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Cellular And Environmental Facets Of Cross- Interlinkage Of Bacterial Toxin-Antitoxin Systems: An Overview

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Abstract
Toxin-antitoxin (TA) systems are widely present in bacterial genomes. They consist of stable toxins and unstable antitoxins that are classified into distinct groups based on their structure and biological activity. TA systems are mostly related to mobile genetic elements and can be easily ac- quired through horizontal gene transfer. The ubiquity of different homologous and non-homologous TA systems within a single bacterial genome raises questions about their potential cross-interactions. Unspecific cross-talk between toxins and antitoxins of non-cognate modules may unbalance the ratio of the interacting partners and cause an increase in the free toxin level, which can be deleterious to the cell. Moreover, TA systems can be involved in broadly understood molecular networks as transcriptional regulators of other genes' expression or modulators of cellular mRNA stability. In nature, multiple copies of highly similar or identical TA systems are rather infrequent and probably represent a transition stage during evolution to complete insulation or decay of one of them. Nevertheless, several types of cross-interactions have been described in the literature to date. This implies a question of the possibility and consequences of the TA system cross-interactions, especially in the context of the practical application of the TA-based biotechnological and medical strategies, in which such TAs will be used outside their natural context, will be artificially introduced and induced in the new hosts. Thus, in this review, authors discuss the prospective challenges of system cross-talks in the safety and effectiveness of TA system usage in view of exploring biotechnological and biomedical applications likely to be worth-interesting for human welfare.

Keywords: *Bacterial Toxin-Antitoxin Systems, Cross- interlinkages, Cellular factors, Environmental facets.*

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I. Introduction

Toxin-antitoxin (TA) systems are intracellular elements corresponding to a non-secreted un-fluctuating toxin, mostly a protein and a hypersensitive to degradation antitoxin, likely to be either a protein or an RNA molecule. The classification of TA systems concomitant with their mode of action have been specifically illustrated in **Figure I**, and are thoroughly reviewed [1].

The toxin-antitoxin systems can be easily acquired through horizontal gene transfer, and at least some of them can possibly cross-regulate. Therefore, there is an apparent possibility that the TA modules' activity can be disturbed by several factors in microorganisms living in their natural environment. For example, that activity can be affected through interference with the TA modules of other bacteria persisting within the environment or with the antitoxins encoded within phages' genetic material. Moreover, TA systems can be involved in broadly understood molecular networks: for instance, as transcriptional regulators of other genes, when antitoxin or TA complex binds the promoter region and affects transcription of a given gene [2-4], or as factors directly affecting other genes' expression through the toxin activity (e.g., due to mRNA degradation by the RNase toxin) [5,6]. This implies a question on the possibility and consequences of the TA system cross-interactions with stress environment, especially in the context of the practical application of TA-based biotechnological and medical strategies.

Moreover, multiple TA systems encoded within bacterial chromosomes could be considered a significant part of the genetic pool to form new components essential for bacterial adaptation in new ecological recession. Considering the fact that the selective pressure may provoke TA system differentiation, as well as their convergent evolution leading to cross-talk between non-cognate TA systems, the security of TA-dependent biosafety control methods should be especially taken into consideration. Therefore, the evolutionary potential of TA cassettes should also be considered when planning TA-based biotechnological and medical applications, as well as during the development of biosafety and crop preservation strategies.

To conclude, authors are convinced that the TA modules might be considered as both a substantial gene source for the development of beneficial innovative biotechnological tools and approaches and as

unpredictable components of those. Thus, in this review, authors intend to highlight the subject of possible TA systems' cross-talk and discuss the implications of the putative effects of such cross-interactions in the practical use of TA modules under following heads:

