Manuscript Number:

Title: New-found fundamentals of bacterial persistence

Article Type: Review

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Abstract: Persister cells display tolerance to high doses of bactericidal antibiotics and typically comprise a small fraction of a bacterial population. Recently, evidence was provided for a causal link between therapy failure and the presence of persister cells in chronic infections, demonstrating the need for persistence research. A series of recent breakthroughs have shed light on the multiplicity of persister genes, the contribution of gene expression noise to persister formation, the importance of active responses to antibiotic tolerance and the heterogeneity among persister cells. Moreover, the development of in vivo model systems has highlighted the clinical relevance of persistence. The current review discusses these recent advances and how this knowledge fundamentally changes the way in which we will perceive the problem of antibiotic tolerance in years to come.

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Dear Dr. Teitzel,

Hereby we have the pleasure to submit our invited review manuscript (TIMI-D-12-00056) entitled 'New-found fundamentals of bacterial persistence' by Kint *et al.* for publication in *Trends in Microbiology*.

I thank you in advance for your kind attention to this manuscript.

Yours sincerely,

Jan Michiels

New-found fundamentals of bacterial persistence

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Keywords: antibiotic tolerance, toxin-antitoxin modules, noise, heterogeneity, chronic infections

Abstract

Persister cells display tolerance to high doses of bactericidal antibiotics and typically comprise a small fraction of a bacterial population. Recently, evidence was provided for a causal link between therapy failure and the presence of persister cells in chronic infections, demonstrating the need for persistence research. A series of recent breakthroughs have shed light on the multiplicity of persister genes, the contribution of gene expression noise to persister formation, the importance of active responses to antibiotic tolerance and the heterogeneity among persister cells. Moreover, the development of *in vivo* model systems has highlighted the clinical relevance of persistence. The current review discusses these recent advances and how this knowledge fundamentally changes the way in which we will perceive the problem of antibiotic tolerance in years to come.

A survival strategy for bacterial populations

About 0.001 to 0.1 % of the cells of an isogenic bacterial population display tolerance to prolonged treatment with high doses of bactericidal antibiotics, leading to a biphasic killing pattern of bacterial populations upon treatment with bactericidal antibiotics (Figure 1) [1]. This subfraction comprises the so-called ‘persister cells’, first described nearly seventy years ago [2]. In contrast to resistant cells, persisters are genetically identical to susceptible bacteria, constituting phenotypic variants of the wild type. Although their phenotypic tolerance allows them to remain viable in the presence of bactericidal antibiotics, persister cells are generally non-growing [3]. Persisters can be formed either by random switching [3,4] or in response to specific environmental cues [5,6]. Moreover, formation of persister cells is growth-phase dependent: their number is low in exponentially growing cultures and reaches a maximum in stationary phase [3]. Upon removal of the antibiotic pressure, persister cells switch back to a growing state, thereby giving rise to a new population that is genetically identical to the original one. Consequently, the new population is again sensitive to antibiotics (Figure 1) [3]. Several excellent reviews on this topic have been published in recent years [1,7-9]. Rather than aiming to present an exhaustive overview of recent literature on the topic, this review addresses novel insights into emerging fundamental features of bacterial persistence and highlights several breakthroughs that were recently achieved in persistence research.

Physiological basis for phenotypic antibiotic tolerance

According to a decade-old hypothesis, the dormant, non-growing state of persister cells is sufficient to confer antibiotic tolerance, as the inactivity of target functions was thought to preclude the detrimental action of antibiotics [1,10]. Experimental evidence supporting this hypothesis primarily came from transcriptome analyses of isolated persister cells. These showed

that genes involved in metabolic and biosynthetic pathways, energy production and non-essential genes are downregulated in persisters, consistent with a dormant state [10-12]. Notwithstanding the fact that persister cells display some degree of dormancy, it is becoming increasingly clear that passive dormancy per se cannot entirely account for the persister phenotype [9,13]. The observation that isolation of dormant cells based on GFP expression under control of the growth-dependent *rrnB* P1 promoter yielded a subpopulation only moderately enriched in persisters, provided an early clue to the contrary [11]. Additionally, the low rate of translation displayed by *Escherichia coli* persister cells should make them susceptible to aminoglycosides [14,15]. The weak activity of the latter class of antibiotics against persister cells also suggests the contribution of other mechanisms to antibiotic tolerance rather than dormancy as sole explanation.

