The interplay of the type II TA system with other processes

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PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27770v2 | CC BY 4.0 Open Access | rec: 9 Jun 2019, publ: 9 Jun 2019

Abstract

The type II toxin antitoxin (TA) system is the most well-studied TA system and is widely distributed in bacteria, especially pathogens such as *Mycobacterium tuberculosis*. Type II TA system plays an important role in many cellular processes, including maintaining the stability of mobile genetic elements, and bacterial altruistic suicide in response to nutritional starvation, environmental stress and phage infection. Interactions between toxin proteins and antitoxin proteins are critical for the regulation and function of type II TA systems; indeed, the understanding of their function is mainly derived from interaction and regulation of paired TA system proteins. Nonetheless, investigating interaction between unpaired TA system proteins, and the interaction between TA system proteins and other functional proteins, are becoming more common and have provided new insight into the complexity of its regulatory mechanism. In this review, we outlined the cross-interaction between TA system proteins, and the interaction between TA system proteins and other functional proteins, and we are trying to explain novel mechanism of TA system in the regulation of cellular activities. On this basis, we further discussed the knowledge and physiological implications of the relevant aspects of TA system research.

Keywords: type II toxin-antitoxin, cross talk, interaction, regulation

1. Introduction

The toxin-antitoxin (TA) system can be harboured on plasmids or chromosomes [1, 2]. Almost all bacteria contain TA systems, and some bacteria carry many TA systems (approximately 80 pairs in *Mycobacterium tuberculosis*) [3, 4]. A typical TA system encodes a stable toxic element (toxin) and its unstable antidote (antitoxin), a protein or RNA that inactivates the toxin under normal growth conditions; under stress conditions, the antitoxin concentration decreases or becomes imbalanced compared to the toxin concentration, leading to an increase in toxin activity [5]. According to the nature of the antitoxin and toxin and antitoxin mechanism, TA systems can be divided into six types, I-VI [5]. The composition of these six types of TA systems is well summarized in a previous review [6].

Of the many TA systems, the type II system is the most widely studied. The number of type II TA systems varies greatly in different bacterial species and even in the same species [3, 4], though in general, both toxin and antitoxin genes are conserved, with toxin proteins being more conserved than antitoxin proteins among different bacteria [7]. To date, 12 type II TA system subfamilies have been identified based on toxin amino acid

sequence homology [8], including *mazEF* [9], *relBE* [10], *yefM-yoeB* [11], *parDE*, *higBA*, *vapBC*, *phd/doc*, *ccdAB*[3], *ω-ε-ζ* [12], and *mqsRA* [13, 14] subfamilies and the *parE/relE*, *ccdB/mazF* superfamilies [8]. The antitoxin component of type II systems, which is usually small and unstable and therefore easily degraded by proteases, binds to promoter DNA through its N-terminus to regulate transcription of the operon in which it is located [15-17]. Antitoxins may belong to different DNA-binding protein families, as those located in an operon of the same toxin family can belong to 2 to 4 different DNA-binding protein families [18]. As the toxin protein can conditionally regulate antitoxin protein binding to DNA, acting as a transcriptional co-repressor or de-inhibitor [19], the TA operon is highly inhibited during logarithmic growth [20, 21]. In most cases, the antitoxin gene is located upstream of the cognate toxin gene, favouring expression of the former. However, there are some exceptions to this arrangement; for example, the toxin gene *higA* of *higBA* TA system is located upstream of the antitoxin gene *higB* [22, 23].

Although the biological functions of most TA systems identified thus far remain unclear, the biological functions of some type II TA system modules have been elucidated in several aspects such as maintaining the stability of mobile genetic elements or plasmids and bacterial altruistic suicide in response to nutritional starvation, environmental stress, and phage infection [24]. The *ccdAB* TA system found on the *Escherichia coli* F plasmid is an example of killing after isolation: *ccdA* kills plasmid-free cells that do not produce antitoxins, thereby maintaining plasmid stability [25]. In

the absence of nutrients, especially amino acids, the toxin protein of the *relBE* system rapidly degrades nascent mRNA , resulting in a reduced rate of protein synthesis that helps to conserve energy and nutrients. Alternatively, the mRNA produced can be eliminated by the toxin protein, readjusting protein synthesis to adapt to a new environment without translation of proteins no longer needed [26]. Similarly, the *mazEF* toxin antitoxin system mediates bacterial programmed cell death in response to exposure to internal and external environmental factors [27]. *mazEF* is also considered to be part of the abortive phage infection system, which induces programmed cell death to prevent invaders from reproducing through an altruistic defence mechanism [28]. The emergence of antitoxins can retard or neutralize the above phenomenon, reversing cell function to a normal state [25-28].