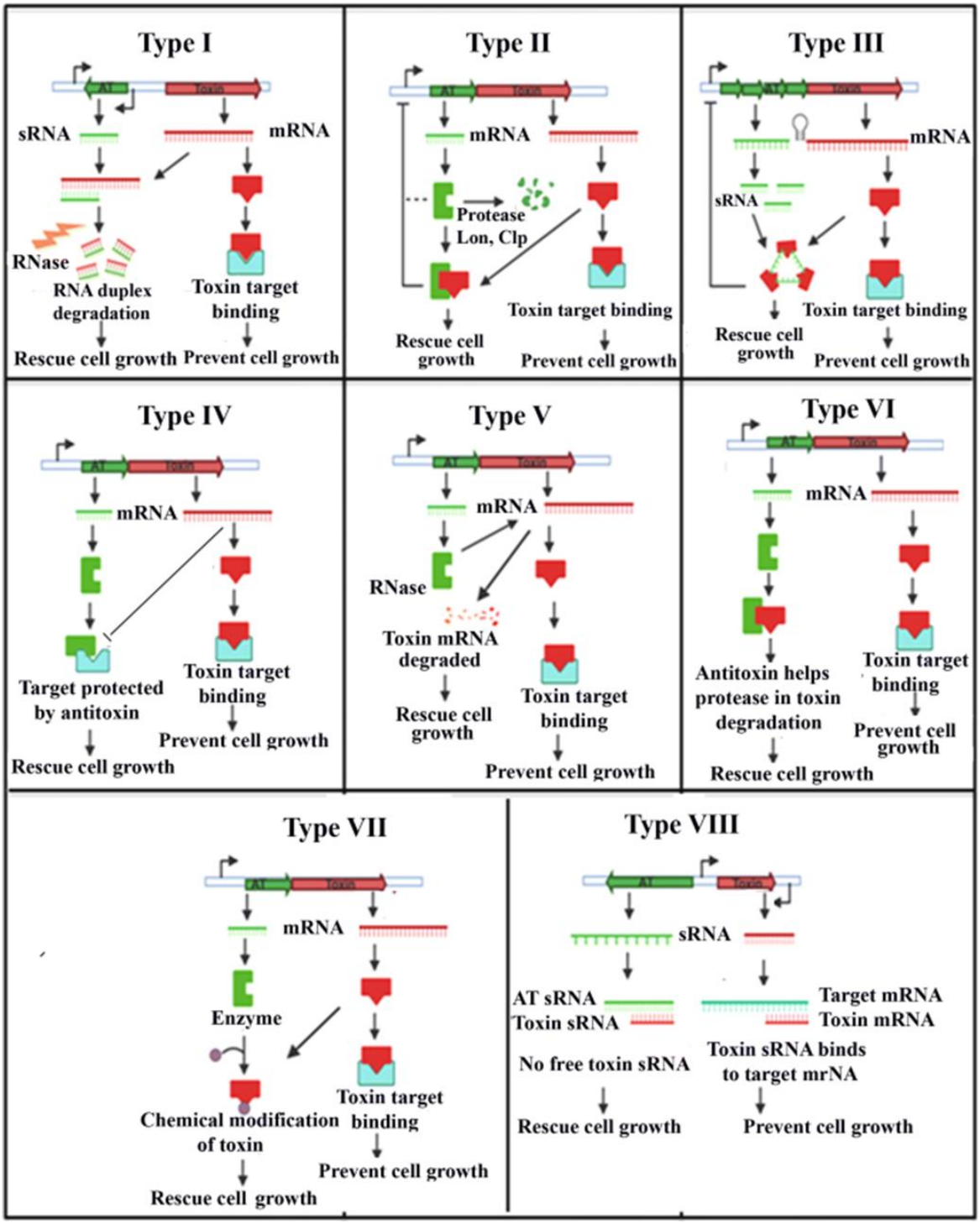

Figure I: The Categorization of the toxin-antitoxin systems.

[1] Activation and Biological Function of the Bacterial Toxin Antitoxin Systems

Toxin-Antitoxin System Classification and Distribution Issues: Since TA discovery, there have been many attempts to classify these systems; however, an ever-increasing number of new records made each classification method imperfect and unsatisfying due to the great structural and functional diversity of toxin and antitoxin components. Based on the nature and mode of action of the antitoxin, TA systems are currently classified into eight types, numbered according to their discovery [7-13; **Figure 1**]. Type II is the most diverse and numerous,

while the other TA types are much less heterogeneous, with types V and VI being represented by only one example so far. However, even this classification system is not perfect, as some instances of cross-type features are observed. An example is *darTG*, a type II/IV TA hybrid system in which the DarG antitoxin inhibits expression of the DarT toxin via direct interaction, like in type II TAS, but also acts on the target of DarT removing toxic modification from the DNA, like in type IV TAS [23,24]. Moreover, some kind of shuffling between different types of TA systems, in terms of the structure and functionality of their components, has also been observed. Namely, ToxN toxin from *toxIN* type III TA structurally belongs to the CcdB/MazF super-family of type II toxins, while SymE type I toxin possesses structural similarities to MazE/AbrB super- family of type II antitoxins, indicating that a toxin may evolve from an antitoxin [14,15]. Furthermore, the YeeU antitoxin of the type IV YeeUV TA system is structurally similar to the ribonucleolytic type II toxins YoeB and RelE, showing another example that toxins and antitoxins can evolve from each other. As a proof of concept, an artificial toxin has been created from the native type V GhoS antitoxin with only two amino acid changes, and subsequently, two disparate antitoxins derived from type III ToxI and type II MqsA have been made to neutralize its toxicity [16]. Based on primary sequence and three-dimensional structure similarity, type II toxins and antitoxins have been grouped into families and super-families, which are not related, while sharing some functional connections. Several bioinformatic analyses revealed that the binding of toxins to specific antitoxins is often evolutionarily unpredictable and reflects the swapping of functional domains between different TA families or even between TA counterparts. Such is the case of the Phd antitoxin, which exhibits the same structural fold as the YefM antitoxin; however, both of them inhibit structurally unrelated toxins, i.e., Doc and YoeB, respectively.

