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Heterogeneous bacterial persisters and engineering approaches to eliminate them

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Bacterial persistence is a state in which a subpopulation of cells (persisters) survives antibiotic treatment, and has been implicated in the tolerance of clinical infections and the recalcitrance of biofilms. There has been a renewed interest in the role of bacterial persisters in treatment failure in light of a wealth of recent findings. Here we review recent laboratory studies of bacterial persistence. Further, we pose the hypothesis that each bacterial population may contain a diverse collection of persisters and discuss engineering strategies for persister eradication.

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Introduction

Bacterial persistence is a phenomenon in which a subpopulation of cells survives antibiotic treatment [1•,2•,3–7]. In contrast to resistant bacteria, persisters do not grow in the presence of antibiotics and their tolerance arises from physiological processes rather than genetic mutations in a subpopulation of bacteria. Persistence was first described by Joseph Bigger in 1944 [8] while attempting to sterilize cultures of pathogenic *Staphylococcus aureus* with penicillin. He found that a small number of cells 'persisted' and could later form colonies even after treatment with high antibiotic concentrations.

The possible clinical implications of persisters were apparent: antibiotics might not sterilize infections and remaining bacteria could later cause recurrence once treatment ended [9••]. Early clinical studies of *in vivo* persistence in *S. aureus*, *S. pneumoniae*, and *M. tuberculosis* demonstrated that the phenotype was indeed an important and distinct problem in the treatment of infections [9••,10••]. Driven by an abundance of recent laboratory findings [11–24], there is renewed interest in clinical persistence [25,26••], which has led to the demonstration that high-persistent mutants can arise during treatment of chronic infections [26••]. Here, we review some of the recent laboratory studies of bacterial persistence in *E. coli* [1•,2•,3] and propose that persistence might be explained by variance in the many processes governing stress responses and antibiotic lethality, suggesting that a single population of bacteria contains a collection of distinct persisters.

hipA and the dawn of persister genetics

The first paper in persister genetics was published in 1983 by Moyed and Bertrand, who presented the results of a mutagenesis-and-selection scheme designed to create mutants with high persistence to penicillin [27]. After 24 independent attempts, they created four high-persistence strains, two of which were found to have mutations in the same gene, named *hipA* (for 'high persistence'). 1% of the *hipA* mutant cells persisted treatment with multiple antibiotics targeting peptidoglycan synthesis [28], representing a 100-fold increase over wild-type persistence. Rather than being resistant, an isogenic population of *hipA* mutants instead had a higher frequency of persisters. Noting that antibiotics that inhibit peptidoglycan synthesis are most lethal immediately following a round of DNA replication, the authors hypothesized that the mutant *hipA* arrests the cell cycle before entry into this susceptible phase. Studies have indicated that both the native *hipA* [29] and mutant *hipA* [30], which is considered a gain-of-function mutation [29,31], appear to target peptidoglycan synthesis, and that such inhibition may explain persistence [32,33]. However, though native *hipA* expressed heterologously can induce persistence under multiple conditions, the chromosomal *hipA* only appears to play a role in persistence in stationary phase [22,29], which raises the question of the importance of *hipA* in persistence generally.

Recent studies add to the early physiological research by suggesting that *hipA* is a kinase that inhibits translation by phosphorylating the EF-Tu elongation factor. Experiments showed incorporation of radio-labeled ATP into *hipA* when the two were incubated together, an effect abolished by mutations to specific residues in a predicted binding pocket, suggesting that *hipA* has the capability of

autophosphorylation [34]. Later studies suggested that *hipA* interacts with and phosphorylates translation elongation factor EF-Tu [35], and two separate crystallography studies suggest that *hipA* possesses a novel phosphorylation mechanism [35,36]. This phosphorylation event was posed as a possible mechanism for *hipA*-induced persistence in which *hipA* phosphorylates EF-Tu, leading to translational inhibition and subsequent tolerance to antibiotics. Whether this is the mechanism for the high persistence of mutant *hipA* is an open question, and further work testing this hypothesis is required as translational inhibition alone does not induce ampicillin persistence [31] and *hipA* has not been implicated in tolerance to aminoglycosides [27]. Connecting these recent biochemical insights to the physiological responses elicited by the native and mutant *hipA* will be crucial to understanding the role of this protein in bacterial persistence.

