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Bacterial toxin-antitoxin systems: Novel insights on toxin activation across populations and experimental shortcomings

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ABSTRACT

The alarming rise in hard-to-treat bacterial infections is of great concern to human health. Thus, the identification of molecular mechanisms that enable the survival and growth of pathogens is of utmost urgency for the development of more efficient antimicrobial therapies. In challenging environments, such as presence of antibiotics, or during host infection, metabolic adjustments are essential for microorganism survival and competitiveness. Toxin-antitoxin systems (TASs) consisting of a toxin with metabolic modulating activity and a cognate antitoxin that antagonizes that toxin are important elements in the arsenal of bacterial stress defense. However, the exact physiological function of TA systems is highly debatable and with the exception of stabilization of mobile genetic elements and phage inhibition, other proposed biological functions lack a broad consensus. This review aims at gaining new insights into the physiological effects of TASs in bacteria and exploring the experimental shortcomings that lead to discrepant results in TAS research. Distinct control mechanisms ensure that only subsets of cells within isogenic cultures transiently develop moderate levels of toxin activity. As a result, TASs cause phenotypic growth heterogeneity rather than cell stasis in the entire population. It is this feature that allows bacteria to thrive in diverse environments through the creation of subpopulations with different metabolic rates and stress tolerance programs.

1. Introduction

During cell division, post-segregational killing ensures the faithful transfer of plasmids to daughter cells. This process was discovered in the 1980s and is based on a pair of genes normally coded as operons in some plasmids (Fig. 1A) (Ogura and Hiraga, 1983). Expression of these genes gives rise to a toxin with growth inhibitory activity and an antitoxin that antagonizes toxin activity or expression usually by the formation of a toxin-antitoxin complex (Gerdes et al., 1986). When TA-encoding plasmids are lost, the intracellular level of antitoxins rapidly decreases due to their high proteolytic susceptibility, resulting in the accumulation of stable toxins which end up causing more or less severe metabolic constraints (Tsuchimoto et al., 1988). Thus, TASs create a strong selective pressure for the maintenance of TA-encoded plasmids, as they reduce the competitiveness of cells that failed to inherit them (Bravo et al., 1988). However, it should be noted that the term post-segregational killing suggests the death of cell siblings that do not inherit TA-containing plasmids, there is no evidence for toxin mediated cell death.

Many years after the discovery of TASs, it was found that they are ubiquitous in the genome of nearly all prokaryotes (Masuda et al., 1993; Aizenman et al., 1996; Shao et al., 2011; Leplae et al., 2011). The number of TA loci varies among different bacteria genera and species and even within closely related strains from the same species. For instance, *Mycobacterium tuberculosis* carries more than 88 TA modules (Ramage et al., 2009), while *Mycobacterium smegmatis* has only eight of these systems (Zhang et al., 2022a). The identification of new TA genes is rapidly expanding, in part due to the development of new bioinformatic tools, such as TADB 2.0 (Xie et al., 2018), TASmania (Akarsu et al., 2019), BtoxDB (Barbosa et al., 2015), RASTA (Sevin and Barloy-Hubler, 2007), FlaGs (Jimmy et al., 2020), TAGMA (Klimina et al., 2020), SLING (Horesh et al., 2018) and T1TAdb (Tourasse and Darfeuille, 2021).

Distinct toxins have different molecular activities. They promote growth inhibition by degradation, post-translational modification or interaction with their cellular targets as show in Table 1 and reviewed in Harms et al. (2018).

Chromosomal TASs are thought to confer stress tolerance by

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repressing metabolic activity via toxin activation (Fig. 1B) (Tamman et al., 2014). The liberation of toxins from inhibitory TA-complexes is tightly regulated and occurs before and during the course of the stress response, yielding cells with diverse metabolic capacities to efficiently face adverse situations (Keren et al., 2011).

The mediation of persistence has been proposed as an attribution of TASs (Kim and Wood, 2010). Persistence is a physiological state in which small subpopulations of bacteria transiently assume a pronounced non-heritable stress resilient phenotype (Jung et al., 2019). Although the physiological programs conferring increasing fitness can be very complex, one of the main features of persisters is a low metabolic state that may arise due to toxin activity (Fig. 1C) (Van den Bergh et al., 2017). Persistence represents a formidable obstacle to antibiotic treatment because these molecules normally require strongly metabolically active cells to exert their antibacterial effects (Balaban et al., 2019).

In addition to persistence, the bacteriostatic activity of toxins protects against phage infection by interfering with the production of mature virions, and thus, their propagation within the clonal bacterial populations (Vassallo et al., 2022; Laura Fernández-García et al., 2023). The first demonstration of TA mediated phage defense came from the Wood group in the late 90 s, who observed that the type I TAS *hok/sok* prevented T4 propagation within *E. coli* cultures (Pecota and Wood, 1996). Since then, the number of TA loci implicated in phage defense has rapidly increased as reviewed in (Kelly et al., 2023; Song and Wood, 2020c).

The metabolic adjustment imposed by toxins have also been associated with other essential physiological processes, namely, biofilm formation (Wang and Wood, 2011), virulence (Lobato-Márquez et al., 2016) and stabilization of pathogenic islands (Wozniak and Waldor, 2009; Yao et al., 2015; Peltier et al., 2020). There is also the possibility that TASs in some microorganisms, as for instance in *Pseudomonas aeruginosa*, behave as selfish elements with no physiological function and are maintained in the host chromosome due to the addicted effect imposed by toxins.

2. Classification of TASs

TASs are classified into eight groups (type I to VIII), according to the molecular nature and mode of action by which antitoxins counteract toxins (Song and Wood, 2020b). Except for the recently discovered type VIII in which the toxins are RNAs, in all other TA classes, toxins are made of proteins. Antitoxins are either non-coding RNAs (type I, III and VIII) or proteins (type II, IV, V, VI and VII) (Harms et al., 2018). Most type I TASs code for membrane pore-forming toxins. Their inhibition occurs at the post-transcriptional level by base-pair interaction with a non-coding antisense RNA antitoxin, which blocks toxin translation and/or