More recent studies suggest an important role for active mechanisms in antibiotic tolerance, specifically the suppression of oxidative stress, universally caused by bactericidal antibiotics (Box 1) [16,17]. Lowered cellular levels of hydroxyl radicals resulting either from less efficient production of reactive oxygen species (ROS), as was suggested for *E. coli* persister cells [18], or from increased activity of detoxifying mechanisms, could counteract the bactericidal action of antibiotics, thereby causing tolerance (Figure 2a). Consistent with the latter hypothesis, it was shown that H₂S induces antibiotic tolerance in several bacterial species including *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* by alleviating oxidative stress through sequestration of ferrous iron and stimulation of anti-oxidant activities of catalases and superoxide dismutases (SODs) [17]. Additionally, a recent study on *P. aeruginosa* persister cells revealed the necessity of a starvation-induced mechanism that actively limits hydroxyl radical levels to prevent cell death caused by bactericidal antibiotics [16]. More specifically, activation

of the stringent response in planktonic stationary phase cells or biofilms increases the cellular levels of the oxidative stress defense elements catalase and SOD and represses production of the pro-oxidant molecule hydroxy-2-alkylquinoline (Figure 2a). Both of these effects are required for the observed antibiotic tolerance. Although hydroxy-2-alkylquinoline production is not conserved among Gram-negative bacteria, similar effects on hydroxyl radical levels and oxidative defense systems were observed upon activation of the stringent response in antibiotic-treated *E. coli* cells. This suggests a common role for oxidative stress control in antibiotic tolerance [16].

Induction of persister formation by antibiotics

In addition to the accepted notion that persisters are present in a given population prior to antibiotic treatment, recent work by the Lewis group showed that, surprisingly, persistence can additionally be induced by the very same cue (Figure 2b). More specifically, *E. coli* persister cells can actively be generated following treatment with antibiotics that trigger the SOS response such as ciprofloxacin [5]. Mutants unable to mount an SOS response display a significantly reduced persister fraction upon treatment with DNA-damaging antibiotics [5,19]. Conversely, a *lexA* mutant in which the SOS response is constitutively active shows an increased persister fraction [5].

SOS-mediated induction of the persistent state was shown to be dependent on increased expression of the *tisB* toxin in *E. coli*. Translation and subsequent activation of TisB are inhibited by asRNA pairing of *tisB* mRNA with its cognate antitoxin *istR-I* (see also Box 2) [20]. Evidence for the involvement of TisB in SOS-mediated persistence is provided by the inability of the SOS response-activating antibiotic ciprofloxacin to induce persister formation in a *tisB* deletion strain [21]. Furthermore, deletion of *istR-I* and a concomitant increase in TisB

levels cause an increase in persistence [21]. Based on the knowledge of the mode of action of TisB, a model for SOS-induced persistence was proposed [21]. Upon induction of the SOS response, the LexA-mediated repression of *tisB* is abolished. Since *istR-I* expression is not under LexA control, the *tisB/istR-I* mRNA ratio increases, causing free TisB to accumulate. TisB, a small hydrophobic peptide, is inserted into the inner membrane, an event leading to disruption of the proton motif force causing cellular ATP levels to drop (Figure 2b) [22]. The lowered cellular ATP concentration contributes to the onset of dormancy and hence to antibiotic tolerance [21].

