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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

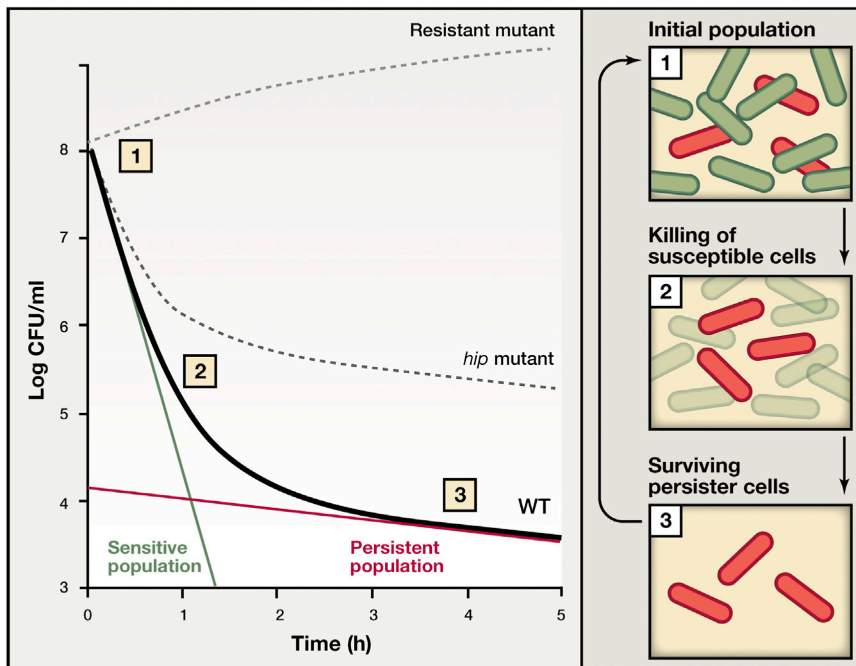

Figure 1. Killing Kinetics during Treatment with a Bactericidal Antibiotic

(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

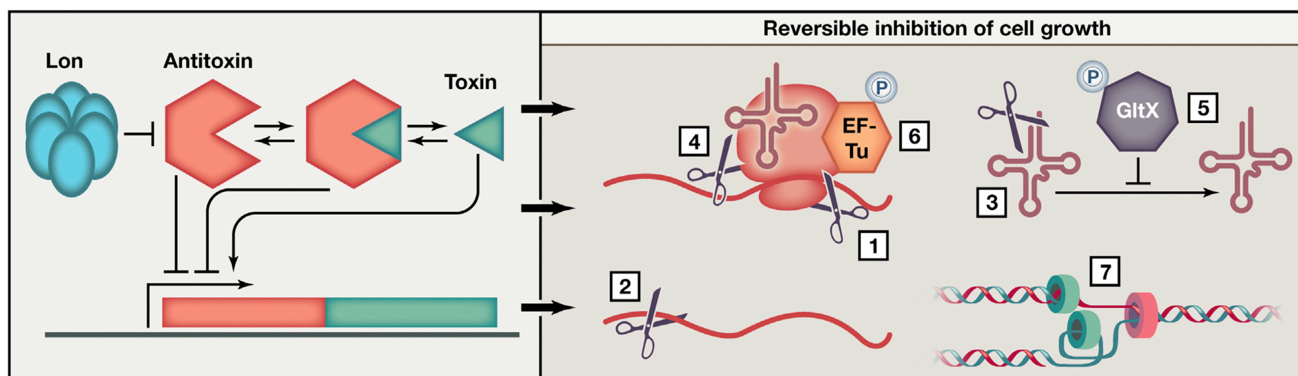

Figure 2. Canonical Type II Toxin: Antitoxin Genes and Their Cellular Targets

Schematic showing the genes and control loops of a canonical type II TA module. The protein antitoxin neutralizes the toxin by forming a tight complex with it. The TA operon is repressed by the antitoxin and by the TA complex. When the level of free toxin is low, the TA complex binds strongly and cooperatively to the promoter region and inhibits transcription. In contrast, transcription is derepressed by an excess of free toxin, a regulatory mechanism called conditional co-operativity (Overgaard et al., 2009). The antitoxins are degraded by cellular proteases (Lon or Clp). Hence, protease activity controls toxin activity by determining the level of antitoxin.