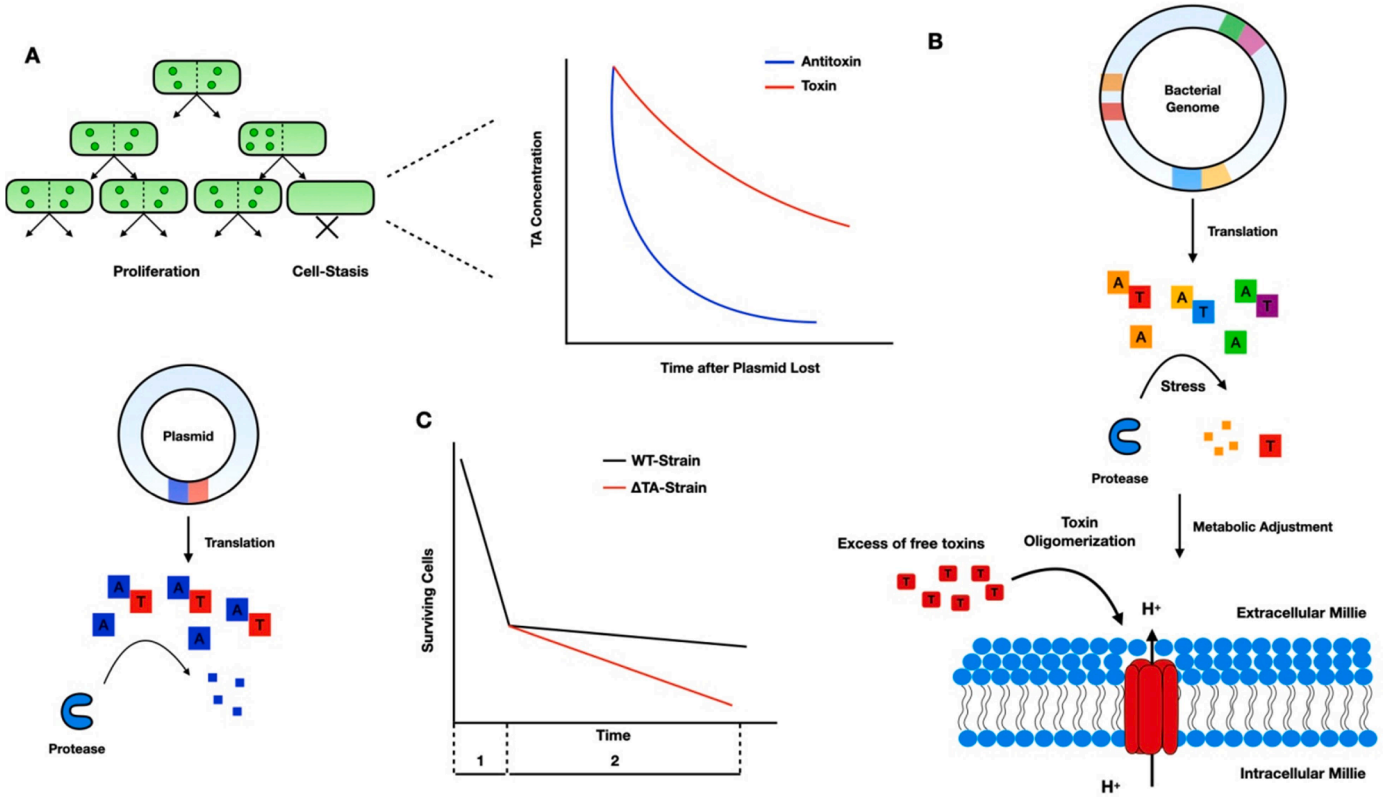

Fig. 1. Overview of plasmid and chromosomal encoded TAS. A) Some plasmids contain TA genes that enhance their stability within the host cell. Toxins and antitoxins are co-expressed and due to the high affinity between them (red squares: toxins; blue squares: antitoxins), TA-complexes are formed, inactivating the toxins. The turnover rate of the antitoxin is higher than that of the toxin because antitoxins are proteolytically unstable. Thus, the inhibition of toxins requires the constant expression of TA genes. The degradation of antitoxins occurs only when they are not bound to their cognate toxins. During cell division, plasmids can be lost and the expression of TA genes is interrupted. In cells without plasmids-encoded TAs, the level of the antitoxins drops significantly, so free toxins cause harsh metabolic inhibition that impairs the proliferation of such subpopulations. **B)** As in plasmid-encoded TAs, chromosomal-encoded toxins and antitoxins are co-expressed forming inactive TA-complexes under normal growth conditions. Toxin activation is triggered by environmental or nutritional stresses. Under such conditions, the toxin disengages from the TA complex, and promotes the slowdown of several metabolic processes (Table 1). The figure in the bottom right corner shows a toxin that interacts with the bacterial cytoplasmic membrane, promoting the formation of pores and proton gradient dissipation. **C)** The exposure of cells to stress yields a biphasic killing curve (black curve). The two phases are labeled in the graph as 1 and 2. In the initial period of treatment all susceptible cells rapidly die, yielding a sharp exponential drop in the number of viable cells – phase 1. In the remaining time, corresponding to phase 2, the recalcitrant persisters die, but with a considerably lower death kinetic. Persisters display low metabolic rates, that is at least partially caused by the action of toxins. Indeed, TA-deleted strains generate fewer persister cells than the WT (red curve) while bacteria with toxin-activating mutations generate high numbers of persisters (blue curve).

Table 1
Molecular activities of *E. coli* K-12 MG1655 TASs and their impact on metabolism.

TA module	Toxin activity	Cellular process impacted
RelBE, YefM/YoeB, YafNO, DinJ/YafQ, HigBA, PrlF/YhaV, MqsRA, MazEF, ChpSB, HicAB and RnlAB, SymER	Ribonuclease	Inhibition of translation by RNA degradation (mRNA, tRNA or rRNA)
HipBA	Kinase	Inhibition of translation-elongation by phosphorylation of glutamyl-tRNA synthase
TopAI/YihQ	Interaction with topoisomerase I	Inhibition of replication/ transcription
DarTG	ADP-ribosylation of virus DNA	Inhibition of phage DNA replication
TisB/IstR, SymER, ShoB/ OhcC, DinQ/AgrB, Idr/ Rdl, Hok/Sok and Ibs/ Sib	Interaction with inner membrane	Insertion into the membrane with dissipation of proton motive force
RalRA	Endonuclease	Activation of SOS response through DNA double-strand break
YafW/Ykfl, CbeA/CbtA and YpjF/YfjZ	Interaction with MreB and FtsZ	Inhibition of cell division and elongation

stimulates toxin degradation (Brantl, 2012; Soutourina, 2019). Like type I, type III antitoxins consist of non-coding RNAs, but in this case the toxin is neutralized by the formation of a toxin-protein:antitoxin-RNA complex (Kang et al., 2018). In type II TASs, the toxin is inhibited by the formation of a highly stable protein-protein complex with the cognate antitoxin (Zhang et al., 2003). Type II is probably the most abundant and the best characterized class of TASs and contain many families of toxins with different molecular activities such as kinases, ribonucleases (ribosome and ribosome independent), acetyltransferases and gyrase inhibitors as reviewed in Zhang et al. (2020). In type IV TASs, a direct toxin-antitoxin interaction does not occur, but the antitoxin is a protein that competes with the toxin for the binding to the substrate (Horesh et al., 2020; Wen et al., 2017). In type V TASs, the antitoxin is an RNAse that degrades the toxin mRNA (Wang et al., 2012). In contrast, in type VI TASs, the antitoxin protein acts as an adaptor that promotes the interaction between the toxin and a protease (Aakre et al., 2013). Antitoxins from Type VII are enzymes that modulate toxin activity by post-translational modifications such as cysteine oxidation, phosphorylation and AMPylation (Wang et al., 2020; Marimon et al., 2016). As to the recently discovered type VIII TASs, the toxin is a small RNA that is inhibited by interaction with an antisense RNA antitoxin (Choi et al., 2018). There are also tripartite TASs, known as toxins-antitoxin chaperones (TACs), whose mechanisms of inhibition resembles that of type II TASs although in the TA complex there is a chaperone bound to the antitoxin to prevent its proteolysis and aggregation (Bordes et al., 2016; Sala et al., 2013).

Type II is the most well-studied class of TASs. This review mostly refers to Type II TASs, unless otherwise indicated.

3. Mechanisms of toxin regulation

Uncontrolled activation of toxins may cause permanent cell stasis. To avoid this situation, toxin activity is tightly regulated (De Bruyn et al., 2021). Although the mechanisms curbing toxin activity vary among different TA pairs, it is possible to recognize common patterns of regulation, such as those discussed below.