Involvement of toxin-antitoxin modules and toxins in persister formation

Since the identification almost 30 years ago of *hipA*, encoding the toxin part of the *hipBA* toxin-antitoxin (TA) module [23], as the very first gene involved in persister formation, several lines of evidence have demonstrated the involvement of chromosomally encoded TA modules (Box 2) in bacterial persistence. Microarray studies revealed increased expression of numerous TA elements in persisters of *E. coli* [10,11] and *Mycobacterium tuberculosis* [12]. Additionally, it was shown that overexpression of several toxins in excess of their corresponding antitoxins leads to an increase in persister fraction in *E. coli* (Table 1) [10,11,24-26]. Finally, the recently discovered pathway for antibiotic-induced persister formation in *E. coli*, discussed in the previous paragraph, relies on increased expression of the TisB toxin [21]. By shutting down potential antibiotic targets, toxins may produce tolerant cells (Figure 2c) [10].

In contrast to the strong effects exerted by overexpression of several type II toxins on *E. coli* persister levels, single deletions of these chromosomal TA-encoding operons generally do not affect persistence [10,24,26]. An exception is the *mqsRA* TA locus of which deletion was shown to significantly repress *E. coli* persister production [27], although this result was contradictory to

a previous report [11]. The preservation of an intact persistence phenotype despite deletion of a specific persister gene has been attributed to the redundancy of persister genes in general [1,28] and of type II TA modules in particular [11], implying that a decrease in persister fraction would only be detectable in a strain in which several persistence genes are deleted simultaneously. Combined deletion of four or more type II TA modules indeed progressively reduces the formation of persister cells in *E. coli*, irrespective of the order in which the genes are deleted. Simultaneous deletion of 10 TA modules dramatically reduces the persister fraction [24]. Interestingly, a single deletion of *tisB*, encoding the only type I toxin shown to be involved in persistence so far, results in a decreased *E. coli* persister fraction [21].

The activity of toxins belonging to type II TA modules is neutralized by forming tight complexes with their cognate antitoxins. Hence, degradation of the unstable antitoxin frees and thereby activates the toxin, causing antibiotic tolerance. It was shown that the Lon protease, of which the activity is induced by high levels of the stringent response alarmone (p)ppGpp, plays a major role in toxin-induced persistence by inactivating type II antitoxin molecules [24,29,30]. Consistent with this hypothesis, deletion of *lon* leads to a massive reduction in the number of *E. coli* persister cells surviving treatment with different classes of bactericidal antibiotics against different antibiotics [24]. Modest overexpression of this protease on the other hand, significantly increases the persister fraction in *E. coli* [24].

Induction of the persistent state by chemical signaling

While persistence is often referred to as a population-level insurance policy [31,32], the problem of population-level decision-making as to whether or not to switch to the persistent state has largely been ignored. Bacterial communication through diffusible signaling molecules might provide an answer to this matter. Quorum sensing, allowing bacteria to sense their cell density

through detection of specific signaling molecules, is of particular interest considering the growth-phase dependency of persister formation. Although this form of bacterial communication seems not to be involved in *E. coli* persistence [33], the *P. aeruginosa* persister fraction increases in response to quorum sensing-related signaling molecules such as pyocyanin and *N*-(3-oxododecanoyl)-L-homoserine lactone (Figure 2d) [34].

Recently, another indication for the involvement of chemical signaling in persistence was reported by the Collins group, as they showed that indole contributes to the induction of persistence in *E. coli* (Figure 2d) [6]. Indole is a bacterial signaling molecule that is produced during stationary phase as a consequence of nutrient limitation [35]. Increasing indole levels in the growth medium results in an elevated persister fraction, tolerant towards different classes of antibiotics. In addition, deletion of *tnaA*, encoding a protein that catalyzes the conversion of tryptophan to indole, results in a decrease in persister numbers [6]. Strikingly, the cellular response to indole, measured as the expression of GFP under control of the indole-responsive promoter of the *tna* operon, correlates with the probability of a single cell to become persistent [6]. Microarray analyses revealed the involvement of the OxyR and phage-shock pathways in this mechanism of persister formation. A model was proposed in which indole activates the OxyR and phage-shock pathways in a fraction of the bacterial population, thereby contributing to antibiotic tolerance. Population-level decision-making is achieved through heterogeneous sensing of the signaling molecule indole, giving rise to bistability [6].

