Trends in Genetics

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REVIEWS

Review

General Mechanisms Leading to Persister Formation and Awakening

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All bacterial populations harbor a small fraction of transiently antibiotic-tolerant cells called persisters. These phenotypic variants compromise successful antibiotic treatment because they are held responsible for the relapse of many chronic infections. In addition, studies employing experimental evolution have demonstrated that persistence contributes to the development of antibiotic resistance. Persisters are typically described as dormant cells. However, recent findings indicate a role for active mechanisms in the formation and maintenance of the persister phenotype. This review summarizes novel insights into the molecular mechanisms of persister formation and awakening, focusing on changes in cell physiology mediated by persistence effectors.

Highlights

Experimental evidence is accumulating for the contribution of persister cells to the recalcitrance of chronic infections and the increased development of antibiotic resistance.

Target inactivity and dormancy caused by energy loss, halted DNA replication, and blocked translation contribute to persister formation.

Target inactivity and dormancy cannot fully explain the complex nature of persister cells because some persisters rely on intrinsic mechanisms to repair antibiotic damage or to lower the intracellular antibiotic concentration.

Depending on the persistence effector at play, persisters can have distinct physiologies that contribute to their heterogeneity within bacterial populations.

In vitro evolution experiments have shown that persistence is a highly evolvable trait.

The Importance of Persistence

When a bacterial infection is treated with an antibiotic, complete sterilization of the population is hardly ever achieved. The best-known cause of treatment failure is the presence of antibiotic-**resistant** (see Glossary) mutants. However, even if bacteria are susceptible to the antibiotic, populations always harbor a small fraction of cells that are **tolerant** to the lethal activity of the antibiotic. This subpopulation consists of so-called persister cells that are genetically identical to the bulk of the population but differ in their tolerance phenotype. Persisters are most often described as **dormant** cells that survive antibiotic treatment because of **target inactivity** [1], although studies on more active **persistence** mechanisms have questioned this view [2–4]. In general, the antibiotic tolerance of persisters, combined with their ability to reinitiate growth after treatment, may result in therapy failure and relapse of the infection [1,5].

Persisters have been identified in almost every bacterial species investigated to date, and even populations of eukaryotic microorganisms harbor similar phenotypic variants. Several lines of evidence support a relation between persistence and clinical therapy failure for major microbial pathogens such as *Pseudomonas aeruginosa* [6], *Staphylococcus aureus* [7], *Mycobacterium tuberculosis* [8], uropathogenic *Escherichia coli* [9], and *Candida albicans* [10]. Especially when shielded from the immune system, as is the case in biofilms or inside host cells, persisters might prove difficult to eradicate despite prolonged antibiotic treatment [11,12]. In addition, evolutionary adaptation of persister levels has been reported *in vitro* (Box 1) as well as during treatment of infections. Indeed, clinical isolates from chronic infections by *P. aeruginosa*, *C. albicans*, and uropathogenic *E. coli* that have been under prolonged antibiotic pressure often exhibit increased persister levels, whereas isolates from acute or early-stage infections do not (see Outstanding Questions) [6,9,10].

The clinical implications of persister cells likely reach beyond relapsing infections. Because persisters constitute a subpopulation of cells that are protected from lethal antibiotic stress, they remain viable in the presence of antibiotics and can accumulate resistance mutations. This idea was already put forward long ago [13], but was later reformulated and supported by mathematical simulations [14] and experimental evidence. Sebastian *et al.* showed that *de*

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Box 1. Persistence Is a Highly Evolvable Trait

The strong evolvability of microorganisms facing antibiotic stress is well established by theoretical predictions as well as by mounting experimental evidence. Whereas genetic resistance rapidly evolves in the continuous presence of sublethal antibiotic concentrations [23,109], high-dose periodic treatment is predicted to favor strains exhibiting high persister levels [25,110,111]. In agreement with these predictions, several independent studies have reported the selective advantage of high-persistence strains during frequent exposure to high antibiotic doses. In an early study performed by Moyed and Bertrand, a high-persistence mutant *hipA7* was selected by repeated application of high ampicillin doses to a population of mutagenized *E. coli* [112]. Importantly, this mutation was later found in clinical isolates of urinary tract infections [113]. More recent research showed a fast increase of population-wide antibiotic tolerance when wild-type *E. coli* and *S. aureus* were intermittently treated with ampicillin and daptomycin, respectively [114,115]. A comparable study demonstrated that daily high-dose aminoglycoside treatment rapidly selects for *E. coli* strains with strongly increased persister levels that match the frequency of environmental fluctuations [24]. Notably, similar evolutionary dynamics were observed in some major bacterial pathogens [116]. Taken together, these experiments indicate that persistence is a highly evolvable trait that can be shaped by periodic antibiotic therapy.

