Leading Edge
Review

Cell

Molecular Mechanisms Underlying Bacterial Persisters

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All bacteria form persisters, cells that are multidrug tolerant and therefore able to survive antibiotic treatment. Due to the low frequencies of persisters in growing bacterial cultures and the complex underlying molecular mechanisms, the phenomenon has been challenging to study. However, recent technological advances in microfluidics and reporter genes have improved this scenario. Here, we summarize recent progress in the field, revealing the ubiquitous bacterial stress alarmone ppGpp as an emerging central regulator of multidrug tolerance and persistence, both in stochastically and environmentally induced persistence. In several different organisms, toxin-antitoxin modules function as effectors of ppGpp-induced persistence.

Introduction

The frequent failure of antibiotic treatment is an acute public health problem. The most apparent reason is that the successful use of any therapeutic agent is compromised by the development of bacterial resistance. Indeed, it was not long after the beginning of the antibiotic era that the first resistant organisms appeared (Abraham and Chain, 1940). It is now known that bacteria develop resistance toward most if not all antibiotics that are used clinically. Moreover, the wide-ranging use of antibiotics in the general population, agriculture, farming, and hospitals has increased the rate with which multidrug-resistant bacteria appear. Although antibiotic resistance is a major culprit, there are less obvious reasons for antibiotics to fail. One reason is that bacteria can escape the lethal action of antibiotics by entering a physiological state in which the antibiotics do not kill them, a phenomenon known as bacterial persistence. This phenomenon was first observed by Joseph Bigger, who discovered that penicillin often failed to sterilize flask cultures of exponentially growing *Staphylococcus aureus* cells (Bigger, 1944). Bacterial persistence can be quantified by following the killing kinetics upon the addition of a bactericidal antibiotic to a growing culture. As shown in Figure 1, addition of a bactericidal antibiotic rapidly killed the vast majority of the cells in a growing bacterial culture. However, after a few hours of treatment, the killing rate decreased dramatically. The tail of the killing curve revealed that the clonal population contained rare cells that were transitory tolerant to the drug and thereby managed to survive (Lewis, 2010). Here, the term "bacterial persistence" will refer to the phenomenon that isogenic populations of antibiotic-sensitive bacteria produce rare cells that transiently become multidrug tolerant.

Bacterial persistence is distinct from antibiotic resistance in the sense that, unlike resistant mutants, persister cells do not proliferate in the presence of the bactericidal agent but, randomly in time, switch back to a growing state, as revealed by the second slope of the biphasic killing curve (Figure 1). Consequently, when the antibiotic was removed, the cells gave rise to a population that was as sensitive as the original one and produced a similarly small proportion of persister cells (Keren et al., 2004a). This observation demonstrated that, as opposed to resistance, persistence is a noninherited phenomenon. The killing efficiencies of most of the clinically used antibiotics depend strongly on the physiological state of the target bacterium. For instance, slow-growing (or dormant) bacteria having a low metabolic activity are partly or completely refractory to killing by most antibiotics. This is because antibiotics usually kill bacteria by corrupting essential, active targets. In slow-growing cells, these targets are recalcitrant to the inhibitory action of the antibiotics and, hence, the bacteria become temporarily drug tolerant. Thus, it was proposed early on that persisters are cells that have entered a state of low metabolic activity, here referred to as dormant or slow-growing cells. Bigger's findings were largely ignored, but in the past decade a growing number of reports have focused on the persistence phenomenon that has been observed with all tested bacteria, including pathogens (Lewis, 2010). Thus, the phenomenon may contribute significantly to the failure to treat chronic and relapsing infections successfully with antibiotics.

Genetic Basis of Bacterial Persistence

hipA, the First "Persister" Gene

It has been proposed that bacterial persistence reflects accidental decline toward cell death (Nyström, 2003). Another view envisages that, while bacterial persistence is noninherited, the propensity to form persister cells is nevertheless a genetically evolved trait (Kussell and Leibler, 2005). Experimental support for the latter view is gradually accumulating. In the 1980s, Harris Moyed revisited the issue of antibiotic persistence by isolating