All of the above examples highlight the great plasticity of TA components and support the idea that antitoxins and toxins were assembled on the basis of the "mix and match" rule, an event that happened multiple times in the course of evolution [17,18]. There are also at least two examples linking TA modules and bacterial secretion systems, suggesting that toxins could be converted into secreted effectors. Namely, *Bartonella* T4SS and *Xanthomonas* T3SS protein effectors derive from FicT and ζ toxins, respectively [19,20].

The number of identified TA loci varies greatly among different bacterial species. The data acquired from TAfinder, which is a type II TA prediction tool integrated into Toxin-Antitoxin DataBase (TADB 2.0), show that 66% of the analyzed species harbor at least one to up to twenty loci in a single strain, while 20% of species carry even more than twenty toxin-antitoxin modules. According to this database, the current record is held by *Microcystis aeruginosa* NIES-843 cyanobacterium, with its 113 predicted TA elements, followed by the human pathogen *Mycobacterium tuberculosis* H37Rv, in which 76 TA elements have been predicted [21]. Since TADB 2.0 database contains only records that match type II TA families that have been previously experimentally characterized, it could be expected that the total number of chromosomal TA elements in prokaryotic genomes is much larger. Accordingly, the TASmania database predicts as many as 423 and 269 putative TA components in the genomes of *M. aeruginosa* NIES-843 and *M. tuberculosis* H37Rv, respectively [4]. This shows that our knowledge of TA systems can be compared to the tip of the iceberg, where the majority of data is yet to be uncovered. Moreover, different analyses indicate that the TA systems are inconsistently and unevenly distributed across prokaryotic genomes and generally are not conserved in closely related species or even strains [5,22,23]. Analyses of the flanking genes and broader genetic context of different *E. coli* genomes suggest that their TA modules have been independently integrated and propagated within species through horizontal gene transfer mechanisms and homologous or non-homologous recombination events, which may be a general rule in the distribution of TA cassettes [24].

Mechanisms of TA Systems' Activation: Expression of the toxin and antitoxin encoding genes has to be balanced to ensure that the toxic effect of each toxin molecule is neutralized by its associated antitoxin. Disruption of the toxin and antitoxin intracellular equilibrium results in the TA system activation. Until recently, the TA system activation was equated with transcriptional induction of the TA operon, especially since it was observed that many TA modules are transcriptionally activated during diverse cellular stresses [25–28]. However, the latest data indicated that although stress can induce TA transcription, it is not necessarily accompanied by toxin activity [29,30]. Therefore, in this review, we define the TA system activation as a situation where the toxin is active and affects its cellular target, leading to bacterial growth inhibition or even cell death.

Likewise, a transcription-based mechanism of toxin activation can be true for certain type I TA systems. Namely, this type of activation was suggested for the type I *hokW-sokW* module of the Sakai prophage 5 (Sp5) encoded within the genome of *E. coli* O157:H7 Sakai strain. It was demonstrated that mitomycin C (MMC) treatment of bacteria resulted in Sp5 induction, combined with a diminution of *sokW* RNA and an increase in HokW toxin level. The MMC damages DNA and promotes RecA protein activation, which was shown to be engaged in DNA repair processes and lambdoid prophage induction. It was proposed that the depletion of *sokW* was mainly caused by transcription inhibition. Interestingly, an increase in HokW level was the combined effect of *sokW* depletion and some indefinite RecA-dependent processes. The HokW protein bound

to the cell membrane promoted its depolarization and, as a result, cell growth retardation. It was observed that bacterial growth retardation after Sp5 induction was significantly lower in an *E. coli* strain with the *hokW-sokW* operon deletion, which suggests an effective HokW activation during Sp5 prophage lytic development [31].On the other hand, examples of TA system activation that is probably mainly based on antitoxin degradation were described as well. Recently published data suggest that in the case of type II systems, *parDE*, and *mqsRA*, the toxin can be activated by ClpAP and ClpXP-mediated antitoxin degradation, respectively. In both cases, the process of antitoxin recognition and targeting to proteolysis was regulated by an additional factor [32,33].