novo resistant mutants emerge during long-term exposure of *M. tuberculosis* persisters, a process provoked by oxidative stress [15]. Another study demonstrated that intermittent ampicillin exposure of *E. coli* selects for increased population-wide tolerance or persistence before resistant mutants arise [16]. Furthermore, an independent study not only established a positive correlation between persister levels and the emergence of resistance in different natural *E. coli* strains, but also between persistence and mutation rates [17]. Mutational processes in the surviving persister subpopulation may be accelerated by the activation of stress responses. Indeed, several stress responses, such as the stringent response and the SOS response, are known for their role in both persister formation and stress-induced mutagenesis [2,18–20]. Furthermore, the evolutionary path towards resistance might be easier to take for a strain that already carries a persistence mutation because several persistence genes have also been implicated in resistance. Examples are genes encoding drug efflux pumps [21] and operons such as *nuo* and *opp* [22–24].

Persistence is often considered as a **bet-hedging** strategy [25]. Indeed, although investing in non- or slowly growing persister cells slightly reduces the overall population fitness under optimal growth conditions, these phenotypic variants are well adapted to survive future stressful environments, thereby saving the population from extinction [26]. In line with this view, mathematical models commonly assume that persistence is governed by phenotypic switching that occurs stochastically [27]. It has been hypothesized that persistence is induced when the concentration of a persister protein exceeds a certain threshold (Figure 1). In addition to stochastic fluctuations, several environmental stimuli (Box 2) induce persistence by increasing the average expression level of persister proteins, allowing more cells to reach the threshold level [5,28].

In this review we discuss recent insights into the molecular mechanisms leading to persistence. Proteins or peptides that directly contribute to the persistence phenotype will be referred to as persistence effectors. We focus on the most prominent mechanisms of these effectors and on the alterations they induce on cell physiology in relation to the persistence phenotype. In addition, the latest findings on persister awakening are summarized.

How To Become a Persister Cell?

Since their discovery, persister cells have typically been described as dormant cells that survive antibiotic exposure because of target inactivity [1,29]. A landmark study demonstrated that a bacterial cell that has stochastically ceased growth survives ampicillin treatment and resumes growth following antibiotic removal [27]. Furthermore, persisters from an exponential-phase culture were shown to have a lower metabolic activity than their antibiotic-sensitive kin [30]. Many reports indicate that persistence effectors that depolarize the membrane, induce ATP efflux, block translation, or inhibit DNA replication are associated with cellular dormancy and/or target inactivity.

Glossary

Aminoglycoside: antibiotics of this class, such as amikacin, induce protein mistranslation. Their uptake into the bacterial cell depends on the membrane potential.
Bet-hedging: a survival strategy that relies on phenotypic heterogeneity in isogenic populations. The presence of phenotypic variants increases the chances of survival under harsh conditions, thereby optimizing population fitness in changing environments.
Dormant: dormancy refers to a metabolically inactive state of the cell.
Elongation factor EF-Tu: this factor is essential for the selection and binding of a ribosome to a charged tRNA, eventually resulting in protein synthesis.
Fluoroquinolone: antibiotics of this class, such as ofloxacin, inhibit DNA synthesis by targeting topoisomerases and cause DNA double-stranded breaks.
Hungry codon: a triplet sequence in an mRNA where the translation machinery stalls because of an insufficient supply of the corresponding charged tRNA.
β-Lactam: antibiotics of this class, such as ampicillin, target cell-wall synthesis by blocking peptidoglycan crosslinking. They are only effective against growing cells and ultimately induce cell lysis.
Persistence: a transient phenotypic trait attributed to a subset of cells within a clonal microbial population, resulting in increased survival following lethal antibiotic treatment. These antibiotic-tolerant bacteria are called persisters.
Polysome: a complex generated when an mRNA binds to ribosomes during the process of translation.
(p)ppGpp: guanosine tetraphosphate/pentaphosphate, an alarmone of the stringent response that is produced during nutrient stress or amino acid starvation. It causes the downregulation of genes responsible for growth, and the upregulation of genes involved in stress responses, amino acid biosynthesis, and nutrient uptake.
Resistance: caused by a genetic mutation, resistance results in an increased minimal inhibitory concentration (MIC). This allows cell growth, as the mutation overcomes the effect of the drug.