Single-cell decision-making

Chemical communication by diffusible signals may result in fluctuations in individual cells' microenvironments, thereby leading to a heterogeneous response at the single-cell level [6]. However, a more fundamental mechanism that is frequently put forward as the explanation for

cell individuality is stochasticity in gene expression arising from fluctuations in transcription and translation despite stable environmental conditions [36]. As a consequence, the expression of genes can vary drastically within a clonal population of cells [37]. The origins of variation in gene expression are twofold. Intrinsic noise is inherent to the nature of the biochemical process of gene expression and depends on the rates of translation, mRNA and protein degradation [37]. This form of noise defines to which extent the activities of two identical gene copies fail to correlate [38]. Extrinsic noise is global to a single cell and as such independent of the specific gene assessed, but varies from one cell to another [37,38]. Several factors including ribosome abundance, micro fluctuations in the cellular environment and asymmetric distribution of proteins upon cell division contribute to this form of noise [37].

Persister cells represent only a very small fraction of cells within an isogenic bacterial population. The persister fraction typically reaches 0.001 % of cells in *E. coli* growing exponentially, up to maximally 0.1 % in stationary phase [1]. This feature, combined with the fact that persister cells can be formed spontaneously prior to antibiotic treatment [3,4,39], led to suggest that persisters are formed as a consequence of stochastic processes [10,33]. However, as persister formation is also favored under specific conditions such as the entry into stationary phase, the presence of indole or the activation of the SOS response, persister formation is also affected by a deterministic component [1,21]. Starting from these observations, it was hypothesized that the switch to the persistent state depends on the cellular concentration of some key persistence regulatory proteins. At the population level, the expression of these proteins is characterized by a mean expression level and stochastic fluctuations around this mean. The mean expression level, which can be highly regulated by environmental triggers, represents the deterministic component. Conversely, stochastic fluctuations are believed to be random in each

cell, thereby generating heterogeneity within the population. It is assumed that cells in which the expression level of a key persister protein exceeds a certain threshold, will switch to the persistent state [10,40]. In this case, it is not the small difference in expression itself that drives the switch to a different phenotypic state, but rather the amplification of this elevated signal which can only occur in cells that show a stochastically higher expression [8,37]. This so-called ‘noise amplification’ ultimately leads to bistability, being the co-occurrence of two or more phenotypically distinct subpopulations of cells within a population [41].

Experimental evidence supporting the importance of stochasticity in gene expression for the formation of persister cells is only just emerging. It was shown that in agreement with the stochasticity hypothesis, the population distribution of HipA expression is transformed into a bimodal phenotypic pattern once the concentration of HipA exceeds a certain threshold [42]. This threshold of expression exceeds the HipA level needed to block cellular growth and can vary depending on the antitoxin level. Once HipA levels raise above the threshold, variability in expression is enhanced within the bacterial population allowing a fraction of cells to arrest their growth, as a measure for persistence, for prolonged times [8,42]. It was suggested that the effects of other TA modules on persistence are also threshold-based [8].

Heterogeneity within a persister population

Although most studies on bacterial persistence consider the persister fraction as a homogeneous entity, it is becoming increasingly clear that there is no such thing as ‘the’ persister fraction [9]. This was first brought to attention by the Balaban group upon categorizing persister cells as either type I or type II based on the phenotypes of two previously identified persistence mutants *hipA7* and *hipQ* [4]. Persister cells produced by the *hipA7* mutant, which are generated spontaneously in stationary phase of planktonic growth, are designated type I. These represent

non-growing cells and their number is directly proportional to the total number of stationary-phase cells. Upon regrowth in fresh medium, these cells restart growth after an extended lag time [4]. The *hipQ* mutant on the other hand, carrying an unknown mutation [23], generates type II persister cells. These constitute a subpopulation of slowly growing cells that constantly divide, albeit at a much lower rate as compared to non-persister cells [4].