Cellular targets of the main toxins are shown in the right panel. (1) RelE inhibits translation by cleavage of mRNA at the ribosomal A site (Christensen and Gerdes, 2003). (2) MazF cleaves mRNAs in single-stranded regions (i.e., MazF, MqsR, HicA) (Christensen-Dalsgaard et al., 2010; Jørgensen et al., 2009; Zhang et al., 2003). (3) Enteric VapCs inhibit translation by cleavage of intitiator $tRNA^{fMet}$ (Winther and Gerdes, 2011). (4) VapC20 of *M. tuberculosis* inhibits translation by cleavage of the Sarcin-Ricin loop of 23S rRNA (Winther et al., 2013). (5) HipA of *E. coli* inhibits translation by phosphorylation of glutamyl-tRNA synthetase (Germain et al., 2013). (6) Doc of prophage P1 inhibits translation by phosphorylating EF-Tu (Castro-Roa et al., 2013). (7) CcdB (a MazF homolog) and ParE (a RelE homolog) inhibit DNA replication by inhibiting DNA gyrase (Bernard and Couturier, 1992; Jiang et al., 2002).

type II toxins dramatically increased persistence (Keren et al., 2004b; Maisonneuve et al., 2011; Shah et al., 2006; Vázquez-Laslop et al., 2006). That TA genes might be involved in persistence was further supported by the finding that TA mRNAs were among the most upregulated transcripts in the persister fraction isolated from a *hipA7* mutant (Keren et al., 2004b). A similar TA mRNA profile was observed in dormant cells collected from a growing population of wild-type *E. coli* cells by cell sorting (Shah et al., 2006). These observations prompted a genetic analysis of the 11 known type II TAs in *E. coli* (all of which encode mRNases except *hipBA*): deletion of any one of these loci had minor effects on the persistence level of planktonic *E. coli* cells (Keren et al., 2004b; Kim and Wood, 2010; Maisonneuve et al., 2011). By contrast, deletion of all 10 mRNase-encoding type II TA loci dramatically reduced persistence, showing that TA loci contribute cumulatively to persistence (Maisonneuve et al., 2011). Mathematical modeling of the connection between TAs and persistence supported this interpretation (Fasani and Savageau, 2013). In contrast, only one type I TA system has been shown to influence persister cell formation directly (Dörr et al., 2010).

Mechanisms of Persister Cell Formation

Stochastic Formation of Persister Cells: A Bet-Hedging Strategy

Adaptive mechanisms have evolved to sense harmful environmental conditions and enable cells to respond adequately to mitigate the detrimental effects. Nevertheless, a pre-existing phenotypic diversity (i.e., mixture of distinct phenotypes) that confers fitness advantages under specific harmful condition would provide another solution to survive fluctuating environments (Kussell and Leibler, 2005). Such types of cellular heterogeneity are often referred to as bet-hedging strategies (Dubnau and Losick, 2006; Veening et al., 2008). To elucidate the nature of bacterial persistence, it was crucial to know whether persister cells differ from others cells before exposure to antibiotics. Due to the tiny frequency of persister cells in a bacterial population (between 10^{-6} and 10^{-4}), this task has been challenging to achieve. However, the last decade has seen the development of powerful tools that have enabled the analysis of single cells en mass by microscopy, thereby surmounting the limits associated with observations of ensembles of cells. In particular, these technological advances have enabled the identification and analysis of very rare cells that are phenotypically different from the main population. First, Stanislav Leibler and colleagues investigated the persistence of *E. coli* at the level of single cells (Balaban et al., 2004). Using fluorescent-based microscopy combined with microfluidics, they were able to record the behavior of individual cells before, during, and after treatment by a β-lactam antibiotic (ampicillin) and subsequently follow the genealogy of the persister cells. Such measurements were made possible only by the use of a *hip*A7 mutant of *E. coli* that produces ~1,000-fold increased level of persisters (Balaban et al., 2004). This approach validated Bigger's initial hunch that persisters comprise a pre-existing subpopulation that are temporarily in a slow or nongrowing state and are thereby tolerant to bactericidal antibiotics (Balaban et al., 2004). These observations refuted the hypothesis that *E. coli* persisters are mainly formed as a response to antibiotics and raised the important question of the underlying molecular mechanism.