Nevertheless, there are several TA examples, including a fused type II TA module ($capRel^{JS46}$) and a two-partite *mazEF* module, where it is the direct binding of different phage capsid proteins to one of the TA system components that trigger the toxin activation [34–37]. Furthermore, Zhang *et a*l. proposed lately that the protease-mediated toxin activation could also be the result of the protease's direct binding to the TA complex, followed by the toxin release—even without the actual antitoxin proteolysis [37].To conclude, it appears that there are several possible molecular pathways of toxin activation depending on the TA type and/or its biological role and that the process may be highly complicated and dependent on multiple cellular and environmental factors.

Contradictions Regarding Biological Function of TA Systems: Regardless of the concerns about the mechanism of TA systems' activation, these modules were claimed to be engaged in a number of different processes and phenomena, such as genomic stabilization (including plasmid propagation and prophage lysogeny control), abortive phage infection, stress response modulation, virulence, and biofilm formation. However, lately, the most popular opinion is that despite the prevalence of TA systems, the majority of TA modules have little or no biological role. It is highly possible that at least some of the TA systems act as selfish entities, ensuring their own transmission, even if this has either no or a negative effect on the host fitness [38–40]. Since a lot of contradictions on this subject have accumulated over the years of research, currently, the actual **biological role of TA modules** is under intense debate [41–46]. For example, it has been shown that contrary to former reports [47–49], TA modules are not required for persister cell formation, neither in *S. enterica* nor in *E. coli* [50–52]. Moreover, there is a body of evidence that transcriptional activation of TA-encoding genes observed during stress is not necessarily accompanied by toxin activity [53].

On the other hand, several studies have shown convincing examples favoring the hypothesis of the TA system activity as a bacterial physiology modulator [54–56]. Thus, although a growing body of data suggest that TA modules are rather selfish genes, their role as the stress response or virulence modulators during microorganism-host interactions cannot be entirely excluded.

It is believed that in the case of plasmids, the TA systems have a clear role in their vertical inheritance by selectively inhibiting or slowing down the growth of plasmid-free daughter cells due to the toxin's activity (the so-called **addiction phenomenon**) [57]. The first such module, named *ccdAB*, was discovered in the F plasmid of *E. coli* and was later shown to mediate the genetic stabilization of this mobile element by the PSK mechanism (Postsegregational Killing) [58,59]. According to this mechanism, degradation of the labile antitoxin and the lack of its *de novo* synthesis leads to the release of the stable toxin in the daughter cells devoid of a plasmid. The toxin then interacts with its intracellular target, leading to cell death or inhibition of metabolic processes. Thus, as progeny is eliminated from the population when the plasmid is lost, bacteria become "addicted" to TA modules located on plasmids. Similarly, multiple examples of TA-mediated stabilization of other types of mobile genetic elements (MGE) have been shown [60–64]. It was shown that the cross-interaction between these chromosome and plasmid-born proteins prevented stabilization of the plasmid within bacterial cell populations (the so-called **anti-addiction phenomenon**) [65].

Another example of the TA systems' recognized biological function is their role as **phage-defense modules**. The subject of TA cassettes' activity as the phage-defense modules and the phage counter-defense systems was thoroughly reviewed recently [66]; below, we are giving just a general description. Inhibition of phage propagation is well documented for TA cassettes of different types. For example, is was shown that the R1 plasmid-encoded type I *hok-sok* module has a role in T4 phage exclusion. This module is probably activated due to an sRNA antitoxin expression inhibition combined with its degradation after host transcription shut off [85]. The Hoc protein then binds to the cell membrane leading to its depolarization and premature cell death; thus, this reduces the amount of phage progeny and limits phage propagation. A similar mechanism of activation was proposed for the type III $toxIN_{Pa}$ module from *P. atrosepticum* in the course of φM1 infection [67]. Likewise, the *E. coli* gene expression switch off by the T4Δ*dmd* phage leads to abortive infection mediated by the toxins of type II chromosomal *rnlAB* and plasmid-encoded *lsoAB* systems. It was observed that both systems are activated during the late stage of infection in a protease (ClpXP and/or Lon) dependent manner [68,69]. Interestingly, phage inhibition can be probably caused not only by the toxin activity but also due to the inhibition of phage gene transcription mediated by binding of the antitoxin to the phage DNA, as was proposed for the type II *crlAT* system of *P. aeruginosa* WK172 strain [70].The