Dormancy and target inactivity do not, however, fully explain the complex physiology of persisters. First, not all non-growing cells are persisters [30,31]. Second, several studies have demonstrated that persisters arising during stationary phase exhibit higher metabolic activity than sensitive cells [32,33]. Third, it was found that non-growing *Salmonella* persisters inside macrophages maintain transcriptional and translational activity as well as actively secrete compounds to withstand the host immune response [34]. Finally, target inactivity does not explain why persisters experience DNA damage after treatment with **fluoroquinolones** [4,35]. Persistence effectors that result in a lower intracellular antibiotic concentration or an intrinsic defense against the antibiotic could explain how cells with active targets counter antibiotic killing.

Target inactivity: the target of the applied antibiotic is inactive, resulting in ineffective antibiotic treatment.
tmRNA: an RNA molecule that functions both as a tRNA and an mRNA, rescuing stalled ribosomes by adding an alanine to the polypeptide and providing a template mRNA encoding an alanine-tag, which is a signal for degradation.
Tolerance: the term population-wide antibiotic tolerance is used throughout the paper to describe the ability of the entire bacterial population to survive antibiotic exposure which would otherwise be lethal. By contrast, persistence is restricted to a subset of cells within a clonal microbial population that survive antibiotic exposure. At the single-cell level, however, tolerance describes the ability of a single cell to survive drug exposure.
Toxin–antitoxin (TA) module: a genomic module encoding a stable toxin that disrupts an essential cellular function, and an unstable antitoxin that neutralizes the action of the toxin.

Persistence through Decreased Cellular Energy Levels

The membrane potential is an electrochemical gradient across the bacterial inner membrane and comprises a difference in proton concentration (chemical potential) and a difference in charge (electric potential). This gradient results in a proton motive force (PMF) which allows transfer of protons across the cytoplasmic membrane, thereby generating ATP by ATP synthase [36]. Persistence has been associated with reduced levels of cellular energy generated through this process. Chemically perturbing intracellular ATP levels using arsenate results in an elevated persister fraction [37]. Similarly, pretreating populations with the protonophore CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) before antibiotic treatment collapses the chemical potential, resulting in an increased persister level [38]. Furthermore, pretreatment of cells with salicylate collapses the membrane potential through a mechanism that relies on reactive oxygen species (ROS), thereby inducing persistence [39].

The membrane potential and cellular ATP levels could affect persistence in several ways. In general, low energy levels result in reduced synthesis of proteins, DNA, and peptidoglycan. This presumably explains the increased survival following treatment with **aminoglycosides**, fluoroquinolones, and **β-lactams** because their respective targets are less or even not active [37]. Indeed, by circumventing this target inactivity using an antibiotic that does not rely on cellular energy, such as acyldepsipeptide 4 (ADEP4) that activates the ClpP protease and results in uncontrolled proteolysis, persister cells can be eradicated if the antibiotic is used in combination with another drug [40]. In addition to a direct effect on antibiotic efficacy, ATP is required for protein solubility and quality control. Lowered intracellular levels of ATP have been reported to result in protein aggregation [41,42]. Because aggresomes contain essential proteins involved in central cellular processes, cells switch to a dormant state. This state can be reverted by DnaK- and ClpB-mediated protein disaggregation [42].

Several biological effectors are known to induce persistence in *E. coli* by influencing the membrane potential [33,43–45]. Toxins TisB and HokB are part of genomic **toxin–antitoxin (TA) modules** that consist of a toxin, inhibiting an essential cellular function, and an unstable antitoxin, counteracting the activity of the toxin [46]. Both toxins are part of a type I TA module, implying that their corresponding antitoxin is an RNA molecule that inhibits the function of the toxin by blocking translation and/or by triggering breakdown of the toxin mRNA [46]. TisB and HokB are small peptides that form pores in the bacterial inner membrane [33,47]. For HokB, the link between pore formation and persistence has been experimentally verified [33]. Although HokB and TisB have similar modes of action at first sight, there are important differences. Whereas TisB forms small, anion-selective pores that decrease the PMF [47], HokB pores are larger and of a dynamic nature [33]. When the membrane potential is high, mature HokB pores are formed and result in ATP leakage, which is associated with persistence. Therefore, in contrast to TisB, membrane depolarization alone is not sufficient for HokB to induce persistence [33]. DNA damage increases *tisB* transcription [43], whereas the signal for induction of *hokB* transcription is unknown [5,44].

