Review

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Bacterial persisters: formation, eradication, and experimental systems

Sophie Helaine and Elisabeth Kugelberg

Section of Microbiology, Medical Research Council (MRC) Centre for Molecular Bacteriology and Infection, Imperial College London, Armstrong Road, London SW7 2AZ, UK

Persisters are multidrug-tolerant bacteria that could account for the relapse of infections. For a long time, persisters have been assumed to be nonreplicating dormant bacteria, but the growth status of these recalcitrant cells is still debated. Toxin–antitoxin (TA) modules have an important role in the formation of persisters and several studies show that they can form in response to different triggers. These findings, together with the invention of new tools to study persisters, could have important implications for the development of novel therapeutics to eradicate persisting subpopulations.

Bacterial persisters and persistent infections

Persisters are bacterial cells that are multidrug tolerant as a consequence of a transient switch. Joseph Bigger discovered these cells in 1944 when he was unable to completely sterilize a *Staphylococcus* culture with penicillin [1]. Contrary to resistant bacteria that arise from heritable mutations, persisters represent a small subpopulation caused by nonheritable phenotypic variation; persister cells are genetically identical to non-tolerant kin. Because resistance to antibiotics emerged shortly after their introduction into clinical practice, the study of antibiotic resistance quickly surpassed interest in the antibiotic tolerance of persisters. Persister cells are thought to be responsible for the recalcitrance of many bacterial infections to antibiotic treatment, which often result in recurrent courses of antibiotics. Such infections pose a significant public health problem. Hence, the interest in persister cells has increased dramatically over the past 5 years [2–9].

A variety of bacteria cause persistent infections, including *Mycobacterium tuberculosis*, *Salmonella*, *Chlamydia*, *Brucella*, *Borrelia*, *Pseudomonas*, pathogenic *Escherichia coli*, *Staphylococcus*, and *Streptococcus* [10,11]. Among women with an acute urinary tract infection, 20–30% will have a recurrent infection within 3–4 months [12], and 15% of patients with typhoid fever, who appear to be treated successfully, suffer from relapse [13]. However, a causal link between persisters and persistent infections was missing for a long time [14], until researchers showed that high-persister mutants were selected in patients with cystic fibrosis who had experienced repeated courses of antibiotic therapies [15].

Corresponding author: Helaine, S. (s.helaine@imperial.ac.uk).
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Growth arrest is the most established hypothesis to explain multidrug persistence displayed by subpopulations of numerous bacterial species exposed to a variety of environments, although other mechanisms have been shown to explain transient tolerance of bacterial cells [8,16]. TA modules, which are widely spread among bacteria, are involved in the generation of nonreplicating subpopulations and are probably the major actors of the persistence phenomenon in response to starvation. Upon stress, the antitoxins are degraded, which results in internal release of the toxins (several of which encode mRNases) and thereby transient blockage of main cellular processes, such as protein production or DNA replication, and eventually halted bacterial division. If bacteria in growth arrest are exposed to antibiotics, they often survive the treatment, because antibiotic target sites are inactive. Eradication of persisters could possibly be used to tackle recurrent infections, and recent efforts have been developed to reach this goal. This review focuses on the latest findings on growth status of persisters, the role of TA modules in persister formation, and the environmental signals that can trigger them. Finally, because conventional microbiological tools used to reveal viability or killing of bacterial populations are not relevant to study true dormant subpopulations, we discuss the need for new tools that will facilitate the study of persisters.

Is persistence due to growth arrest?

When Bigger discovered *Staphylococcus aureus* persister cells he was the first to propose that these were non-growing bacteria. However, this was not shown experimentally until six decades later when the combination of microfluidics and microscopy enabled the study of persistent *E. coli* at the single cell level [17]. In this study, a subpopulation of non-growing bacteria was identified prior to exposure to antibiotics and a fraction of those individual cells survived and successfully resumed growth after antibiotic removal. Several studies have inferred that persisters are non-growing dormant bacteria, in which many antibiotic target sites are inactive, thereby explaining ineffectiveness of antibiotics [18,19]. This view has been challenged in recent years and several studies report evidence for various active mechanisms of persistence (rather than lack of bacterial growth) such as detoxification through efflux pumps or lower antibiotic uptake [20]. Because this review article focuses on the growth status of bacterial persisters, we will only include studies that have directly tested this aspect of persistence.

