNA methylation observed in mammalian cells, could also contribute to resistance^{3,4}. However, recent studies have shown that bacteria can

survive and grow in the presence of antibiotics without undergoing genetic changes, a phenomenon collectively known as phenotypic resistance^{5-II}. Surveys suggest that phenotypic resistance is wide-spread, contributing significantly to antibiotic treatment failure¹²⁻¹⁵. Unlike genotypic resistance, however, phenotypic resistance is not easily detected by conventional susceptibility tests and evades diagnosis by sequencing¹⁶. No well-defined physiological markers have been identified to explain its emergence. Phenotypic resistance is distinct from bacterial persistence, where dormant variants tolerate antibiotic treatment by transiently halting growth¹⁷. Unlike these tolerant cells, phenotypically resistant cells continue to grow and multiply in the presence of antibiotics, having significant effects on pharmacodynamics.

¹Department of Physics, Emory University, Atlanta, GA, USA. ²Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, GA, USA. ³Division of Infectious Diseases, Department of Medicine, Emory University, Atlanta, GA, USA. ⁴Antibiotic Research Center, Emory University, Atlanta, GA, USA. ⁵These authors contributed equally: Wesley Stine, Tatsuya Akiyama. 🖂 e-mail: minsu.kim@emory.edu Recent advances in our understanding of phenotypic heterogeneity offer valuable insights into this enigma. Over the past decade, it has become clear that genetically identical cells can exhibit distinct phenotypes^{18,19}, because molecular processes in the cell are inherently stochastic^{20–22}. Notably, gene expression turned out to be 'noisy', which leads to rapid variations in gene product levels within cells, which some studies view as a bet-hedging strategy^{18,23}. Due to these variations, the phenotype of genetically identical cells can differ from cell to cell.

Similar observations have been made in antibiotic treatments of isogenic populations. Despite uniform antibiotic exposure, some cells succumbed and were killed while others replicated^{12,14,15,17,24}, which not only complicates antibiotic treatments but also alters the evolution of resistance²⁵. This phenomenon was observed across a wide spectrum of antibiotics regardless of their mode of action²⁶, indicating it is a general principle of bacterial response to antibiotics. With the recognition that stochastic gene expression drives cell-to-cell heterogeneity, recent studies used stochastic branching processes to model cell growth and death under antibiotic exposure as random events, predicting random variations in population size during antibiotic treatments²⁶⁻²⁸.

Yet, this perspective dismisses the heterogeneity as mere noise in a population, overlooking potential regulatory mechanisms that may actively modulate the heterogeneity to enhance population fitness under antibiotic stress. If these mechanisms persist near the clinical breakpoint, i.e., the threshold concentration defining the limit of antibiotic susceptibility²⁹ (Supplementary Text 1), they can contribute to antibiotic resistance, having significant impacts on antibiotic diagnosis tests and treatment strategies^{13,16}. Importantly, because these processes do not involve genetic changes, they will be manifested as phenotypic resistance.

In this study, we focused on this heterogeneity to establish phenotypic resistance and understand its propagation dynamics within a population. Specifically, we exposed an isogenic population of *E. coli* cells to a β -lactam antibiotic, cefsulodin (which inhibits the synthesis of bacterial cell wall³⁰) at its breakpoint concentration. We tracked cell growth and death at single-cell resolution in real time, while simultaneously tracing their family relationship and documenting their growth/death patterns in the genealogical trees. Given the well-known role of efflux pumps in antibiotic resistance³¹, we inactivated them by knocking out a major component of efflux pumps, *tolC*. We then analyzed the cell survival patterns probabilistically using statistical methods and developed a population model to quantitatively understand the observed statistical properties. The results collectively provide evidence for phenotypic resistance, while demonstrating its intricate inheritance dynamics through cell-to-cell heterogeneity.

Results

Single-cell-level analysis of cell growth and death

Traditional diagnosis of antibiotic resistance provides an incomplete picture about antibiotic susceptibility. For example, the breakpoint of a β -lactam antibiotic, cefsulodin, is 32 µg/ml^{32,33}. A wild-type *E. coli* culture grows to the saturating density at this breakpoint, thereby being categorized as resistant²⁹ (Fig. 1a; see Supplementary Fig. 1 for growth curve). However, single-cell-level imaging shows that a substantial number of cells are susceptible and are killed at this concentration (Supplementary Movie 1, left panel). Death of these cells, which occurs concurrently with the growth of others, causes the number of live cells in a population to fluctuate seemingly randomly (Fig. 1b). Such population fluctuations have been observed for various other bactericidal drugs as well²⁶.