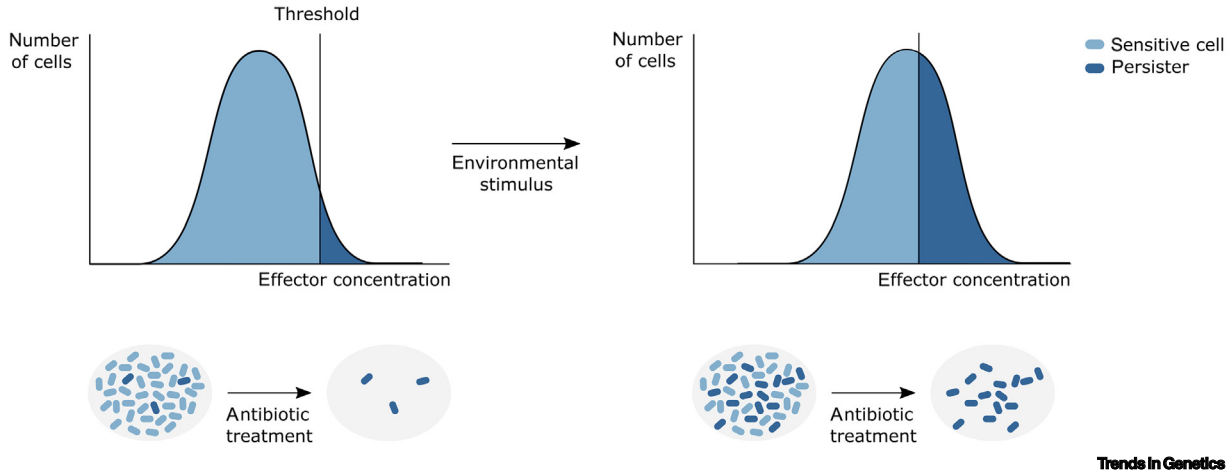

Figure 1. Persistence Is Governed by Stochastic Switching as well as by Deterministic Signals. A small proportion of cells in a bacterial population stochastically switch to the persister phenotype that is characterized by the ability to survive antibiotic treatment. Switching occurs when the concentration of a persistence protein exceeds a threshold value. Different environmental stimuli alter the distribution of effector levels, thereby affecting the level of persisters in a population (Box 2).

Persistence by Halting DNA Replication and/or Transcription

Persister cells that display tolerance towards fluoroquinolone and β-lactam antibiotics can emerge as a result of inhibited DNA replication. Increased levels of the stringent response alarmone **(p)ppGpp** (Box 2) prevent DNA replication and transcription by inhibiting negative supercoiling of DNA, resulting in tolerance towards ofloxacin [48]. In addition, CspD is involved in persistence because it inhibits both the initiation and elongation of DNA replication, presumably by binding to single-stranded DNA [49]. Shutdown of DNA replication is accompanied by growth inhibition and thus increased survival after ampicillin treatment [50].

Persistence by Blocking Translation

Chemically inhibiting protein synthesis using tetracycline results in a strong increase in the number of persister cells [38]. In addition, persisters are enriched in populations with reduced transcription of 16S rRNA [51]. Impairing the translation machinery is obviously correlated with an increased tolerance towards aminoglycosides. In addition, β-lactams become less effective owing to growth inhibition resulting from halted translation. How translation-inhibiting effectors affect fluoroquinolone tolerance is intuitively less clear because inhibition of protein synthesis does not directly lead to a shutdown of DNA replication [52]. In this case, secondary effects are presumably at play. For example, the toxin HipA induces persistence by inhibiting translation, and concomi-