It is well known that all cells are subjected to both intrinsic and extrinsic molecular noise that influences gene expression in individual cells (Elowitz et al., 2002). However, noise by itself is not able to trigger pronounced phenotypic diversity unless regulatory enhancement processes (i.e., positive feedback) are involved (Eldar and Elowitz, 2010). This so-called noise amplification

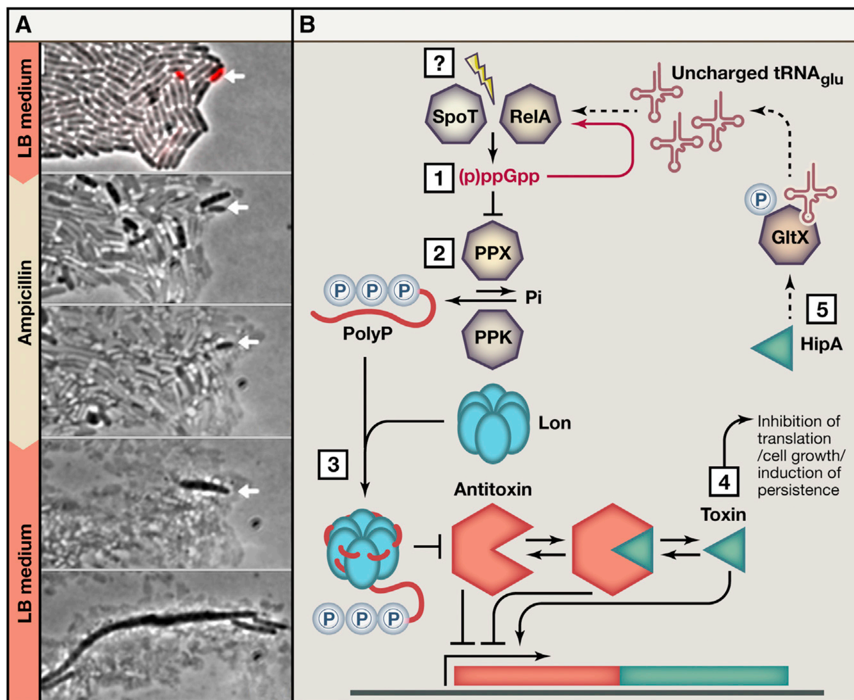

Figure 3. Mechanism underlying ppGpp-Controlled Bacterial Persistence

(A) Time-lapse microscopy experiment showing that single *E. coli* cells with a high level of [ppGpp] as revealed by expression of the "ppGpp-proxy" RpoS-mCherry survive antibiotic treatment.

(B) Current model for how ppGpp stochastically induce persistence in *E. coli*. (1) Activation of RelA or SpoT in single cells promotes accumulation of ppGpp. (2) The ppGpp-dependent inhibition of exopolyphosphatase (PPX), the cellular enzyme that degrades PolyP, promotes PolyP accumulation by the constitutively active polyphosphate kinase (PPK). (3) PolyP combines with and stimulates Lon to degrade all 11 type II antitoxins of *E. coli* K-12. (4) Free and activated toxins inhibit translation and cell growth and thereby induce persistence. (5) Speculative positive-feedback loop that ensures even more synthesis of ppGpp. The model predicts that degradation of HipB enables free HipA to phosphorylate glutamyl-tRNA synthetase (GltX) and inhibits charging of $tRNA^{Glu}$. The resulting uncharged tRNAs enter the ribosomal A site and trigger RelA-dependent synthesis of (p)ppGpp. Adapted from Germain et al. (2013) and Maisonneuve et al. (2013).

process leads to the emergence of bistability (or metastability): that is, the establishment of two (or more) phenotypically stable subpopulations of cells that coexist in a genetically identical population. Moreover, heterogeneity may exist even within such subpopulations, thereby possibly explaining that persister subpopulations generated by similar underlying molecular mechanisms may exhibit antibiotic-specific levels of persistence.

Theoretical and experimental single-cell analysis of *hipBA* showed that a threshold level of HipA was necessary to induce dormancy. Moreover, the quantity by which this threshold was surpassed determined the time of HipA-induced dormancy (Rotem et al., 2010). Thus, fluctuations of toxin levels above and below the threshold determine the onset as well as the duration of growth arrest, resulting in the co-occurrence of dormant and growing cells. Moreover, mathematical simulations of type II TAs indicated that their general architecture and regulation (Figure 2) provide the potential for a robust stochastic switch between the ON and OFF states. This switch can be driven either by the proteolysis of the antitoxin, stochastic fluctuation or by any change in the growth rate (Cataudella et al., 2012, 2013; Fasani and Savageau, 2013; Gelens et al., 2013; Lou et al., 2008). Moreover we and others described that transcription of evolutionary independent TA operons, such as *relBE*, *vapBC*, and *phd/doc*, is regulated by a phenomenon called conditional cooperativity. As described further below, conditional cooperativity refers to the ability of a given toxin to act as both a corepressor and a derepressor of its own TA operon (Afif et al., 2001; Garcia-Pino et al., 2010; Overgaard et al., 2008; Winther and Gerdes, 2012) (Figure 2). It is thus possible that conditional cooperativity provides a means of tight control of the bistable switch to gene expression and toxin activation (Cataudella et al., 2013).