In previous studies, these fluctuations were modeled using a stochastic branching process²⁶⁻²⁸, which assumes that each cell's fate is uncorrelated. To test this assumption, we analyzed the fluctuations in greater depth. Specifically, in addition to counting live cells, we

followed the family relationship of these cells, thereby constructing a genealogical tree for each colony. This tree visualizes which cells grew and which died (Supplementary Movie 1, right panel), displaying the variations in antibiotic susceptibility among individual cells. An experiment encompassing -30 colonies yielded -30 genealogical trees, collectively comprising ~4000 cells on average, per experiment. The experiment was independently repeated thrice.

To statistically assess the variations in antibiotic susceptibility, we introduced a survival parameter *X*. If a cell was susceptible and killed, which is visualized as the termination of the lineage in the tree, we assigned X=0 to the cell. If a cell survived antibiotic exposure and completed the replication (i.e., unsusceptible), X=1 was assigned to the cell. In our genealogical trees, this event is visualized as the split of a lineage.

Cell survival is correlated between kin

We then quantified how the survival parameter *X* is correlated between cell pairs with varying degrees of relationship. Conventionally, the degree of relationship refers to the number of generational connections between two individuals. However, this conventional notation is ambiguous. For example, first cousins, great aunts, and great-nieces all have the degree of four. To uniquely define the relationship, we developed another notation (Fig. 2a). The first number in the square bracket is the number of generations to go up to the common ancestor, and the second is the number to go down to get to the related cell. For example, a first cousin, great aunt, and great niece are denoted by [2, 2], [3,1], and [1,3], respectively. The sum of these two numbers equals the conventional degree of connection.

We then performed a pairwise Pearson correlation of X for each relationship. The correlation coefficient would be zero if the fates of two cells were independent of each other. However, we found positive correlations (Fig. 2b), which means that if a cell survives, the other cell is more likely to survive. The correlation was the highest between sibling cells and decreased for more distant relationships (Fig. 2b).

We wondered how the survival pattern changes when a key factor for resistance is inactivated. Efflux pumps extrude antibiotic molecules out of cells³¹. Notably, the TolC-Acr efflux complex has broad substrate specificity, contributing to multidrug resistance³⁴. We inactivated this pump in *E. coli* by knocking out *tolC*. This strain was no longer able to grow at the cefsulodin breakpoint (Fig. 1a), which underscores the importance of this efflux. We therefore lowered the cefsulodin concentration to 13 µg/ml such that the ratio of growing and dying cells in the $\Delta tolC$ strain matches that in WT: the mean survival $<X > _{\Delta tolC} = 0.64 \pm 0.04$, which is comparable to $<X > _{WT} = 0.66 \pm 0.01$. Pairwise Pearson correlation analysis again shows positive correlations of survival in the $\Delta tolC$ strain (Fig. 2c). The decrease of the correlations at distant relationships was more gradual in the $\Delta tolC$ strain than in the WT strain (Fig. 2b, c).

We next examined the genealogical tree vertically. Applying Pearson correlation analysis along the lineages is not informative because, for a cell to be present, its direct ancestors (e.g., mother or grandmother) would always have survived antibiotic treatment. Instead, we analyzed how cell survival changes over time as a tree branches out. Due to the binary fission of bacterial cell division, a cell always has an old pole and a newly formed pole. Upon division, one cell inherits the old pole, carrying on the lineage, while the other receives the new pole. We calculated the cell age by the number of generations, Z, through which the older pole had been passed down. Previous work offered contradictory predictions about the effects of cell age on fitness. Some studies indicate a decline in fitness with pole age^{35,36}, while others highlight a preferential accumulation of TolC in older poles, which enhances the fitness of aged cells⁹. We analyzed cell survival according to their age, $\langle X \rangle_7$. We found that $\langle X \rangle_7$ increased with an increasing Z for both WT and $\Delta tolC$ strains (Fig. 2d). The plot also shows that this increase is comparable between $\Delta tolC$ and WT. This