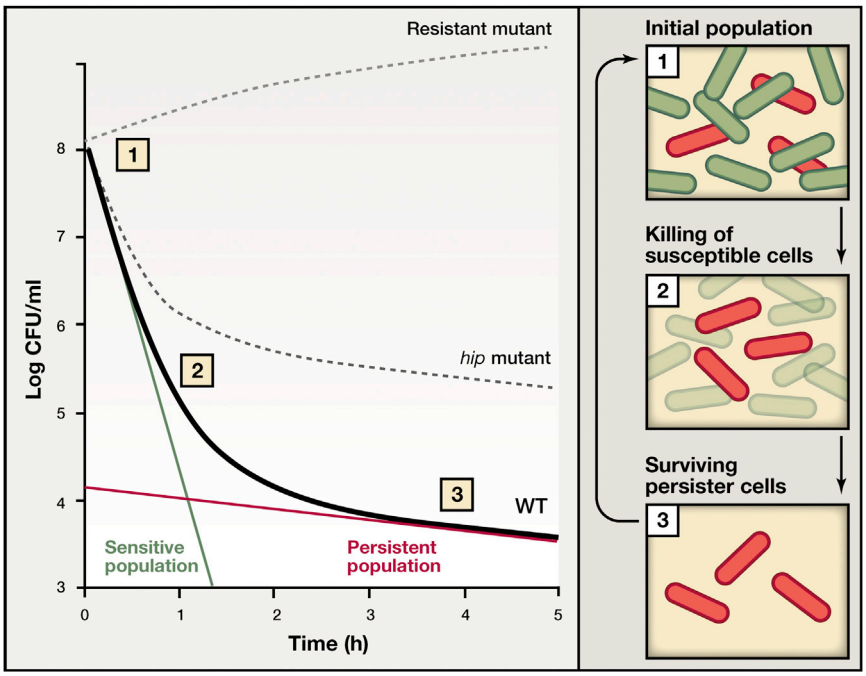

Figure 1. Killing Kinetics during Treatment with a Bactericidal Antibiotic

(1) Lethal dose of a bactericidal antibiotic is added at time zero to a growing population of sensitive, genetically identical bacteria. The experiment reveals a characteristic biphasic killing curve. (2) The slope of the initial phase reveals the susceptibility of the bulk of the population. The initial log-linear relationship reveals an exponential killing kinetics (green line). (3) The slope of the second inactivation phase (red line) reveals the existence of a persister subpopulation that is killed with a much slower kinetics. Killing kinetics for a high persister mutant (*hip*) strain producing a highly elevated number of persisters is also shown (dark dashed line). After removal of the antibiotic (pointed by the arrow flanking the right panels), persister cells resume growth and give rise to progeny cells that are genetically identical to the cells of the original population and, therefore, as drug-sensitive as the original cells. The gray dashed line indicates how a drug-resistant mutant strain would support growth under these conditions. Adapted from Lewis (2010).

mutants from *E. coli* that reproducibly formed high frequencies of persister cells (Moyed and Bertrand, 1983). Indeed, intermittent application of high doses of bactericidal antibiotics to a population of chemically mutagenized bacteria gave rise to the isolation of stable *hip* (high persister) mutants (Moyed and Bertrand, 1983; Wolfson et al., 1990). Notably, one gain-of-function allele, called *hipA7*, enhanced persistence up to 1,000-fold. This showed that the level of persisters could be increased as a result of a heritable mutation (Figure 1). The *hipA7* allele consisted of two separate nucleotide substitutions in *hipA*, a gene of 440 codons (Black et al., 1991; Moyed and Broderick, 1986). The *hipA* gene is preceded by *hipB* that encodes an autorepressor of *hipBA* transcription (Black et al., 1991, 1994). Overproduction of HipA inhibited cell growth by attenuation of translation, DNA replication, and transcription and strongly enhanced tolerance to bactericidal antibiotics (Korch and Hill, 2006). HipB interacts directly with HipA and inhibits its activity. These observations led to the suggestion that *hipBA* constitutes a bona fide toxin-antitoxin (TA) locus (Korch et al., 2003). The direct inhibition of HipA by HipB and the reduced interaction between HipB and HipA7 readily explained the *hipA7* phenotype because it would lead to hyperactivation of HipA that, in turn, would trigger persistence (Rotem et al., 2010; Schumacher et al., 2009). However, this explanation seemed at variance with the observation that HipA7 was less toxic than wild-type HipA (Korch and Hill, 2006), and the molecular mechanism behind the phenotype of the *hipA7* allele was not fully explained. Later analysis revealed that HipA is a eukaryote-like Ser/Thr kinase whose kinase activity was required for both inhibition of cell growth and the stimulation of persister cell formation (Correia et al., 2006). We and others discovered recently that HipA inactivated glutamyl tRNA synthetase (GltX) by phosphorylation (Germain et al., 2013; Kaspy et al., 2013). Inhibition of GltX stimulated accumulation of uncharged $tRNA^{Glu}$ and synthesis of ppGpp. In turn, the high level of ppGpp dramatically increased the persistence level (Germain et al., 2013). These observations raised the obvious and important question of how ppGpp mediates persistence.

Toxin-Antitoxins and Persistence

Since the discovery of *hipA* as a bona fide persister gene, numerous research articles support the notion that persistence of the model organism *Escherichia coli* depends on TA loci (Dörr et al., 2010; Keren et al., 2004b; Maisonneuve et al., 2011; Shah et al., 2006; Vázquez-Laslop et al., 2006). Prokaryotic TA loci code for two components, a stable "toxin" (always a protein) that inhibits cell growth and a labile "antitoxin" (either RNA or protein) that regulates toxin activity. The genetic architecture and the nature of regulation of TA activity gave rise to division of TAs into five classes. Type I and III TA loci encode small RNA antitoxins that counteract the toxins at the translational (antisense RNA) or posttranslational levels (direct toxin binding), respectively. Type II TA loci encode an antitoxin protein that combines with and neutralizes the toxin by direct interaction. A general organization and regulation of type II TA loci is presented in Figure 2. In type IV TAs, the antitoxins protect the toxin targets instead of inhibiting the toxin directly (Masuda et al., 2012). Type V antitoxins are site-specific endoribonucleases that inhibit toxin expression by cleavage of toxin-encoding mRNAs (Wang et al., 2012). In this Review, we will mainly focus on the role of type II TA loci in persistence. Interestingly, ectopic overproduction of type II toxins not only very efficiently inhibited cell growth, but also induced a nongrowing state from which the cells could be rapidly resuscitated by the induction of cognate antitoxin genes (Christensen-Dalsgaard and Gerdes, 2006; Christensen-Dalsgaard et al., 2010; Pedersen et al., 2002). Because of the ability to severely inhibit cell growth, it has been of particular interest to identify the cellular targets of the toxins. The targets of the toxins that have been identified are summarized in Figure 2. Important in this context, several reports showed that overproduction of