NON-STANDARD FORMAT CHAPTER

A Historical Perspective on Bacterial Persistence

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Historical Perspective on Bacterial Persistence

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Summary/Abstract

Bactericidal antibiotics quickly kill the majority of a bacterial population. However, a small fraction of cells typically survives through entering the so-called persister state. Persister cells are increasingly being viewed as a major cause of the recurrence of chronic infectious disease and could be an important factor in the emergence of antibiotic resistance. The phenomenon of persistence was first described in the 1940s, but remained poorly understood for decades afterwards. Only recently, a series of breakthrough discoveries has started to shed light on persister physiology and the molecular and genetic underpinnings of persister formation. We here provide an overview of the key studies that have paved the way for the current boom in persistence research, with a special focus on the technological and methodological advances that have enabled this progress.

Key words

Persisters, persistence, antibiotic tolerance, dormancy, antibiotics, review

The Early Days

The first report on the survival of a small fraction of streptococci cells following treatment with penicillin dates from 1942 [1]. Two years later, Joseph Bigger established that addition of penicillin to staphylococci does not result in complete sterilization of all cells in a clonal population. One out of a million cells survived even prolonged treatment with antibiotics. He appropriately named the surviving cells persisters [2]. More recently, it was shown that in most bacterial species, the majority of cells is efficiently killed by relatively low concentrations of bactericidal antibiotics. However, killing shows a biphasic pattern and beyond a certain threshold, further increasing the concentration of the antibacterial does not result in complete clearing of the culture (Figure 1) [3].

(FIGURE 1 HERE)

For forty years following its discovery, the persistence phenomenon was largely neglected, at least by molecular geneticists. This was partly due to the fact that the clinical relevance of persister cells was not clear. In contrast, the threat posed by inherited antibiotic resistance was generally recognized, adding incentives to resistance research. The problem was compounded by technical challenges that inevitably accompany the study of a transient phenotype that is associated with only a very small fraction of cells.

A breakthrough discovery came in the early 1980s, from research carried out by Harris Moyed during a sabbatical leave in the lab of Alexander Tomasz [4]. Mutagenesis of *E. coli* populations with ethyl methanesulfonate (EMS) led to the identification of three high persistent (*hip*) mutants exhibiting 10- to 10,000-fold

increased persister fractions upon incubation with penicillin [4,5]. Moyed's pioneering work led to the identification of two mutants hit in the *hipA* locus that up until now remains the best-studied persister gene [6-10]. Furthermore, because of their increased persister fraction, *hipA* mutants have frequently been used as a tool in persistence research. Crucially and for the first time, *hipA* mutants enabled the direct observation of persister cells. Using a combination of microfluidics and live cell microscopy, Nathalie Balaban recorded how persisters survived killing by antibiotics through dormancy and subsequent resuscitation [11]. In addition, the *hipBA* locus is a representative for other toxin-antitoxin (TA) loci that are now intensively studied in relation to persistence. TA modules consist of a stable toxin, typically targeting essential cellular functions, and an unstable antitoxin, which counteracts the activity of its cognate toxin [12,13]. TA systems were originally identified on plasmids, where they play a role in plasmid maintenance, yet a significant number of TA loci are chromosomally encoded and these have been implicated in persistence [14]. Examples include RelE [6], MqsR [15-17], TisB [18,19], MazF [20] and YafQ [21]. Interestingly, with the notable exception of *Salmonella* persisters residing within macrophage vacuoles [22], deletion of a single toxin generally does not affect persistence. This can partly be explained by redundancy of TA systems in most bacteria. Deletion of multiple TA systems, on the other hand, causes a decrease in *E. coli* persistence [23].