Toxin-antitoxin protein interactions are critical for the function of type II TA systems. While most studies have focussed on paired toxins and antitoxins, there is scant literature on the protein-protein interactions between unpaired toxins and antitoxins, and between TA system proteins and other functional proteins. The purpose of this review is, therefore, to describe and discuss the interaction between unpaired toxins and antitoxins and direct protein-protein interactions between TA systems and other functional proteins, along with the cellular functions mediated by such interactions. This review will help in the prediction of relevant targets or substrates, enrich our understanding of TA system functions, and provide clues for new antimicrobial design.

2. Cross-interaction between Type II TA systems

The distribution of TA systems varies largely among different bacteria, with most bacteria having many different sources [29]. The redundancy of TA systems in bacteria is worthy of discussion, and some studies have shown that many type II TA system toxins are similar in function and have inhibitory effects on bacterial growth [30]. Genomic sequence analysis indicates that extensive functional domain exchange has occurred between TA systems during evolution, and different families of TA systems are linked by complex evolutionary relationships [3, 18], allowing for cross-interaction between different systems, with important roles in transcriptional activation, bistability formation, cell growth, environmental stress response, and retention of different TA systems.

2.1 A Type II TA system cross-regulates the transcription of its operon gene

Amino acid starvation induced many changes in the physiological processes of bacterial cells, and guanosine tetraphosphate (ppGpp) synthesized by RelA (ppGpp Synthase I) plays a major regulatory role [31]. *relBE* is the second Rel locus in *E. coli*, and Christensen et al found that the *relBE* promoter is rapidly and strongly activated during amino acid starvation and that this activation is independent of ppGpp but relies on the Lon protease [32]. In turn, Lon-mediated degradation of RelB releases RelE, which globally inhibits mRNA translation without interfering cell viability [32] (Fig. 1). It was recently discovered that the Doc toxin of a different TA system can also activate

the RelE mRNA interferase *in vivo*, without a known role for other TA system loci [33] (Fig. 1). Doc binds to the 30S ribosomal subunit and reversibly induces *E. coli* cell growth stagnation by inhibiting the extension of translation. In addition, expression of MazF is induced under conditions of nutrient deficiency [34, 35] (Fig. 1). However, this mutual activation of the TA system is not unique but also exists in both *Salmonella* and *Shigella*. The *vapBC* locus is also activated by amino acid starvation, and ectopic expression of VapCs derived from these two bacteria leads to growth inhibition, which can be reversed by the cognate antitoxin [18, 36]. Expression of *vapC* in *E. coli* results in cleavage of the mRNA at the stop codon, and interestingly, this cleavage depends on the *yefM/yoeB* locus, the VapC-activated YoeB mRNA interferase. Translational inhibition by VapC mediates degradation of the YfM antitoxin by Lon, and degradation of YefM leads to activation of YoeB and mRNA cleavage [37] (Fig. 1). Cross-transcriptional activation between different TA toxins results in mRNA degradation and disproportionate toxin production, constituting potential positive feedback regulation. Indeed, cross-interacting TA systems may form complex regulatory networks that activate each other [30, 38]. Regardless, further research is required to determine whether this continuous TA activation and propagation mechanism is widespread in TA system-rich *M. tuberculosis*.

2.2 Effect of cross-interaction of type II TA system proteins on environmental stress responses and bacterial cell growth

Although the TA system in bacteria is not essential for normal cell growth, it does play a key role in survival in response to environmental stress and antibiotic stress. Toxin proteins inhibit bacterial growth by targeting a variety of important cellular physiological processes such as conversion from normal growth to dormancy and drug resistance [5]. The homologous TA system forms a bistability complex, and individual toxins lose this status when the antitoxin protein is degraded. Some reports have indicated that a variety of homologous TA systems or TA systems from different families can form a complementary bistability complex to cope with environmental pressure to regulate growth [39].