Fig. 1 | **Growth of** *E***.** *coli* **exposed to cefsulodin. a** Images of wild type and $\Delta tolC$ cultures after 24 h of incubation with different concentrations of cefsulodin. See Supplementary Fig. 1 for growth curves. The experiment was repeated three times, and similar images were obtained. **b** Representative growth of wild-type cells exposed to the breakpoint concentration of cefsulodin. We inoculated multiple *E*. *coli* colonies on solid media containing the breakpoint concentration of cefsulodin and counted the number of live cells in - 30 colonies. The number of live cells from 4 colonies over time is shown here as an example. Different colors indicate different colonies. Two additional biological replicates were performed, and similar population fluctuations were observed.

increase in the $\Delta tolC$ strain suggests that there are additional resistance factors beyond TolC that accumulate to enhance cell survival.

Inheritance of robust survival

The observed correlation and age-dependence of cell survival suggest the phenotypic inheritance of antibiotic resistance. To further validate this, we next analyzed the various configurations of cell survival patterns along the lineages. Specifically, in our analysis, survival refers to a cell that completes its division and produces two daughter cells. However, if only one or no daughter cell survives antibiotic treatment, the mother cell's survival does not contribute to overall population growth. We therefore categorized the survival of a mother cell as robust only if both of its daughter cells survived. We identified the robust mother cells in our dataset and marked them as 'R'. We additionally denoted the number of generations considered in the analysis by N. In this case, since we consider the robustness of one generation (only mother cells, without considering grandmothers or great grandmothers yet), N is equal to 1; see Supplementary Fig. 2a (and its caption) for graphic illustration. We then determined the frequency of robust mother cells, $F(R_{N=1})$; see Supplementary Fig. 2e table. We compared it to the frequency expected by random chance, $F_0(R_{N=1})$, which we calculated based on the probability for two daughter cell survival being $\langle X \rangle^2$; see Supplementary Fig. 2e table. We plotted their fold difference, i.e., $F(R_{N=1})/F_0(R_{N=1})$, in Fig. 3a, which shows that it is slightly greater than 1.

To examine the heritability of robust survival, we next assessed how likely a daughter cell is robust when its mother cell is robust. For this daughter cell to be robust, both of its own daughter cells must survive the antibiotic treatment. Defining the number of this second generation of daughter cells that survive antibiotic treatment as *D*, we quantified the conditional probability, $P(D=2 | R_{N=1})$, i.e., the probability of two daughter cell survival (D=2) conditioned upon one prior generation of robustness $(R_{N=1})$. See Supplementary Fig. 2b for a graphical illustration of $P(D=2 | R_{N=1})$. As shown in Fig. 3b, this conditional probability was marginally higher than the non-conditional probability, P(D=2), which is denoted by a dashed line in Fig. 3b.

Enrichment of robust lineages

Heritability became evident when we considered multiple generations. We first predicted that the positive effect of robust mothers on daughter cells would elevate the frequency of two consecutive generations exhibiting robustness, denoted as $F(R_{N=2})$. Indeed, this frequency was much higher than the frequency expected by random chance, $F_0(R_{N=2})$, with a fold difference greater than that observed for one generation of robustness (Fig. 3a). We also found that the daughter cells originating from the two robust generations are more likely to be robust than daughter cells from one robust generation (Fig. 3b). This trend continued for three consecutive generations of robustness (N=3), which were even more over-represented than expected by random chance, with a fold difference greater than that observed for two robust generations (Fig. 3a). Moreover, daughter cells originating from three robust generations exhibited an even higher likelihood of robust survival compared to those from two generations (Fig. 3b). These data indicate positive feedback on robust lineages, where robust lineages become progressively more likely to produce robust offspring, thereby increasing their representation in the population.

We additionally found that once cells ride the tide to produce robust lineages, these lineages are less likely to go extinct. This effect was uncovered when we analyzed how likely a robust cell gives birth to non-robust daughter cells. Here, being non-robust means either one of their daughter cells (i.e., daughter's daughter cells) die, D = 1 (Supplementary Fig. 3, right panel), or both dies D = 0 (Fig. 3c), with the latter indicating the termination of the lineage branch; see Supplementary Fig. 2c, d for graphic illustration. We observed a decreasing probability of termination with each additional generation of robustness (Fig. 3c). This highlights a trend where robust lineages are more likely to persist and propagate.