A Model for Stochastic Induction of Bacterial Persistence in E. Coli

We addressed recently the question of whether TA activation in a small fraction of cells could be connected to persister formation. Using transcriptional fusions between two representative TA loci (*relBE* and *yefM yoeB*) and *gfp*, we discovered that TA operon transcription was stochastically turned on at low frequencies in exponentially growing *E. coli* cells (Maisonneuve et al., 2013). The switching rate was similar to that of the frequency of persistence. Using microfluidics and fluorescent reporter gene fusions, we established that the TA operon ON cells were phenotypically associated with cessation of growth. These rare cells were tolerant to high doses of β-lactam antibiotic and could resume growth once the drug was removed (Maisonneuve et al., 2013). These observations established that the toxins encoded by TA genes are the effectors of bacterial persister cell formation.

We also established that ppGpp, the common regulator of the bacterial stringent response, controls *E. coli* persister formation by stochastically switching to a high level in single cells (Maisonneuve et al., 2013) (Figure 3). We then uncovered a linear, hierarchical signaling pathway connecting TA activity to ppGpp that invokes inorganic polyphosphate (PolyP) and Lon protease as explained in detail in Figure 3 (Maisonneuve et al., 2013; Maisonneuve et al., 2011).

RelA (the main enzyme responsible for ppGpp accumulation during amino acid starvation in *E. coli*) is positively stimulated by ppGpp, its own product (Shyp et al., 2012). Predictably, such positive feedback would sustain rapid increases of [ppGpp] that, in turn, would amplify both intrinsic (i.e., transcription and translation rates) and extrinsic noise (fluctuations of [PolyP] and thereby in Lon activity) that could activate toxins and thereby contribute to persister cell formation (Figure 3). The fact that no

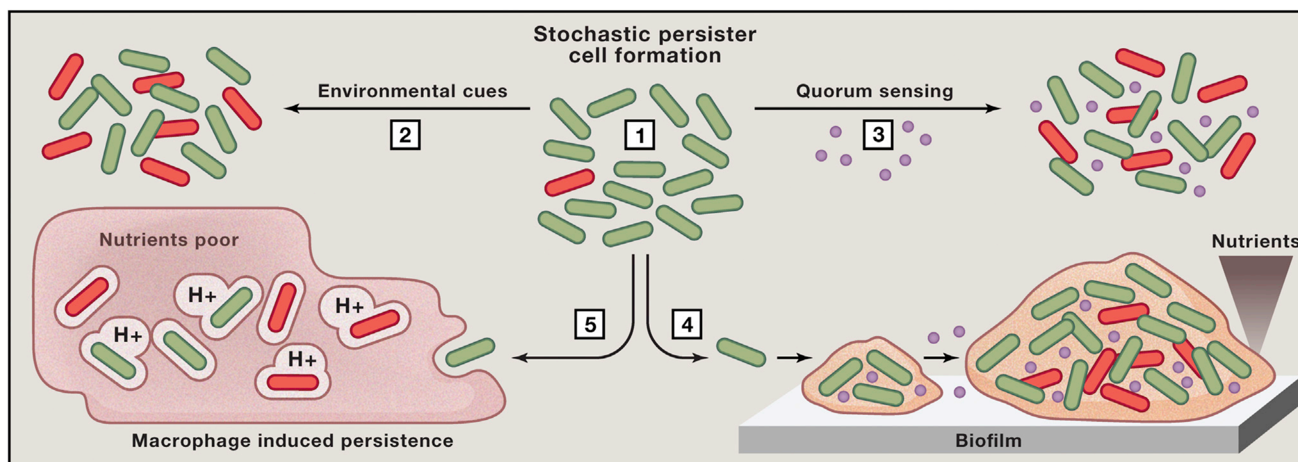

Figure 4. Overview of Physiological and Environmental Cues Stimulating Persister Cell Formation
(1) Bacterial persisters can arise stochastically in unstressed bacterial cultures as a bet-hedging strategy. (2) Environmental insults (i.e., starvation, oxidative and acid stress, heat shock) provoking persister cell formation. (3) Social engagement through quorum sensing promotes persister cell formation. (4) Heterogeneous and diffusion-limited biofilm microenvironments enhance persistence. (5) Host-pathogen interaction also induces formation of persisters.

apparent environmental inputs were required for *E.coli* persister formation raised the possibility that such cells were generated as a consequence of purely random stochastic processes. Obviously this does not preclude a role for environmental factors in persistence. In fact, external factors could have a modulatory or a stimulatory effect on the rate of switching by affecting a deterministic component. For instance, it is well established that the fraction of persisters in cultures of both Gram-negative and Gram-positive bacteria increases substantially with cell density (Keren et al., 2004a; Maisonneuve et al., 2013). At stationary phase, when the bacteria stop increasing in number, or when growing in biofilm, at least 1% of the bacterial cells become antibiotic tolerant. Thus, we suggest that rare cells in exponentially growing populations may encounter a "micro-starvation" environment that triggers ppGpp synthesis and, thus, persister formation (Maisonneuve et al., 2013).