M. tuberculosis produces three pairs of *relBE*-like proteins: RelBE, RelFG, and RelJK [40]. Previous study found that in addition to direct interaction between paired proteins, unpaired proteins also interact in the three *relBE* systems. For instance, interaction of the antitoxin RelB with the toxins RelG and RelK allows for co-expression of the antitoxin gene with the two toxin genes to neutralize the virulence of the latter, and both toxin proteins promote binding of the antitoxin protein to DNA. We also found physical and functional interaction between the antitoxin RelF and the toxins RelE and RelK, though co-expression of this antitoxin gene with the two toxin genes enhanced their virulence [41]. Interestingly, another study found the antitoxin RelF and toxins RelE

and RelK to be induced in macrophages after infection by *M. tuberculosis* [40]. Therefore, this cross-regulation may play a synergistic role in preventing macrophage immune clearance, and when the same toxin or antitoxin is in short supply, other toxins or antitoxins will help in resisting environmental stress. In addition, Zhu et al reported physical interaction between *M. tuberculosis* toxin MazF-mt3 and antitoxin VapB and found that the toxicity of MazF can be neutralized by non-homologous VapB to restore bacterial growth [42]. The observed cross-interaction between these TA modules broke the myth of "one toxin and one antitoxin". Coincidentally, cross-physical interaction occurs between MazEF and RelBE in *Bifidobacterium longum*. Moreover, expression of MazF1 alone inhibits growth in *E. coli*, which is inhibited by non-homologous RelB antitoxin against toxin mRNA degradation [43]. A similar pattern of synergistic regulation has recently been reported in pneumococci. The pneumococcal strain R6 has three pairs of TA modules, *yefM-yoeB* and two pairs of *relBE*, and neutralization of the *relBE* toxin does not occur in a typical manner of TA systems in that overexpression of the paired antitoxin does not neutralize the activity of the toxin [26, 34]. Deletion of *yefM-yoeB*, *relBE* or all TA systems in strain R6 sensitizes these bacteria to oxidative stress; in addition, biofilm formation is significantly impaired, though the growth rate is not significantly affected. These results indicate that two pairs of TA systems in pneumococci perform the same role, similarly contributing to bacterial oxidative stress and biofilm formation [44]. Therefore, we can speculate that when environmental

stressors are removed, the newly synthesized antitoxin of a pathogen TA system will complex with the existing cognate or a different source of toxin to reverse toxicity, restore normal growth, and promote adaptation.

2.3 Formation of a bistability state and persistence in a type II TA system

Numerous TA systems are associated with bacterial drug resistance and persistence [45-47], the latter of which is observed when a small percentage of bacteria survives after antibiotic treatment [48]. Despite being a form of bacterial tolerance, persistence is not related to gene mutation or acquisition and will result in a dormant state of replication, which is different from resistance. Many of the toxins of type II TA modules degrade mRNA without causing cell death and instead promote a transition to a dormant or semi-dormant state [2]. In general, the TA system exists in a bistable state, and recent studies have shown that under unstable conditions, a variety of different TA systems can be coupled to generate this state, which is converted under normal and toxic conditions [49, 50]. In a previous study, Fasani et al constructed single and multiple TA system models assuming that translational inhibition of toxins requires activation of all TA modules in the cell; if the determined action of the toxin is independent of other TA systems, the rate of retention is very sensitive to the number of TA systems, and the frequency of persistence in wild-type *E. coli* was between 10^{-6} to 10^{-5} [51]. Thus, a change in the total

number of TA systems in cells can significantly alter the frequency of persistence, whereas adding a similar TA system can increase the frequency. Therefore, if the bacteria successfully recover from the dormant state, synchronous conversion of all TA systems is required, and the process is accompanied by a sharp decrease in the number of TA systems [52]. The specific mechanisms by which TA systems function together remain elucidated.

3. Type II TA system proteins interact with other functional proteins and regulate cellular activities

The prevalent TA system has multiple roles in bacterial physiology. Indeed, type II TA systems target cell wall synthesis, tRNAs, ribosomes, biofilm formation, DNA gyrase, and membrane potential [53]. In addition to the role of the toxin proteins, TA system components interact directly with other functional proteins to coordinate cellular physiological processes. Overall, TA systems are regulated by precise and complex genes, and in some bacteria, TA systems regulate bacterial growth by interacting with other functional proteins.