We made contrasting observations when we investigated the fate of non-robust lineages, particularly the extreme case where there is only a single surviving branch, and all other cells die in the lineage, denoted by ~ R_N (Supplementary Fig. 4a diagram). We observed that these non-robust lineages are much less likely to produce robust daughter cells than robust lineages (Supplementary Fig. 4a). This is supported by a statistical test which shows that P ($D = 2 | ~ R_N$) is significantly lower than P ($D = 2 | R_N$); See Supplementary Fig. 4 caption. Instead, these daughter cells are much more likely to die (Supplementary Fig. 5a). This is supported by a statistical test, which shows that P ($D = 0 | ~ R_N$) is significantly higher than P ($D = 0 | R_N$); See Supplementary Fig. 5 caption. Therefore, the lineages that failed to exhibit robustness were more likely to terminate.

The lineages we analyzed above represent two extreme patterns of ancestral history (either all cells survive or die). In the population, however, there are other possible combinations of ancestral survival (e.g., an aunt survives but one cousin dies, etc.). Here, we sought to analyze how daughter cells originating from different ancestral combinations survive differently to gain insight into the inheritance of resistance across the population. Because we recorded the fate of every cell in our dataset, we can explore this relationship for each combination of ancestral history. However, the diversity of these combinations is vast; for instance, within just three generations of an ancestral lineage, up to eighty different combinations of cell fate are possible. To manage this complexity and extract meaningful insights about inheritance, we turned to information theory. Briefly, we used Shannon entropy to quantify the uncertainty³⁷., i.e., missing information in predicting the fates of two daughter cells, $H(D) = -\sum_{D=0,1,2} p_D \ln p_D$, where p_D refers to the probability that D daughter cells survived. In addition, we can determine these probabilities conditioned upon the fates of ancestors, whose pattern we denote by Y. For example, we can determine the probability of daughter cells surviving or dying given the fate of their aunts (Y), i.e., when an aunt was alive $(y_{aunt} = 1)$ or dead $(y_{aunt} = 0)$. The reduction in



the entropy with the knowledge of ancestral survival, H(D) - H(D|Y), widely known as mutual information³⁸, reveals how much information ancestral survival history provides to the fates of daughter cells. This analysis showed that, in WT, the information content is low and remains constant across multiple generations (Fig. 3d and its caption), meaning that at the population level, the survival of daughter cells is minimally influenced by their lineage history. This finding appears to contrast with the pronounced lineage-dependent dynamics of robustness we identified earlier, where robust lineages exhibited a clear pattern of inherited resistance. This contrast indicates that the resistance inheritance that confers advantages to robust lineages is unique to these lineages and does not uniformly apply across the entire population.

When we repeated this analysis for the $\Delta tolC$ strain, we saw a different trend: the information content about the offspring survival increases with ancestral history in $\Delta tolC$ (Fig. 3d). This agrees with our above observation that pairwise correlations of cell survival lasted for more distant relationships in the $\Delta tolC$ strain than the WT strain (Fig. 2b, c). However, we note that this increase is similar in magnitude to the upper bound of uncertainty theoretically calculated according to the ref. 39 (shown as error bars in the figure). Furthermore, despite the increase, the information content remain still very low (Fig. 3d) and negligible at the population level.

Fig. 2 | Cell survival is correlated. a Labeling scheme of relationship. The first number in the square bracket is the number of generations to go up to a common ancestor, and the second is the number to go down to get to the related cell. The sum of these two numbers is equal to the conventional degree of separation. **b**, **c** Pearson correlation of the survival parameter, *X*, for wild-type (WT, red) and $\Delta tolC$ (green) strains. The lines are from the model fit; see Fig. 4 for the model. In WT, the correlation was the highest between sibling cells and decreased for distant relationships. The decrease was more gradual in the $\Delta tolC$ strain. Three biological repeats were conducted. Small open circles indicate data from each replicate. The horizontal bars indicate their weighted means. The number of cells with known fates (used to calculate the correlation) differed between the replicates; the three biological replicates yielded 2529, 5737, and 4246 cells with known fates for WT, and 2535, 2261, and 10070 cells for $\Delta tolC$. The brackets indicate the relationship with equal degrees of connection. To statistically assess whether the obtained correlation coefficients are significantly greater than zero (i.e., no correlation), we performed linear regression analyses on the survival parameters (X) between related cells and calculated corresponding p-values; see Supplementary Text 3.1. **d** We sorted cells according to cell age, Z, and calculated the survival $\langle X \rangle_7$ for different ages. The population-mean survival $\langle X \rangle$ was subtracted: $\langle X \rangle = 0.66$ for WT and 0.64 for $\Delta tolC$. Small open circles indicate $\langle X \rangle_7 - \langle X \rangle$ for each biological replicate. Solid red circles or green squares indicate the mean of these replicates (for WT and $\Delta tolC$, respectively). To calculate the error bar, we compared the standard deviation of data from three biological replicates and the standard error for a binomial distribution with the number of cell pairs used and plotted whichever was larger. The plot shows that $\langle X \rangle_Z - \langle X \rangle$ increases with increasing Z. The slope of this increase was found to be 0.0168 \pm 0.0035 for WT, and 0.0118 \pm 0.0033 for the $\Delta tolC$ strain. See Supplementary Text 3.2 for statistical analysis.

