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Active efflux in dormant bacterial cells – New insights into antibiotic persistence

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ABSTRACT

Bacterial persisters are phenotypic variants of an isogenic cell population that can survive antibiotic treatment and resume growth after the antibiotics have been removed. Cell dormancy has long been considered the principle mechanism underlying persister formation. However, dormancy alone is insufficient to explain the full range of bacterial persistence. Our recent work revealed that in addition to 'passive defense' via dormancy, persister cells employ 'active defense' via enhanced efflux activity to expel drugs. This finding suggests that persisters combine two seemingly contradictory mechanisms to tolerate antibiotic attack. Here, we review the passive and active aspects of persister formation, discuss new insights into the process, and propose new techniques that can facilitate the study of bacterial persistence.

1. Introduction

Bacterial persisters, first reported by Joseph Bigger in 1944 in his study of the killing effects of penicillin on *Staphylococcus aureus* (Bigger, 1944), are defined as a very small subgroup of cells that can survive antibiotic treatment and resume growth after the removal of antibiotics. Persisters are genetically identical to the rest of the bacterial population, different from the well-known concept of antibiotic resistance resulting from genetic mutations or horizontal gene transfer. The phenomenon of bacterial persistence is widely observed from in the laboratory and the clinic, as well as in the natural environment. Interest in studying persisters has greatly increased in recent years due to their roles in chronic infections and the potential links to antibiotic resistance. However, to characterize this rare and transient bacterial population is challenging, and we still know very little about bacterial persisters.

Previous investigations have suggested that cell dormancy is the leading mechanism underlying persister formation (Balaban et al., 2004; Lewis, 2007) and the contributions from the toxin-antitoxin (TA) loci are highly important (Balaban et al., 2004; Dorr et al., 2010; Keren et al., 2004; Vazquez-Laslop et al., 2006). In the TA module, the toxin is stable and can disrupt fundamental cellular pathways, resulting in a dormant state of the cell. By contrast, the antitoxin is labile and can conjugate the toxin to abolish its toxicity. TA modules are probably triggered by stress responses through the alarmone guanosine tetraphosphate (ppGpp) pathway (Maisonneuve et al., 2013). Additionally, the SOS response (Dorr et al., 2009) and several global regulators (Hansen et al., 2008) play roles in dormancy and persister formation. However, recent studies have indicated that some active pathways also participate in bacterial persistence.

1.1. Clinical significance

The existence of persister cells is thought to be the cause of many chronic bacterial infections (Conlon et al., 2015; Lewis, 2010, 2007; Stewart and Cookson, 2012). Treatment of these chronic infections, such as the diseases induced by *Mycobacterium tuberculosis*, *Salmonella enterica* serovar *Typhimurium* and *S. aureus,* is extremely difficult because of the reservoir of persister cells (Conlon et al., 2015; Lewis, 2010, 2007; Stewart and Cookson, 2012).

In the medical treatment of tuberculosis, incomplete eradication leads to pathogens remaining in the patients. This residual bacterial population gives rise to refractory tuberculosis (Davies, 2001). Although this recalcitrance can be partly ascribed to genetic mutation-conferred drug resistance, accumulating evidence suggests that bacterial persistence is the major reason (Wayne, 1994). Many *M. tuberculosis* persisters are found to reside in a microenvironment called a granuloma (Davies, 2001; Honer zu Bentrup and Russell, 2001). The granuloma microenvironment provides challenging conditions for *M. tuberculosis*, including low pH, high

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oxidative stress, and limited nutrients, which have been shown to induce persister formation (Davies, 2001; Honer zu Bentrup and Russell, 2001; Marakalala et al., 2016; Mok et al., 2015; Rubin, 2009). Upon encountering this stressful environment, *M. tuberculosis* cells cease to grow, resulting in a non-replicating state that protects them from being eradicated by antibiotics (Davies, 2001; Voskuil et al., 2003). It has been reported that the proportion of persister cells can be up to ~1% of the entire *M. tuberculosis* population (Keren et al., 2011), indicating the significant role of persistence in drug tolerance.

Similar to *M. tuberculosis*, *S. Typhimurium* is another pathogen that demonstrates the importance of non-heritable persistence in disease progression. *S. Typhimurium* causes systemic and enteric infections in a wide range of hosts, represented by acute and chronic typhoid fever (Behnsen et al., 2015; LaRock et al., 2015; Monack et al., 2004; Wain et al., 2015). Ingested bacteria reside within professional phagocytes, particularly in macrophages and are tolerant to many adverse conditions, including antibiotic treatment (LaRock et al., 2015; Monack et al., 2004; Rivera-Chavez and Baumler, 2015; Wain et al., 2015). Recently, a fluorescent single-cell analysis identified *S. Typhimurium* persisters during infection, induced immediately after uptake by macrophages (Helaine et al., 2014; Helaine et al., 2010). The study demonstrated that in addition to antibiotics, the intracellular environment of macrophages could also induce the formation of non-replicating persisters that could provide a reservoir for relapsing infection (Helaine and Kugelberg, 2014). Furthermore, in some macrophages, the non-replicating *S. Typhimurium* persister cells represented the sole intracellular population (Helaine et al., 2010). Another recent study showed that some toxins, including relE-5, sehA, relE and vapC, were highly expressed during *S. Typhimurium* infection of macrophages (Silva-Herzog et al., 2015). Therefore, it is possible that the *S. Typhimurium* persisters could tolerate the stresses imposed by the host environment, as well as escaping the killing effects of antibiotics (Helaine and Holden, 2013; Helaine and Kugelberg, 2014; Silva-Herzog et al., 2015).