3.1 MazF homologues interact with DNA topoisomerase I to regulate bacterial growth

The Rv1495-encoded protein in *M. tuberculosis*, a homologue of *E. coli* MazF, is considered to be a toxin with ribonuclease activity [54]. In a previous study, we found

interaction between the Rv1495 protein and DNA topoisomerase I (MtbTopA), an enzyme that cleaves one of strands of DNA to prevent negative supercoiling during transcription [55]. Mycobacterial MazF inhibits the DNA helicase activity of MtbTopA and its ability to bind to double-stranded DNA. In turn, MtbTopA affects MazF mRNA cleavage (Fig. 2), though this cleavage activity of MazF does not affect its interaction with MtbTopA. Expression of the Rv1495-N-terminal (29-56) fragment alone can inhibit the growth of *Mycobacterium smegmatis* [56]. This protein-protein and protein-peptide interaction reveals a new mechanism by which the TA system regulates bacterial growth, providing important clues for the development of effective antibiotics against topoisomerase I. Overall, compounds that activate or mimic bacterial toxins may be developed as a new antibiotic. In addition, the MazEF system may be involved in the long-term dormancy of pathogens in human skin or tissue, and the MazEF system exhibits some additional growth-regulatory functions that are essential for bacterial persistence. Interaction of MazF with functional proteins may allow bacteria to escape the host immune system and survive in the adverse macrophage environment. However, there are few studies in this area, and more in-depth research is needed to understand the mechanism of action.

3.2 Type II TA system proteins interact with transcription factors to regulate bacterial growth

Different signalling molecules control gene expression during different stages of cell development as well as cell viability. For example, in *Myxococcus xanthus*, the signalling molecule (p) ppGpp induces and activates early-developmental genes under starvation conditions and transmits to extracellular A-signal to regulate expression of various genes, including the *mrp* operon. When the cells begin to aggregate, the C-signal of the short-range signal is taken over [57-59]. Conversely, programmed cell death is mediated by the MazF toxin [60]. Interestingly, Nariya and Inouye studied the TA system in the genome of *M. xanthus* and found that it contains only a single MazF toxin and no homologous MazE-like antitoxin, suggesting that the investigated strain may have other proteins that interact with MazF to exert antitoxin functions [61]. As identified by yeast two-hybrid screening and *in vitro* pull-down experiments, MrpC can form a complex with MazF to inhibit programmed cell death (Fig. 2). Importantly, the *mrpC* gene is required for development [58] and is a key early transcriptional activator of the FruA gene, another essential developmental regulator; this is similar to the response regulator in the two-component signalling system, which plays an important role in regulating gene aggregation and sporulation [62] (Fig. 2). The synergistic response of the regulatory factor and the independent transcription factor MrpC is an unusual gene regulation mechanism that effectively neutralizes the toxicity of MazF to ensure proper control of gene expression and cell fate during *M. xanthus* development [63-65].

In addition to the cross-reactivity of the RelBE system toxin-antitoxin system protein, we found direct interaction between RelJ/RelK (Rv3357/Rv3358) and the transcriptional repressor SirR (Rv2788) [66]. SirR is capable of exerting a similar function on the toxin RelK (Rv3358) to regulate the DNA-binding activity of the antitoxin RelJ (Rv3357), thereby attenuating the inhibitory effect of RelJ on its promoter. Furthermore, SirR may be able to replace the antitoxin RelJ (Rv3357) to reverse bacterial growth inhibition by overexpression of RelK (Rv3358). Conversely, RelJ/RelK (Rv3357/Rv3358) attenuates the inhibitory effect of the transcriptional repressor SirR (Rv2788) on its own promoter [66]. This interaction reveals a novel regulatory function for transcription factors in bacteria and a new regulatory mechanism of the toxin-antitoxin system, and we hypothesize that SirR can maintain minimal cell growth when the concentration of antitoxin is low. The SirR transcription factor is located upstream of the iron-absorbing ABC transporter gene operon. Although the effect of direct interaction among the target genes regulated by SirR is not discussed herein, in the absence of nutrients or under environmental stress conditions, the pathogen TA system can temporarily inhibit specific cellular processes and prevent bacterial growth, returning the cell to normal infection processes when favourable conditions occur. Identifying more examples of transcription factors involved in the TAS system will require further investigation.

