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Review

Toxin-antitoxin systems and its biotechnological applications

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Toxin-antitoxin (TA) systems are important genetic modules composed by two elements: a toxin, that is always a protein, and an antitoxin, that can be a RNA or a protein and neutralizes the toxic effect of toxin. These systems are widespread in bacteria and archaea, found on plasmids and chromosomes. According to the nature of the antitoxin and its mode of interaction with the toxin, TA systems are grouped into five types. In general, the antitoxin is less stable than the toxin and is rapidly degraded in special conditions, leaving the toxin free to act on its cellular targets. TA modules are important in several events in cell physiology such as plasmid maintenance, formation of persister cells, stress resistance, protection from bacteriophages and regulation of biofilm formation, acting on crucial cellular processes including translation, replication, cytoskeleton formation and membrane integrity. TA systems components have proven to be very useful in biotechnology, being used to enhance cloning selection and protein expression in living bacterial cells. Furthermore, they are also considered as promising targets for the development of antibacterial drugs and can be used in gene therapy. Here, we reported current aspects and the application of TA modules in biotechnology research.

Key words: Bacterial toxin-antitoxin (TA) systems, toxin, antitoxin, post-segregational killing (PSK).

INTRODUCTION

Toxin-antitoxin (TA) loci encode two-component systems that consist of a toxin and an antitoxin that neutralizes its effect (Yamaguchi et al., 2011). Toxin molecules tend to be stable whereas antitoxin molecules are more susceptible to degradation by specific proteases. Thus, the life times of both toxin and antitoxin molecules are different and to prevent the toxic effects of toxin molecules, antitoxin have to be produced continuously (Leplae et al., 2011).

Plasmids have been shown to encode a variety of regulatory mechanisms to their stable maintenance in growing populations of bacteria. TA systems have been initially discovered on plasmids as mechanism of plasmid maintenance by death of bacterial daughter cells which did not receive a copy of the plasmid during the cell division (Jaffe et al., 1985; Gerdes et al., 1986). Thus, they confer plasmid maintenance through a process called post-segregational killing (PSK). Later, TA systems were also found on bacterial and archaeal chromosomes (Pandey and Gerdes, 2005). The role of TA system

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Abbreviations: TA, Toxin-antitoxin; **PSK,** post-segregational killing; **SD,** Shine-Dalgarno.

systems encoded by chromosomes is far less understood. They have been suggested to stabilize linked genes, genomic islands and integrated conjugative elements (Wozniak and Waldor, 2009; Szekeres et al., 2007; Tsilibaris et al., 2007; Van Melderen, 2010). Besides acting on plasmid maintenance, TA systems act on others important events in cell physiology such as formation of persister cells (Maisonneuve et al., 2011), stress resistance (Aizenman et al., 1996), protection from bacteriophages (Fineran et al., 2009) and regulation of biofilm formation (Wang and Wood, 2011).

The toxin molecules perform regulatory functions acting on cellular replication and translation processes. TA systems can have many cellular targets that include the DNA replication machinery (Jaffe et al., 1985; Miki et al., 1992; Roberts et al., 1994), mRNA (Yamaguchi and Inouye, 2009), tRNA (Garcia-Contreras et al., 2008), 30S ribosomes (Neubauer et al., 2009), 50S ribosomes (Zhang and Inouye, 2009; Zhang et al., 2009; Zhang and Inouye, 2011) and cytoskeletal proteins (Tan et al., 2011). The toxin molecules have a variety of actions such as inhibition of DNA gyrase (Bahassi et al., 1999), cleavage of RNA (Yamaguchi and Inouye, 2009), transfer of phosphate groups (Mutschler and Meinhart, 2011), phosphorylation of proteins (Schumacher et al., 2009; Correia et al., 2006) and inhibition of ATP synthesis (Unoson and Wagner, 2008). Bioinformatics approaches have helped to search for new TA systems and have demonstrated a tremendous abundance of these systems in bacterial and archaeal genomes (Leplae et al., 2011).

The aim of this article was to review current aspects and classification of TA systems and discuss main insights and perspectives about its applicability in biotechnology.

CURRENT ASPECTS AND CLASSIFICATION OF TA SYSTEMS

According to the nature of the antitoxin and the mode of interaction between toxin and antitoxin, TA systems are currently grouped into five types: types I, II, III IV and V (Mruk and Kobayashi, 2013; Unterholzner et al., 2013). Toxin is always a protein but the antitoxin can be a RNA (types I and III) or a protein (types II, IV, and V).