A recent hypothesis suggests that variability in cellular processes among single cells generates a collection of cells with distinct physiologies. According to this theory, persister cells arise specifically from stochastic fluctuations in lethality-governing and persistence-mediating processes. Since gene expression varies between different persister cells as well, each persister cell adopts a unique cellular status, in part reflected by its own specific tolerance properties [9]. This implies that for each bacterial population, the fraction of persisters surviving treatment with a specific bactericidal antibiotic differs from the persister subpopulation surviving treatment with a different antibiotic. This hypothesis is supported by experimental evidence demonstrating that artificially increasing the persister fraction by overexpressing specific persistence genes such as the toxins RelE and MqsR in *E. coli*, only affects the persistence phenotype towards a specific set of antibiotics. In the case of MqsR, overexpression increases tolerance to ofloxacin and cefotaxim but not to the aminoglycoside tobramycin [11]. Conversely, overexpression of RelE also increases the persister fraction towards tobramycin in addition to cefotaxim and ofloxacin [10]. Another study, in which a set of natural *E. coli* isolates was assessed for persistence following exposure to three different classes of bactericidal antibiotics revealed significant strain-specific variation in persister formation. Intriguingly, increased persistence to one drug was uncorrelated with increased persister formation towards any other drug for a given strain, indicating substantial variation in mechanisms underlying persistence [43]. Taken

together, it is clear that the mechanism of persister formation contributes to their specific antibiotic tolerance profiles. Since single persister cells may be formed through a unique set of persistence-inducing mechanisms, the tolerance spectrum of individual persister cells is greatly variable.

In addition to the unique tolerance spectrum of single persister cells, their heterogeneity manifests itself in other properties. It was shown that a subfraction of *E. coli* and *S. aureus* persister cells can be sensitized for aminoglycosides by the addition of specific metabolites [15]. Metabolites that enter the upper glycolysis, such as mannitol, potentiate a fraction of persister cells for the action of aminoglycosides. These metabolic stimuli do not revert the persister cells into a growing state but rather directly induce the proton motif force, coinciding with the facilitated uptake of aminoglycosides [15]. The observation that this metabolite-enabled killing only reduces the persister fraction instead of eradicating it, again suggests the existence of different kinds of persister cells within a persister subpopulation [9]. Evidently, this complicates any effort directed at eradicating persister cells since it diminishes the chance of identifying a single ‘silver bullet’ that can kill all persisters [43]. In addition, it points to hidden layers of complexity that underlie the molecular basis of persistence, possibly confounding the presently known mechanisms.

Clinical relevance of the persistence phenotype and *in vivo* studies of bacterial persistence

The clinical relevance of microbial persistence is gradually emerging now that evidence has been provided for a causal link between therapy failure and the presence of persister cells [44,45]. Their transient insensitivity to bactericidal antibiotics combined with the ability to revert from this multi-drug tolerant to an antibiotic-sensitive state in which cells rapidly divide, makes them of substantial importance for the recalcitrance of chronic infections [1,7].

Consequences of persister cells surviving antibiotic treatment are at least twofold. Firstly, the continuous presence of viable cells during consecutive rounds of antibiotic treatment is suggested to contribute to the emergence of antibiotic resistance [7,46]. Crosstalk between resistance and persistence, as was reported in *P. aeruginosa*, might further complicate treatment of infections [47]. Secondly, experimental evidence indicates that prolonged exposure to antibiotics leads to the selection of high-persistent mutants. More specifically, late *P. aeruginosa* isolates from cystic fibrosis patients showed a significant increase in persister levels without displaying any antibiotic resistance [44]. Similarly, high-persistent strains of the eukaryotic pathogen *Candida albicans* were isolated from long-term carriers of this pathogen [45]. The latter also demonstrates that the impact of persistence is not restricted to prokaryotes only. Its relevance reaches even further as reversibly drug tolerant cells, sharing remarkable similarities with bacterial persisters, have been identified in cancer cell populations (Box 3) [48-51].