anti-bacteriophage activity of TA modules may also be associated with **competi- tion between bacteriophages**. Activation of the fused type II *capRel*^{SJ46} system, encoded within the temperate prophage SJ46 genome, results in the lytic SECφ27 phage abortive infection. CapRel^{SJ46} is activated by direct binding of the SECφ27 phage Gp57 capsid protein, which probably induces conformational changes that, in turn, activate toxicity. The active CapRel^{SJ46} protein phosphorylates tRNAs and thus inhibits both the host and phage translation.

In contrast, some bacteriophages encode **pseudo-antitoxins** that can act to prevent the above-described TA-based anti-phage strategy [71-74]. For example, it was shown that the wild-type T4 bacteriophage encodes a *dmd* antitoxin which directly interacts with the RnlA and LsoA toxins, neutralizing their toxic effect. Moreover, T4 encodes the PinA protein that directly binds and inhibits the Lon protease activity, which may affect the TA modules' activation process. Almost exactly the same mechanism of action was proposed for the 4.5 gene product of T7 phage that prevents type II system *sanaTA*-mediated abortive infection [75], for the *pseudo-toxI* sRNA encoded within phage φTE of *P. atrosepticum* [90] and for the anti-DarT1 factor encoded within some T-even phages [76]. It was also proposed that the bacterium-phage interaction constitutes a strong se- lective pressure for bacteriophages susceptible to TA-mediated exclusion to acquire a cross-interacting pseudo-antitoxin. On the other hand, an opposite trend was predicted for the host bacteria. Namely, it was suggested that the rise of a TA-resistant escape phage would drive the selection of non-cross-interacting toxins in bacteria. All in all, it seems clear that the biological composition of the bacterial environment is the main factor affecting the TA modules' activity and evolution.

[2] Modes of the Toxin-Antitoxin System Cross-Talk

The ubiquity of different homologous and non-homologous TA systems within a single bacterial genome raises questions about their potential cross-interactions. Phylogenetic studies show that proteins of distantly related TA systems usually do not exhibit any cross-talk, whereas proteins of close homologs are sometimes able to interact with non- partners [77]. However, the latter situation presumably exemplifies an intermediate step in the evolution pathway toward complete divergence and insulation of paralogous systems in the same genome. This is confirmed by the observation that identical TA cassettes are mutually exclusive and virtually do not coexist in the same cell.

Given the danger brought by toxins to the bacterial cell and the fact that numerous different toxin-antitoxin cassettes can co-occur in a single genome on chromosomes, plasmids, or bacteriophages, it was crucial for TA systems to develop mechanisms that allow them to avoid non-specific, random cross-reactions. On the other hand, it was also critical for paralogous TA pairs to proceed to a safe evolution of insulation which is inevitably directed by genetic drift and selection based on the toxin effect. Thus, the biggest challenge for the interacting proteins is to keep the desired interaction and, at the same time, to avoid unwanted cross-talk, which can be dangerous to a cell. This balance is achieved by a subset of specificity-determining residues, which are responsible for the stabilization of cognate interactions and the disturbance of non-cognate ones [78,79].

The exact number of amino acid residues controlling the specificity of toxin-antitoxin pairing in the studied examples varies. In the case of two insulated *parDE* systems, three amino acid substitutions were shown to confer the toxin-antitoxin interaction specificity. Each of these key interface positions was shown to form a positive element that promotes the cognate interaction, while two of them serve as negative elements that protect from the non-cognate interaction. However, substituting just one of these residues was sufficient to produce promiscuity [80]. Similarly, a single Asp83Tyr substitution in the Txe toxin of the type II *axe-txe* system was shown to relax the specificity enough to allow its interaction with the YefM antitoxin of the homologous *yefM-yoeB* TA system [81].