3.1. Transcriptional control of TASs

Except for type I and VII, TA genes are organized in operons and are co-transcribed. In most cases, the toxin open-reading frame (ORF) is typically positioned downstream of the antitoxin ORF (Guglielmini and Van Melderen, 2011). This configuration ensures that the antitoxin is first transcribed and translated to efficiently neutralize the toxin as soon as it emerges from the ribosome (Deter et al., 2017). In some rare cases, the order is reversed, i.e., the toxin ORF is located upstream to the antitoxin ORF, as in *higBA* (Liu et al., 2020), *hicBA* (Turnbull and Gerdes, 2017) and *mqsRA* (Brown et al., 2013) operons.

For the classic gene order, antitoxin followed by toxin gene, the transcription is regulated by a single promoter located upstream the antitoxin gene. In this case, the level of transcription is proportional to the intracellular toxin:antitoxin ratio as described below. In contrast, for the inverse gene order, toxin followed by toxin gene, the transcription is additionally regulated by internal promoters that allow for an excess of antitoxin transcription independent of toxin transcription (Turnbull and Gerdes, 2017; Boss et al., 2013; Hayes and Kędzierska, 2014).

Some TASs with classic gene order display weak Rho independent transcriptional terminators immediately after the antitoxin ORF. These regulatory sequences often cause premature TA transcription termination, resulting in an excess of antitoxin transcripts (Sarpong and Murphy, 2021; Goeders et al., 2016).

Transcription of type II TASs is auto-regulated by the cognate TA-complex in a mechanism named conditional cooperativity (Fig. 2) that allows cells to modulate TA transcription according to the intracellular toxin/antitoxin ratio (Garcia-Pino et al., 2010). Most studies use TA transcription as a reporter for gauging stress-mediated molar excess of toxins in a process that is summarized as follows. Antitoxins carry toxin binding domains that can form multiple stoichiometric complexes with their cognate toxins (Manav et al., 2019) and DNA binding domains, which interact with cognate TA operons causing self-transcriptional repression (Garcia-Pino et al., 2010; De Jonge et al., 2009; Kumari and Sarma, 2022). Under steady-state growth conditions, there is an excess of antitoxin molecules over the cognate toxins. Under such conditions, cells carry TA-complexes with a stoichiometry of toxin and antitoxin molecules that can strongly bind to DNA, efficiently inhibiting TA transcription (Garcia-Pino et al., 2010). The strongest expression of TA genes takes place when the cellular level of toxins considerably exceeds that of antitoxins. This occurs during stress-response when protein translation is impaired and/or general protease activity is elevated, causing a strong drop in the antitoxin level. Under these conditions, TA-complexes carrying an excess of toxins are formed, which are unable to interact with the cognate TA-operon (Kędzierska and Hayes, 2016). This feedback mechanism enables the replenishment of the intracellular antitoxin pool following a toxin-mediated metabolic slowdown. It is important to note that some TASs, such as *dinJ-yafQ* (Ruangprasert et al., 2014), *mqsRA* (Kim et al., 2010) and *higBA* (Jadhav et al., 2020) of *E. coli* are regulated solely by antitoxins rather than by TA-complexes and are not subjected to conditional cooperativity.

Transcription of some TASs is also controlled by external regulators. For instance, the expression of several TASs in different microorganisms are controlled by LexA, the SOS response repressor (Dörr et al., 2010; Fernández de Henestrosa et al., 2002; Courcelle et al., 2001; Vogel et al., 2004; Weel-Sneve et al., 2013; Singletary et al., 2009). In *S. aureus*, the transcriptional regulator SarA, which controls the expression of some virulence genes, also regulates the transcription of the type I toxin *sprG2* (Oriol et al., 2021) and *mazEF* (Donegan and Cheung, 2009). In *E. coli*, several external regulators are involved in TA transcription: (i) *rnlAB* expression is controlled by IscR, a transcriptional regulator that is involved in various metabolic processes and stress responses (Otsuka et al., 2010); (ii) *paaA2/parE2* expression is controlled by the transcription regulators PaaR2, YdaS and YdaT (Jurėnas et al., 2021); (iii) *hicAB* is positively regulated by a catabolite repressor protein and the competence factor Sxy (Turnbull and Gerdes, 2017).

3.2. Post-transcriptional control of TASs

Several ribonucleolytic toxins regulate the expression of their own

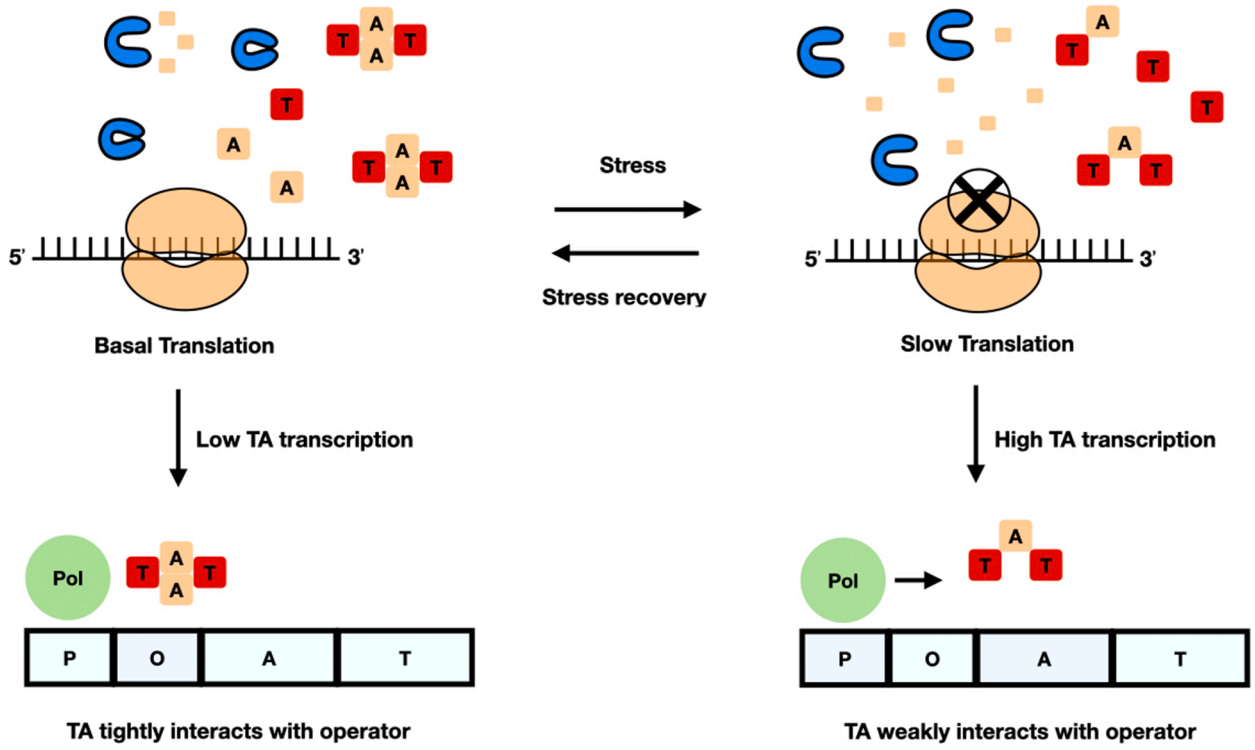

Fig. 2. Transcription of TA operons through conditional cooperativity. It is shown a TA operon with the toxin ORF (T) downstream of the antitoxin (A) ORF. The operator region (O) interacts with TA-complexes, whereas the polymerase (green circle) binds to the promoter region (P). Antitoxins (yellow squares) can form protein complexes with toxins with distinct stoichiometries. Under exponential growth conditions, TA-complexes efficiently bind to TA operons blocking RNA polymerase (green circle) activity, resulting in low levels of TA transcription. Under adverse conditions, antitoxin concentration drops due to an increase in general protease activity (blue irregular circle) and translation slowdown, resulting in deficient TA-complexes (with stoichiometric amounts of toxins and antitoxins that cannot efficiently interact with the TA operon promoter) enabling the transcription of the bicistronic toxin-antitoxin mRNA and the consequent development of a new equilibrium between toxins and antitoxins.