Up until now, the lack of *in vivo* model systems has precluded the accumulation of experimental evidence to support hypotheses on the importance of bacterial persistence for the treatment of chronic infections and for the emergence of antibiotic resistance. Recently however, two infection models in mouse were successfully developed to validate *in vitro* results concerning sensitivity of persister cells to aminoglycosides and the importance of the stringent response for persister formation, respectively [15,16]. In a first experimental setup, the effect of metabolites on the bactericidal action of aminoglycosides with respect to sensitivity of persister cells, was assessed on a chronic biofilm infection of uropathogenic *E. coli* in the urinary tract of mice [15]. In a second setup aimed at determining the significance of starvation responses to antibiotic

tolerance, the effectiveness of treating a *P. aeruginosa* biofilm infection caused by either a wild-type strain or a mutant defective for the stringent response was compared [16].

These successful animal models constitute a breakthrough for *in vivo* persistence research, enabling a new level of validation of *in vitro* results. Crucially, they also provide a platform for proof-of-concept studies aimed at developing innovative strategies to combat the growing scourge of chronic infections.

Concluding remarks

The transient multi-drug tolerant state of bacterial persister cells greatly contributes to the recalcitrant nature of chronic infections. Previously, persisters were considered to be in a dormant, inactive state displaying an overall stress tolerance. However, the recent insights into this phenomenon, presented in the current review, show that this view is radically changing. Persister cells appear to establish active defense systems towards oxidative stress imposed by bactericidal antibiotics, through specifically induced mechanisms. Furthermore, the antibiotic tolerant phenotypes and specific tolerance profiles are heterogeneous among cells within a single persister subpopulation. This heterogeneity imposes fascinating challenges for the future development of advanced techniques for persister research at the single-cell level such as single-cell transcriptome analysis.

More insight into the fundamental features of persister cells is necessary to ultimately develop rational strategies for persister elimination. This is complicated by the recently discovered correlation between antibiotic treatment and induction of persister formation. The recent development of *in vivo* persistence model systems however, will allow further analysis of these and other *in vitro* results.

Acknowledgements

C. I. K. is recipient of a fellowship from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). The work was supported by grants from the KU Leuven Research Council (IDO/07/010, IDO/09/010) and the Fund for Scientific Research - Flanders (FWO) (G.0299.07, G.0413.10, G.0471.12). We apologize for not including important work from other investigators in the field of bacterial persistence. These omissions were due to space limitations.

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Boxes

Box 1: Common killing mechanisms of bactericidal antibiotics

Bactericidal antibiotics are classified according to their target pathways and subsequent modes of action. β -lactams (penicillin, ampicillin) inhibit cell wall synthesis, fluoroquinolones (ofloxacin, ciprofloxacin) inhibit DNA gyrase causing double-stranded DNA breaks and aminoglycosides (gentamicin, kanamycin) bind the 30S ribosomal subunit, thereby inhibiting translation [52]. Although primary targets differ, treatment with lethal doses of these diverse bactericidal antibiotics leads to a common mechanism of cell death in Gram-positive as well as Gram-negative bacteria, through the production of ROS such as superoxide and hydroxyl radicals [53,54].

Primary drug-target interactions stimulate oxidation of NADH via the electron transport chain and this in turn promotes superoxide formation. Superoxide has the ability to damage iron-sulfur clusters, thereby generating free ferrous iron. Fe^{2+} is oxidized in the ‘Fenton reaction’ leading to the generation of Fe^{3+} and hydroxyl radicals [54]. In the case of aminoglycosides, insertion of mistranslated proteins into the membrane leads to activation of the *cpx* two-component envelope stress response system which in turn activates the *arc* two-component system involved in regulation of metabolic and respiratory systems. The combined cellular effects of these systems is responsible for provoking oxidative stress leading to hydroxyl radical production [55].

The highly toxic ROS generated following treatment with bactericidal antibiotics damage DNA, lipids and proteins, thereby contributing to cell death [56]. However, a recent report suggests that this generalized oxidative damage is not the main cause of ROS cytotoxicity. Instead, cell death seems predominantly elicited by specific oxidation of the guanine nucleotide pool and subsequent use of the thus generated 7,8-dihydro-8-oxoguanine (8-oxo-G) and its

deoxyribonucleotide derivative 8-oxo-dG [57]. In case of β -lactams and fluoroquinolones, integration of closely spaced 8-oxo-dG during DNA replication causes lesions, which can give rise to double-stranded DNA breaks through incomplete base excision repair. The bactericidal action of aminoglycosides may additionally originate from mistranslation due to the presence of 8-oxo-guanosine in cellular RNA. These mistranslated proteins in turn activate the pathway for another round of ROS generation [57].