Box 2. Environmental Stimuli Induce Persistence

In addition to the stochasticity involved in biological processes, persister levels are also modulated by several environmental stimuli [5]. For example, DNA damage induces the SOS response, leading to the formation of persisters by stimulating the expression of TisB toxin [43]. Furthermore, oxidative stress affects persistence by increasing the expression of the AcrAB–TolC efflux pump, resulting in a lower intracellular antibiotic concentration [88]. However, in contrast to these two examples, the link between environmental stress and the final effector of persistence is in many cases unknown. Several studies have demonstrated a link between starvation or nutrient transitions and increased numbers of persister cells [31,48,108,117]. The stringent response alarmone (p)ppGpp has been identified as a central hub in this type of induced persistence [48,53,117,118]. (p)ppGpp inhibits translation and replication, and affects transcription by downregulating genes necessary for rapid growth and by upregulating genes involved in amino acid biosynthesis, stress survival, and nutrient uptake [119,120]. In addition to nutritional stress, pre-exposure to many sublethal stresses such as heat stress, acid stress, antibiotic stress, and hyperosmotic stress has been shown to increase persister levels [56,121–123].

tantly induces synthesis of the stringent-response alarmone (p)ppGpp. In turn, (p)ppGpp prepares the cells for nutrient-poor conditions and thereby halts DNA replication [53,54]. Thus, a block in translation might indirectly be associated with inhibited DNA replication, resulting in tolerance towards fluoroquinolones.

Biological effectors inhibit translation in various ways, thereby inducing persistence. A first example is the translation **elongation factor EF-Tu**, which is highly abundant in protein aggregates that are generated in stationary phase. This sequestering of EF-Tu might lead to inhibition of translation, and thus to the induction of persistence [41]. The first enzyme in the methionine biosynthetic pathway (MetA) is also prone to aggregation under various stress conditions, resulting in methionine limitation [55]. Because methionine is required for translation initiation, aggregation of MetA has been linked to the induction of persistence [56].

Other persistence effectors that inhibit translation are a component of type II TA modules. In these systems, the protein antitoxin inhibits the protein toxin by direct binding [46]. Upon stress or through stochastic variation, the toxin/antitoxin ratio may increase, which activates the toxin and in many cases induces persistence [28]. Type II toxins typically target mRNA, ribosomal proteins, rRNA, or tRNA. Cleavage of mRNA can be ribosome-dependent, indicating that mRNA is cleaved during translation. Examples are the toxins YafQ and RelE, which bind to the 50S and 30S ribosomal units, respectively [57–61]. Ribosome-independent mRNA cleavage is performed by the toxins MazF and MqsR [62–65]. In addition to mRNA cleavage, type II toxins can also inhibit ribosome assembly. PasT directly inhibits 70S ribosome assembly by binding to the 50S subunit [66,67], whereas MazF and MqsR prevent the formation of new ribosomes indirectly because MazF cleaves precursor rRNA whereas MqsR cleaves both 16S rRNA and 23S rRNA before secondary structure formation takes place [68,69]. It has been suggested that MazF also cleaves 16S rRNA [68,70], although this claim is under debate [62,69]. Finally, some toxin effectors target tRNA. For example, HipA phosphorylates the conserved serine at position 239 of GltX aminoacyl-tRNA synthetase. This inactivates the enzyme and results in the formation of **hungry codons** that trigger the formation of (p)ppGpp [71,72]. The latter in turn induces growth arrest and persistence to β-lactam antibiotics by downregulating the synthesis of ribosomal proteins, rRNA, and tRNA [73–77]. Another tRNA-targeting mechanism is adopted by TacT, a toxin expressed in *Salmonella* Typhimurium. TacT inhibits translation by acetylating the amino group of tRNAs on the A (aminoacyl) site of the ribosome, rendering the amino acid unavailable for the nascent polypeptide [78,79].

Persistence by Intrinsic Defense against Antibiotic-Induced Damage

In contrast to the paradigm of persisters being dormant cells with inactive targets for antibiotic killing, several lines of evidence suggest that persister cells accumulate at least some antibiotic-induced damage. Tolerance towards aminoglycosides can then partly be explained by reduced protein misfolding as a result of stochastic variations in chaperone activity [80]. A seminal study has shown that persisters acquire DNA damage upon treatment with the fluoroquinolone ofloxacin [4]. The capacity to repair this damage is, among others, dictated by the abundance of the DNA repair machinery and was demonstrated to be crucial for persistence [4,9]. In a follow-up study, it was shown that persisters execute a timed series of processes in the post-antibiotic recovery period, consisting of DNA repair, DNA synthesis, and growth [35].