The Rise of Persistence Research

Following the discovery of *hipA*, persistence as a field of study steadily gained attention. This was partly due to the acknowledgement of its clinical significance (summarized by [24]). In 1944, Bigger already alluded to the role of persisters in the

resuscitation of chronic infections [2]. Decades later, Kim Lewis postulated that persisters might contribute to the recalcitrance of biofilm infections [25,26]. This is of particular interest as biofilms are known to withstand antibiotic treatment, thereby causing chronic infections [27]. Subsequently, mathematical modelling demonstrated that persistence could extend the duration of antibiotic treatment, thereby causing treatment failure and promoting the emergence of resistance [28]. Finally, two studies have unambiguously demonstrated that prolonged antimicrobial therapy selects for high-persistent strains of *Candida albicans* during candidiasis and of *Pseudomonas aeruginosa* during cystic fibrosis lung infections [29,30]. In addition, the role of persister cells in the development of resistance is becoming increasingly clear [31]. Apart from providing incentives to further intensify persistence research, these findings also promoted the search for anti-persister therapies. At present, several strategies have been described, but their *in vivo* effectiveness remains to be investigated. Examples include the use of resonant activation [32], electrochemical currents [33], cadaverine [34], metabolites [35,36], antimicrobial peptides [37], brominated furanones [38-41] and activated ClpP [42] (summarized by [43]).

Apart from increased interest due to the clinical importance of persistence, the development of novel techniques also caused persistence research to boom. An overview of these novel techniques is provided below.

Screening approaches

Over the years, several screening procedures have been developed that led to the identification of persister genes. In a first approach, a non-redundant *E. coli* knockout library was screened for mutants with altered persistence [44]. Persister cells of individual mutants were quantified by treating a stationary-phase culture with

ofloxacin and plating the surviving cells on agar medium containing amdinocillin. As the number of spontaneous amdinocillin-resistant mutants is a fraction of the original number of cells, this obviates the need for dilution steps and greatly reduces the laborious task of screening several thousands of strains.

A second screening approach employed a *P. aeruginosa* plasposon knock-out library. Individual mutants were grown until stationary phase and treated with either ofloxacin to kill non-persister cells or water, the latter serving as a control. Subsequently, samples were diluted and incubated in an automated plate reader (Bioscreen C, Oy Growth Curves Ab Ltd), allowing the optical density of 200 samples to be measured simultaneously as a function of time. Given the linear relationship between the number of cells in an inoculum and the lag phase, this allowed for the selection of mutants displaying altered persister levels [45].

Both screenings led to the identification of a number of interesting persister genes including some global regulators. In addition, not a single mutant lacking persisters was identified. As a general conclusion, these screenings therefore provided evidence pointing to the multiplicity of persister formation mechanisms.

In a final approach, a random overexpression library was generated in *E. coli*. Cells from the recombinant library were pooled and logarithmically growing cultures of library clones were exposed to multiple rounds of exposure to ampicillin. This led to the enrichment of mutants with increased probability of persister formation and ultimately to the identification of *glpD* as a genuine persister gene [46].

Single-cell studies

As persistence is a phenotypic trait expressed in only a subfraction of a population, advances in single-cell research signified an era of vast new possibilities. First used

by the Balaban group [11], transparent microfluidic devices proved instrumental for microscopic examination of persister cells [47-49]. The strength of this technique lies in the possibility to monitor individual cells for prolonged periods of time while adapting growth conditions. For example, normal growth conditions can be alternated with antibiotic treatments in order to kill non-persister cells. This allows to pinpoint persister cells surviving treatment. Subsequently, the history of persister cells can be traced back through the recorded images. Several studies have used this technique to demonstrate preexisting heterogeneity in bacterial populations [11], to characterize the dormant state of single persister [47], to monitor persistence formation following administration of indole [48] and to correlate high TA expression to cessation of growth [49].

Also developed by the Balaban group, a colony-appearance assay was elaborated to quantify single-cell persister lag phases [50]. Experiments demonstrated that a threshold concentration of toxin molecules is required for induction of persistence.