Since the description of the *hipA* mutation, *hipA* has become the persister gene of choice and has been studied extensively [22,27,29–31,34–39], playing an important role as a model system for the study of bacterial persistence [11–13,40,41]. Though *hipA* launched the field of persister genetics, numerous other genes and processes have since been found to contribute to bacterial persistence, as we discuss below.

More than one way to make a persister

There have been many laboratory studies on persistence in the past decade, many of which have uncovered previously unrecognized conditions and processes contributing to the phenotype. Here, we focus on three of these: heterogeneous growth, nutrient limitation, and the SOS response.

Heterogeneous growth

Heterogeneity in growth rates has been shown to play a role in bacterial persistence [11,13,20]. Studies have demonstrated that non-growing and dormant cells are more likely to persist antibiotic treatment than actively growing cells. Evidently, the growth rate of *E. coli* fits a bimodal distribution, in which two distinct populations, growing and non-growing, co-exist in the same culture [20]. Cell-sorting experiments showed that dormancy of the non-growing population correlates with persistence as dormant populations have 20 times more persisters than normally growing populations. This additionally suggests that dormancy does not entirely explain persistence as many dormant cells are not persisters and many persisters are not dormant.

Interestingly, the dormant cells in one of these studies appear to be narrower than normally growing cells, suggesting that stationary-phase cellular dwarfing [42], bacterial self-digestion, may play a role in persistence. These dormant cells may be stationary-phase remnants that have yet to wake up rather than population variants that chose to cease growing, and it would be interesting to examine whether serial passage of a culture eliminates this population [21].

Nutrient limitation

Under laboratory conditions, the environment and physiology of bacteria change considerably once they have exhausted the majority of nutrients, a situation referred to as stationary phase [42]. It has been shown that persisters predominantly form as cultures approach and enter stationary phase [11,21] and they are lost from cultures that have been serially passed through rich media conditions to promote early exponential-phase growth [21]. These data suggest that persisters examined in rapidly growing populations may be relics of the overnight culture used for culture inoculation, though this clearly does not account for persisters that arise from induction of processes such as *hipA* overexpression or the SOS response (discussed below).

A recent study highlights the importance of stationary phase in bacterial persistence by demonstrating that the age of inocula has a dramatic effect on persister levels [43•]. This work suggests that the differences in persistence observed upon perturbing certain genes can be explained by the amount of time spent in stationary phase before inoculation, indicating that these genes may not be important contributors to persistence. The age of the bacterial inoculum is rarely controlled for in persister methods, and this study strongly suggests that it should be.

SOS response

The SOS response, a major stress response system in bacteria that is induced by DNA damage [44], has recently been implicated in persistence [23,24,45]. Strains in which the SOS response cannot be activated have decreased persistence to DNA-damaging quinolone antibiotics. It was further shown that induction of the SOS response by subinhibitory levels of mitomycin C increased persistence [23,45], demonstrating that bacteria can adjust their tolerance to antibiotics by sensing and responding to stressful environmental conditions. The antibiotic concentration dynamics appear to be crucial for SOS-induced persistence as only 1% of those that survive treatment with high antibiotic concentrations had induced the SOS response. This work implies that persistence is in some cases inducible, which adds to models of persistence as a random switching event [11].

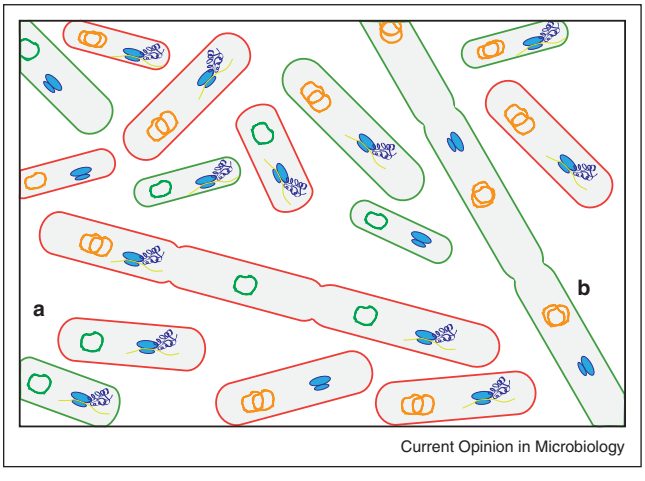
Persisters and physiological heterogeneity

The diversity of the pathways implicated in bacterial persistence suggests that, in addition to there being more than one way to make a persister, there may be different types of persisters. This raises the possibility that each persister has its own specific tolerances to antibiotics.