Minimal requirement for a quantitative model

We next sought to develop a minimum model to explain key aspects of our data. Previously, we and others described the dynamics of populations exposed to an antibiotic using a branching process^{26–28}. This process was mathematically described as a two-state model, i.e., where a cell can be in either an alive or a dead state, where the state of each cell is independently determined. While this model can explain the heterogeneous response of cells to antibiotics, it fundamentally assumes no correlation in these responses between cells. However, we observed the kinship correlation (Fig. 2b, c), which is central to lineage-dependent response to antibiotic exposure. To construct a model that generates the kinship correlation, we incorporated the phenomenon of robust survival observed in our experiments into the model, introducing a phenotypically healthy state (Fig. 4a). Cells in this state give rise to daughter cells equipped to withstand antibiotic exposure, and thus, both daughter cells survive. Conversely, while a phenotypically vulnerable cell can still divide, its daughter cells might succumb to antibiotic exposure, entering a dead state. In our model, a cell maintains its state during its lifetime but can change the state at the time of division. When a cell divides, a daughter cell's state is determined independently of the other. A daughter cell can either retain the same state as her mother or transition to an adjacent state. For example, a healthy mother cell might yield healthy daughter cells or produce vulnerable ones due to antibiotic damage. A vulnerable mother cell could produce healthy (recovery), vulnerable, or dead (killed) daughter cells. See Fig. 4a and its caption for details.

To test whether this process can quantitatively explain the observed cell fate correlation, we constructed a population model. Typical pharmacodynamic models track the number of live cells without considering their phenotypic states⁴⁰⁻⁴³. In our model, we distinguished live cells into healthy and vulnerable states as discussed above. We described this phenotypic heterogeneity using a population projection matrix (Eq. 11 in Supplementary Text 2). One challenge in applying this matrix to a genealogical tree was that dead cells no longer produce offspring, which convolutes the tree structure. We therefore introduced a dummy state as a placeholder in the matrix to maintain the tree structure. Applying this modified population projection



matrix into the genealogical tree (Eq. 16), we can calculate the probability distribution of phenotypic states in the offspring originated from healthy or vulnerable cells (Eq. 21-22), thereby predicting how different phenotypes propagate along lineages. In our case, we were specifically interested in understanding the kinship correlation of cell survival. We therefore applied this matrix to pairs of cells to calculate the probabilities for different combinations of their fates, e.g., both survive, one survives, or neither survive (Eq. 23-30). We then used

Fig. 3 | The propagation of lineage-dependent survival. In our analysis, survival refers to a cell which completes its division and produces two daughter cells (D). Both of these daughter cells might be killed by antibiotic treatment before their own division (D = 0), one daughter cell might survive (D = 1), or both might survive (D = 2). The latter is categorized as robust survival. **a** We identified the robust mother cells in our dataset, determined their frequency, $F(R_{N=1})$, and compared it with what would be expected by random chance, $F_0(R_{N=1})$. Their fold difference (i.e., ratio, WT and $\Delta tolC$ in red and green, respectively) is plotted here. See Supplementary Fig. 2 Table for raw data. We then determined the frequency for two consecutive generations of robustness $F(R_{N=2})$, i.e., robust grandmother, mother, and aunt. The frequency for three consecutive generations of robustness $F(R_{N=3})$ was determined in a similar manner. Three biological repeats were conducted. Small open circles indicate data from each replicate. Horizontal bars indicate their weighted means. Error bars indicate their standard errors. b We calculated the conditional probability, i.e., probability that daughter cells are robust given (i) their mother cell was robust R_{N=1}, (ii) two generations of robustness R_{N=2}, and (iii) three generations of robustness, $R_{N=3}$: $P(D=2 | R_N)$. The dash line refers to the nonconditional probability that two daughter cells survived by chance $P(D=2) = \langle X \rangle^2$. **c** Conditional probability that both daughter cells die (D=0) given different generations of robustness. d Information stored in ancestral survival history. The error bar represents the upper bound of uncertainty theoretically calculated according to the ref. 39. See Supplementary Text 3 for statistical analyses.