A major drawback of microfluidic devices, or more precisely of microscopy, is the limited number of cells that can be studied simultaneously. This can be circumvented by using flow cytometry, allowing thousands or even millions of cells to be evaluated in a high-throughput manner. A shortcoming of this technique is the inability to continuously monitor individual cells over time. Nonetheless, flow cytometry has been successfully used to study the kinetics of persister awakening [51]. In addition, while Bigger postulated that persisters are in a dormant, non-dividing state [2], flow cytometry has been used to demonstrate that dormancy is not a requirement for entry into the persister state [52]. Finally, a recent study performed by the Holden group showed how to characterize the dynamics of intracellular bacterial replication at the single cell level. They used a fluorescence dilution technique to quantify the number

of replication cycles of internalized *Salmonella* [53]. This showed the existence of different *Salmonella* subpopulations in bone marrow-derived macrophages including a non-replicating but metabolically active subpopulation, comprising the persister cells, possibly capable of resuming growth and causing relapsing infections [22]. Similarly, the Bumann group exploited a DsRed variant called TIMER^{bac}, which spontaneously changes color from green to green/orange over time, as a dynamic growth rate reporter to identify persister cells *in vivo* [54].

Transcriptomics

Insight into global transcriptional changes in persister cells came from several elegant studies by the Lewis group. To enrich for persisters, all three approaches conveniently employed the metabolic inactivity of these cells. In the first report, logarithmically growing populations of the high-persistence *E. coli* mutant *hipA7* [4] were treated with ampicillin, thereby lysing non-persister cells. Isolated RNA was enriched for mRNA, labeled and hybridized to *E. coli* GeneChips [6]. Similarly, gene expression profiling of persisters was performed after treating an exponentially growing population of *Mycobacterium tuberculosis* with D-cycloserine and collecting surviving persister cells by centrifugation. Transcriptome analysis was performed by microarray hybridization [55]. The third study followed a slightly different approach. It was based on the assumption that persisters are dormant cells with low levels of protein synthesis and corresponding low levels of rRNA transcription. *E. coli* persister cells were isolated by linking the *rrnB* promoter to a gene encoding an unstable fluorescent protein. In so doing, persister cells are dim as compared to normal cells in the population, which allows for the isolation of persisters using Fluorescence

Activated Cell Sorting (FACS). cDNA was prepared from purified RNA and hybridized to spotted *E. coli* DNA microarrays [56].

Based on the studies cited above, stress response pathways as well as TA loci were shown to be highly expressed in isolated persister cells. On the other hand, biosynthetic functions including energy production were downregulated [6,55,56].

Experimental evolution

The use of experimental evolution for elucidating antibacterial resistance mechanisms is a widely used method. A recent study by the Balaban group used this technique for enriching a population with persisters by repeated exposure of a bacterial population to high concentrations of antibiotics. This resulted in evolved strains showing very high persister fractions caused by fixed specific genetic mutations. The increased survival appeared to be the result of an adjustment in the single cell lag-time distribution, which was correlated with the extent of the antibiotic exposure interval [57]. They implemented the ScanLag method, which allows the simultaneous measurement of lag times of hundreds of cells [58]. These findings resulted in a new theory regarding persister cells and their ability to adapt to high doses of drugs called tolerance by lag.

Modeling

Apart from these wet lab techniques, mathematical modeling has provided interesting insights [28,59-62] (summarized by [63]). Briefly, two main strategies can be discerned: the first one relies on estimating the switching rates between persister and non-persister growth states and assumes this process to take place continuously and stochastically (e.g. [11,28,59,61,64]). The balance between both switching rates

provides a straightforward way to model a given persister level, although ignoring exactly what determines the switching rates. The second strategy focuses on the molecular mechanisms of persister formation by TA systems, with the ratio of (free) toxin over antitoxin ultimately determining, at the single-cell level, the decision to switch to the persister state (e.g. [60,65,66,50]). A crucial factor in this type of models is the generation of phenotypic bistability at the population level, typically requiring noisy gene expression and noise amplification through positive feedback mechanisms [67]. Both modeling strategies have their merits, and until a more integrated approach is presented, the choice between both will depend on the goal and specific focus of the study at hand.