and of non-related TA transcripts. For instance, in *E. coli*, MqsR enriches *ghoT* transcripts by specific degradation of the *ghoS* antitoxin mRNA (Wang et al., 2013). The MazEF system is differentially regulated in *E. coli* and *M. tuberculosis*. Whereas *E. coli* MazF preferentially degrades its own transcript, *M. tuberculosis* MazF6 and MazF9 RNases display high affinity for mazE mRNA, characterizing a negative and a positive feedback loop, respectively. These results illustrate that homologous TASs in different organisms are subjected to different forms of regulation and should therefore yield distinct physiological outcomes. (Tiwari et al., 2015)

Several post-transcriptional control mechanisms were described for several TASs in distinct microorganisms, for instance: in *S. aureus* the mRNA of the type I antitoxin *spF1* has an elevated stability under high osmolarity in comparison to the cognate toxin mRNA (Pinel-Marie et al., 2021); the type I *aapA1* toxin mRNA of *H. pylori* is more stable under oxidative stress relative to the cognate antitoxin mRNA (El Mortaji et al., 2020); mRNA edition of type I *hokB* toxin progressively increases in response to *E. coli* culture density, leading to more active versions of HokB (Wilmaerts et al., 2019a; Bar-Yaacov et al., 2017); the activity of type I toxin mRNAs is not only regulated by interactions with sRNA antitoxins but also by enzymatic processing through distinct phosphorylases and ribonucleases (Berghoff and Wagner, 2017; Masachis and Darfeuille, 2018); the efficient interaction between some type I antitoxin sRNAs with their cognate toxin mRNA requires the protein chaperone Hfq (Brennan and Link, 2007; Waters and Storz, 2009).

3.3. Translational control of TASs

TASs are mainly controlled at the translational level to ensure an excess of antitoxins and formation of physiological TA-complexes (Deter et al., 2017). The most common control mechanism is based on the presence of high affinity ribosome binding sites in the antitoxin mRNA that results in higher levels of antitoxin translation relative to that of toxins (Li et al., 2014). Additionally, some toxin transcripts contain non-canonical start codons (Masachis and Darfeuille, 2018; Durand et al., 2012b) or unusually strong Shine Dalgarno sequences (Sarpong and Murphy, 2021; Daou-Chabo et al., 2009; Durand et al., 2012a) that cause a reduced rate of toxin translation relative to that of the antitoxin.

3.4. Post-translational control of TASs

Type II toxins are sequestered in an inhibitory toxin-antitoxin complex. Usually, the antitoxin blocks the toxin active site or acts as an allosteric regulator (Schumacher et al., 2009; Kamada et al., 2003). Unlike toxins, most antitoxin proteins have intrinsically disordered regions (IDRs) that render them more susceptible to proteolytic degradation through stress activated proteases like Lon and ClpXP (Bordes and Genevaux, 2021; Dubiel et al., 2018; Muthuramalingam et al., 2016; Donegan et al., 2010; Christensen et al., 2004). Besides, In addition, toxin activity is also influenced by environmental conditions because any adverse situation that results in slowdown of protein translation will increase the cellular toxin/antitoxin ratio (Ramisetty, 2020).

Distinct post-translational control mechanisms have also been described: (i) MazF cleaves its own mRNA (Nikolic et al., 2018; Tiwari et al., 2015); (ii) the type III toxin ToxN cleaves its own sRNA (Short et al., 2018); (iii) HipA is inactivated by autophosphorylation (Correia et al., 2006); (iv) HEPN is neutralized by oligoAMPylation (Yao et al., 2020), while the type I toxin Fic-1 requires autoAMPylation to become active (Lu et al., 2016); v) some antitoxins are proteolytically more

unstable under specific stress conditions (Vos et al., 2022); vi) host specific factors disrupt TA complex interactions (Cui et al., 2022; Zhang et al., 2022b; LeRoux et al., 2022; LeRoux and Laub, 2022); vii) some toxins are neutralized by unrelated antitoxins (Zhu et al., 2010).

The activity and the physiological outcomes of TASs are also regulated by several proteins. For example, the acetylation of tRNAs promoted by TacT can be reversed by two deacetylases (Cheverton et al., 2016). After RelE activation, metabolic recovery is dependent on tmRNAs for rescuing stalled ribosomes (Christensen and Gerdes, 2003). The insertion of the type I toxin HokB into the plasma membrane depends on a complex interplay between an oxidoreductase, a disulphide isomerase and a protease (Wilmaerts et al., 2019a). An RNA ligase was identified that counteracts the degradation of rRNAs promoted by MazF (Temmel et al., 2016). The type I toxin TimP has a putative *sec* signal indicating that its insertion into the membrane might be dependent on the activity of the sec-translocon (Andresen et al., 2020).

4. Considerations about toxin activation variability within bacterial populations

Recent research has pointed out that toxin activity does not manifest strongly and homogenously across all bacteria in a population. This recurrent subtle effect of TASs is in line with the fact that some studies could not identify a physiological outcome mediated by toxins when employing population average methods or techniques unable to capture subtle toxin activity. Next, we discuss that either at physiological or stress conditions, the toxin activity is tightly controlled and results in a mosaic of cells with very different metabolic rates.

4.1. Strong toxin activation is unlikely

Toxin activation is usually triggered by stress but it may also occur under optimum growth conditions. This latter scenario is possible owing to molecular noise in gene expression, which leads to a large fluctuation in the concentration of individual proteins across cell populations. Unbalances in the toxin/antitoxin ratio across unstressed cell cultures might create phenotypic growth diversification, i.e., the establishment of cell populations with distinct toxin activities, and distinct metabolic capacities. Indeed, this phenomenon has been observed for several TASs, such as *yefM/yoeB* (Goormaghtigh et al., 2018), *hipBA* (Schumacher et al., 2015), *ecnAB* (Claudi et al., 2014), *mazEF* (Schuster et al., 2015), *hha/tomB* (Jaiswal et al., 2016), *vapBC30* (Srinivas et al., 2020) (Wu et al., 2019) and *paaR2-paaA2-parE2* (Jurėnas et al., 2021). For instance, in *E. coli*, subpopulations with high expression of *hipBA* (Schumacher et al., 2015) and *yefM/yoeB* (Goormaghtigh et al., 2018) have been observed, with *hipBA* subpopulations yielding increased survival rates following antibiotic exposition. (Balaban, 2004). . Similarly, subpopulations displaying high *mazEF* and *vapBC30* expressions exhibited pronounced antibiotic resilience in *M. tuberculosis* cultures (Srinivas et al., 2020; Chi et al., 2018). Under optimal growth conditions, TA-deleted strains of *M. tuberculosis* (Patil et al., 2021; DeJesus et al., 2017), *Pseudomonas putida* (Rosendahl et al., 2020) and *Salmonella typhimurium* (Helaine et al., 2014) exhibited competitive advantage over the corresponding WT strains. Such results suggest that TASs in these organisms generate toxin-dependent metabolic less active cells with lower fitness under favorable conditions. Additionally, small changes in the transcriptome and proteome were found between the WT and a Δ*mazEF* strains of *S. aureus,* which is a strong evidence for the activation of MazF (Bezrukov et al., 2021). Finally, proteomic analysis detected an enrichment of the toxin EcnB in slow-growing and antibiotic-recalcitrant subpopulations of *Salmonella* cells (Claudi et al., 2014).

