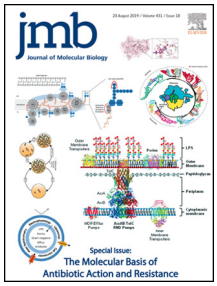

Clarifying the Link between Toxin–Antitoxin Modules and Bacterial Persistence

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Abstract

While most of a bacterial population is killed upon antibiotic exposure, a fraction transiently exhibits a multidrug-tolerant phenotype termed antibiotic persistence. This phenomenon enables the bacteria to escape killing by drugs and is presumed to be, at least partly, responsible for the recalcitrance of many bacterial infections. For this reason, understanding mechanisms allowing a fraction of a bacterial population to become transiently multidrug-tolerant represents an essential step to eradicate these persisting subpopulations. Toxin–antitoxin (TA) systems were proposed as perfect candidates to control this phenomenon since these elements are often mutated in high-persistence screens and overexpression of these toxins often increases persister frequency in a defined population. However, the accumulation of evidence and counter-evidence for the role of TA systems in bacterial persistence has led to general confusion in the field. In this review, we summarize evidence that link TA modules to antibiotic bacterial persistence. Then, we discuss the limitations of work on these stress-responsive modules as well as bacterial persistence in general.

Introduction

Upon exposure to antibiotics, a fraction of any isogenic bacterial population is able to survive and generate viable offspring capable of resuming growth following removal of antibiotics from the environment. These cells, referred to as persisters, are rare cells that transiently display a multidrug-tolerant phenotype. This phenomenon was shown for the first time in 1942 by Hobby and colleagues [1], who observed that a small part of a *Staphylococcus* population was not killed upon penicillin treatment. Only 2 years later, Bigger [2] focused his work on these surviving bacteria that he named persisters. In his pioneering work, he suggested that these cells were temporarily in a non-dividing state. In addition, he discovered that these persisters generated offspring, which were as easily killed by penicillin as the original culture. Despite the potential involvement of these phenotypic variants in relapsing infections, antibiotic persistence was ignored for decades despite the confirmation of Bigger's observations in other bacterial species [3–6]. Moyed and Bertrand [7] explored the genetic basis of antibiotic persistence for the first time by isolating mutants of *Escherichia coli*, which produce a higher fraction of persisters in the population. Twenty years later, Balaban and colleagues [8] used a strain carrying one of these gain-of-function alleles, commonly known as *hipA7*, to track bacterial persisters in a microfluidic device. In their work, they elegantly showed that *hipA7* persisters were growth-arrested bacteria in the bacterial population, thus confirming Bigger's suggestion that lack of growth was enabling bacteria to survive exposure to penicillin. Shortly after, HipA was shown to be a toxin that causes self-inhibition of growth in the absence of its cognate HipB antitoxin, making HipAB a toxin–antitoxin module (TA module) [9,10]. These discoveries linked TA modules and bacterial persistence for the first time. Such bipartite systems are ubiquitous and highly represented in bacterial genomes [11–15]. They are believed to be stress-response elements that can cause transient

bacterial growth arrest and therefore appear to be the perfect candidates to control formation of growth-arrested bacterial persisters [16]. In the years following these publications, both antibiotic persistence and TA systems have become much more extensively studied. Evidence and counter-evidence for the role of TA systems in persister formation have accumulated, leading the community to question their contribution to this phenomenon [17–22]. In this review, we critically adress the importance of TA modules by reviewing the current body of evidence linking these molecular players to bacterial persistence in enterobacteria. Then we address the current limitations, bias and future challenges of the field.