Lon protease degrades all known antitoxins of *E. coli* K-12 (Gerdes and Maisonneuve, 2012), including HipB (Hansen et al., 2012). Because HipA activation leads to ppGpp synthesis (op. cit.), an increased level of HipA activity is predicted to generate a positive-feedback loop that ensures even more synthesis of ppGpp (Figure 3). This increase could also contribute to stabilize the TA ON state and could thereby prolong the persister state. However, a *hipBA* deletion strain did not exhibit a reduced level of persisters (Falla and Chopra, 1998; Hansen et al., 2008; Korch et al., 2003), and more research is required to clarify this issue.

Environmental Induction of Bacterial Persistence

A *plethora* of environmental signals increase persister levels among isogenic bacterial populations. For instance, the fraction of persisters increases during nutrient limitation and during diauxic carbon-source transition (Amato et al., 2013; Bernier et al., 2013; Fung et al., 2010; Keren et al., 2004a; Leung and Lévesque, 2012; Nguyen et al., 2011). Pretreatment with subinhibitory concentrations of antibiotics, oxidative stress, heat shock, or DNA-damaging agents all increase antibiotic tolerance as part of the bacterial adaptive response (Dörr et al., 2010; Leung and Lévesque, 2012; Möker et al., 2010; Vega et al., 2012; Wu et al., 2012) (Figure 4).

Social engagements can promote cell individuality among bacterial populations through chemical signaling communication, a phenomenon called quorum sensing (QS) (Bassler and Losick, 2006). The strong increase in persistence as a bacterial culture reaches late exponential phase resembles the dynamics of QS (Keren et al., 2004a; Lewis, 2010). Interestingly, addition of spent media from stationary cultures induced a significant increase of persisters of a growing culture of *P. aeruginosa*, but not of *E. coli* or *S. aureus* cultures (Möker et al., 2010). Indeed, *P. aeruginosa* modulates the persister frequency in response to the QS-signaling molecules pyocyanin and acyl-homoserine lactone (Möker et al., 2010). However, deletion of the biosynthetic pathway for pyocyanin had no effect on persister cell formation, raising the possibility that additional or compensatory signaling systems control the persister phenotype in this organism. In addition, the cariogenic organism *Streptococcus mutans* generates persister cells that are positively influenced by the stress-inducible QS pheromone peptide (CSP) (Leung and Lévesque, 2012) (Figure 4).

More recently, bacterial communication through indole was suggested to induce persister cell formation in *E. coli* (Vega et al., 2012). Indole, produced under nutrient-limited growth conditions, altered the probability of a single cell to become persister (Vega et al., 2012). Remarkably, indole also induced persister formation in a *Salmonella* Typhimurium culture, even though this bacterium does not normally produce indole. Thus, persister modulation by chemical signaling is not limited to intraspecies communication and could also confer a cross-species bet-hedging strategy by interception of nonnative bacterial-signaling molecules (Vega et al., 2013).

Signaling Pathways behind Induced Persistence

The "alarmone" ppGpp is almost ubiquitous in the bacterial domain and plays a central role in environmentally induced

persister cell formation. It was discovered as the effector molecule of the stringent response that is activated by a host of different environmental stresses in almost all bacteria. In brief, the stringent response reprograms cellular metabolism from rapid to slow growth by simultaneously inhibiting and stimulating the expression of ~500 genes in *E. coli* (Durfee et al., 2008; Traxler et al., 2008). Importantly, ppGpp binds directly to RNA polymerase, thereby conferring inhibition of stable RNA (rRNA and tRNA) promoters and stimulation of amino acid biosynthetic promoters. ppGpp also stimulates accumulation of RpoS, the master regulator of the general stress response. Many of these changes also occur when bacteria enter stationary phase, and in general, these response networks are essential for bacterial survival and for virulence (Dalebroux et al., 2010). Interestingly, when the two genes of *E. coli* coding for the enzymes that produce ppGpp, *relA* and *spoT* (coding, respectively, for ppGpp synthetases I and II), are deleted, persister cells become extremely rare in exponentially growing planktonic cultures (Korch et al., 2003; Maisonneuve et al., 2013). Moreover, basal levels of ppGpp are required to sustain tolerance to cell-wall active antibiotics of the Gram-positive pathogens *E. faecalis and S.aureus* (Gaca et al., 2013; Geiger et al., 2014).