Mathematical modeling of persistence poses several advantages. Experiments that are not feasible in the lab can be simulated to predict the outcome. It also allows to explain empirically observed persister levels in terms of the parameters encompassed by the model, and why varying some parameters has more impact on persistence than others. Consequently, evolutionary forces that shape persister levels can be identified, which should help to devise strategies to avoid high persister levels emerging in the clinic.

State of the Art and Future Perspectives

Recently, the field of microbial persistence research has exploded, as evidenced by a host of publications in top-tier journals [10,22,23,35,48-50,54,68-70]. Currently, it is generally accepted that persister cells are present in a bacterial population preceding antibiotic treatment [71]. It is postulated that their formation results from noisy gene expression [72] as was first suggested by Kim Lewis [6]. However, over the years, several stimuli have been shown to induce persistence. For example, sub-inhibitory

concentrations of fluoroquinolones are known to induce persistence via activation of the *tisAB/istR* TA locus [18,19]. Other examples include quorum sensing molecules [73,74], carbon source transitions [70] and nutrient deprivation leading to activation of the stringent response [75]. As was earlier described for HipA [76], a recent model ascribes TA-regulated persistence to stochastic fluctuations in cellular concentrations of the alarmone (p)ppGpp. High (p)ppGpp levels activate TA loci through a regulatory cascade requiring inorganic polyphosphate and Lon protease targeting protein toxins [49]. For an elaborate discussion on the role of these mechanisms in persistence, the reader is referred to some excellent reviews on the topic [3,77-81].

Adding to the significance of these studies is the recent observation of a phenotypically distinct subpopulation of transiently drug-tolerant persisters in cancer cell populations. These cells are held responsible for “fractional killing” upon chemotherapy [82]. Cell-to-cell variations in protein levels were suggested to contribute to this phenomenon in which each round of therapy kills some but not all of the cells in a tumor [83]. There is a striking analogy between bacterial and cancer cell-derived persistence as both phenomena reflect a transiently phenotypic heterogeneity causing multi-drug tolerance and recurrence of disease symptoms upon removal of treatment [84]. Added insight into bacterial persistence may therefore impact research areas far beyond infectious disease.

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Figure captions

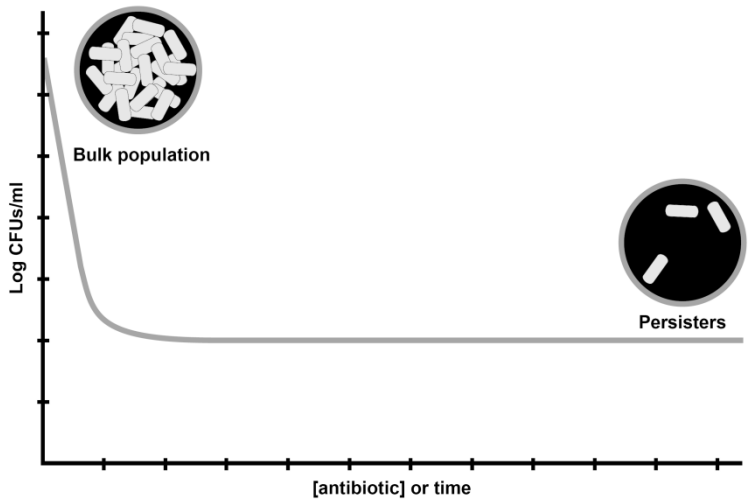

Figure 1: Illustration of persistence. The majority of cells in a bacterial culture is efficiently killed by relatively low concentrations of antibiotics. However, beyond a certain threshold, a killing plateau is observed as only persister cells remain viable. When regrown in fresh medium, the surviving cells generate a population as sensitive to the antibiotic as the original population.