TA Systems

Biology of TA modules

TA modules are usually composed of two elements: a stable toxin that intoxicates the cell by interfering with an essential process such as translation, for example, and an unstable antitoxin that neutralizes its cognate toxin [21,23]. Originally, TA modules were described as "addiction modules" enabling plasmid maintenance after cell division by a process termed post-segregational killing [24–26]. Daughter cells that lose the plasmid are killed by the toxin (or at least stop proliferating) since the unstable antitoxin needs to be continuously produced from the plasmid to prevent intoxication by the toxin [24–26]. TA modules were also shown to provide phage resistance by a mechanism referred to as abortive infection [27]. By hijacking bacterial transcription and translation, phages limit the production of the unstable antitoxin, which leads to self-intoxication by the toxin. Consequently, phage proliferation is limited by the strong impact that the toxin has on bacterial physiology [27]. TA loci are also commonly found in bacterial chromosomes, but their distribution varies considerably, even between closely related bacterial species [11–15]. Until now, six types of TA module have been described, each defined by the neutralization mechanism of the antitoxin. Type I and type II modules are the most abundantly reported in the literature. Type I antitoxins are anti-sense RNAs that block translation of the mRNA of their cognate toxins, therefore inhibiting translation of the toxin. In type II TA systems, antitoxins are proteins that neutralize their cognate toxins by direct binding. In the other families of TA system, the antitoxin has been shown to be a toxin-binding sRNA (type III), a competitor of the toxin target (type IV), an endoribonuclease degrading the toxin mRNA (type V) or a proteolytic adapter of the toxin (type VI) [21,23,28].

Regulation of TA modules by stress responses

TA systems are mostly described as stress-responsive modules that are activated in specific conditions. Even if most of them are self-regulated operons, this self-regulation is modulated by major stress regulators. Two major stress responses have been linked to the regulation of TA modules: the stringent and the SOS responses (Fig. 1).

The stringent response is triggered in response to nutrient limitation and is mediated by the (p)ppGpp alarmone [29]. Accumulation of (p)ppGpp affects essential processes such as transcription and translation, and thereby reprograms the cell to enable adaptation and better survival under harsh conditions [30–32]. Several groups have reported upregulation or activation of TA systems upon establishment of the stringent response. For example, the addition of serine hydroxamate, a potent inducer of the (p)ppGpp alarmone, to growth medium causes mRNA levels of the *hicAB* type-II TA module to increase sharply in *E. coli* [33]. Similarly, isoleucine starvation leads to high levels of (p)ppGpp and upregulates a large number of type II TA modules including *hicAB*, *dinJ/yafQ*, *mazEF*, *mqsRA*, *relBE* and *yafNO* [18,34]. In addition to type II TA modules, the (p)ppGpp alarmone also upregulates the type I toxin HokB [35]. Interestingly, (p)ppGpp does not only promote expression of TA modules but also participate in the activation of the toxin, for example, by affecting the stability of the cognate antitoxin [35,36].

The SOS response is triggered by DNA damage induced by a broad set of conditions including oxidative stress, UV irradiation or exposure to antibiotics [37–39]. Accordingly, the SOS response is primarily involved in the activation of DNA repair systems, but also triggers type I (*tisB/istR-1*, *symE/symR*, *dinQ/agrB*, *hokE/sokE*) and type II (*yafNO*, *dinJ/yafQ*) TA modules [37,39–46].

Antibiotic Persistence

To understand how TA modules could participate in the survival of bacteria upon antibiotic exposure, it is important to differentiate three distinct phenomena—bacterial resistance, tolerance and antibiotic persistence [47,48]. Resistance is an inherited trait that allows a clonal bacterial population to grow in the presence of a specific antibiotic. The minimal inhibitory concentration (MIC) for this specific antibiotic is higher for a resistant strain compared to that of a susceptible strain, and therefore, MIC is a good readout of bacterial resistance [48]. In contrast, both tolerance and persistence allow bacteria to transiently survive antibiotic exposure. For this reason, these two phenomena are easily confused in the literature, although they have major differences. Tolerance is