Total dormancy of a subpopulation is an attractive model for persistence as it simplifies the phenotype and suggests a possible unified theory of persistence. However, this model does not fit the growing body of experimental findings demonstrating diversity in both the tolerance to different antibiotics and the genes that contribute to persistence. This diversity suggests that the processes involved in stress responses and those required for antibiotic lethality are not simultaneously controlled within individual cells as part of a physiological program leading to a persistent state. As a result, the persistent subpopulation may not be uniformly distinct, physiologically speaking, from the susceptible population.

An alternative model is that each population contains a diversity of persisters based on fluctuations and variability in cellular processes (Figure 1). This diversity hypothesis suggests that persistence may be a result of multiple distinct cellular physiologies within a population. The apparent population split between tolerant and persistent cells when treated with antibiotic would then be masking an abundance of different mechanisms and associated phenotypes by which cells evade antibiotic lethality. Given the potential variance in cellular processes [46•,47–49], it seems plausible that persistence could result from stochastic fluctuations in lethality-governing processes. Whether persister states explored through fluctuations can be stabilized by epigenetic DNA modifications, which play an important role in other heterogeneous phenotypes [50], remains an open question. This hypothesis suggests that each bacterial culture contains a collection of distinct persister subpopulations, each with its own unique tolerance mechanisms. The study of such persistent heterogeneity is beyond many current experimental methods and will benefit greatly from increased investigation of single cells [51–53].

Figure 1



A plurality of persisters. Within a group of persisters, there may be significant diversity based on multiple, tolerance-conferring mechanisms. Membrane potential can be active (red) or reduced (green). DNA may be under active replication (orange circles) or not (green cicrles). Ribosomes (in blue) may be translating proteins or inhibited. Cell division is blocked in some cells. For example, cell **a** has normal translation, high membrane potential, but inhibited DNA synthesis, whereas cell **b** has inhibited translation, low membrane potential, active DNA replication, and stalled cell division.

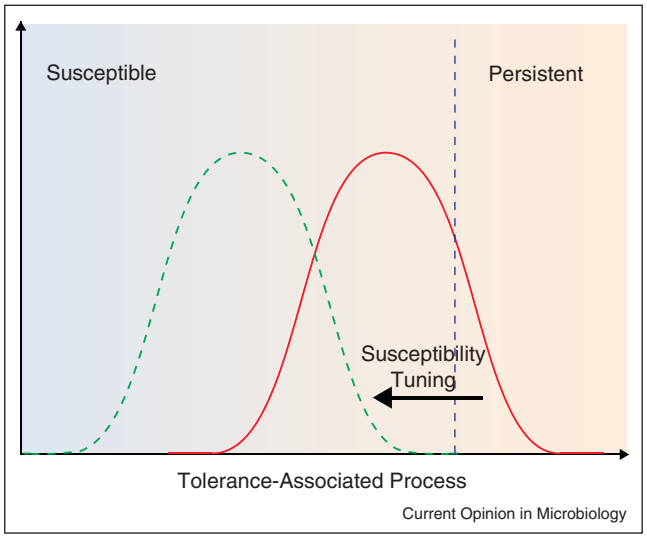
Following this reasoning, experimentally determined persister levels are the result of the distributions of stress–response and lethality-governing processes, which suggests that environmental conditions and genetic perturbations affecting these processes alter persistence. Experiments that perturb 'persister genes' impact related processes and tune the distributions of processes governing antibiotic tolerance. Given the role of stochastic fluctuations in large populations studied in the lab, persistence may be a statistical certainty, regardless of the exact persister level, the antibiotic used, or the particular tolerance mechanisms present. As a result, a model of bacterial persistence as a unified and distinct state may be impossible. Though persisters may currently be beyond comprehensive mechanistic study, attempts to eliminate persisters may not require full attention to the complicated set of processes leading to persistence.

