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Stochastic response of bacterial cells to antibiotics: its mechanisms and implications for population and evolutionary dynamics

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The effectiveness of antibiotics against bacterial infections has been declining due to the emergence of resistance. Precisely understanding the response of bacteria to antibiotics is critical to maximizing antibiotic-induced bacterial eradication while minimizing the emergence of antibiotic resistance. Cell-to-cell heterogeneity in antibiotic susceptibility is observed across various bacterial species for a wide range of antibiotics. Heterogeneity in antibiotic susceptibility is not always due to the genetic differences. Rather, it can be caused by non-genetic mechanisms such as stochastic gene expression and biased partitioning upon cell division. Heterogeneous susceptibility leads to the stochastic growth and death of individual cells and stochastic fluctuations in population size. These fluctuations have important implications for the eradication of bacterial populations and the emergence of genotypic resistance.

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Introduction

Although it is commonly assumed that genetically identical cells would display the same level of antibiotic susceptibility or resistance, this is not well supported by experimental observations. When a bacterial culture is plated on solid media containing increasing concentrations of a bactericidal drug, the percentage of colony-forming cells, that is, plating efficiency (PE), decreases gradually [1,2]. Consider an intermediate concentration at which the PE was 10%. This means that 10% of plated cells *resisted* antibiotic exposure and grew to form colonies, whereas 90% of plated cells were susceptible, suggesting heterogeneous susceptibility. If these survivor cells had acquired resistance through genetic mutation or horizontal gene transfer (genotypic resistance), then the subsequent plating of these survivor cells to solid media with the same antibiotic concentration would result in PE substantially higher than the original population. Instead, when these 10% survivors were re-plated, the PE remained similar (~10%), indicating that their resistance (i.e. ability to grow in presence of antibiotic) is transient and easily lost within several generations [3••].

Heterogeneity in antibiotic susceptibility is also observed in commonly used susceptibility tests. The minimal inhibitory concentration (MIC) is defined as the lowest antibiotic concentration that inhibits population growth [4]. It is often used as a simple diagnostic measure of antibiotic susceptibility to predict binary outcomes (whether a bacterial population would grow or not at a given antibiotic concentration). Despite this simple definition, the MIC value is known to vary substantially even for the same reference strains [5,6] (e.g. MIC of colistin for a quality control *Escherichia coli* strain can range 0.25–1 μg/mL [7]). While different practices in laboratories could contribute to the variation, the variation persists even between the identical technical replicates in a single experiment, suggesting that antibiotic susceptibility is inherently heterogeneous [8••,9,10].

Time-lapse single-cell microscopy enables us to track growth and death of individual cells in real time and has been instrumental for establishing cell-to-cell heterogeneity in antibiotic susceptibility. Single-cell-level monitoring confirms that when exposed to antibiotics, some cells are killed, while other cells resist the antibiotic exposure and grow even at the MIC [3••,11]. However, these growing cells resisting the antibiotic exposure could spontaneously die in the next few generations (i.e. resistance is transient). In the field of antibiotic resistance, the term *phenotypic resistance* is used to describe a subpopulation of cells which grows in the presence of antibiotics without genetic alteration [12]. Phenotypic resistance we describe here is different from bacterial persistence, where cells become tolerant to antibiotics by virtue of not growing [13,14]. Additionally, there are several other characteristics that distinguish resistance from

persistence, which are well articulated in a recent review paper of persistence [15]. Likewise, we apply the term phenotypic resistance to cells that continue to grow in the presence of antibiotics.

Mechanisms underlying cell-to-cell heterogeneity in antibiotic susceptibility

Heterogeneity in phenotypic traits within a clonal population and its mechanism have been studied previously. When a *lac* operon inducer (e.g. TMG) is added to an *E. coli* culture, a subpopulation primed to utilize lactose emerges [16,17]. Studies have established stochastic gene expression, such as fluctuations in the mRNA copy number, as a mechanism for phenotypic heterogeneity [18–20]. Additional studies found that stochastic gene expression results in heterogeneity in phenotypic traits [21,22].

Several studies demonstrated the importance of stochastic gene expression in the heterogeneity in antibiotic susceptibility. The cell envelope, particularly the outer membrane of gram-negative bacteria, limits the entry of antibiotics into the cytoplasm. Porins enable antibiotic molecules to cross the outer membrane [23,24]. The expression of porins is shown to be stochastic; some cells express higher levels of porins than others, which increases their sensitivity to antibiotics [25]. In addition, intracellular antibiotic concentration is modulated by efflux pumps, which extrude antibiotic molecules out of cells. AcrAB-TolC is one of the resistance-nodulation-division (RND) family of efflux pumps associated with multi-drug resistance in gram-negative bacteria [26,27]. The promoter activity and the abundance of AcrB were shown to be heterogeneous affecting the antibiotic susceptibility of individual cells [28•,29]. Of note, recent studies have shown that the gene copy number can increase in a subpopulation of cells exposed to antibiotics [30•]. This form of genotypic resistance could further amplify the variation in the abundance of gene products, making antibiotic susceptibility of individual cells more heterogeneous.