S. aureus is a harmful pathogen implicated in both hospital- and community-associated infections (Malani, 2013). It is one of the most common pathogens responsible for wound, endovascular and bone infections (Conlon, 2014; Grady and Cullen, 2003; Malani, 2013). *S. aureus* can cause chronic infections with a high rate of relapse due to its ability to adapt to the local microenvironment (Conlon, 2014; Trouillet-Assant et al., 2016). The formation of persister cells helps *S. aureus* to survive the stressful environment encountered during the infection of humans (Trouillet-Assant et al., 2016). The existence of *S. aureus* persisters may explain the reason why long-term antibiotic treatment is often required to eradicate the infection (Conlon, 2014).

In addition to *M. tuberculosis*, *S. Typhimurium* and *S. aureus,* there are many other pathogenic bacteria that can cause chronic recalcitrant infections, including brucellosis caused by *Brucella spp.* (Ahmed et al., 2016; von Bargen et al., 2012), lung infections in cystic fibrosis patients caused by *Pseudomonas aeruginosa* (Hazan et al., 2014; Mulcahy et al., 2010), urinary tract infections caused by uropathogenic *Escherichia coli* (Niu et al., 2015; Norton and Mulvey, 2012), and melioidosis caused by *Burkholderia pseudomallei* (Butt et al., 2014; Nierman et al., 2015). Whether bacterial persisters play vital roles in these chronic diseases warrants in-depth investigation.

1.2. Passive and active aspects of persister formation

Cell dormancy has long been considered the fundamental mechanism underlying bacterial persistence (Balaban et al., 2004; Rotem et al., 2010). Although antibiotics still bind to their targets in dormant cells, the drugs cannot exert their lethal effects due to the inactivation of downstream pathways. The molecular mechanisms involved in cell dormancy have been widely studied, including the toxin-antitoxin modules (Balaban et al., 2004; Maisonneuve et al., 2011), SOS response pathways (Theodore et al., 2013), oxidative stress response pathways (Vega et al., 2012; Wu et al., 2012), and global regulators leading to slow metabolism (Hansen et al., 2008). However, dormancy alone cannot fully explain persister formation. Although persister cells are found to be dormant in most cases, there are examples showing that persister cells can actively grow under both *in vitro* and *in vivo* conditions (Adams et al., 2011; Orman and Brynildsen, 2013). Additionally, in our recent work, we found that active efflux is another important mechanism that contributes to bacterial persistence (Pu et al., 2016). In the following text, we will review the passive and active cellular processes that are closely involved in persister formation.

2. 'Passive defense' of persisters

2.1. Molecular mechanisms of dormancy

The molecular mechanisms of persister formation have been studied for decades. Whole-genome screening and a transcriptome analysis found that persister formation is linked to a large number of genes and pathways, and these mechanisms are highly redundant (Baba et al., 2006; De Groote et al., 2009; Girgis et al., 2012; Hansen et al., 2008; Keren et al., 2004; Shan et al., 2015; Spoering et al., 2006). The standard loss-of-function experiments that are performed to identify functional genes leading to the persister phenotype failed to find a single gene knockout that completely abolished persister formation (Girgis et al., 2012; Maisonneuve et al., 2011; Shan et al., 2015). An alternative methodology involves overexpressing genes and evaluating which gene(s) can induce increased level of persisters. Based on a combination of these studies, numerous dormancy-related genes have been identified (Keren et al., 2004).

2.2. Toxin-antitoxin modules

The first identified persister gene was *h*igh-*p*ersistence A (*hipA*). Together with *hipB*, it forms the *hipBA* operon that was later found to be a Type II toxin-antitoxin (TA) locus (Korch et al., 2003; Moyed and Bertrand, 1983). Of the many TA loci expressed by *E. coli*, *hipA* is the most widely studied and has greatly advanced our understanding of how TA systems facilitate persistence. The roles of *hipA* were determined by isolating a stably heritable mutant carrying a gain-of-function allele called *hipA7*, which contains two amino acid substitutions (G22S and D291A) and leads to a 1000-fold increase in the number of persisters (Black et al., 1994; Black et al., 1991; Korch et al., 2003). HipA is a protein co-transcribed with the DNA-binding protein HipB, which together form the HipB-$(HipA)_2$-DNA complex, a higher-order oligomer (Schumacher et al., 2015; Schumacher et al., 2009). The HipA7 variant exposes the HipA active site, ablating HipA-HipA dimerization, and renders the toxin more effective, resulting in an enhanced persistence phenotype (Schumacher et al., 2015).

In general, TA loci encode a toxin that causes dormancy and growth arrest of the cell, and an antitoxin that neutralizes toxin activity. These TA systems are currently divided into six classes according to their different mechanisms of regulation and are termed the Type I–VI TA systems (Kedzierska and Hayes, 2016; Page and Peti, 2016; Schuster and Bertram, 2016). In Type I and III TA systems, the antitoxin is a noncoding RNA; in Type II, IV, V and VI TA systems, the antitoxin is a protein. Most toxins act as RNases and can cleave cellular mRNA to induce persister formation when they are not neutralized by antitoxins (Gerdes and Maisonneuve, 2012).