these probabilities to determine the Pearson correlation coefficients of cell survival for various degrees of relationship (Eq. 39). The predicted values are plotted as a line in Fig. 2b, c, which quantitatively matches the experimentally observed coefficients (columns in Fig. 2b, c). Importantly, the fit of the model to the data identifies the rates of transition between different states (Fig. 4b). It is plausible that cells' response could be modeled better with more than three states, which we plan to test in our future studies. However, our model convincingly demonstrates that a three-state model is sufficient to explain the broad correlation pattern that we observed.

We next compared the rates of transition between WT and $\Delta tolC$ strains. WT exhibited a higher damage rate than the $\Delta tolC$ strain (Fig. 4b), although this difference was not large enough to be statistically significant (Fig. 4b caption). The damages rates between these two strains could differ due to our experimental protocol that the cefsulodin concentration used for WT was higher than that for the $\Delta tolC$ strain (the concentration for each strain was chosen such that <x> is similar between them). Interestingly, WT exhibited a recovery rate that is about one order of magnitude higher than $\Delta tolC$ (Fig. 4b), indicating frequent forward-backward transition. Conversely, the lower rates of transition in the $\Delta tolC$ strain indicates that cells maintain their phenotypic states longer. This insight agrees with higher correlation of survival between distantly related cells (Fig. 2b, c) and the higher information content in ancestral history in the $\Delta tolC$ strain (Fig. 3d).

Discussion

Antibiotic susceptibility is typically characterized by how a population of bacteria as a whole responds to antibiotics. Heterogeneous responses of isogenic cells, therefore, complicates antibiotic diagnostic tests and treatments^{13,16}. In particular, the survival of a small subpopulation can lead to antibiotic treatment failure and the recurrence of infections. Our work elucidates how phenotypic resistance contributes to this phenomenon. We characterized heterogeneous cell growth and death under antibiotic exposure. We first observed that antibiotic-exposed populations exhibit seemingly random population fluctuations (Fig. 1), consistent with previous findings²⁶. When we recorded cell growth and death in genealogical trees and analyzed their patterns probabilistically, we observed intricate trends. Cell survival was not independent but correlated among kin (Fig. 2). Robust cells produce robust offspring, thereby enriching the robust lineages (Fig. 3). This robustness can be inherited from generation to



Fig. 4 | A minimal model to explain the survival correlation and inheritance. a We developed a three-state population model that includes a phenotypically healthy state. In our model, a cell maintains its state but can change the state at the time of division. Daughter cells can either retain the same state as her mother or transition to an adjacent state independently. For example, healthy mother cells might yield healthy daughter cells or produce vulnerable ones due to antibiotic damage. A vulnerable mother cell could produce healthy (recovery), vulnerable, or dead (killed) daughter cells. **b** We fit the data (lines in Fig. 2b, c) with this model to determine the transition rates (WT and $\Delta tolC$ in red and green, respectively). Small open circles show the rates determined for three independent experiments. The columns and error bar indicate the means and standard deviation from the independent experiments. A two-tailed heteroscedastic *t* test on the rates obtained from three biological replicates between WT and $\Delta tolC$ shows the *p*-value = 0.18, 0.31, and 0.01 for the damage, kill, and recovery rates, respectively, indicating statistical significance for the recovery rate.

generation (Fig. 3). Information theory indicates that this inheritance is unique to the robust lineages and is obscured in the population-level analysis (Fig. 3d), highlighting a challenge in studying this phenomenon using a population-averaging approach. Collectively, our findings demonstrate intricate dynamics of phenotypic resistance propagating selectively in a minority population, offering novel insights into nongenetic mechanisms underlying antibiotic resistance. Importantly, we observed this phenomenon in K-12 *E. coli*, a strain not evolved in the clinical setting, which suggests that this is an innate characteristic of bacteria.