As an example of the hyper versatility of antitoxins, Kurata and co-authors described an antitoxin protein domain, DUF4065, that is able to form complexes with dozens of different cognate toxins. Moreover, through directed evolution, they were able to select variants that neutralize even a noncognate and non-homologous toxin with just two amino acid substitutions. Such antitoxins were called PanA, while this hyper-promiscuous domain has been named Panacea to underline its universality [82]. On the other hand, in their recent paper, Grabe and co-authors found that insulation of three *tacAT* paralogs from *Salmonella* spp. is conferred by the presence and size of a specific helix within the toxin structure. They also revealed that TacA antitoxins display remarkable flexibility in their structural organization, allowing the formation of additional interfaces within the toxin to accommodate this helical element. These additional helices on the TA interfaces may provide a safe space for the evolution of insulation between paralogous *tacAT* systems [83]. To summarize, the presence of promiscuous variants enabled by the super plasticity of the antitoxins seems to promote diversification which is crucial for the safe expansion of TA systems during evolution. Moreover, additional TA interfaces between both interacting partners act as elements supporting

neutralization and compensate for imperfect fits between the highly evolving regions of the toxin and antitoxin.

[4] Toxin-Antitoxin Systems—Biomedical Applications

Despite controversies on the TA systems' biological role and uncertainty about particular mechanisms of these modules' activation and their evolutionary pathways, attempts at the development of promising TA-based technologies for medical use have been made. Here, we divided the most propitious TA-based approaches into three categories: antimicrobial strategies (I), TA-based gene therapy strategies for viral diseases (II), and cancer (III) treatment.

[5] Practical Applications of Toxin-Antitoxin Systems in Biotechnology

Several different TA-based strategies have also been developed for diverse biotechnological applications. Here, we divided the most interesting TA-based approaches into six purpose-based categories: vector stabilization (I), counterselection (II), single protein production (III), positive selection of inhibitors (IV), containment control (V), and bacterial infections control in plants (VI). The TA-based biotechnological strategies were extensively reviewed recently [84-86], and thus in the following sections, we will mostly focus on discussing putative risks associated with their use and the impact of potential cross-interactions on possible applications.

Plasmids are important tools in diverse biotechnological applications. However, segregational instability of these mobile genetic elements, especially for the low copy number plasmids, may lead to their quick loss from the bacterial population. Typically, due to the metabolic burden, plasmid-harboring cells exhibit a reduced growth rate relative to the plasmid-free population. Therefore, when plasmid instability occurs, the faster- growing cells devoid of plasmid outcompete their plasmid-bearing counterparts. Thus, stable maintenance of the engineered DNA molecules comprises a significant concern in the industrial employment of microorganisms for protein or DNA production, as well as in the preparation of gene therapy constructs. For this, antibiotic-resistance genes are routinely employed; however, the use of antibiotics for medical applications and on the industry scale raises safety concerns of the world-wide regulatory authorities.

One of the tested alternatives for maintaining plasmid stability without the use of antibiotic resistance genes is toxin-antitoxin systems loci which have been successfully used as plasmid stabilization cassettes in several various approaches [87,88]. Basically, all of them rely on different stability of the toxin and the antidote components. However, the success of PSK-mediated (post segregational killing) plasmid stability depends on a sufficiently high concentration of the active toxin and on the mechanism by which the toxin affects cell survival and proliferation. Each subsequent cell division leads to the dilution of the toxin, further decreasing its ability to exert PSK. When the cell finally escapes from PSK-mediated killing, there is no mechanism to stop such plasmid-free cells from taking over the whole bacterial culture. Thus, not every TA system will be suitable as a stability determinant, and therefore the right choice is crucial here.

Several different TA modules were tested, although, in more recent applications, the *E. coli* type I *hok-sok* and enterococcal type II *axe-txe* are the ones that are frequently used. For instance, the *hok-sok* was employed to stabilize a plasmid introduced into probiotic *E. coli* Nissle 1917 to detect cancer cells in the urine, whereas the *axe-txe* was successfully used in the same bacterial strain to maintain plasmids constructed for cancer immunotherapy [89,90]. Interestingly, a study that included a mathematical model describing PSK, supported by both in vivo and in vitro experimental data, has shown that the *axe-txe* system provides greater plasmid stability than the *hok-sok* TA module [91].

For biotechnological applications, it is important to take into consideration that diverse plasmid-borne TA systems differ in their plasmid maintenance ability and specificity. Some of them are strictly specific to certain species or even strains. For instance, the *pemIK* modules were able to maintain plasmids in various strains of both *E. coli* and *K. pneumoniae*, whereas the *ccdAB* cassettes demonstrated higher specificity towards their own host strain [92]. Moreover, some TA toxins cause the host death upon loss of the plasmid, whereas some only hinder bacterial culture growth, acting as bacteriostatic agents, as was shown for the Hok and VapC toxins, respectively [93,94]. However, this effect probably mainly depends on the toxin concentration within the cell and on the timing of toxin induction under experimental conditions. Furthermore, depending on the mechanism of the TA system activation, the possibility of its application can be limited to a particular bacterial species or even a bacterial strain. For example, the lack of specific proteases engaged in particular antitoxin degradation and disruption of the TA complexes may prevent the PSK effect of the toxin. On top of that, as mentioned above, some toxins exhibit toxicity only in particular strains. For instance, the chromosomal YoeB toxin from *Agrobacterium tumefaciens* was shown not to be toxic in *E. coli*, yet it inhibited the growth of its native host [95].