Most studies on persistent tuberculosis infections have been based on the assumption that a part of the *M. tuberculosis* population is nonreplicating or dormant, although no direct observation of nonreplicating persisters during infection has been provided. Recently, this consensus was challenged by two independent studies indicating that mycobacteria persisted antibiotic treatment while replicating. In the first study, persistent *Mycobacterium marinum* was identified in macrophages of infected zebrafish larvae [16]. Following this observation, *M. marinum* and *M. tuberculosis* persisters were examined using *in vitro* cultured macrophages. The loss of a nonpropagative plasmid, as a reporter of intracellular replication in the presence of antibiotics, was monitored; this showed that antibiotic-tolerant bacteria were actively growing. The basis for antibiotic tolerance in this experiment was efflux-mediated elimination of the antibiotics. Several efflux pumps are induced during macrophage infection because these are involved in adaptation to intracellular growth [16]. In the second, more recent study, replicating isoniazid-tolerant persisters of *Mycobacterium smegmatis* were identified and analysed at the single cell level [8]. Isoniazid inhibits cell wall biogenesis, but only after activation by the bacterial catalase KatG. The study showed that KatG is produced in pulses, which results in a population with heterogeneous susceptibility to isoniazid, without any link to growth rates [8]. These are elegant examples of replicating persisters in mycobacteria. However, in the second study, the molecular mechanism of drug tolerance is rather specific to a particular drug and it still needs to be determined if the KatG-negative and isoniazid-tolerant cells exhibit multidrug tolerance.

The importance of growth arrest in bacterial persistence has been carefully investigated in a study of antibiotic tolerance of *Pseudomonas aeruginosa* biofilms [6]. A large fraction of biofilm-forming bacteria experience growth arrest as a consequence of nutrient starvation. Nguyen *et al.* [6] compared the antibiotic susceptibility of a mutant deficient in the stringent response (starvation stress response) with that of a wild type strain under conditions where both bacteria were forced into growth arrest by exposure to serine hydroxamate, which is a serine analogue that induces starvation. The authors showed that activation of the stringent response, and not merely the passive effects of growth arrest, induces antibiotic tolerance in *P. aeruginosa*. This tolerance was mediated by the induction of antioxidant defences, which is in line with the idea that all bactericidal antibiotics share a common killing mechanism by stimulating the generation of reactive oxygen species (ROS) [21]; therefore, increased antioxidant defences would increase survival of the bacterial population. Although this ROS paradigm of unified killing mechanism by antibiotics was attractive to explain the role of oxidative stress defences in bacterial persistence [22], each point of the paradigm has now been critically challenged by three reports (Figure 1) [23–25]. These refutations of the ROS paradigm do not directly challenge the involvement of antioxidant defences in bacterial persistence. However, single cell analysis of an antibiotic-treated *E. coli* population showed no correlation between the ability of the bacteria to tolerate the treatment and their intracellular concentration of ROS [24]. The mechanism by which antioxidant defences promote persistence needs to be investigated further.

Whereas direct evidence of nonreplicating and replicating persisters has recently accumulated in *in vitro* models, direct evidence for the long suspected presence of non-growing persisters of a bacterial pathogen in its host has been lacking for a long time. However, such non-growing persisters have now been reported for *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) during systemic infection of mice [26,27]. Phagocytosis of *Salmonella* by host macrophages dramatically increased the formation of non-growing bacterial persisters compared with the basal levels of persisters that form during growth in laboratory medium [26].

Although a few studies show that some antibiotic-sensitive mycobacteria can persist antibiotic treatment while replicating [8,16], bacterial growth arrest and dormancy seem to be the most general means by which bacterial subpopulations in a variety of environments can tolerate exposure to a variety of multiple antibiotics. However, for *E. coli* it was recently shown that growth arrest was not sufficient to promote bacterial persistence, but it increased the likelihood of a cell to tolerate antibiotic exposure [28].

TA modules and their role in persister formation

Forty years after the discovery of persistent bacterial cells, an *E. coli* mutant with increased ability to form persisters was isolated *in vitro*. Mutants were generated by chemical mutagenesis and then selected by transient ampicillin treatment. Survivors were analysed and most of the mutations mapped to the high persister gene *hipA* [29]. Subsequently, *hipA* was shown to encode the toxin part of a TA module [7]. This toxin is a serine-threonine kinase inhibiting the glutamyl-tRNA synthetase (GltX) by phosphorylating its ATP binding site, and therefore preventing aminoacylation and triggering activation of the stringent response [30,31] (Figure 2). The involvement of TA systems in persistence has been supported by two transcriptomic studies on *E. coli* persisters, which revealed that persistent cells display increased expression of TA modules [32,33].

TA operons encode a non-secreted toxin, which inhibits essential cellular functions such as RNA translation, DNA replication, and cell wall or ATP synthesis, leading to growth arrest of the bacterial cell [34]. In normal circumstances, an antitoxin neutralizes the toxin so that bacterial cell growth is unaffected. Five different types of TA modules have been described depending on how the antitoxin neutralizes the toxin, and these were recently reviewed elsewhere [35]. So far, the formation of persisters has mainly been linked to expression of type II TA modules, which are characterized by a protein antitoxin that binds and inhibits the activity of the toxin.

A comprehensive study from 2011 showed that successive deletion of all 10 mRNA interferases toxins of the 15 type II TA loci of *E. coli* progressively reduced the level of persisters, suggesting that persistence is a phenotype contributed by highly redundant TA loci. All the cognate antitoxins of those toxins were shown to be substrates of the ubiquitous Lon protease and bacteria lacking *lon* displayed an even lower level of persisters [36]. However, TA