Type I toxin-antitoxin systems

In the type I TA system, the antitoxin is an antisense RNA which suppresses toxin effect by binding to its mRNA (Fozo et al., 2008; Fozo et al., 2011). The toxin and RNA antitoxin genes are almost always on opposite strands of the DNA. The genes are transcribed separately and the RNA antitoxin binds to the toxin-encoding mRNA to form a duplex, which inhibits the translation process of the toxin-encoding mRNA.

Two models are currently described as regulatory mechanisms in type I TA system (Brantl, 2012): I) The antitoxin RNA is complementary to the Shine-Dalgarno (SD) sequence of the toxin mRNA preventing ribosome binding and translation initiation (Figure 1A); II) Toxin-encoding mRNA and RNA antitoxin are complementary at their 3' ends promoting toxin-encoding mRNA degradation via RNAse III (Figure 1B).

The toxin is typically a small hydrophobic toxic protein (less than 60 amino acids) containing a potential transmembrane domain which induces pores into cell membranes, leading to cell death. However, many toxins do not promote a bactericidal effect, but interfere with phage propagation, modulate the cell membrane or prevent mature particle formation (Brantl, 2012).

Type I TA systems were first identified on plasmids and are required for their replication and maintenance. Chromosomal type I toxins were discovered but their role remains unclear. In Gram-negative bacteria, toxin families (all have predicted transmembrane domains) have been identified such as Ldr, Hok, TisB, ShoB and EHEC. In Gram-positive bacteria, the first type I toxin-antitoxin system, Fst/RNA II system, was reported in *Enterococcus faecalis* and Fst-like toxin-antitoxin systems were also found on plasmids from *E. faecalis*, *Lactobacillus curvatus* and *Staphylococcus aureus*, the chromosome of *E. faecalis*, *Lactobacillus casei* and *Staphylococcus saprophyticus* and a phage from *Lactobacillus gasseri* (Durand et al., 2012).

Type II toxin-antitoxin systems

In the type II TA system, the antitoxin is a protein which neutralizes the toxicity of the toxin protein by binding directly to it (Figure 2) (Gerdes et al., 2005; Leplae et al., 2011). Antitoxin-encoding mRNA and toxin-encoding mRNA are synthesized from the same promoter and are translated to produce both proteins. The toxin and its cognate antitoxin form a stable TA complex, which blocks toxin function and autoregulate the TA module. Antitoxin alone can also autoregulate the TA system, but more weakly than the TA complex (Yamaguchi and Inouye, 2011). Under stress conditions, antitoxins are digested by stress induced proteases, releasing the toxin from the TA complex to attack its cellular target and resulting in cell growth interruption or eventual cell death.

Type II toxin-antitoxin systems were first discovered on plasmids where they contribute to plasmid maintenance. Subsequently, were found several genetic modules encoded on chromosome. This modules has been described to act on important cell events such as cell persistence, response to stress and biofilm formation (Maisonneuve et al., 2011; Aizenman et al., 1996; Wang and Wood, 2011; Yamaguchi and Inouye, 2011). The toxin proteins are generally longer than the antitoxins (around 100 amino acid residues) and have endoribonuclease, protein kinase, phosphotransferase activity or generate double-strand breaks poisoning the DNA