In light of TA modules' cross-talk, there is a substantial danger that potential cross- interactions

between pre-existing chromosomal TA modules and the one being introduced on the plasmid may abolish cellular addiction to the plasmid. For instance, *E. coli* chromo- somal *yefM-yoeB* complex was shown to cross-regulate the expression of a plasmid-encoded *axetxe* cassette, which is often chosen as a stability module, as mentioned above [96].

Counter- selection Systems Based on Toxins of TA Modules: The major challenge in different DNA cloning strategies is the low efficiency of inserting introduction into plasmid vectors. To increase the chances of isolating recombinant DNA molecules, two different strategies that employ toxin-antitoxin cassettes have been developed. In the cloning systems that are commercially available, StabyCloning™ from Delphi Genetics Inc. and Gateway™ recombination cloning system provided by Invitrogen, the CcdB toxin that targets *E. coli* DNA gyrase has been used for positive counter-selection of recombinants [97]. In the first method, the toxin gene is present on the bacterial host chromosome, while the fragment of the cognate antitoxin is located within the vector. The missing part of the antitoxin gene needs to be incorporated by PCR into the insert. In this way, only recombinant DNA molecules in the right orientation of the insert will provide the active CcdA antitoxin to counteract the genome-encoded CcdB toxin [98,99]. On the other hand, the Gateway™ strategy is based on the site-specific recombination system derived from bacteriophage λ. A specifically prepared vector contains a gene encoding the CcdB toxin flanked by *attP* sites, which should be replaced during cloning by the insert bearing corresponding *attB* sites incorporated in a PCR reaction. In this way, the host cell will survive only when the vector undergoes successful recombination [100–102].

Both of the above-described strategies rely on the CcdB toxin activity. However, there are examples of cross-interactions between chromosomally encoded and plasmid-encoded *ccdAB* modules that may result in the termination of the toxin's activity [103]. Thus, this method is not universal, as it cannot be designed for bacterial strains that harbor cognate antitoxin; therefore, a thorough analysis has to precede the choice of the cloning system.

Toxin of TA Cassette in a Single Protein Production Technology: Another strategy that employs the toxin of the TA module is the single protein pro- duction system (SPP) developed by Suzuki and co-workers and later commercialized by Takara Bio company [104]. This technology is based on *E. coli* MazF toxin acting as a sequence-specific endoribonuclease that recognizes and cleaves ACA sites in vitro [105]. In this method, a gene of interest must be designed to be free of ACA sequences. Upon MazF induction, most cellular mRNAs are degraded, resulting in protein synthesis shut down except for the engineered gene of interest. This allows for efficient production of the desired protein. This technique is especially useful for toxic protein production, as well as for efficient and low-cost isotope labeling of proteins required for NMR studies [106].

Still, subsequent studies have revealed that *E. coli* MazF specificity is more relaxed and that this nuclease cleaves single and double-stranded RNA in the NAC sequence, where N is preferentially U or A [107]. Moreover, some other members of the MazF family recognize different and sometimes longer sequences. For instance, *B. subtilis* MazF recognizes UACAU, while ChpBK is another MazF family toxin of *E. coli*—XACY, where X can be U or G, while Y is U, A, or G [108-110]. On the other hand, PemK, a homolog of MazF encoded by plasmid R100, preferentially cleaves single-stranded RNA at the A residue in the UAH sequence, where H is C, A, or U [111]. Finally, secondary RNA structures also play important roles in recognition by MazF since not all RNAs are cleaved by this toxin, even if recognition sites are present. On top of that, it was shown that ectopic expression of *mazF* may activate other TA systems, such as *relBE* [112]. It could be easily imagined that such a situation during protein production might affect SPP strategy because potentially induced other TA toxins could degrade mRNA of the protein to be overproduced. Additionally, again, this method is not universal as it has to be designed for a specific bacterial strain that does not possess cognate antitoxin and any other antidote which could abolish the MazF toxicity.