To analyze how phenotypic resistance spreads in a population, we developed a population model that incorporates single-cell-level heterogeneity within a genealogical structure (Fig. 4). This model quantitatively captures lineage-based dynamics of phenotypic propagation. Typical pharmacodynamic models assume that cells respond uniformly to antibiotics, predicting the number of live cells in a population. In contrast, our model demonstrates how cellular heterogeneity and its inheritance alter population dynamics during antibiotic treatment, providing a new framework for understanding pharmacodynamics. Furthermore, this modeling approach holds significant potential for the field of population ecology, particularly in contexts where phenotypic diversification and inheritance play a critical role.

Application of our model to different cell survival patterns between WT and $\Delta tolC$ provides new population-level insights into the molecular details of the major efflux pump. TolC, forming a complex with Acr, extrudes antibiotic molecules, keeping the intracellular antibiotic concentration low³¹. Interestingly, recent studies found that the cell-to-cell heterogeneity in TolC expression, as well as of Acr expression, results in heterogeneous response of cells to antibiotics^{8,44–46}. Our model suggests that these molecular properties are manifest at the population level as a high rate of recovery from antibiotic damage in WT. It would be interesting to further investigate how their expression patterns alter the propagation of phenotypic resistance. In particular, the designs of efflux gene promoters can be altered to increase or decrease the heterogeneity in the expression of efflux pumps⁴⁷. Future studies could benefit from using such promoter designs.

We believe our findings are particularly timely, aligning with the growing interests in advancing single-cell-level antibiotic susceptibility diagnosis. The past few years have witnessed a significant rise in cutting-edge approaches, which leverage microfluidics and microdroplets in combination with optical, electrochemical, or isothermal techniques to measure the mass, size, and morphology of single cells exposed to antibiotics⁴⁸⁻⁵¹. These new developments will greatly facilitate the thorough quantitative analyses demonstrated here. In parallel, our findings can guide the refinement and application of these techniques to fulfill their potential. For example, measuring bacterial responses to antibiotics over multiple generations is critical to evaluate phenotypic resistance. The dynamics of cell growth and death can be analyzed quantitatively to deduce the rates of transition into and out of the distinct phenotypic states (Fig. 4). Such detailed knowledge will provide a more complete view of population dynamics under antibiotic exposure, which will improve antibiotic treatments. Another exciting development in the field is single-cell omic techniques, which are rapidly improving to decrease the number of bacterial cells needed to make transcriptomic and proteomic measurements^{52,53}. Focusing these techniques on the robust lineages can reveal the differential molecular profiles that underlie the inheritance of phenotypic resistance.

Methods

Experimental procedure

Bacterial strains and growth conditions. *E. coli* K-12 NCM3722^{54–56} and its Δ *tolC* derivative (NMK320)⁵⁷ were grown in LB broth (Miller) supplemented with 10 mM glucose and 1 mM MgSO₄. Briefly, a single colony was inoculated into media in borosilicate glass culture tubes and incubated at 37 °C with shaking (250 rpm) in a water bath shaker overnight. The next morning, the culture was diluted with fresh media to an optical density (OD₆₀₀) of ~0.001 and incubated in a water bath shaker at 37 °C with shaking.

Time lapse imaging of bacterial growth. Time lapse microscopy was performed as previously described^{58,59}. When the culture reached $OD_{600} > 0.05$, cells were placed on a 35 mm glass-bottom petri dish (Cellvis) and covered with a 1.5% agarose pad containing LB, 10 mM glucose, 1 mM MgSO₄, and cefsulodin. Cells were imaged every 5 min using an inverted fluorescence microscope (Olympus IX83) with an oil immersion phase-contrast 60 × objective seated inside an incubator chamber (InVivo Scientific) pre-warmed to 37 °C. The microscope was controlled with MetaMorph software (Molecular Devices).

Wild-type cells were grown with a cefsulodin concentration $32 \mu g/mL$ and the $\Delta tolC$ mutant with $13 \mu g/mL$. A total of 91 WT colonies (37 living and 54 extinct) and 112 $\Delta tolC$ colonies (38 living and 74 extinct) were analyzed. In these colonies we had a total of 12,512 WT cells and 14,866 $\Delta tolC$ cells.