It has been suggested that the induction of ROS production is a common mechanism by which bactericidal antibiotics kill their target cells [81], although this view is still a matter of scientific debate [82–84]. Nevertheless, detailed examination of isolated *E. coli* persisters revealed a lower level of hydroxyl radicals upon antibiotic treatment compared to their sensitive kin [85]. This suggests that persister cells either accumulate less ROS or harbor scavenger enzymes such as

superoxide dismutase, catalases and alkyl hydroperoxide reductases that degrade ROS [86]. The latter is the case for persisters in *P. aeruginosa* biofilms, which display increased levels of stringent-response-mediated antioxidant scavenger enzymes and reduced production of the pro-oxidant 4-hydroxy-2-alkylquinoline (HAQ) [2].

Persistence by Lowering the Intracellular Antibiotic Concentration

Several mechanisms contribute to a decreased intracellular antibiotic concentration in persister cells. Multidrug efflux genes have higher expression levels in *E. coli* persisters, resulting in reduced intracellular concentrations of β-lactam antibiotics [87]. In addition to varying expression levels, asymmetrical distribution of AcrAB–TolC efflux pumps following cell division, or the induction of AcrAB–TolC by oxidative stress, might contribute to population heterogeneity [88,89]. Although the expression of efflux pumps is a well-known resistance mechanism and the role of efflux pumps in persistence is under debate, cells surviving treatment in this case have characteristics of persisters because they do not grow in the presence of the antibiotic and their phenotype is not heritable [87].

A different persistence mechanism was observed in *Mycobacterium smegmatis* cells surviving exposure to the prodrug isoniazid. The latter is activated by the hydroperoxidase KatG, and decreased expression of *katG* reduces the intracellular concentration of the active drug [3]. *M. smegmatis* persisters were shown to harbor low levels of KatG, presumably due to stochastic fluctuations in gene expression. This stochasticity might originate from fluctuations in ROS produced by the respiratory metabolism because *katG* is induced upon oxidative stress [3].

Intracellular antibiotic concentrations can also be reduced by a collapse of the membrane potential because the PMF regulates aminoglycoside uptake [90]. Accordingly, repolarizing the membrane using metabolites such as mannitol or glucose decreases the persister fraction in planktonic populations of *E. coli* as well as in *P. aeruginosa* biofilms [90–92]. Importantly, these cells are still tolerant towards other antibiotics, implying that repolarization does not trigger persister awakening but instead results in increased uptake of aminoglycosides [91].

How To Revert the Persistent State?

Treating persister cells with antibiotics results in a slow decline in the number of viable cells over time, suggesting that persisters can 'wake up' and become sensitive to the antibiotic. When conditions are favorable, the awakened cells are able to grow and establish a new population [5]. Awakening of persisters is believed to occur stochastically, although it is currently unclear which processes are involved [93]. In addition to stochastic awakening, it is hypothesized that environmental signals, such as fresh medium, trigger awakening [94,95], for example by replenishing cellular ATP levels. Recently, Pu *et al.* demonstrated that replenishment of cellular ATP levels enabled the chaperones DnaK and ClpB to dissolve the aggregates that are associated with the induction of persistence [42]. In line with this view, several chaperones are found to be upregulated in persister cells, and deletion of the corresponding genes decreases the persister fraction [80,87,96–101], pointing towards defective awakening.

The literature hints at possible awakening mechanisms for toxin-induced persister cells. For several toxins, antitoxin-mediated detoxification was reported. For example, expression of the antitoxin MazE neutralizes MazF-induced persistence [35], and RelE- or MazF-induced growth arrest is reverted by the expression of their respective antitoxins, RelB and MazE [60,102]. However, protein antitoxins are intrinsically unstable, and it remains puzzling how *de novo* synthesis of antitoxins would occur in a cell with a stalled translation machinery. A possible explanation, supported by the observation that expression of *mazF* does not change the number of **polysomes**, is that a low level of translation still takes place in these cells [69]. Furthermore, several toxins,

such as MazF, target mRNA in a codon-specific manner, and mRNAs lacking this codon might still be translated [103].