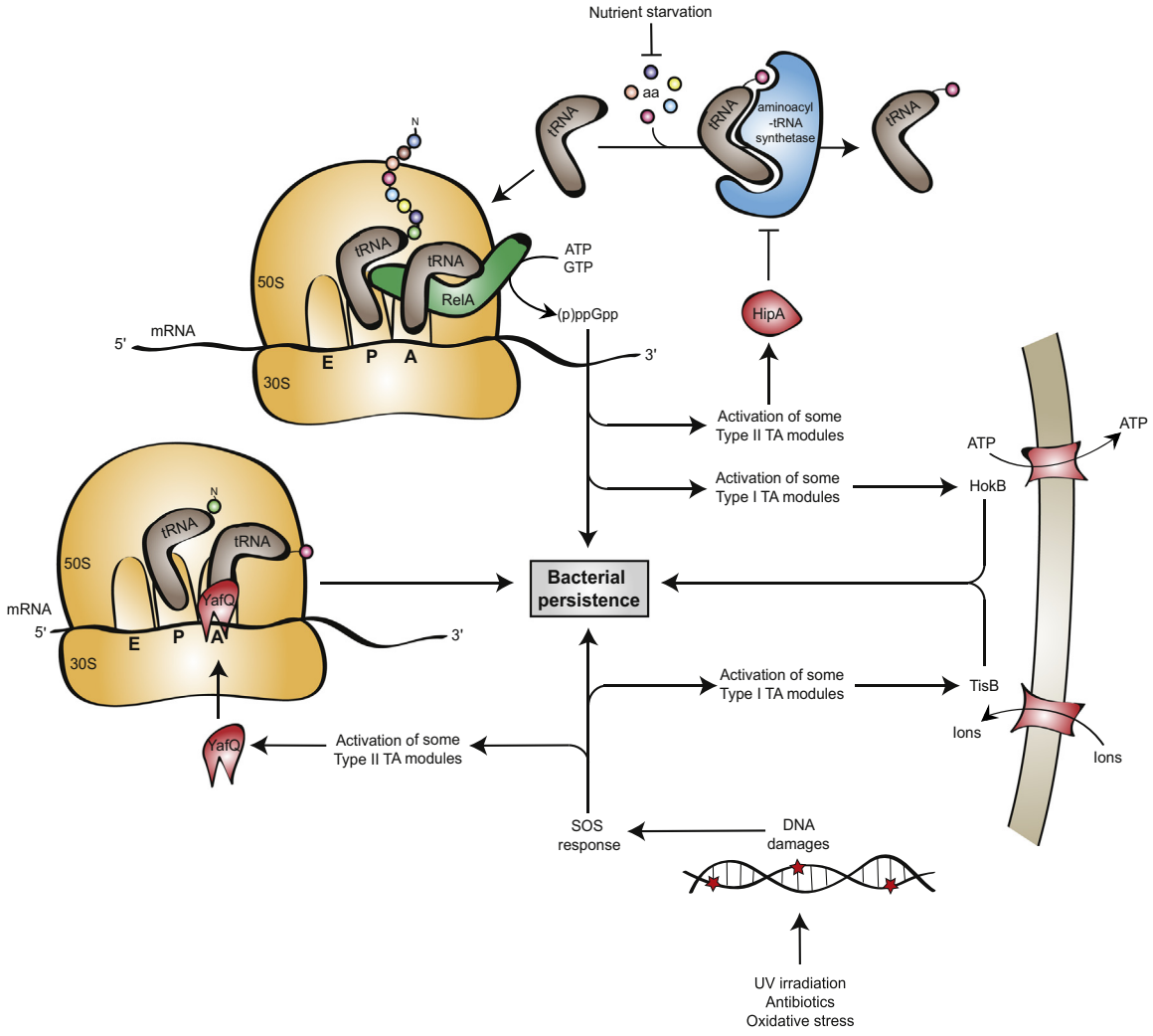

Fig. 1. TA modules are activated by stress responses that contribute to bacterial persistence. Under amino acid starvation or intoxication by HipA toxin, uncharged tRNA in the ribosomal A site promotes the accumulation of (p)ppGpp through RelA, leading to an increase of persister cells in the population. Interestingly, (p)ppGpp also activates specific type I and type II TA modules that contribute to bacterial persistence such as the type I toxin HokB. In the same way, the SOS response triggers the activation of specific type I and type II TA modules that participate in the increase in persister cells in the microbial population.