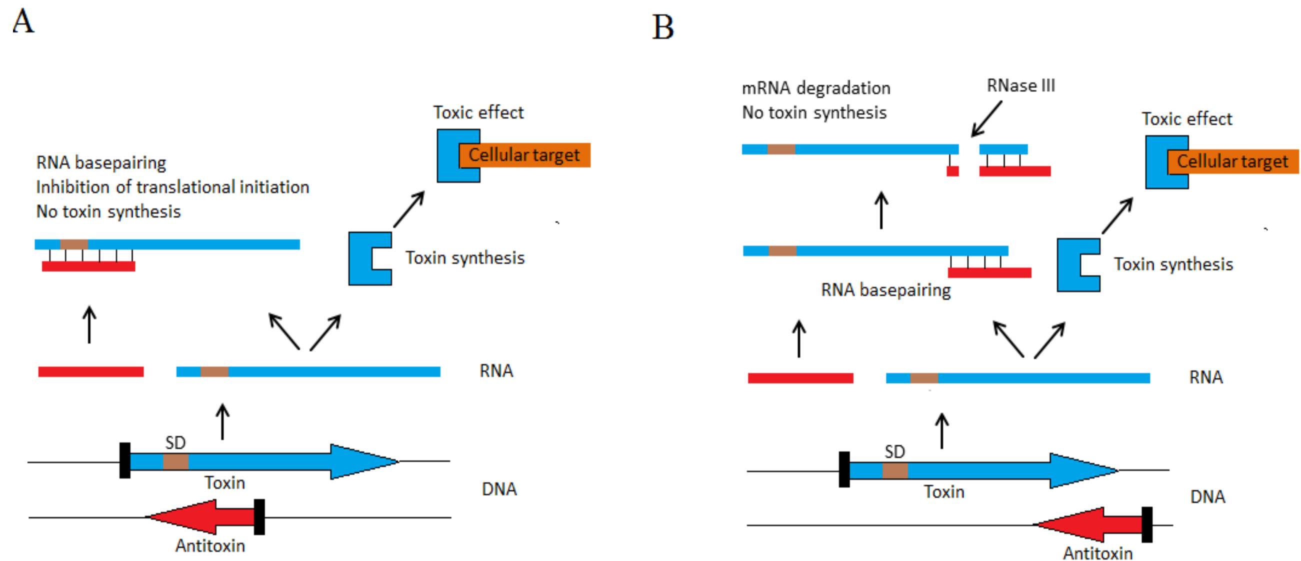

Figure 1. Regulatory mechanisms employed by type I toxin-antitoxin systems. A, Translation inhibition. Antitoxin RNA hides Shine-Dalgarno sequence of the toxin-encoding mRNA and prevents translational initiation; B, mRNA degradation. Toxin-encoding mRNA and RNA antitoxin are complementary at their 3' ends promoting degradation via RNAse III. SD (brown rectangles) and black rectangles denote Shine-Dalgarno sequence and promoters, respectively.

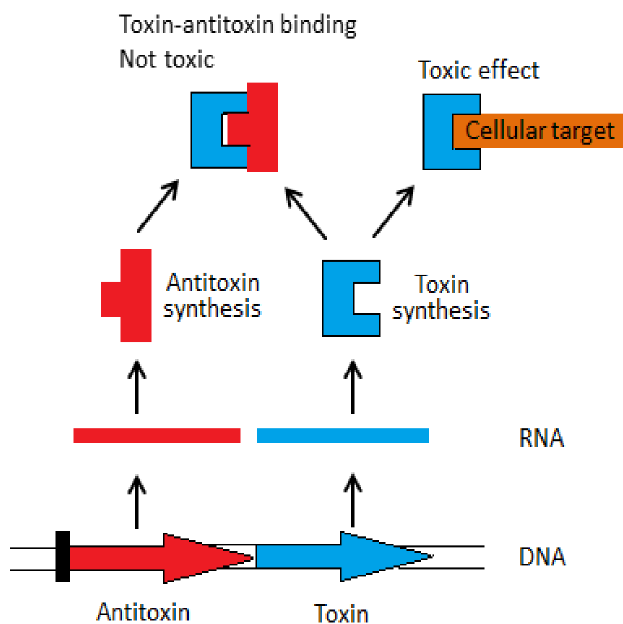

Figure 2. Regulatory mechanisms employed by type II toxin-antitoxin systems. Both toxin and antitoxin are translated from same promoter to produce proteins. The antitoxin neutralizes the toxicity of the toxin protein by binding directly to it. Black rectangle denotes promoter.

gyrase (Van Melderen and Saavedra De Bast, 2009; Van Melderen, 2010). For type II bacterial TA systems, toxin components have been grouped into 12 superfamilies (ParE/RelE, Zeta, VapC, Doc, CcdB/MazF, HipA, GinA, GinB, GinC, GinD, VapD and YafO) and antitoxins into 20 superfamilies (HigA, FizA, FizB, FizC, FizD, FizE, FizF, FizG, FizH, FizI, FizJ, FizK, Phd, VapB, VapX, YdcD, RelB, CcdA, Epsilon and $ParD_{RK2}$) (Leplae et al., 2011).