More recently, Amato et al. (2013) proposed a model explaining how, in *E. coli*, the "ppGpp network" may lead to persistence (Amato et al., 2013). Their model was derived from observations seen under diauxie—that is, growth with two different carbon sources, usually sugars. In the transition phase between the utilization of two sugars, the cells elicited the stringent response and ppGpp increased. In keeping with our model (Figure 3), Amato et al. found that diauxie-induced persistence also depended on ppGpp. However, the diauxie-induced persistence appeared to be specific for a fluoroquinolone and depended on the transcription factor DksA and nucleoid-associated proteins. Because only single deletions of TA genes were tested, the study was inconclusive as to the involvement of these genes in diauxie-induced persistence.

During pathogenesis, bacteria often form biofilms within which the bacterial cells are embedded in a three-dimensional matrix, providing protection during harmful conditions. In biofilms, bacteria often encounter limited access to nutrients and therefore elicit the stringent response (Figure 4). Consistently, multidrug tolerance of *P. aeruginosa* and *E. coli* grown in biofilms depended on ppGpp (Bernier et al., 2013; Maisonneuve et al., 2013; Nguyen et al., 2011). Interestingly, the model proposed by Nguyen and colleagues highlights an active mechanism involving the stringent response (Nguyen et al., 2011). However, it was not tested whether TAs were required for the ppGpp-mediated increase in persistence. Importantly, we observed that starvation failed to induce persistence in biofilms of *E. coli* cells unable to activate stringent response, lacking PolyP, Lon, or TA genes (Maisonneuve et al., 2013).

During pathogenesis, bacteria also encounter a variety of stresses in their hosts that may trigger persister cell formation. Single-cell microscopy of *S.* Typhimurium showed that, upon macrophage phagocytosis, some bacteria initiated replication, whereas others entered a nonreplicative state, even within the same macrophage (Helaine et al., 2010). Part of these nonreplicative cells were in a persister-like state that enabled them to survive long-term, host-induced damage and exposure to antibiotics (Helaine et al., 2014) (Figure 4). The *Salmonella*-containing vacuoles of macrophages are relatively acidic and nutrient poor (Dandekar et al., 2012). Consequently, bacteria within these vacuoles elicit the stringent response and become persisters (Figure 4). Consistent with this conjecture, *Salmonella* mutants impaired in the stringent response exhibited a highly reduced macrophage-induced persister formation. Moreover, macrophage-induced persisters depended on both Lon protease and TA genes (Helaine et al., 2014), thus lending support to the notion that ppGpp-induced persister formation is not confined to *E. coli*. Rather, these observations indicate that *S.* Typhimurium uses a mechanism very similar to that of *E. coli* growing planctonically to induce persisters (Maisonneuve et al., 2013). However, unlike *E. coli* persisters, the Lon-dependent persister induction pathway of *Salmonella* responded specifically to signals encountered within host cells (Helaine et al., 2014).

In addition to the stringent response, activation of the bacterial SOS response, a signaling pathway that upregulates DNA repair functions, appears to be associated with bacterial persistence. Indeed, *E. coli* mutants with defects in SOS response genes (i.e., *lexA*, *recA*, or *recB*) exhibited reduced persister levels when treated with fluoroquinolones (Dörr et al., 2009). This work also suggested an active and inducible mechanism of persister formation mediated by the SOS response (Dörr et al., 2009). Induction of the SOS response increased persistence to fluoroquinolones dramatically. This increase depended on the DNA-damage-inducible TisB toxin encoded by a type I TA locus (Dörr et al., 2010). TisB is a small, 29 amino acid polypeptide that associates with the cell membrane and decreases the proton motive force and ATP levels (Unoson and Wagner, 2008). These physiological effects may directly or indirectly lead to TisB-dependent multidrug tolerance.