homogeneously displayed by the whole population, whereas persistence is only found in a fraction of a clonal population (Fig. 2). For example, bacterial populations that are slow growing, due to either their environment or a genetic alteration, are tolerant to beta-lactams because this class of antibiotic only kills actively growing cells [49]. Consequently, a longer exposure to the antibiotic is required to observe the same level of killing as that obtained with a susceptible strain [50]. Therefore, the minimal duration for killing (MDK) was proposed as a measure for comparing the tolerance levels between two populations [48]. For example, the MDK_{99} represents the minimum duration of an antibiotic treatment required to kill 99% of a bacterial population. In contrast to resistance and tolerance, antibiotic persistence is only displayed by a fraction of an entire population and will therefore not influence the MIC or the MDK of this population (Fig. 2). To reveal antibiotic persistence, a kill curve as a function of time is required. The presence of persisters is manifested through a second phase in the killing kinetics where persisters die at a much slower rate than their susceptible kin [48] (Fig. 2B). It is important to note that the frequency of persister cells in a bacterial population increases in response to numerous stresses such as starvation or DNA damage [20] (Fig. 1). This phenomenon is referred to as “triggered persistence” in contrast to “spontaneous persistence,” which is believed to occur at a

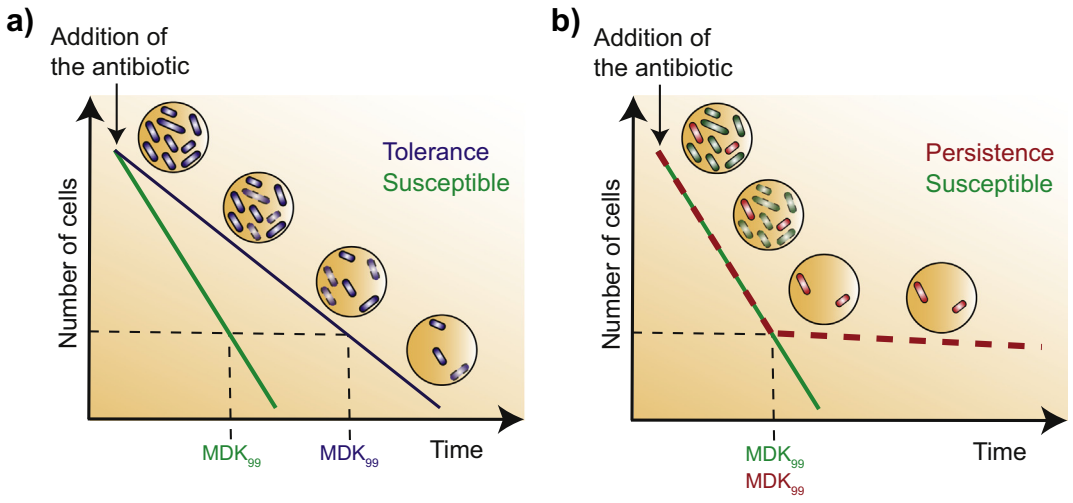

Fig. 2. The difference between bacterial tolerance and persistence in response to antibiotic exposure. Populations containing susceptible (green), tolerant (blue) and persistent (red) bacteria display different killing curves. The minimal duration of killing (MDK_{99}) measures the duration of antibiotic exposure required to eradicate 99% of the population and enables discrimination between tolerance and persistence. (a) Tolerant bacteria are less sensitive to antibiotics, and therefore, a longer antibiotic exposure is required to kill 99% of the population (MDK_{99}) than a susceptible strain. (b) Bacterial populations containing persister cells display a biphasic killing curve that can be divided in to two phases. First, non-persister cells are killed at the same rate as a susceptible strain. Second, the tail of the killing curve reveals the presence of persister cells in the microbial population.

considerably lower rate in a bacterial population in the absence of any particular stress (Fig. 3).