Exit from the persister state can also be accomplished via an antitoxin-independent route that reverts the effect of the toxin on cellular physiology instead of directly inhibiting the toxin itself. tRNAs acetylated by TacT are, for example, deacetylated by the peptidyl-tRNA hydrolase Pth, thereby contributing to the growth resumption of persisters [78]. In this case, Pth is likely present before TacT-induced translational inhibition [104]. Other antitoxin-independent detoxifying mechanisms are known, but their specific role in the awakening of persister cells has not yet been explored. For example, rapid detoxification of RelE-induced growth-arrested cells depends on **tmRNA** (SsrA), which rescues stalled ribosomes and tags nascent polypeptides for proteolysis [60]. Interestingly, depletion of SsrA causes a decrease in the persister fraction [105], and this may result from impaired persister awakening. MazF-induced growth arrest is rescued by the RNA ligase RtcB that ligates cleaved 16S rRNA fragments [106], although the effect of MazF on 16S rRNA is under debate [68]. However, how the activities of these additional proteins that counteract the toxin are regulated and how they drive exit from the persister state remain outstanding questions.

The above-mentioned mechanisms of persister awakening focus on reverting the influence of the effector on cellular physiology, either directly or indirectly. An additional way for persister cells to reactivate is based on how well they are able to repair antibiotic-inflicted damage. It was shown that treatment with fluoroquinolones results in DNA damage [4], and that awakening of surviving persisters depends on DNA repair mechanisms [35]. Furthermore, proteome analysis of TisB-induced persisters after antibiotic treatment revealed that these cells upregulate proteins that are important for recovery [107]. In this view, persisters may be considered as the few lucky cells that acquired the right proteome to establish awakening.

Altogether, two general mechanisms contributing to persister awakening can be proposed. First, the capacity of a cell to cope with antibiotic-inflicted damage determines whether or not a cell resumes growth [35]. Second, persister awakening is based on how persistence was induced. For example, in some cases ATP must be replenished to dissolve protein aggregates, in others acetylated tRNAs must be deacetylated [42,78]. It is currently unclear whether persisters from the second category also need to manage antibiotic-inflicted damage, but both mechanisms might contribute simultaneously to awakening.

Concluding Remarks

Intensive research over the past 20 years has revealed an unexpected richness of molecular mechanisms leading to the formation of persister cells in different bacterial species. Several of these mechanisms result in target inactivity and/or global cellular dormancy, and comprise mechanisms such as reduced cellular energy, halted DNA replication, and blocked translation. Other mechanisms are of a more active nature, and include intrinsic defense against antibiotic-induced damage or reduced intracellular antibiotic concentration (Figure 2, Key Figure). Although described here as discrete mechanisms, persistence presumably results from the interplay between multiple processes.

Because several mechanisms induce persistence at the molecular level, the persister population itself can be considered to be heterogeneous. The latter explains why persister levels often vary following treatment with different types of antibiotics. Persistence is not only governed by stochastic fluctuations in effector concentrations but also by environmental triggers which can increase the concentration of effectors, for example by activating a stress response. However, the cellular processes upstream of the effectors are often poorly understood. Although not the topic of this review, global changes in metabolism, for example as a result of nutrient availability,

Outstanding Questions

Which persister mechanisms are at play in natural strains during infection? Is evolutionary adaptation of persister levels important in an *in vivo* setting?

Can therapies directed against persister cells contribute to combating the ongoing antibiotic crisis?

Which environmental triggers induce specific persistence effectors, and which regulatory pathways are involved in the activation of these effectors? How is the activity of these effectors translated to global metabolic processes and physiology?

Which molecular mechanisms contribute to persister awakening? Can we study these processes at the single-cell level?