It has been proposed that generation of reactive oxygen species (ROS) is central to cell killing by bactericidal antibiotics (Kohanski et al., 2007; Kohanski et al., 2008). If true, mechanisms that reduce the intracellular level of ROS should increase drug tolerance. For instance, treatment with H_2S, a gas known to quench ROS production by iron ferrous sequestration, promotes multidrug tolerance in a broad range of bacteria (Shatalin et al., 2011). Moreover, activation of catalase, superoxide dismutase, and antioxidant enzymes that are known to reduce cellular level of ROS was associated with multidrug tolerance in planktonic and biofilm associated bacteria. However, recent work demonstrating that bacteriocidal antibiotics also kill bacteria under anaerobic conditions is at variance with the hypothesis that ROS are major killing agents generated by antibiotics (Keren et al., 2013; Liu and Imlay, 2013). That ROS are dispensable for the action of bactericidal antibiotics was supported by several additional reports (Ezraty et al., 2013; Mahoney and Silhavy, 2013). However, these observations are of course not inconsistent with the proposal that ROS generated by antibiotics interacting with their targets play a minor role in bacterial cell killing.

Recently, it was reported that a subpopulation of *M. smegmatis* cells survived treatment by the bactericidal drug isoniazid (Wakamoto et al., 2013), a prodrug that requires activation by the bacterial enzyme catalase. Using microfluidics, these

authors elegantly showed that expression of catalase varied stochastically in single cells. Thus, the surviving subpopulation of bacteria was able to grow in the presence of isoniazid because it did not activate the drug. We suggest that such drug escape is better viewed as an atypical case of drug avoider, rather than the multidrug tolerance phenotype exhibited by slow-growing persister cells.

Resuscitation of Persistent Bacteria

To generate viable offspring, persister cells must be able to resume growth. The rate of resuscitation of persisters is revealed by the slope of the curve in the second phase of the classical persister experiment shown in Figure 1. Ideally, the low killing rate reflects the resuscitation rate because persisters reverting to grow in the presence of the antibiotic are killed. The constant rate of killing suggests that resuscitation is a stochastic event occurring randomly in time (Rotem et al., 2010). The molecular mechanisms behind the switching back to growth after dormancy are largely unknown due mainly to the lack of experimental approaches that can be employed to attack the problem. Thus, at present, the mechanistic basis behind resuscitation of persisters can only be speculated upon. In the persistent state, the cellular translation rate is low. The low global translation rate is assumed to support a high toxin/antitoxin ratio because the antitoxins are less stable than the toxins. In turn, the T/A ratio favors derepression of TA operon due to conditional cooperativity and sustains toxin production in persisters (Figure 2). Mathematical modeling argued that conditional cooperativity may also function to quench toxin activity rapidly when the signal (high levels of ppGpp, or Poly(P)) that triggers antitoxin degradation is diluted out (Cataudella et al., 2012, 2013). Neutralization of the toxins, in turn, allows for resumption of cell growth.

Other active events may be required for a rapid quenching or alteration of the original signal, and we speculate that inhibitors of Lon, Poly(P), or ppGpp synthesis help persisters to resuscitate. We are currently addressing this possibility experimentally. Obviously, a better understanding of the outgrowth mechanism may facilitate the design of strategies that reduce persistence.

Clinical Relevance of Persister Cells

Given that persisters are produced by many pathogenic bacteria such as *E. coli*, *S. aureus*, *M. tuberculosis*, *P. aeruginosa* and the fungus *Candida albicans* (Dhar and McKinney, 2007), the implications of microbial persistence to antibiotics failure are believed to be significant. Theoretical arguments initially supported a role for bacterial persistence in the outcome of antibiotic therapy. Indeed, mathematical modeling predicted that persister cells would (1) prolong the time before an infecting population of bacteria is cleared and (2) preclude the eradication of infection (Levin and Rozen, 2006). As described previously, Harris Moyed observed that repetitive application of high doses of bactericidal antibiotics to a population of mutagenized *E. coli* cells led to the selection of *hip* mutants that stably produced increased levels of persisters (Moyed and Bertrand, 1983). Because periodic application of high doses of antibiotics is used routinely for treatments of chronic infections, it was assumed that the emergence of *hip* mutants could provide a selective advantage to the pathogens and would be causally connected to recalcitrance of the disease. Indeed, such *hip* mutants were selected in vivo during the course of antimicrobial therapy in infections caused by *C. albicans* (Lafleur et al., 2010) and *P. aeruginosa* (Mulcahy et al., 2010). Thus, the persistence phenomenon undoubtedly underlies many recurrent clinical infections. Furthermore, it was suggested that persisters could support and even enhance the development of heritable genetic resistance. Indeed, the same stress response pathway that is important for the generation of persisters can also accelerate random mutation or horizontal gene transfer, thereby promoting the generation of antibiotic-resistant mutants (Al Mamun et al., 2012; Beaber et al., 2004). The importance of persisters in the recalcitrance of infectious diseases urges us to design new drug strategies to kill both actively dividing cells and persisters effectively.