Implication of TA Modules in Bacterial Persistence

Role of type II TA modules in bacterial persistence

To isolate mutants with a high frequency of persister cells, Moyed and Bertrand [7] exposed *E. coli* to successive rounds of ampicillin treatment and discovered mutations in *hipA*, the gene encoding the toxin of the HipAB type II TA module. One of the high-persister alleles they found, *hipA7*, contains two substitutions in the HipA toxin (G22S and D291S), which led to an increase in survival upon exposure to ampicillin in comparison with the parental strain, but without any change in MIC [9]. The increased survival of the *hipA7* strain is due to a higher frequency of persister cells in the population [8]. Two independent research groups showed that HipA is a serine–threonine kinase that phosphorylates the aminoacyl $tRNA^{glu}$ synthetase

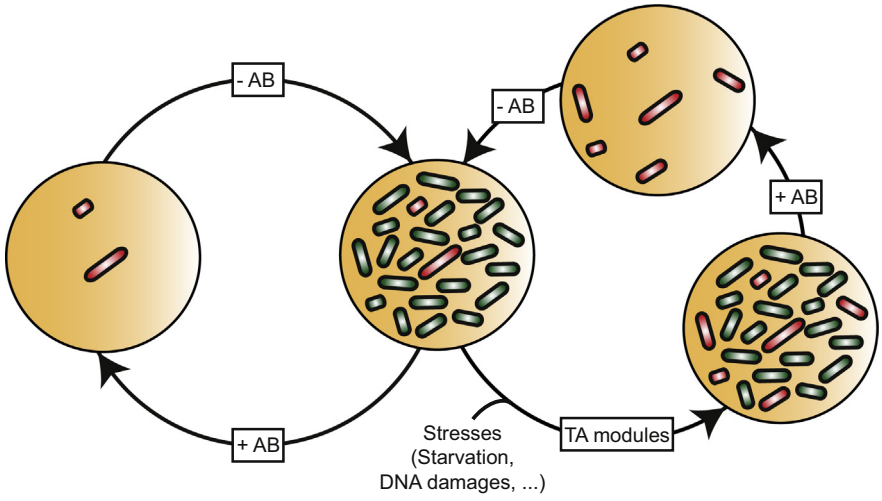

Fig. 3. Increased frequency of persister cells can be triggered by stress responses. Upon activation of stress responses induced by starvation or DNA damage, specific TA modules are activated and contribute to bacterial persistence by increasing the frequency of persister cells in the population under these harsh conditions.

GltX, leading to accumulation of uncharged $tRNA^{glu}$ in the cell. Consequently, the presence of this "hungry" uncharged tRNA in the ribosomal A site activates the production of the (p)ppGpp alarmone by RelA [9,51–53] (Fig. 1). Interestingly, a recent study showed that, in addition to GltX, HipA also phosphorylates additional targets such as ribosomal proteins that could contribute to the formation of persister cells [54]. However, the HipA7 toxin variant was shown to exclusively phosphorylate GltX, supporting that phosphorylation of this unique target is sufficient to lead to a high persister phenotype in *E. coli* [54].

Besides the higher number of persisters observed by Balaban in exponentially growing bacteria for a *hipA7* mutant, the wild-type HipAB module was also shown to contribute to bacterial persistence in other conditions such as stationary phase and biofilm populations [55]. It is noteworthy that the activity of HipA triggers production of (p)ppGpp, which has been shown to promote antibiotic persistence (Fig. 1). Indeed, mutants devoid of the (p)ppGpp alarmone have a decreased survival upon antibiotic exposure [56]. Therefore, the role of HipA in stimulating persister formation may be via its kinase activity on direct targets, or indirectly via the induction of (p)ppGpp and the stringent response. Strikingly, the genomic analysis of *E. coli* isolates from patients with recurrent urinary tract infections revealed the selection of *hipA7* variants in patients over time, supporting the relevance of *hipA* as a "persister" gene [57].