Under adverse situations, the enhancement of antitoxin degradation could massively increase the subset of bacterial populations with very high toxin activities. However, there is no report of strong and homogenous activation of toxins even under stress conditions. Instead, the ratio of subsets of cells with high toxin activity is just moderately increased in the course of stress responses (Fig. 3A). For instance, activation of the SOS response increases the transcription of the type I toxin *tisB*, but only results in small subpopulations with membranes depolarized by TisB (Berghoff et al., 2017). Independent studies detected only small subpopulations of *E. coli* with high MazF activity following exposition to distinct adverse situations (Nikolic et al., 2017; Cao et al., 2021). A remarkable heterogeneity of *relBE* expression levels within an *E. coli* culture was identified under conditions resembling amino-acid starvation. Challenging this culture to ampicillin eradicated more efficiently cells with lower *relBE* expression (Svenningsen et al., 2019b). Finally, the exposure of *E. coli* cells to phage T2, which activates the MqsRAC tripartite system (Vassallo et al., 2022), caused heterogeneity in growth rates among different populations probably due to distinct levels of MqsR toxin activation in individual cells (Laura Fernández-García et al., 2023).

Controlled expression of toxins resembling physiological conditions does not lead to harsh cell stasis, further supporting that it is implausible for biological systems to acquire toxin dominant states. For example, independent studies have shown that the MazF toxin of *M. tuberculosis* (Barth et al., 2019), *M. smegamatis* (Barth and Woychik, 2020) and *E. coli* (Bezrukov et al., 2021; Nikolic et al., 2022; Nigam et al., 2020) promoted proteome shifting rather than global inhibition of translation. Similarly, in *M. tuberculosis*, some proteins involved in stress survival pathways and virulence were highly translated after low levels of VapC4 expression (Barth et al., 2021). In *B. subtilis*, physiological levels of the toxin ζ induced heterogeneous physiological responses, such as induction of efflux pumps and up-regulation of *relA* (Lioy et al., 2012). Native expression of the type I toxin *timP* in *Salmonella* did not cause cells stasis but rather a mild membrane stress (Andresen et al., 2020). Finally, proteomic analysis of HipA-induced *E. coli* cells provided an indication of active translation during antibiotic persistence with high expression of ribosomal and stress-related proteins (Semanjski et al., 2021). It is important to emphasize that the studies cited above employed population average methods to study the physiological effect of toxins. It is highly likely that controlled toxin expression also enhances the subsets of metabolic less active cells, which can only be captured using single cell measurement methods.

Presumably, massive toxin activation would result in hard-to-revert cell stasis, in which the host loses its ability to actively respond to environmental changes through differential protein expression. Therefore, the successful integration of TA genes in the host chromosome requires that the toxin is subjected to strong homeostatic control, so that even harsh stress conditions would not increase toxin activity to undesirable high levels (Fig. 3A). In fact, it was demonstrated that toxins are readily inactivated, mostly by promoter mutations, when antitoxin genes are silenced or when only the toxin gene is acquired by horizontal gene transfer (Fernandez-Garcia et al., 2019). As a result, research on TASs demands the employment of sensitive techniques and analysis of single cells to capture the nuances of toxin effects. Accordingly, Srinivas et al. demonstrated that in *M. tuberculosis* the formation of persister cells is mainly upheld by the establishment of subpopulations with high levels of TA genes expression. However, collective average measurements did not detect variations in the expression of these genes (Srinivas et al., 2020). Many other experimental practices that should be considered in order to elucidate the role of TASs in bacteria are discussed below.

The next section discusses how the regulation of TASs restricts the full induction of toxins while supporting the emergence of distinct growth phenotypes within genetically uniform cell populations.

4.2. Tight regulation of TASs yields phenotypic heterogeneity

Phenotypic switches are possible when the concentration of a specific regulator, such as a toxin, exceeds a certain threshold in a cell culture individuals (Fig. 3B) (Fasani and Savageau, 2013). Whether the cellular level of free toxins suffices to cause phenotypic variability