Key Figure

Molecular Mechanisms of Persister Formation

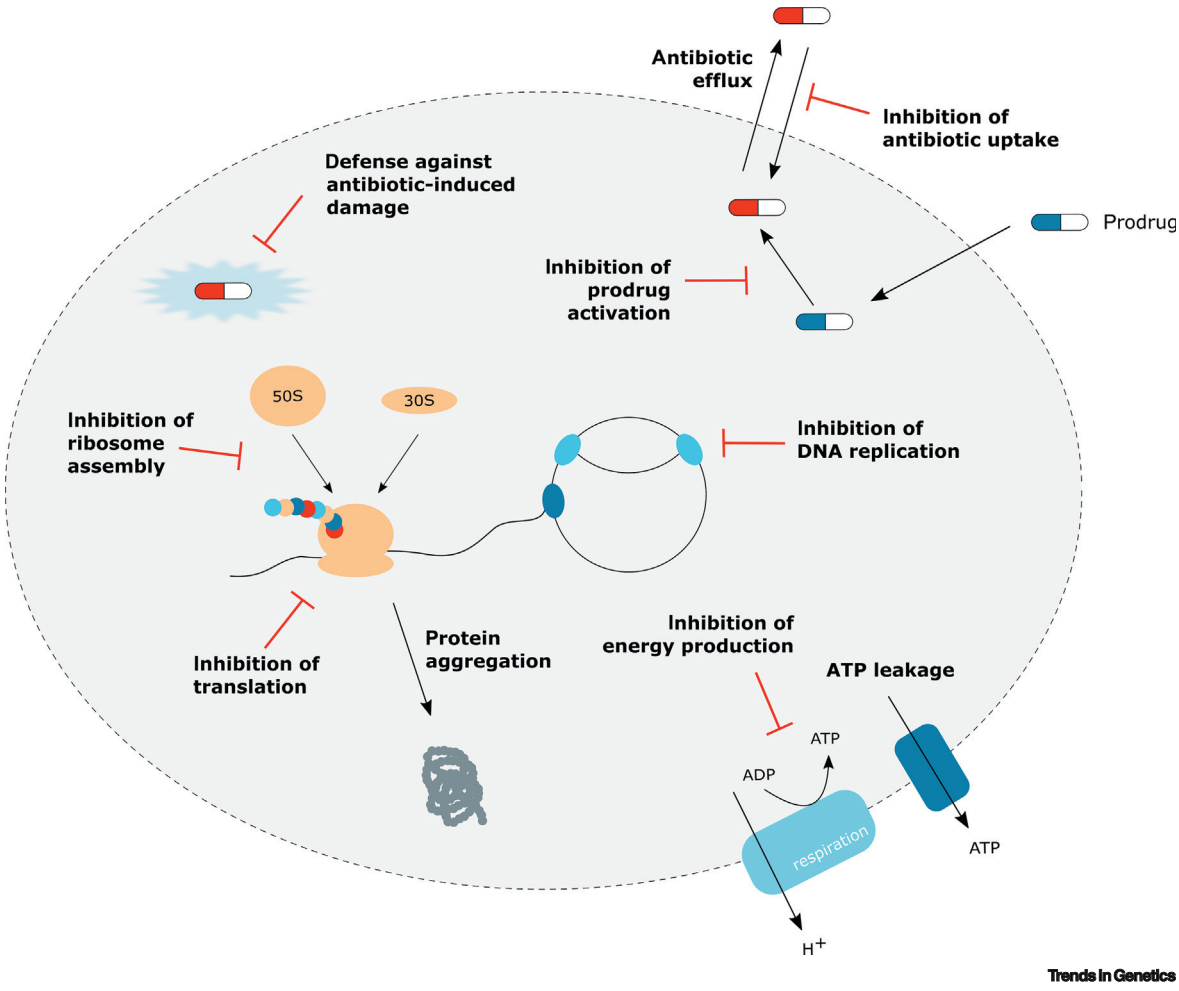

Figure 2. Several cellular targets of persistence effectors have been implicated in persister formation. Reduced cellular energy levels can be the result of inhibition of ATP production or leakage of ATP out of the cell. Alternatively, persistence can be elicited by halted DNA replication. Furthermore, translation can be reduced in persisters either directly by disrupting mRNA, tRNA, rRNA or ribosomal proteins, or indirectly by impeding ribosome assembly. Several essential processes can also be affected simultaneously because proteins involved in these processes may aggregate under persistence-inducing conditions. In other cases, persisters are characterized by reduced intracellular drug concentration due to inhibition of antibiotic uptake, antibiotic efflux, or to low levels of prodrug-activating enzymes. In addition, persisters can express active defense mechanisms against antibiotic-induced damage.

have also been implicated in the formation of persisters [108]. Future research should be directed towards delineating molecular mechanisms from trigger to effector. In addition, insight into persister awakening is steadily growing and much progress is to be expected in the coming years. A better understanding of both entry and exit from the persister state would contribute to a better perception of the complex nature of persisters, which might spur the development of efficient strategies to eradicate them.

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