Engineering treatments for persisters

The clinical importance of developing anti-persister strategies is self-evident, though there have been few attempts to target the elimination of persisters. It has been suggested that drugs and methods could be developed to target the genetic determinants leading to persister formation so as to prevent or reverse persistence [2•]. Given the number of genes involved in persistence, such an approach may prove difficult. Toward development of treatments for a diversity of persisters, it may be advantageous to focus instead on the mechanistic aspects of bactericidal antibiotics. Regardless of how they form, persisters must tune certain processes, such as peptidoglycan synthesis in the case of *hipA*-associated persisters, in order to avoid the lethal effects of antibiotics. Many of these processes are known and have been characterized for commonly used, bactericidal antibiotics [54•]. The distributions of these processes can be tuned by external stimuli, an approach that could be aided by single-cell based methods such as fluorescently activated cell sorting. Tuning these processes would alter the distribution of susceptibility in a population, thereby rendering persisters vulnerable to the antibiotics that target such processes (Figure 2). Though easier said than done, we have recently demonstrated the success of such an approach.

As protein translation occurs in persisters [12], we reasoned that persisters should be susceptible to aminoglycoside antibiotics, which are ribosome-targeting, bactericidal antibiotics [55,56]. Persisters are however not susceptible to aminoglycosides [18,22], and we suspected that this could be owing to decreased proton-motive force (PMF), as it is well established that PMF is required for

Figure 2



Tuning tolerance-associated processes. Cells in a population have varied activity of a particular tolerance-associated process (red distribution). As a result, though most cells can be killed by an antibiotic, a portion of the population is persistent. By tuning the process, and thereby shifting its distribution (green dotted distribution), the persistent population can be made susceptible to an antibiotic.

aminoglycoside uptake [57•]. Through specific metabolic stimuli (e.g. mannitol, fructose), we induced PMF in persisters, thereby enabling aminoglycoside uptake and killing [58•]. This approach specifically induced processes necessary for aminoglycoside killing as the metabolic stimuli did not facilitate persister killing by quinolones or β-lactams. Not only was this approach successful in laboratory cultures, but it was also effective at eliminating *E. coli* and *S. aureus* biofilms and improving treatment in a mouse chronic, urinary-tract-infection model. Hence, through mechanistic understanding of aminoglycoside lethality and by tuning persister physiology, we were able to engineer a clinically viable treatment for eradicating persisters.

Given the wealth of knowledge on how bactericidal antibiotics kill bacteria, it should be possible to develop a variety of similar mechanism-based approaches for treating persisters. Future anti-persister strategies could be engineered by utilizing methods promoting peptidoglycan synthesis or autolysin activity for the β-lactams [59–61], by harnessing methods promoting DNA replication or inhibiting SOS response for the quinolones [62], or by employing methods inducing reactive oxygen species (ROS) or inhibiting ROS-protective genes for all bactericidal antibiotics [63–65]. These methods might utilize external metabolic and chemical stimuli or engineered bacteriophage [66] to evoke the needed physiological responses. Additionally, incorporation of mechanistic knowledge of antibiotic lethality in bacterial persistence may shed light on the tolerance of quiescent cancer cells to chemotherapeutic agents [67–69].

Conclusion

Studies over the past decade have implicated a multiplicity of processes contributing to bacterial persistence. Given the physiological complexity of each bacterial cell, it seems plausible that persistence may be the result of fluctuations and variance in different tolerance-associated processes. This suggests, that in a single bacterial population, there may be many different types of persisters, each with distinct mechanisms for evading the lethal effects of bactericidal antibiotics. Advances in sequencing and single-cell technologies will help test this hypothesis and aid standard techniques in the elucidation of persister physiology. This hypothesis further suggests the importance of incorporating mechanistic understanding of antibiotic modes of action into models of persistence. The wealth of available knowledge and insights into the action of antibiotics could be utilized to develop clinically viable, mechanism-based methods for eradicating persisters.

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