Eradication of Bacterial Persistence in Future Treatment Regimens

As our mechanistic understanding of persister cell formation expands, new strategies for tackling the problem must be developed. As mentioned above, persisters are often functionally isolated as slow-growing or dormant cells. Consequently, efforts have been devoted to develop strategies that minimize heterogeneity by nudging resuscitation of dormant cells and thereby rendering the bacterial population homogeneously susceptible to antibiotics. Using metabolic network analysis, Collins' group found that simple sugars like mannitol, fructose, or glucose in combination with gentamicin (a generic aminoglycoside antibiotic) are able to decrease the number of persisters by >1,000-fold in both Gram-negative (*E. coli*) and Gram-positive (*Staphylococcus aureus*) bacterial populations (Allison et al., 2011). Interestingly, these metabolites did not restore normal growth of persisters but instead energized the bacterial cell membrane by increasing the proton motive force that, in turn, increased the uptake rates of aminoglycosides. This metabolic adjuvant approach is probably limited to aminoglycosides, as the approach failed to reduce the level of cells tolerant to β-lactam or fluoroquinolone antibiotics. Kim et al. (2011) used high-throughput chemical screening for drugs that selectively kill persisters without affecting normal growth of *E. coli.* This led to the isolation of a compound that facilitated the killing of persisters that survived ampicillin or ciprofloxacin treatments. The compound considerably shortened the lag time needed for persisters to resume growth after antibiotic exposure, suggesting that it stimulates or accelerates reversion of persisters to antibiotic-sensitive cells.

Most bactericidal antibiotics kill bacteria by corrupting a cellular target whose activity is required during active cell growth (e.g., ampicillin kills bacteria by inhibiting cell-wall synthesizing enzymes). An alternative approach is to use antibacterial compounds that corrupt their cellular targets not only in growing cells, but also in resting cells. Acyldeptipeptides represent a class of antibiotics that activate ClpP, the core unit of a ubiquitous bacterial protease complex (Brötz-Oesterhelt et al., 2005). Acyldeptipeptides bind directly to ClpP and unleash unregulated and lethal protein degradation. By conjecture, such proteolytic avalanche should be lethal also for nongrowing bacteria. Kim Lewis' group tested this inference and found that one such acyldeptipeptide (ADEP4) produced complete eradication of

Staphylococcus aureus biofilms in vitro (Conlon et al., 2013). Most importantly, when administered concomitantly with a conventional antibiotic, ADEP4 cleared a deep-seated murine biofilm infection caused by *S. aureus* (Conlon et al., 2013). Finally, silver has been shown to enhance the action of conventional antibiotics against Gram-negative bacteria both in vitro and in a mouse biofilm infection model (Morones-Ramirez et al., 2013). However, the practical implications of this discovery remain to be seen, given that silver is a potent human poison.

Having shown that the stringent response is central to persister induction and virulence, it is conceivable that inhibitors of the stringent response could have therapeutic potentials. The search for such potential inhibitors has already begun. For instance, synthetic ppGpp analogs have been developed that efficiently inhibit the ppGpp synthetase activity of Rel proteins and thereby interfere with the long-term survival of Gram-positive bacteria (Wexselblatt et al., 2012).

Conclusions

Recent insights into bacterial persisters have revealed that multiple mechanisms underlie the phenomenon. Given the physiological heterogeneity and complexity of each cell, it is evident that persisters can emerge as a result of different tolerance-associated mechanisms. However, the general involvement of stress response pathways, in particular the stringent response, suggests that persister formation is an evolved and deterministic bet-hedging strategy used by bacteria to survive rapid and potentially lethal environmental changes. So far, the majority of studies have been restricted to the understanding of antibiotic tolerance. Persisters are also tolerant to heavy metals (Harrison et al., 2005), but the inference that persisters are tolerant to general stresses, such as high osmolarity, heat, or extreme pHs, remains to be examined.

The underlying cellular heterogeneity presents interesting challenges for the development of sophisticated techniques to study single-cell transcriptomics and metabolomics. Although persisters are fundamentally distinct from resistant cells, the stress response mechanisms described here raise the possibility that persisters contribute to or even enable relapsing and chronic infections and may sustain the development of resistance. Present and future insights into persister mechanisms and the action of antibiotics will be used to develop clinical methods to combat the consequences of this phenomenon. Research on bacterial persistence may also assist the study of other medical problems. For instance, similarly to bacteria, cancer cell populations exhibit heterogeneity, as revealed by the existence of slow-growing, drug-tolerant subpopulations that, after treatment, may revert to the malignant, rapidly growing state (Sharma et al., 2010). Thus, similar to bacteria, cancer cells may exploit population heterogeneity to escape drug treatment.

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