3.3 Type II TA system proteins interact with chaperone proteins to maintain protein stability

Chaperones are a class of proteins that assist in intracellular assembly and protein folding [67]. The protein encoded by Rv1957 in *M. tuberculosis* is 19% homologous to SecB in *E. coli*. However, in contrast *E. coli* SecB, the Rv1957 gene is adjacent to the stress response TA system higAB, and transcription of the Rv1957-higAB operon is induced by DNA damage, heat shock, and hypoxia stress [68-70]. In addition, deletion of the antitoxin gene higA alone inhibits bacterial growth, though absence of the entire operon region has no significant effect. Destruction of Rv1957 can also slow bacterial growth [71, 72]. Rv1957 can also directly interact with HigA and protect the antitoxin from protease degradation, thereby promoting its folding and subsequent binding to the HigB toxin to specifically control the HigAB toxin-antitoxin system. Co-expression of Rv1957 and higA in *E. coli* can significantly weaken the toxicity of HigB and restore bacterial growth. This toxin-antitoxin-chaperone three-component system is called TAC (Toxin-Antitoxin-Chaperone). Overall, SecB is the first example of a chaperone protein that regulates a TA system [73-75].

Activation of type II TA system toxins is generally thought to be dependent on degradation of the cognate antitoxin by the stress proteases Lon and Clp [26]. However, as no protease has been found to be involved in the cascade activation of HigB, the SecB-like antitoxin is significantly more competitive than is the protease [74, 75] due to the

structure of the antitoxin. Analysis of the TAC sequence has revealed that the carboxy terminus of the antitoxin contains an extended chaperone addition sequence, ChAD, that can effectively prevent folding of the antitoxin and specifically recruit the SecB-like chaperone. Fusion of the HigA ChAD sequence to an irrelevant recombinant protein or a typical chaperone-independent type II TA system causes chaperone activity towards these proteins, indicating that the ChAD motif in the antitoxin is transferable [76]. Many questions remain regarding the interaction between the TAS system and the chaperone protein, including whether TAC systems exist in some common pathogens, such as *Staphylococcus aureus,* which causes skin infections, and whether these chaperones perform the same function to maintain antitoxin stability. Additional work is needed to elucidate the transcriptional regulatory mechanisms of toxins and the effects of toxin-targeted cellular processes on the physiology and virulence of bacteria. Various TA system proteins and chaperone machinery may be used to optimize the expression and folding of heterologous proteins in prokaryotic hosts or for biotechnology and medicine.

4. Final remarks

The unique and precise regulation of type II TA system toxin proteins highlights the importance of these molecules in bacterial stress responses, and the mechanism of action of this system is complex and interesting due to the cross-regulation between different TA systems, either directly or through protein interactions. Such interaction and

communication of TA systems with other cellular components during bacterial life processes are even more compelling.

With further research, we found that the TA system's ability to sense signals is not fully dependent on ppGpp-regulated protease activity in detoxifying the antitoxin-activated toxin pathway. Therefore, which signalling molecules can directly regulate TA systems? Can toxin-antitoxin proteins act as a direct receptor for signalling molecules? Among the many antitoxin proteins with DNA-binding domains, do global regulators of TA systems and even transcriptional regulators that exert extensive regulatory functions in whole cells exist under certain conditions? These questions are worthy of further study.

The interaction and regulation of type II TA system proteins with key proteins in cellular processes, such as DNA replication, gene transcription, protein synthesis, and cell division, are beginning to be understood, though more interactions and functions must be explored. In particular, the role of the TA system of some pathogenic bacteria rich in type II TA systems is still unclear with regard to autoimmune and host immune processes. Additional questions include the following: Can TA system proteins be modified after translation? Do TA system proteins interact directly with the CRISPR-Cas system to protect against bacteriophage infection and regulate growth? Do TA system proteins interact with host immune proteins to regulate pathogenic capacity?

In recent decades, the emergence and spread of bacterial resistance, especially multi-drug resistant and extensively resistant bacteria, has led to challenges in the

treatment of infectious diseases. In addition to TA system-mediated cell retention resistance, increasing evidence shows that bacterial resistance is associated with genetic maintenance systems. The TA system, similar to the presence of a drug, can be parasitic to bacteria. Abundant TA systems are associated with resistance genes present on the same plasmid, thereby maintaining microbial resistance in the absence of drugs. In addition, it remains unclear whether expression of resistance-related genes is regulated by the TA system or direct interaction with TA system proteins under drug stress, and how TA system proteins sense drug pressure signals needs to be determined.