Image analysis

Cell tracking. Cell division and location of cells were tracked using a TrackMate v7.10.2, a plug-in of Fiji, ImageJ⁶⁰ Cefsulodin induces a variety of cell shapes⁶¹. Instead of segmenting the cells, we simply tracked the birth and location of cell poles at each time frame. Marked cell poles of the same cell in different frames were linked, and old poles of newborn cells were linked to their parents, so that genealogical trees

could be constructed. We marked the moment of death when cells were visibly lysed. In many cases, growth arrest preceded lysis. Approximately one to five percent of growth-arrested cells exhibited growth arrest for a few frames (-1 h) and then resumed their growth. We distinguished these different fates of growth-arrested cells by moving forward or backward in time while reviewing the videos. We made sure that we only marked cells as 'dead' when lysis was observed. Cells were tracked until either a whole population went extinct or the field became so crowded that individual cells could not be reliably distinguished.

A cell always has old and new poles. The age of the old pole was used to define the age of the cell^{35,62}. Because we do not know the pole age of the initial inoculum, the first cells were not included in our analysis. We then assigned a unique identification to each cell representing the age of the cell pole, generation, and kinship by following a bookkeeping protocol similar to the one used in the ref. 35. The detail of this protocol is described in Supplementary Fig. 6.

Statistical analyses. The Pearson correlation coefficient for two datasets is a number between -1 and +1 that measures the correlation between them. For a dataset of size *N* in which each datapoint is represented by two variables, *x* and *y*, the correlation coefficient is given by

$$C = \frac{\sum_{i=1}^{N} (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{N} (x_i - \bar{x})^2} \sqrt{\sum_{i=1}^{N} (y_i - \bar{y})^2}}.$$
 (1)

In order to account for potential experimental bias that might produce correlation, we determined the 'background correlation'; it is reported in the 'C_B' column in Source Data 1. Background cells were born in the same colony as the primary cells, in either the same or an adjacent frame, but separated by seven or more degrees of kinship. We found that the correlation coefficients between the primary cells and the background cells, C_B , were very close to zero. For example, C_B was found to be 0.004, 0.013, and 0.0001 for three replicate plates in the WT sibling correlation. When we performed a two-sided t test to see whether they are significantly different from zero, the *p*-value was 0.27. For aunt correlation, the C_B were 0.0354, 0.006, and 0.0156. The *p*-value from a two-sided *t* test shows the *p*-value of 0.16. We consistently observed such high p-values, indicating no significant experimental bias. For extra caution, we subtracted the background correlation from the above calculated correlation C as follows,

$$C_{net} = \frac{C - C_B}{1 - C_B}.$$
 (2)

The subtracted values are plotted in Fig. 2b, c.

Three-state population model. Details of the model construction were provided with equations in Supplementary Text 2.

Shannon entropy calculation. Shannon entropy³⁷ is defined as $H(D) \equiv -\sum_{D=0}^{D=2} p_D \ln p_D$, where p_D refers to the probability that D daughter cells survived antibiotic treatment (D = 0, 1, or 2). Because we know the fates of every cell, we can calculate p_D and hence Shannon entropy. We then calculated Shannon entropy conditioned upon the fates of cells in the prior generation. Denoting the cell fate in the prior generation by Y, we have $H(D|Y) \equiv \sum p_y H(D|Y=y)$. Here, the summation runs for all different fates of Y. See Supplementary Fig. 7 and the caption for detail explanation of calculating conditional Shannon entropy. The difference between the original and conditional Shannon entropy is equal to mutual information, which is plotted in Fig. 3d. We

corrected the potential bias due to small sample size by using Miller-Madow correction⁶³.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Source data for figures are provided in the Source Data file. Raw data used to generate these source data are provided in https://doi.org/10. 6084/m9.figshare.28877642.v1 Source data are provided in this paper.

Code availability

Our custom-built software is provided in https://doi.org/10.6084/m9. figshare.28877669.v1.

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Author contributions

T.A. and M.K. conceived the study. T.A. designed and carried out the experiments. W.S. and T.A. analyzed and interpreted the data. W.S. developed the mathematical model and fit the data. D.W. provided feedback. D.W. and M.K. secured funding and provided resources. T.A. and M.K. wrote the manuscript. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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