Box 2: Toxin-antitoxin modules

Toxin-antitoxin (TA) modules consist of a stable toxin, typically targeting essential cellular functions such as translation or replication, and an unstable antitoxin, which counteracts the activity of its cognate toxin [29,58]. These systems were originally identified on plasmids where they play a role in plasmid maintenance. However, a significant number of TA modules is chromosomally encoded: 33 chromosomal TA modules have been identified in *E. coli* whereas the *M. tuberculosis* chromosome contains at least 60 [58]. The function of chromosomal TA modules is still a matter of debate. They have been implicated in numerous cellular pathways including starvation-induced cell stasis, stress response, genetic stabilization, programmed cell death and bacterial persistence [59].

TA modules are categorized based on the nature of the antitoxin and their mode of regulation [29,60]. Type I TA modules encode a protein toxin and an antisense RNA antitoxin. Translation of the toxin is prevented by binding of the RNA antitoxin to the toxin-encoding mRNA. Type II TA modules are organized in operons from which both toxin and antitoxin are expressed. The antitoxin is a protein that binds and thereby neutralizes the toxin protein. In addition, the type II TA complex is responsible for autoregulation of expression of the TA operon [29,58,60]. In

type III TA systems, the activity of the protein toxin is inhibited through a direct interaction with an RNA antitoxin [29,61]. In contrast to type I and II TA systems, type III TA modules have hitherto not been implicated in bacterial persistence.

Box 3: Drug-tolerant persists in cancer cell populations

A fraction of 0.3 to 5 % of cells within multiple tumor-derived cancer cell lines can survive prolonged treatment with high concentrations of anti-tumor drugs resulting in fractional killing [48]. These ‘drug-tolerant persisters’ (DTPs) display a significantly reduced sensitivity to several drugs. The tolerant state is reversible and the fact that no mutations accounting for the observed resistance are acquired in the DTPs implicates epigenetic regulation of this phenotype [48].

On the one hand, it was proposed that cell-to-cell variation in protein levels contributes to the onset of drug tolerance [51]. This is consistent with the hypothesis that all of the tumor cells potentially have the ability to stochastically acquire drug tolerance [48]. On the other hand, a specific mechanism involving a distinct chromatin state as well as active signaling through the insulin-like growth factor 1 (IGF-1) receptor was identified. The establishment of a metastable chromatin state within DTPs requires the histone demethylase activity of KDM5A, of which inhibition consequently leads to specific killing of DTPs. Similarly, treatment with IGF-1 receptor inhibitors selectively ablates the drug-tolerant cancer cell subpopulation. Elimination of DTPs through inhibition of these specific processes prevents the development of drug resistance, conferring great relevance to this mechanism [48].

Overall, it seems that a transiently-acquired phenotypically distinct state mediates the temporary drug tolerance of a subpopulation of cancer cells, which could protect the tumor from

eradication by lethal exposures. Of interest, this dynamic phenotypic heterogeneity leading to a reversible state of multi-drug tolerance in a subpopulation of cells is remarkably analogous to the formation of persister cells within bacterial populations [48].

Table 1. TA modules of which toxin overexpression increases the *E. coli* persister fraction

Module	Toxin	Type	Target mechanism	Pathways affected	References
<i>hipBA</i>	HipA	II	Phosphorylation of EF-Tu (putative target)	Inhibition of translation, RNA and DNA synthesis	[23,62-64]
<i>dinJ-yafQ</i>	YafQ ^a	II	Site-specific mRNA cleavage	Inhibition of translation	[25,65]
<i>relBE</i>	RelE	II	mRNA cleavage at ribosomal A site	Inhibition of translation	[10,24,66]
<i>mazEF</i>	MazF	II	Site-specific mRNA cleavage	Inhibition of translation	[24,67,68]
<i>mqsRA</i>	MqsR	II	Site-specific mRNA cleavage	Inhibition of translation	[69,70]
<i>tisB-istR-1</i>	TisB ^b	I	Insertion in inner membrane	Disruption of proton motive force	[20-22]