Studying the TA system not only helps us to understand bacterial physiology but also contributes to the development of new antibacterial therapies. Direct interaction between TA system proteins and functional proteins provides a theoretical basis for the development of new and effective antibiotic proteins with similar structure to the bacterial toxin. On the one hand, this type of drug promotes programmed cell death of pathogens by preventing or reducing the association between a given pair of toxin and antitoxin; on the other hand, it promotes inhibition of functional proteins, thereby interfering with specific cellular processes. This review summarizes and discusses cross-interaction among known TA systems and interaction of these systems with other functional proteins to promote the design of new antibiotics. However, the realization of this goal will undoubtedly keep researchers busy for years to come.

Funding: This work was supported by the National Key R&D Program of China (2017YFD0500302) and the National Natural Science Foundation of China (31730005), the Fundamental Research Funds for the Central Universities (2662016QD007), the National Natural Science Foundation of Hubei Province (2018CFB495), the State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (SKLVEB2016KFKT008).

Conflict of interest: The authors declare that they have no conflicts of interest.

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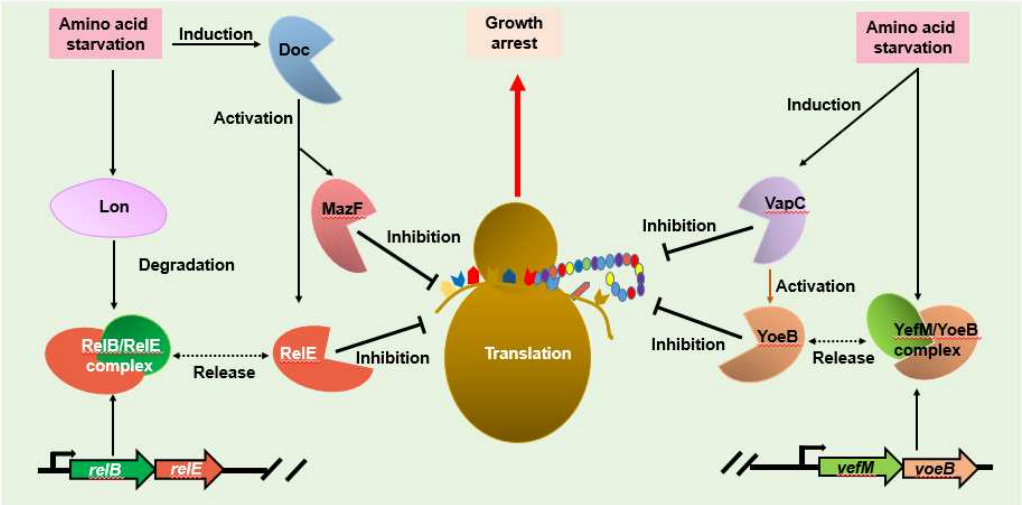

Fig. 1 Toxin Doc and VapC activate toxin proteins in different source TA systems under amino acid starvation conditions.

When amino acids are deficient, *E. coli* Lon protease degrades RelB antitoxin to activate the RelE toxin protein; alternatively, Doc activation activates RelE and MazF toxin proteins, which cleave mRNA and inhibit bacterial growth. In *Salmonella*, the VapC toxin is activated under amino acid starvation, and VapC in turn activates the YoeB toxin. Both toxins inhibit protein translation and arrest bacterial growth.