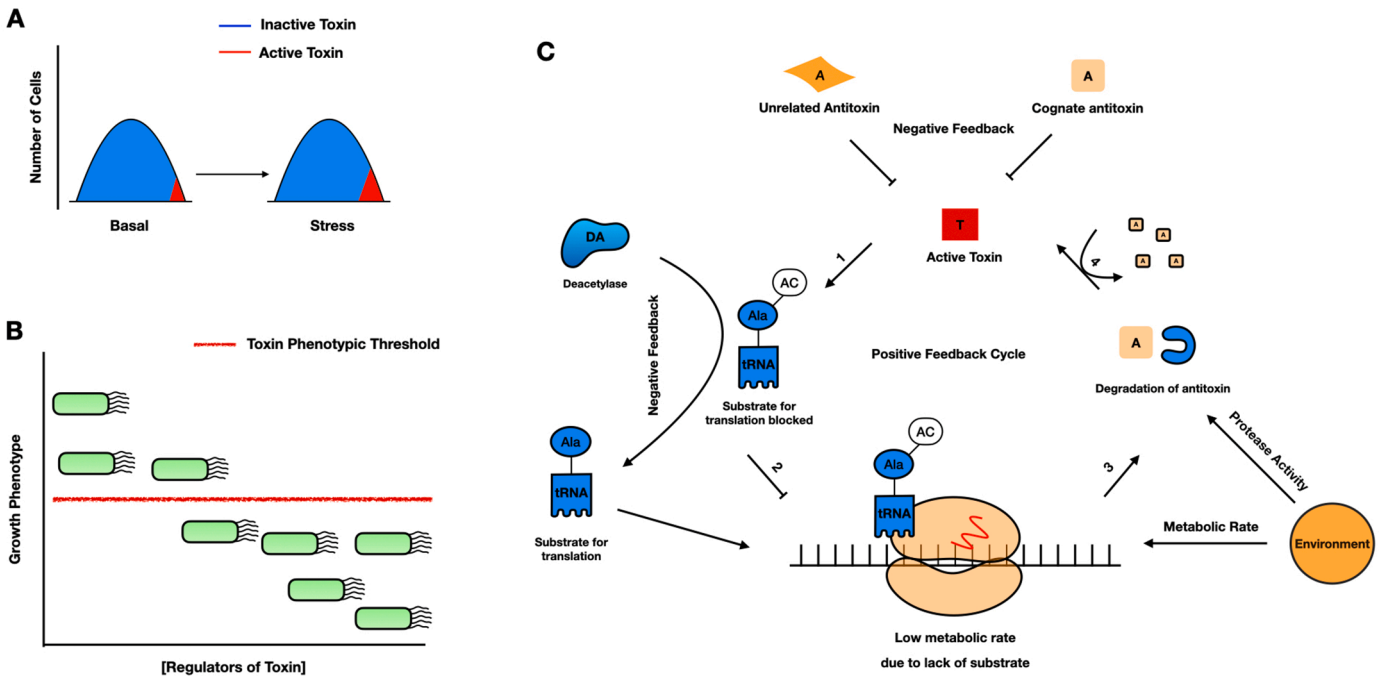

Fig. 3. The complex regulation of TA networks restricts the full induction of toxins and cause phenotypic growth heterogeneity in bacterial populations. **A**) Under exponential growth some cells stochastically develop high levels of toxin activity (red) whereas most cells hold it low (blue). Stress can moderately increase the fraction of high-toxin cells, but a full induction of toxins is never observed across the population. **B**) In order to mediate a phenotype, toxins should overcome inhibition caused by specific antitoxins and also by other elements that restrict their full induction. Each control element has a different impact on the height of the toxin phenotypic threshold. It is expected that cells with lower concentrations of toxin regulators will pass more efficiently the threshold. The extent by which the threshold is overcome will determine the cell growth rate. **C**) Once toxin concentration reaches a threshold it might affect metabolic processes: 1. A toxin with acetyltransferase activity acetylates an amino acid loaded into tRNA. 2. Translation is impaired, preventing peptide synthesis. 3. Given antitoxins instability, they must be constantly translated in order to keep toxins in check. Thus, low cell metabolic rate should favor the activation of toxins via antitoxin degradation by proteases. 4. As the low metabolic status progresses the cellular pool of free toxins increases leading to further inhibition of translation. This cycle represents a positive feedback loop. In addition to cognate antitoxins, other molecules, such as unrelated antitoxins and enzymes that revert the effect of antitoxins, e.g., deacetylases also regulate toxin activity. These regulators establish negative feedback, preventing that toxin concentration reaches a certain level from which the outcomes of toxin activity would be hard to revert. Environmental parameters dictate, for instance, the rates of translation and the activity of stress proteases. The concentration of each single element in a TA network varies across a population, and thus, individual cells have distinct probabilities of displaying high toxin activities. The consequence is that differential regulation of TA networks yields phenotypic growth diversity in a single population. The other levels of TA regulation at transcription, post-transcription and translation contain several factors and mechanisms and should be also considered for phenotype transitions.

depends on the architecture of the TA regulatory network (Rosenblum et al., 2021). Such networks comprise several elements: related and unrelated antitoxins, regulators of TA operon activity, proteases targeting antitoxins, positive/negative feedback loops and unrelated proteins that affect toxin activity (see general mechanisms of toxin activity regulation). Toxins should overcome the threshold set by all controlling elements to promote phenotypic transitions (Rotem et al., 2010). Those considerations are supported by several experimental observations mentioned below.

Flow cytometry analysis revealed that 50 % of an *E. coli* population displayed active type I TisB toxins upon induction of the SOS response. Deletion of the cognate antitoxin *istR* incremented the fraction of depolarized cells to 70 %, indicating that the antitoxin sets an important threshold for phenotypic switch. In the same study it was also observed that deletion of the 5′ untranslated region (5′ UTR) of *tisB* and the antitoxin *istR* increased antibiotic persistence by 11- and 4-fold, respectively, implying that the control exerted by the antitoxin might be weaker than that applied from the 5` UTR (Berghoff et al., 2017). As to HipAB, it was found that cells become persistent when the levels of toxins exceeds a threshold determined by the concentration of the antitoxin HipB (Rotem et al., 2010). Regarding MazEF, it promotes growth heterogeneity in *E. coli* through two distinct post-transcriptional processes (Nikolic et al., 2018). First, MazF cleaves its mRNA, which corresponds to a negative feedback loop that emerges when the toxin is active. Second, the process of growth resumption mediated by MazE, after MazF activation, is heterogeneous across the population. Indeed, growth heterogeneity was neither observed in strains producing an mRNA lacking MazF-cleavage sites (ACA) nor in antitoxin knockout strains, i.e., lacking two toxin regulatory motifs (Nikolic et al., 2018). One last example is the demonstration that the bistability in the tripartite *paaR2-paaA2-parE2* system is dependent on the regulation of the TA operon by an interplay between PaaR2, YdaS and YdaT. Indeed, mutation in the promoter protein binding site abolished phenotypic heterogeneity mediated by this TAS (Jurėnas et al., 2021).

Fig. 3C shows a hypothetical simplified TA network to illustrate the tight control imposed on toxins and the effect of each toxin controlling element on phenotypic transitions. The network contains an acetyltransferase toxin, whose activity is controlled by a cognate, a non-cognate antitoxin and a deacetylase enzyme. This scenario resembles conditions described in (Cheverton et al., 2016). The network also contains positive and negative feedback loops that can amplify and reduce the output of the toxin, respectively. Feedback loops are regulatory motifs often involved in cell fate decisions and well known to generate phenotypic diversification (Mitrophanov and Groisman, 2008). They turn cells hypersensitive to the concentration of the regulator, meaning that small variations of it (e.g., toxins) above a threshold might be sufficient to enable the rise of a phenotype only in some cells of the population. Feedback loops are present in several TA networks (Nikolic et al., 2018; Pinel-Marie et al., 2021; Kasari et al., 2013). When cells reach a certain toxin level, it acetylates amino-acids loaded into tRNA precluding their incorporation into proteins and, consequently, constraining global translation. A slow growing rate mediated by toxins

impacts *de novo* antitoxin production and acts as a positive feedback loop, turning toxin activation more likely due to the high proteolytic instability of antitoxins (Ramisetty, 2020). However, there are several negative regulators of toxins, such as deacetylases that reverse the effect of toxins on substrates and unrelated antitoxins that hold toxins inactive, although they are not as efficient as the cognate toxins (Cheverton et al., 2016). Those regulators counteract the effect of toxins and impose a negative feedback loop (Avendaño et al., 2013), preventing a strong toxin-mediated cell stasis.

The activity of toxins is also dependent on environmental conditions since they may contribute to the activation of stress proteases as well as determine the metabolic rate of cells and, thus, the production of antitoxins (Klumpp et al., 2009). Once toxins turn active, the rate of cell detoxification (e.g. *de novo* antitoxin production, modulation of deacetylase activities and turnover of toxin substrates) will also contribute to phenotypic heterogeneity of the population (Nikolic et al., 2018; Wilmaerts et al., 2019a). All components of TA networks can be differentially (stochastic or deterministic) regulated at all levels (transcription, translation and post-translational) and facilitate/hinders phenotypic switches via toxin activation (Ackermann, 2015). However, no full induction of toxins ever occurs probably because it would negatively impact bacterial phenotype plasticity due to harsh constraining of cells metabolic activity (Svenningsen et al., 2019b).

4.3. Phenotypic heterogeneity conferring fitness advantages

Some authors postulated that TASs increase bacterial fitness by causing cell stasis in the entire population. This assumption conflicts with the fact that the effect of TASs is often modest, which might be insufficient to promote harsh cell stasis but sufficient to cause small changes in metabolic abilities across the population. Even if discovered about 75 years ago, such important facets are still elusive and require further considerations regarding stress survival mechanisms (Kaldalu et al., 2016).

Persisters are cells that survive antibiotic killing, a phenomenon that is mostly attributed to a complete lack of metabolic activity, although without further analysis. However, it has become clear that the phenotype of stress-survivors is highly complex and the maintenance of some metabolic functions is essential (Semanjski et al., 2021; Nguyen et al., 2011; Wakamoto et al., 2013; Wilmaerts et al., 2019b; Renbarger et al., 2017; Orman and Brynildsen, 2013; Hemsley et al., 2014). For instance, cells treated with sub-lethal doses of antibiotics usually yield increasing number of survivors when they are subsequently exposed to lethal doses of the same antibiotic (Arias-Sánchez et al., 2018). It could be assumed that a pre-treatment inducing dormancy would be sufficient to confer bacterial survival by inactivation of their antibiotic targets.