Other than the case of the HipA toxin, one of the most cited pieces of evidence supporting the role of type II TA modules in persister formation was the observation that the deletion of 10 mRNA endonucleases (commonly referred as *ΔTA10* strain in the literature) decreased bacterial persistence in exponentially growing *E. coli* [58]. Recently, it was shown that the decreased persistence in this strain was due to the presence of the ϕ80 bacteriophage, a common laboratory contaminant, in the mutant strain but not the wild-type [22]. Reconstruction of the original *ΔTA10* strain (*ΔmazF*, *ΔchpB*, *ΔrelBE*, *ΔdinJ/yafQ*, *ΔyefM/yoeB*, *ΔhigBA*, *ΔprlF/yhaV*, *ΔyafNO*, *ΔmqsRA*, *ΔhicAB)* revealed that these 10 TA modules do not affect persistence in nutrient-rich conditions [22,59]. Quite understandably, the debate about this work has led the scientific community to question the role of TA modules in bacterial persistence [18,19,22,59,60]. However, this result principally disproves that the 10 TA modules tested play a role in spontaneous persister formation, but does not address the question of a role of any of these stress-responsive elements in control of growth-arrest, and the subsequent antibiotic persistence, under stress conditions.

For example, overexpression of RelE or MazF toxins was shown to sharply increase the survival of *E. coli* under antibiotic exposure, suggesting that these toxins could contribute to bacterial persistence in a condition where their cognate antitoxin is absent [55,61]. A second example is the *dinJ/yafQ* TA module which is involved in antibiotic susceptibility to cefazolin (cephalosporine) and tobramycin (aminoglycoside) in biofilm but not in exponentially growing cells [18,63]. Intriguingly, the implication of this TA module in biofilm tolerance seems restricted to some antibiotics since deletion of *dinJ/yafQ* does not affected tolerance of biofilms to doxycycline (cycline), rifampin (rifampicin) or oxfloxacin (fluoroquinone) [62,63]. Beside *dinJ/yafQ*, a SNP mutation (V10F) in the YafN antitoxin of the YafNO TA module was recently identified in a genetic screen where *E. coli* was subjected to cycles of antibiotic exposure [64]. Tolerance and persistence mutations (with no change in MIC) selected in this genetic screen caused an extended lag phase, suggesting that mutations in the YafN antitoxin could lead to intoxication by YafO. However, further investigations are needed to confirm this hypothesis. Moreover, five other lag phase-extending SNPs (T3N, L7P, A13P, L16R, V20G) were found in VapB, the antitoxin of the VapBC type II TA module, suggesting that activation of VapC could lead to an increase in the number of persisters in the population [64]. In their work, the authors also described mutations in the methionyl-tRNA synthetase (encoded by *metG*), which increase bacterial persistence. Interestingly, the VapC toxin was shown to target $tRNA^{fMet}$, suggesting that mutations in the VapB antitoxin or in MetG could both lead to a decreased availability of charged $tRNA^{fMet}$, ultimately leading to bacterial persistence [65].

Further evidence linking type II TA modules and bacterial persistence was reported in uropathogenic isolates of *E. coli*. In these bacteria, deletion of the *pasTI* type II TA locus alone decreases survival of the microbial population in complex medium after 5 h of ampicillin and ciprofloxacin exposure. Despite these clear observations in UPEC, deletion of *pasTI* in the widely used *E. coli* K12 laboratory strain does not lead to the same phenotype, suggesting that the function of TA modules might be dependent not only on the environmental condition but also on their genetic context [66].