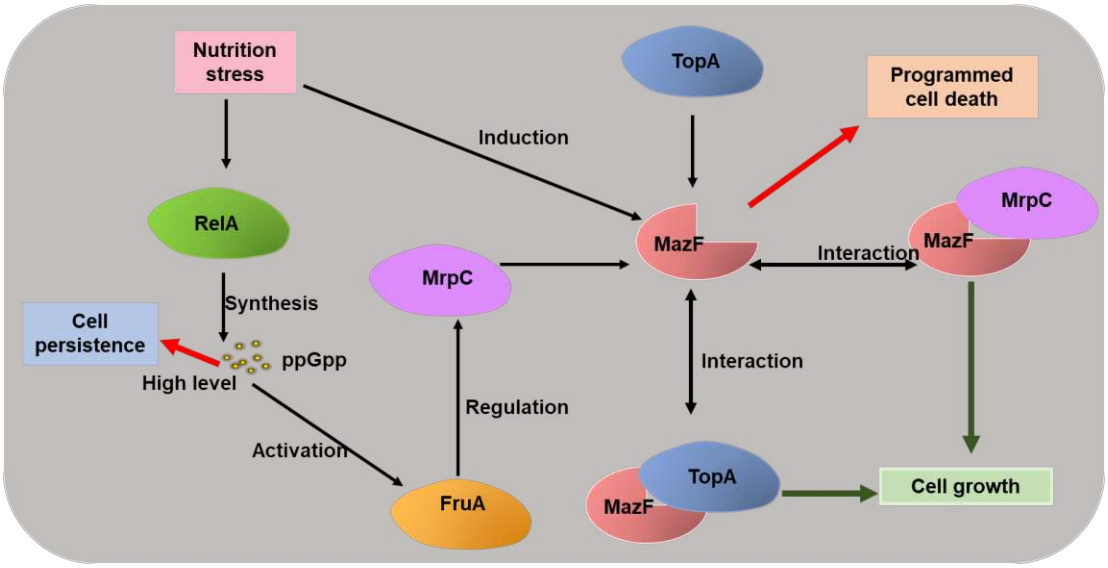

Fig. 2 MazF interacts with functional proteins to regulate bacterial growth.

In the absence of nutrients, the toxin MazF is induced, resulting in programmed cell death. RelA synthesizes ppGpp to activate the early developmental gene cluster mrpC through FruA, which can bind to MazF to inhibit bacterial death, whereas high levels of ppGpp cause bacteria to enter the retention state. In *M. tuberculosis*, TopA interacts with MazF to weaken the virulence of toxins and allows bacteria to grow normally.

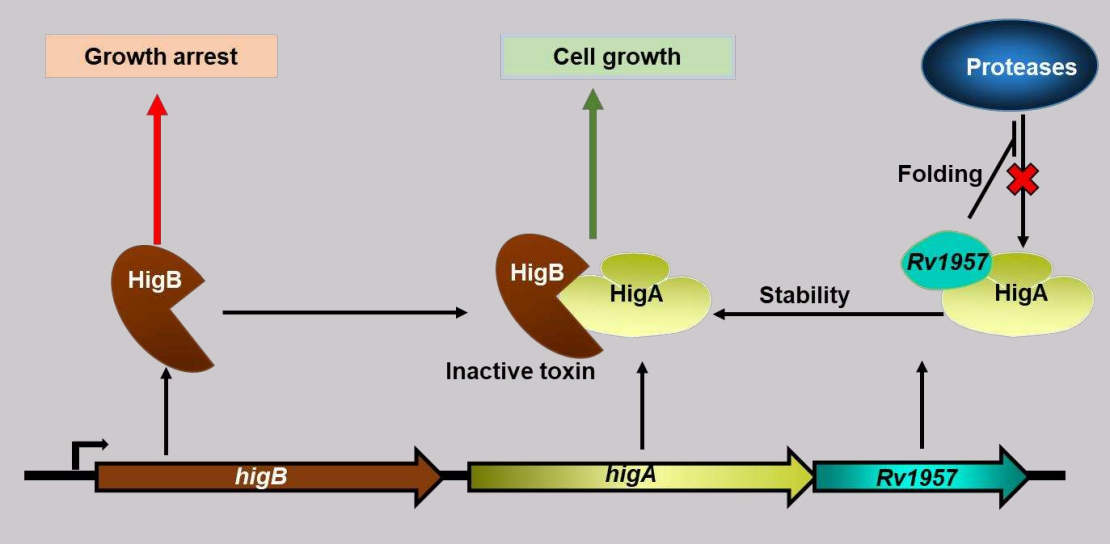

Fig. 3 SecB interacts with HigAB to maintain cell growth

The SecB-like chaperone protein Rv1957 binds to the ChAD structure of the antitoxin HigA, aids in the folding and protein stability of the antitoxin to protect the antitoxin from protease degradation, promotes the binding of HigA to the toxin HigB, and restores bacterial growth.