However, SOS deficient *E. coli* cultures sensitized with sublethal ciprofloxacin doses were almost eradicated by a subsequent lethal dose (Goneau et al., 2014). This result implies that DNA-gyrase remains active in persisters and a functional DNA repair mechanism is essential for antibiotic-tolerance rather than simply dormancy (Goneau et al., 2014). Accordingly, it was recently demonstrated that while some persisters have several biological processes inhibited, their efflux pumps become more active. Indeed, a low metabolic rate in combination with an active antibiotic pumping system were essential for surviving antibiotic exposures (Pu et al., 2016). Finally, it was recently demonstrated *in E. coli* that the MqsRAC system act in concert with the McrBC restriction/modification system to provide protection against phage attack. While the MqsRAC system is activated after phage attack causing a heterogeneity in growth rates within the population, the McrBC restriction/modification system acts probably by specifically cleaving phage DNA (Laura Fernández-García et al., 2023).

As demonstrated in *Salmonella*, the best strategy for facing adverse situations is maintaining a great distribution of different metabolic features within the population instead of just dormant cells. A multitude of subpopulations of *Salmonella* cells with different division rates was identified in the course of mouse infection. Following fluoroquinolone treatment, it was observed that slow dividing cells survived better, but they represented only a very small fraction of the population. In contrast, although subpopulations with high and intermediate levels of metabolism displayed a slightly higher death rate, their initial populations were higher, thus contributing more to the relapses of *Salmonella* infections once antibiotic treatment ended (Claudi et al., 2014).

As a rule, phenotypic growth diversification, which is in full agreement with the putative biological function of TASs, allows cells to explore diverse physiological programs with a variety of stress tolerance mechanisms, increasing survival probability in an broader spectrum of environments (Lee et al., 2019; Gerdes and Semsey, 2016; Fraser and Kaern, 2009; Carja and Plotkin, 2017).

5. Inconsistencies in TASs research

Although stabilization of genetic mobile elements and phage inhibition are acceptable physiological functions of TASs, other biological functions are still debatable. Whereas several studies demonstrated TAS mediated-stress adaptations, others could not identify the lack of a persister phenotype upon TAS deletions (Table S1). In some studies the activity of toxins in vivo could not be detected even after extended periods of stress treatment (LeRoux et al., 2020), leading to the hypothesis that TASs are selfish elements without a dedicated physiological function (Ramisetty and Santhosh, 2017; Magnuson, 2007). Others suggest that the integration of TA loci in the chromosome of some bacteria is disadvantageous (Rosendahl et al., 2020; Ma et al., 2021).

The next sections discusses shortcomings contributing for discrepant experimental results and misperceptions in TA research.

5.1. Redundant mechanisms mediating persistence

Development of a stress-resilient persister phenotype is an evolved adaptive feature of microorganisms that arises through redundant mechanisms and does not rely on any single regulator (Goormaghtigh et al., 2018; Svenningsen et al., 2019a; Shan et al., 2015; Theodore et al., 2013; Tsilibaris et al., 2007; Harms et al., 2017). Several disturbances in the bacterial metabolic homeostasis are sufficient to increment the fraction of persisters: i) overexpression of cell growth inhibitory proteins (Chowdhury et al., 2016; Vázquez-Laslop et al., 2006), ii) treatment with compounds that inhibit ATP synthesis, transcription and translation (Kwan et al., 2013; Shah et al., 2006; Dörr et al., 2009; Winther and Gerdes, 2009), iii) mutations that inactivate transporter systems and enzymes involved in bacterial central metabolism (Van den Bergh et al., 2016; Cameron et al., 2018; Li and Zhang, 2007; Ma et al., 2010; Spoering et al., 2006), iv) Nutrient-starvation, nutrient-shift, poor nutrient-sources (Keren et al., 2004a; Amato et al., 2013; van Heerden et al., 2014), v) environmental cues related to stress as low pH, oxidative-, cold- and heat-stress (Dörr et al., 2010; Kussell et al., 2005; Leung and Levesque, 2012; Wu et al., 2012).

As of today the only accepted model for persister formation is by ribosome dimerization, in which in response to an increase in ppGpp levels, ribosome inactivating factors are produced to promote translation inhibition and metabolism slowdown (Song and Wood, 2020a).

Due to redundant mechanisms of persister formation, the effect of TASs on stress survival can be easily masked by other physiological changes as demonstrated by Pontes et al. (Pontes and Groisman, 2019). These authors observed that *Salmonella* cells lacking 12 TAs formed less antibiotic persisters than the WT strain. However, when growth was previously committed before antibiotic addition, both the WT and knockout strains exhibited almost the same antibiotic tolerance (Pontes and Groisman, 2019). Such results indicated that, though TASs might confer increasing antibiotic tolerance, a metabolic collapse due to the lack of nutrients can increase the frequency of persisters and mask the effect of TASs.

5.2. Distinct activation kinetics among TASs

Certain TASs are only active during specific growth phases. Possible survival defects of ΔTA strains should therefore be analyzed along different time windows. For instance, a Δ*msqR* culture yielded similar persister frequency compared to the WT in the initial 8 h-exposure to antibiotic. The deletion of *mqsR* revealed a more antibiotic susceptible-phenotype only after 24 h of treatment (Wu et al., 2015; Shimizu, 2013). Correspondingly, *M. tuberculosis* toxin HigB1 was described to be more important in the chronic than in the initial stage of host infection (Sharma et al., 2021). In *E. coli*, membrane depolarisation promoted by the type I toxin TisB initiated only after 4 h of antibiotic treatment (Berghoff et al., 2017). Other examples demonstrating widely variable activation kinetics of TASs can be found in (Keren et al., 2004b; Harrison et al., 2009; Zadeh et al., 2022; Jahanshahi and Li, 2020).

5.3. Growth media and growth condition

The frequency of persisters in cell cultures depends on the growth medium. For instance, Harms et al. reported different persister outcomes using different LB-batches, possibly because the composition of nutrients in LB slightly varies from batch to batch (Harms et al., 2017). For *B. pseudomallei*, TA deletion reduced persistence in LB but not in RPMI media (Ross et al., 2020). Additionally, *S. aureus* MazF yielded different levels of substrate cleavage in different growth media, suggesting that toxin activation prevails under specific conditions (Schuster et al., 2015).

HipA7 cultures of *E. coli* usually have higher frequency of pre-formed persisters than the corresponding WT culture. However, when the start-culture is made from an inoculum subjected to a long stationary phase period, WT and *hipA7* strains display similar number of persisters (Luidalepp et al., 2011). Intuitively, overnight culture carries a high number of metabolically less active- and antibiotic-tolerant cells, resulting in an elevated frequency of persisters, that can mask the effect of toxins (Balaban, 2004).