Positive Selection of Inhibitors Based on Toxin-Antitoxin System: The MazEF toxin-antitoxin system has been recently used for positive selection in a strategy of searching for inhibitors of SARS-CoV-2 main protease, Mpro, in microbial cells [113]. In this method, Alalam and co-authors took advantage of the fact that MazF displays toxicity not only toward bacteria but also eukaryotic cells, such as *Saccharomyces cerevisiae*. Thus, the toxin and antitoxin genes were cloned as a fusion separated by a DNA linker coding for the viral protease recognition sequence. In the presence of active protease, the peptide linker is cleaved. Then, the toxin and antitoxin proteins are separated, resulting in growth inhibition by the toxic component because of the higher stability of the released toxin. Therefore, any molecule that abolishes the activity of the corona virus protease will prevent the toxin's release, thereby promoting indicator cell growth. The authors claim that this

system is suitable for screens of a large number of drug candidates, allowing for in vivo evaluation and quick adaptation for new variants.

Toxin-Antitoxin Systems for Biological or Gene Containment Control: With the growing number of successful applications of genetically modified organisms (GMO) or microorganisms (GMM), the programmed or unintended release of engineered bacteria to the natural environment raises biosafety concerns. Thus, active containment systems are necessary for biotechnological applications where GMOs or GMM are used to reduce the possibility of their escape from the desired location. Biological containment reduces the viability of introduced organisms outside of the target habitat or after the completion of their desired task. On the other hand, gene containment control prevents the spread of genetic material outside the host. For the active containment system, two major factors are necessary: lethal function and control element [114-116]. In both strategies, different genetic approaches have been employed, including the use of toxin-antitoxin systems.

The kill switch strategy employing TA modules was also tested in different eukaryotic species. For instance, the RelE toxin gene was successfully cloned under a glucose-repressed promoter in *S. cerevisiae* as a host. In the glucose-limited environment, the transgenic yeast were killed by this toxin, showing that such a strategy could be potentially used to control the fermentation process in the industry [205]. More recently, there were several successful attempts to express TA systems in plants. It was shown that expression of YoeB derived from *S. pneumoniae* cloned under a strictly inducible promoter was lethal to the plant model, *Arabidopsis thaliana*. The toxin induction resulted in plant development defects and necrotic symptoms, which were followed by plant death from the detrimental effects of YoeB as an endoribonuclease in both the bacterial and eukaryotic cells [117]. In a different study, the MazF toxin was expressed in a specific type of cell in the plant *Nicotiana tabacum*, called the tapetum, which are essential for the development of pollen grains. In parallel, the MazE antitoxin was expressed in the other plant tissues to protect them from the toxin's activity. In this way, researchers were able to obtain male sterility in tobacco plants [118].

However, as underlined by the authors of the studies mentioned above, inhibition of cell growth in these systems is not complete, indicating that some cells may escape the toxin's attack, and therefore control of the spread of transgenic organisms would not be risk-free. Moreover, most of the existing biocontainment strategies are not effective enough to achieve both low escape frequency and high efficiency. Mutations inactivating the toxin usually occur within the toxin gene or in the promoter controlling its expression. Therefore, for the long-lasting effect which is necessary for some of these applications, such a situation raises serious concerns. The escape effect is not surprising, considering the increased fitness of mutant strains harboring inactivated toxin genes [119].

II. Conclusion And Future Perspectives

Although cross-interactions between bacterial toxin-antitoxin systems do not appear to be the principal cause affecting the TA systems' biology, they are one of the pivotal elements that drive TA module evolution. In the natural environment, the TA systems' cross-talk usually leads to such systems differentiation. However, in large part, of the proposed biotechnological and medical TA-based applications, an opposite trend occurs. The main reason for this is using components of the TA systems that naturally do not coexist within a single cell, which raises the probability of their cross-talk and forces selective pressure, which is opposite to the one occurring in the natural environment (e.g., by artificial activation of the toxin). Since unintentional TA cross-interactions can significantly affect the outcome of the TA-based methods, these kinds of side effects should be carefully examined before the implementation of TA-based approaches in biotechnology and biomedical sciences. Bacterial toxin-antitoxin (TA) modules are abundant environmental and genetic stress elements that encode a toxin protein capable of inhibiting cell growth and an antitoxin that counteracts the toxin. The majority of toxins are enzymes that interfere with translation or DNA replication, but a wide variety of molecular activities and cellular targets have been described and those may provide new insights to propagate studies along these notice notions and objectives in future.

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