For some antibiotics, the expression of endogenous genes is necessary to function. The abundance of their gene products determines the degree of antibiotic susceptibility. For instance, isoniazid, an anti-mycobacterial drug, requires the activation by catalase-peroxidase, KatG. Expression of KatG was found to be heterogeneous between mycobacterial cells, and its expression level was positively correlated with antibiotic susceptibility of individual cells [31].

Since the timescale of fluctuations in gene expression is relatively short ($\sim$ one generation time) [32,33], the change in antibiotic susceptibility of cells would be ephemeral. However, the timescale could be extended through various mechanisms such as positive feedback. Self-reinforcement of fluctuations by positive feedback can generate and stabilize two distinct states, that is, bistability; see Ref. [34] for a review of positive feedback. When the expression of genes that confer resistance is activated through positive feedback via gene regulatory network [35,36] or innate growth-mediated regulation [37], some cells express high levels of resistance proteins for an extended period of time, which leads to the stable maintenance of phenotypic resistance. Importantly, a feedback does not always require the expression of resistance genes. For example, theoretical work predicted that, for antibiotics with low membrane permeability, a feedback between slow influx and growth-mediated dilution of antibiotic molecules could lead to the emergence of a subpopulation that continues to grow at high (external) antibiotic concentrations [38].

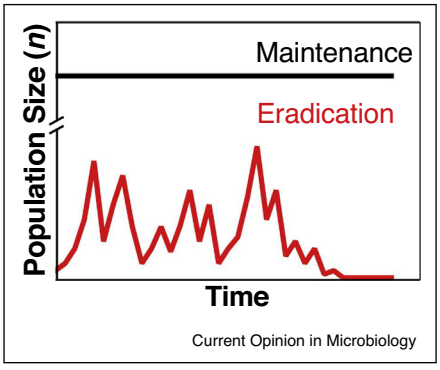
Biased partitioning is another factor that can amplify heterogeneity in antibiotic susceptibility. Not every cellular component is partitioned equally between two daughter cells during cell division. TolC, a component of the AcrAB-TolC efflux pump, preferentially accumulates at an old cell pole of *E. coli* [29]. As a result, cells that inherit an old pole (a.k.a. old daughter cell) have a stronger efflux activity and exhibit higher rates of survival and growth in the presence of antibiotics.

Misfolded and damaged proteins form aggregates when cells are exposed to stresses such as aminoglycosides and heat shock [39,40]. These aggregates are segregated to cell poles and asymmetrically partitioned between cells during division, leading to aging (i.e. loss of fitness) of an old daughter cell, while a new daughter cell (i.e. a cell with new poles), devoid of cellular damage, exhibits rejuvenation [41,42•,43]. Such asymmetric allocation of cellular damage leads to fluctuations in cell growth [40,42•]. Asymmetric allocation of cellular components is essentially 'robbing Peter to pay Paul'. Increasing resistance in some cells comes at the cost of lower resistance in others. It is unclear whether it is beneficial to a population as a whole. A recent analysis shows that asymmetric allocation can increase the overall population fitness in the presence of lethal levels of damage, suggesting asymmetric allocation as an adaptive mechanism enhancing population survival under antibiotic exposure [42•].

Implication of heterogeneous susceptibility for population dynamics

The implication of heterogeneous susceptibility for population dynamics of bacteria has been quantitatively described [3••]. When a bacterial population is exposed to an antibiotic, some cells die, while others grow. The stochastic cell growth and death lead to fluctuations in the number of live cells, n, in a population [3••] (Figure 1). Here, the state, $n = 0$, is equivalent to an *absorbing boundary* in stochastic physics [44,45]. Once a system reaches an absorbing boundary, the system cannot come

Figure 1



Population dynamics of antibiotic-exposed bacteria depend on the population size (n). At the MIC, cell growth rate is equal to death rate ($\lambda = \varphi$). When n is small (i.e. a small population), it is subject to fluctuations and incidentally reaches the absorbing boundary ($n = 0$), which indicates the eradication of the population (red). On the other hand, in a large population, fluctuations are averaged out, and population size is maintained (black).