Interestingly, phosphate starvation, acid and osmotic stress lead to upregulation of expression of some specific type-II TA (including *dinJ/yafQ*, *mazEF*, *mqrSA*, *relBE*, and *yafNO*) but do not increase bacterial persistence in these conditions [18]. However, some of these TAs have been shown to influence bacterial persistence under other stress conditions, suggesting that they have the ability to contribute to bacterial growth arrest [63]. The toxins of these upregulated TAs were not shown to be active (i.e., having a toxic effect) under phosphate starvation, acid and osmotic stress conditions. This suggests that increasing the expression of a TA module might not be sufficient to promote

bacterial persistence if conditions still allow the antitoxin to neutralize its cognate toxin.

Beyond *E. coli*, TA modules were also linked to bacterial persistence in other bacteria such as *Salmonella*. Isolation of high persister mutants in this pathogen after two rounds of ampicillin treatment led to the discovery of the *shpB1* allele, a mutation in the antitoxin of the *shpAB* TA locus (for *Salmonella* High Persister), but the mechanism promoting bacterial persistence in this strain remains totally unknown [67]. In addition to the ShpAB module, 13 other type II modules were shown to contribute to bacterial persistence triggered upon stresses encountered during infection of primary macrophages [68]. Particularly, three acetyltransferase toxins (TacT, TacT2, TacT3) of *Salmonella* have been shown to alter translation through acetylation of aminoacyl-tRNAs [69,70]. Overexpression of these three toxins leads to an increase in the proportion of persisters in the population, as opposed to overall increased tolerance [69,70], and their deletion leads to a decrease in the proportion of persisters recovered upon macrophage infection [68].

What about the role of the other types of TA modules?

Alongside the type II TA modules, other types of TA modules were shown to be involved in bacterial persistence in *E. coli*. For example, membrane-targeting toxins such as TisB, HokB and GhoT increase antibiotic persistence by damaging the membrane, affecting the proton-motive force and the ATP levels [35,39,71,72] (Fig. 1). Absence of the type I toxin TisB leads to decreased survival after ciprofloxacin exposure, whereas the deletion of *istR-1*, its cognate antisense RNA antitoxin, results in a higher number of colonies in the same condition [39]. In addition, overexpression of *tisB* significantly increases the survival of *E. coli* exposed to ciprofloxacin without any change in MIC, supporting the role of this type I TA module in bacterial persistence [39]. Along the same line, the production of the type I toxin HokB provokes a collapse in the membrane potential of *E. coli*, which ultimately leads to bacterial persistence via slow growth [35]. GhoST is a type V TA module where the GhoS antitoxin is an endoribonuclease that cleaves the *ghoT* mRNA, thereby inhibiting toxin production [71,72]. Interestingly, the activation of the membrane-targeting GhoT toxin is dependent on the MqsR type-II toxin, which promotes the degradation of the antitoxin GhoS mRNA under specific conditions to promote bacterial persistence [71].

The two main pathways that were described to activate TA modules, the stringent and the SOS response, are also shown to drastically increase bacterial persistence in conditions where they are activated [38,39,56,62]. Moreover, mutants in gram-negative bacteria that are devoid of these stress responses have a decreased survival in comparison with a wild-type strain upon antibiotic exposure [35,38,39,56,62]. Strikingly, the loss of the TisB toxin in a strain constitutively expressing the SOS response leads to a dramatic decrease of persister cells upon ciprofloxacin treatment, supporting the biological function of TisB toxin in the SOS-dependent formation of persister cells [39].

Current Limitations and Future Challenges

Despite a large body of literature describing TA modules, their relevance in bacterial persistence remains controversial. The main reason is that studying TA modules in addition to their impact on bacterial persistence remains technically challenging and many groups refer to very different phenomena using the same terminology. Consequently, special attention should be given to major drawbacks and limitations in this field.