^a *yafQ* overexpression specifically increases the *E. coli* persister fraction in biofilms [25]

^b *tisB* overexpression only increases the *E. coli* persister fraction in exponentially growing cultures [21]

Figure legends

Figure 1. Occurrence of a fraction of persister cells within a bacterial population. **(a)** The upper panel shows an isogenic population of antibiotic-sensitive bacterial cells (red). Persister cells (black) are phenotypic variants of the wild type that are formed at low frequency in the population (middle panel). Upon antibiotic treatment, the majority of cells is killed off, leaving the tolerant persisters unaffected (lower panel). Removal of the antibiotic pressure (denoted by the arrow alongside the panels) allows the persister cells to resume growth, thereby generating a new population that is genetically identical to the original one. Hence, this new population is again susceptible to the antibiotic. **(b)** Biphasic killing pattern of a sensitive bacterial population upon antibiotic treatment. Following antibiotic treatment, the number of cells in the population (y-axis) initially decreases sharply. In a second phase however, increasing the treatment length or antibiotic concentration ([AB]) (x-axis) kills the remaining persister cells at much slower rate.

Figure 2. Overview of recently gained insights into the nature of persisters and their formation. **(a)** Emergence of antibiotic tolerance by active induction of oxidative stress defense mechanisms under control of the stringent response. Bactericidal antibiotics (AB) induce formation of cytotoxic reactive oxygen species (ROS) that target essential cellular components such as proteins, DNA and lipids. Activation of the stringent response promotes the activation of oxidative stress defense elements such as superoxide dismutases (SODs) and catalases in both *P. aeruginosa* and *E. coli*. In addition, production of the pro-oxidant hydroxy-2-alkylquinoline (HAQ) is inhibited by an active stringent response in *P. aeruginosa*. Overall, this results in lowered cellular ROS levels, which in turn leads to antibiotic tolerance. **(b)** Induction of persister formation by antibiotics. In *E. coli*, treatment with DNA-damaging antibiotics triggers

the SOS response for DNA repair. Cleavage of LexA, the repressor of SOS genes, leads to release of inhibition of *tisB* transcription. As a consequence, free TisB, encoding a toxin part of a type I toxin-antitoxin (TA) module, accumulates. Insertion of TisB in the membrane subsequently disrupts the proton motif force (PMF) causing cellular ATP levels to drop. This leads to dormancy and persistence. **(c)** Formation of persister cells through elevated toxin expression. Type II TA modules are encoded in operon structures (dark and light green arrows). Expression of these operons yields protein toxins (T) (light green) and antitoxins (A) (dark green). Toxic action (mRNA cleavage in case of type II TA modules) is normally inhibited by tight complex formation between the toxin and its cognate antitoxin. However, activation of the Lon protease leads to rapid degradation of the unstable antitoxins, allowing free toxins to accumulate. The cellular concentration of free toxins differs between the cells in a population (illustrated as a normal distribution of toxin concentrations within a population). Although this level is comparable in the majority of cells, a fraction of cells displays a stochastically higher free toxin level (black part under the curve). Toxic action is enhanced in these cells in which the concentration of free toxin exceeds a threshold level (red dotted line). A shutdown of cellular processes as a consequence of toxin action leads to the onset of persistence in a fraction of the population. **(d)** Induction of persistence through chemical signaling. (Left) Nutrient limitation leads to the production of the bacterial signaling molecule indole (green sphere). Heterogeneous sensing of this signal molecule induces persister formation (black cells) in a fraction of cells within an *E. coli* population (red cells). (Right) Quorum sensing-related signaling molecules (QSS) (blue) increase the *P. aeruginosa* persister fraction.

Figure

Figure

(a) **Oxidative stress defense**

(b) **Induction by AB**

(c) **Redundant TA modules**

(d) **Chemical signaling**