5.4. Strain genetic background

Most studies on TASs are focused on a few lab strains, but the biological effect of homologous TASs can be different even on strains of the same species. As argued by Nicolic et al., due to dependence on the regulatory network, even highly toxin homologs from different strains are likely to strong diverge in most biochemical parameters, such as affinity to the cognate antitoxin and activation kinetics, and hence, in phenotypic outcomes (Nikolic, 2019). Indeed, whereas most studies did not reveal a phenotype in *E. coli* MG1655 (Table S1), a single TA deletion was sufficient to decrease competitiveness of uropathogenic *E. coli* strains in the course of infections (Norton and Mulvey, 2012). The expression patterns of two pathogenic adherent-invasive *E. coli* strains challenged with acid stress revealed a 4-fold up-regulation of the type V toxin gene *ghoT-1* for one strain, whereas for the other it was detected a 4-fold down-regulation (Bustamante and Vidal, 2020). As to *S. aureus,* it was demonstrated that *mazF* is upregulated under oxidative stress in methicillin resistant-strains (MRSA) but downregulated in methicillin sensitive-strains (MSSA) (Karimaei et al., 2021). In agreement, an independent study revealed that the expression of *mazEF* is highly variable among 100 MRSA and MSSA clinical isolates (Abd El rahman et al., 2021). Finally, deletion of *mazF* increased the growth rate of a *S.aureus* SH1000 during the exponential phase, but not of a Newman strain, clearly indicating strain variability in response to the toxin (Ma et al., 2019).

5.5. Stress specific activation of TASs

Some TASs are regulated only by specific types of stress. Thus, knowledge regarding the conditions in which TASs are responsive is indispensable when comparing the survival rate between WT and the isogenic TA deletion strain under specific conditions. If the proper stress has not been tested, they will display similar fitness due to the lack of toxin activation. Many cases of stress specific toxin activation for several TASs and in different microorganism are described below.

Several TASs contain LexA binding boxes (Dörr et al., 2010; Fernández de Henestrosa et al., 2002; Courcelle et al., 2001; Vogel et al., 2004; Weel-Sneve et al., 2013; Singletary et al., 2009) and are responsive to DNA damage but not to other types of stress, such as amino-acid starvation or heat. The type I SprF1/SprG1 and AaapA1/IsoA1 modules are involved in osmotic- and oxidative-stress, respectively, due to the high stability of their corresponding functional mRNAs (antitoxin *sprF1* and toxin for *isoA1*) under these specific stress conditions (Pinel-Marie et al., 2021; El Mortaji et al., 2020). Virus capsid proteins interact and disrupt specific TA-complexes, mediating toxin activation. All this explains why only specific TASs promote protection against some specific set of phages, but are not responsive to others (Vassallo et al., 2022; Cui et al., 2022; Zhang et al., 2022b; LeRoux et al., 2022). Finally, the *msqRA* operon is increasingly transcribed in response to oxidative stress due to the proteolytically instability, specially towards the Lon protease, of the antitoxin under such condition (Vos et al., 2022; Wang et al., 2011). Indeed, an independent study demonstrated that the level of the antitoxin MsqA remains constant in response to amino acid starvation and it even increases following heat-shock implying that toxin activation is unlikely under those conditions (Wu et al., 2019).

5.6. Link between TA transcription and toxin activity

Most studies are based on transcriptional analysis in which an increase in TA transcription is assumed to positively correlate with toxin activity, according to the model of TA transcriptional cooperativity (Fig. 2). However, more recently, some studies failed to detect toxin activity despite high levels of TASs been transcribed (Jurènas et al., 2021; Goormaghtigh et al., 2018; LeRoux et al., 2020). Especially Leroux et al. highlight that whether toxins become active should be carefully analyzed. As those authors demonstrated, stress can considerably enhance the transcription of TASs, but a simultaneous assessment of the activity of ribonucleolytic toxin enzymes revealed that they did not become active (LeRoux et al., 2020).

TA transcription is not a proxy of toxin activity for several reasons. First, TA-complexes have dissociation constants in the range of nano-femtomolar (Loris and Garcia-Pino, 2014) and the basal concentration of antitoxins are higher than that of toxins (Deter et al., 2017; Cataudella et al., 2012; Li et al., 2014). Therefore, it is not possible to unequivocally conclude whether, despite extensive stress-mediated protein degradation, the remaining antitoxin moieties would not be sufficient to maintain the toxins inactive. Second, although antitoxins are clearly more proteolytic unstable than toxins (Jaiswal et al., 2016; LeRoux et al., 2020; Dai et al., 2021; Ni et al., 2021; Marsan et al., 2017), there are solid hints that antitoxin degradation does not occur within TA-complexes (Vos et al., 2022; LeRoux et al., 2020; Riffaud et al., 2020; Tandon et al., 2020; Culviner and Laub, 2018). This is indicative that, at least in a short time window, only the cellular pool of free antitoxins is depleted under stress. Finally, the neutralization of toxins by non-cognate antitoxins has also been reported and represents an important factor that can strongly hamper toxin-activation (Nikolic et al., 2018; Zhu et al., 2010; Santos-Sierra et al., 2006). Some toxins are neutralized even by antitoxins from unrelated families (Arbing et al., 2010; Riffaud et al., 2020; Tandon et al., 2020). It is important noticing that this kind of regulation might not be the norm for all toxins and requires further research.

5.7. Lack of sensible techniques for monitoring toxin-activity

The main reason for divergences in investigations focused on TASs is the poor availability of sensitive techniques capable of identifying toxin

activity in vivo. Further studies employing recently developed powerful ones (Srinivas et al., 2020; Wu et al., 2019; Cao et al., 2021; Barth et al., 2021; LeRoux et al., 2020; Jahanshahi and Li, 2020; Dougan et al., 2021; Culviner and Laub, 2018) will increase the reliability of data in this field. Indeed, although RNA-seq based studies could not identify enhanced MazF activity following antibiotic treatment (LeRoux et al., 2020), it was possible by the use of an ultra-sensitive biosensor based on C-SDA and CRISPR/Cas12a (Cao et al., 2021).

6. Concluding remarks

Biological processes are often redundant in order to confer robustness to living organisms (Ghosh and O'Connor, 2017). A prominent example is the way eukaryotic cells control growth and division. There are several checkpoints throughout the cell cycle where the interaction of numerous proteins with hundreds of metabolites dictates cell fate. Consequently, uncontrolled cell-division is rare and does not arise until many mechanisms have failed (Matthews et al., 2021). Likewise, prokaryotes have multiple pathways to control their metabolic rate (Shimizu, 2013).

Although many studies postulate TASs as key regulators of metabolic phenotypic switch, the recent research cited here points out that their physiological effects are subtle. Microorganisms are constantly under selective pressure (Poole, 2012). A regulatory network allowing an easy and strong activation of potent growth inhibitory proteins like toxins, would probably incur high fitness costs to the bacterium. It would be quite counterproductive to constantly corrupt the metabolism so drastically, for example by extensive degradation of mRNA (RNAse toxins) or by strong dissipation of the proton gradient (membrane peptide toxins). A remarkable feature of bacteria, strictly related to their success to colonize diverse niches, is their ability to rapidly change their physiological program. This phenotypic plasticity is possible through transcription and translation, processes that could be strongly impaired by uncontrolled toxin activity. Hence, TASs are implemented in regulatory networks that allow only marginal toxin-activity in small subsets of cells under basal and non-basal conditions. This assertion is in line with the fact that some studies were unable to show the involvement of TASs in stress tolerance. However, by employing more suited and sensitive techniques and improving experimental conditions, the physiological effects of TASs turn evident. Although modest and often masked by some mechanisms that mimic their functions, the impact of TASs on bacterial growth and on their ability to adapt and survive in hostile environments cannot be ignored. TASs are players in a robust and sophisticated regulatory network controlled by multiple regulators and feedback loops that creates extensive stress-related phenotypes in bacterial populations.

Credit author statement

All authors contributed equally for conception, preparation and revision of this review article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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Supplementary materials

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