The first priority is to validate the approach used to distinguish bacterial tolerance and antibiotic persistence to avoid confusion between these two phenomena. Toxins are associated with bacterial persistence for two main reasons. First, their overexpression leads to an increase in survival upon antibiotic exposure without any change in MIC. Second, the increased survival is due to an increase in persister frequency—that can be revealed by a higher tail of a biphasic killing curve—as opposed to increased tolerance of the entire population as observed in increase in MDK_{99} [48] (Fig. 1). It is to be expected that the overexpression of any moderately toxic molecule that decreases the growth rate of an entire population would thereby decrease the rate of antibiotic killing leading to an increased tolerance of that population. To illustrate this point and question the use of overexpression to define toxins as "persister" determinants, Vazquez *et al.* [73] overexpressed unrelated proteins that reduced growth rate (such as DnaJ and PmrC) and showed that cells expressing these proteins display an increased survival to ampicillin and ciprofloxacin exposure, similar to the HipA and MazF TA toxins. However, the MIC was not measured for these strains, and the absence of biphasic killing curves means that the discrimination between tolerance and persistence in this study is not possible [73].

A second priority is to choose appropriate conditions to characterize the role of TA modules in bacterial persistence. Up to now, overexpression of toxins has offered the easiest way to characterize the molecular mechanisms underlying their toxic activity on essential processes such as translation or DNA replication [52,54,69,70,74]. However, it is undeniable that overexpression of toxins on multicopy plasmids can generate artifacts. For this reason, emphasis should be put on identifying

conditions under which TA modules are activated, to allow for meaningful comparisons between wild-type and mutant strains. As discussed previously, the absence of a persister phenotype in the *Δ10TA* strain is not sufficient to draw conclusions about the implication of these TA modules in bacterial persistence since some of these modules contribute to cell survival upon antibiotic treatment in response to specific stimuli, highlighting the importance of using the optimal experimental conditions [22,59].

Finally, one of the major experimental limitations when examining the role of TA modules lies in functional redundancy existing between these actors. For example, a high number of TA modules target the translation machinery to control bacterial growth [21]. On top of that, an interdependency has been shown between different TA modules suggesting a hierarchical organization of these systems [71,75]. Ideally, a strain completely devoid of any TA modules would give the greatest insight into their roles in persistence, but their abundance in genomes of many bacteria has so far made this a challenging task. The *Δ10TA* strain, for example, only covers approximately 25% of the TA modules present in *E. coli* [16,21].

Concluding Remarks

Currently, a large body of evidence supports that TA modules contribute to bacterial persistence. However, studying the involvement of TA systems in this phenomenon is challenging since only a small fraction of the population becomes persister cells. Consequently, two schools of thought have emerged on the question of bacterial persistence. Whereas part of the community considers bacterial persistence as an evolved character, others postulate that this phenomenon might arise from "different kinds of errors and glitches" [20,76]. Despite this, heterogeneity in a microbial population has emerged as a key factor for bacterial persistence [47,77]. In agreement with the literature, we suggest that the role of TA modules is to increase the heterogeneity in the population in response to different stresses such as starvation or DNA damage [21]. Activation of these TA modules in a fraction of the population could impact the entry into, or the length of growth arrest, leading to bacterial persistence [8] (Fig. 3). For this reason, mutations in TA modules have been easily isolated by genetic screens for high persistence phenotypes, as dysregulation of one of these TA modules (through hyperactivation or hyponeutralization of the toxin) can increase the frequency of persister cells in the population [7,64,67]. Finally, it is important to keep in mind that TA modules participate in other phenomena beyond bacterial persistence, as already shown by different studies that highlight their implication in host invasion and in virulence [78,79]. To conclude, although our knowledge of the molecular mechanisms of TA modules becomes more and more detailed, fully understanding how these TA modules participate in bacterial survival in nature is an exciting challenge for future work.

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TA, toxin–antitoxin; MIC, minimal inhibitory concentration; MDK, minimal duration for killing.

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