out of the state and permanently remains at the boundary. Likewise, once population size reaches $n = 0$, the population goes extinct and cannot revive. Because of the stochastic growth and death of cells under antibiotic treatment, n randomly fluctuates and incidentally reaches this state ($n = 0$). When the antibiotic concentration is sublethal, some populations reach $n = 0$ and go extinct, whereas other populations manage to avoid extinction and ultimately flourish. It becomes harder for a population to avoid this absorbing boundary as the antibiotic concentration increases, which results in a gradual decrease in the fraction of live and growing cells. At lethal antibiotic concentrations, all populations eventually get eradicated but the time of eradication varies dramatically. Importantly, due to the stochastic and discrete nature of growth and death, it is challenging to make a deterministic prediction about population eradication under antibiotic treatment. Quantitative prediction may still be made, not deterministically, but probabilistically. The study by Coates *et al.* experimentally measured the probability of population eradication and used a simple model for stochastic growth and death to explain the experimental observation [3••]. A similar model was used in another study to analyze the probability distribution of population eradication time [46]. While these models provide a conceptual understanding of stochastic population dynamics during antibiotic treatment, further studies are needed to evaluate their quantitative predictive power.

The gained knowledge of population dynamics can be extended to improve our understanding of MIC and its relation to antibiotic susceptibility. Typically, cultures with inoculum size of 5×10^5 cells/mL are grown in the presence of antibiotics for 16–20 hours to determine the MIC using a broth dilution method [4]. In recent years, various approaches were developed to rapidly determine the antibiotic susceptibility with a smaller population size using microfluidic devices [47–49]. However, comparing the antibiotic susceptibility from different approaches requires a detailed understanding of how population dynamics vary in different regimes. As discussed above, the MIC is defined as the lowest concentration of antibiotic which inhibits the growth of a bacterial population. Theoretically, this is the critical concentration at which the growth rate of cells λ is equal to the death rate φ ($\lambda = \varphi$) (a.k.a. pharmacodynamic MIC or stationary concentration [50,51]). Under this condition ($\lambda = \varphi$), small populations undergo extinction due to the stochastic population fluctuations and the presence of absorbing boundary (discussed above). In a large population, however, fluctuations become averaged out, and thus the population size is stably maintained (Figure 1). This means that under the same condition that defines the MIC ($\lambda = \varphi$), large and small populations exhibit different dynamics (maintenance versus eradication). Importantly, a recent study demonstrated the involvement of stochastic population dynamics in the inoculum effect, independent of inoculum density [8••].

This raises a question: 'How large should the population size be to escape extinction by random fluctuations?' Unfortunately, there is no clear cut-off value [3••]. When the death rate is small (at antibiotic concentrations far below the MIC), where all cells grow, the population size n increases with minimal fluctuations even for small populations. As the death rate approaches the growth rate (near the MIC), fluctuations are amplified. It has been experimentally shown that an initial population consisting of up to 6×10^5 cell/mL experiences fluctuations near the MIC [3••,8••]. Currently, we do not know whether fluctuations persist in a population with the size relevant to the clinical settings (e.g. $\geq 10^8$ cell/mL in mature infections [52–56]).

Heterogeneous susceptibility can significantly impact the emergence of genotypic resistance as well. For example, cells that transiently gained phenotypic resistance are more likely to mutate and develop genotypic resistance [28•]. Furthermore, the establishment of a resistant population requires the survival and reproduction of the first mutant cell. Even after the acquisition of resistance, the mutant cell is subject to the stochastic cell growth and death (although the death rate is lower than parental cells), which influences the probability of a rare resistant mutant to outgrow and achieve fixation [8••]. Importantly, it was theoretically determined that the fixation probability is affected by how antibiotics are delivered (e.g. whether it was administered constantly or in alteration) [57]. Collectively, these studies highlight the important effects of heterogeneous susceptibility on the emergence of genotypic resistance.

Discussion

In order to develop optimal treatment plans that maximize bacterial eradication while minimizing the emergence of antibiotic resistance, a detailed understanding of antibiotic susceptibility is crucial. Cell-to-cell heterogeneity in antibiotic susceptibility has been observed for a variety of antibiotics against many bacterial species. Studying this phenomenon has been challenging due to the necessity of specialized skill sets for quantitative measurements and analyses at the single-cell level, including advanced microscopy, mathematical modeling of stochastic processes, and statistical assessment. Recent quantitative studies are advancing our understanding of single-cell-level responses and their effects on population behaviors. Interestingly, studies suggest that heterogeneous susceptibility is not limited to bacteria under antibiotic treatment; similar phenomena have been reported for anti-cancer drugs [58,59]. A deeper understanding could have an important implication for subjects beyond antibiotic resistance, including cancer treatment.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Tatsuya Akiyama: Conceptualization, Literature search, Writing - original draft preparation. **Minsu Kim**: Supervision, Writing - reviewing and editing.

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