Type III toxin-antitoxin systems

Type III TA system has an antitoxin RNA but that is not an antisense RNA. The antitoxin RNA interacts directly with the toxin protein (Figure 3) (Fineran et al., 2009; Blower et al., 2012). Toxin gene is preceded by a short palindromic repeat, which is itself preceded by a tandem array of nucleotide repeats that acts as a transcriptional terminator, regulating the relative levels of antitoxic RNA and toxin transcript (Blower et al., 2012).

Bacteria have acquired a variety of anti-phage systems to survive in phage-containing environments. Type III toxin-antitoxin systems were identified as abortive infections systems that protected bacterial population from bacteriophages (Blower et al., 2012). The *toxIN* locus from a plasmid of the *Pectobacterium atrosepticum*, a plant pathogen, was the first type III toxin-antitoxin system identified. In this system, ToxN is the toxin, acting as a ribosome independent endoribonuclease, and the antitoxin is ToxI (Fineran et al., 2009).

Using structure-based homology searches combined with iterative protein sequence comparisons, three independent type III families were identified (designated *toxIN*, *cptIN* and *tenpin*), showing that active type III TA systems are far more diverse than previously known (Blower et al, 2012).

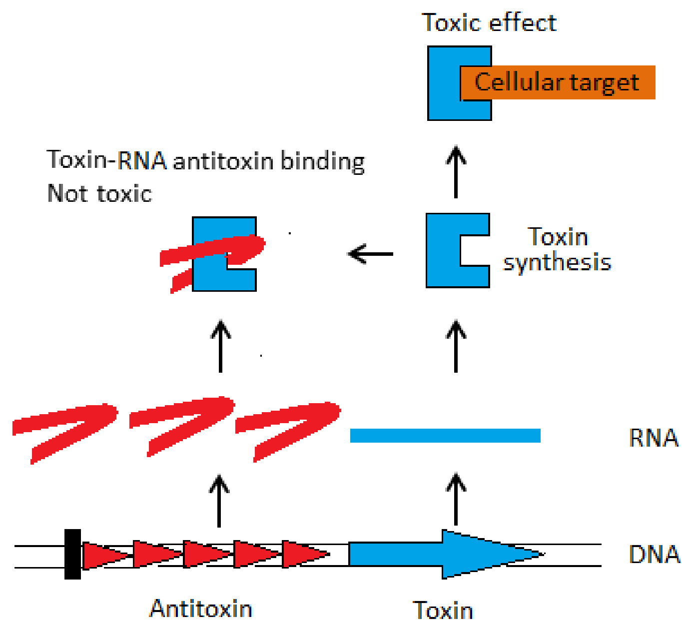

Figure 3. Regulatory mechanisms employed by type III toxin-antitoxin systems. RNA antitoxin directly neutralizes the protein toxin. Black rectangle denotes promoter.

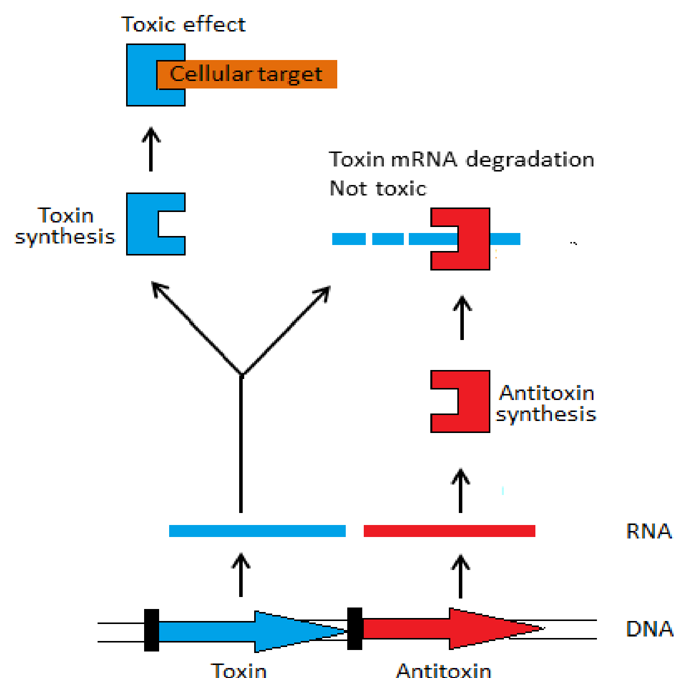

Figure 5. Regulatory mechanisms employed by type V toxin-antitoxin systems. Antitoxin directly cleaves the toxin-encoding mRNA. Black rectangles denote promoters.

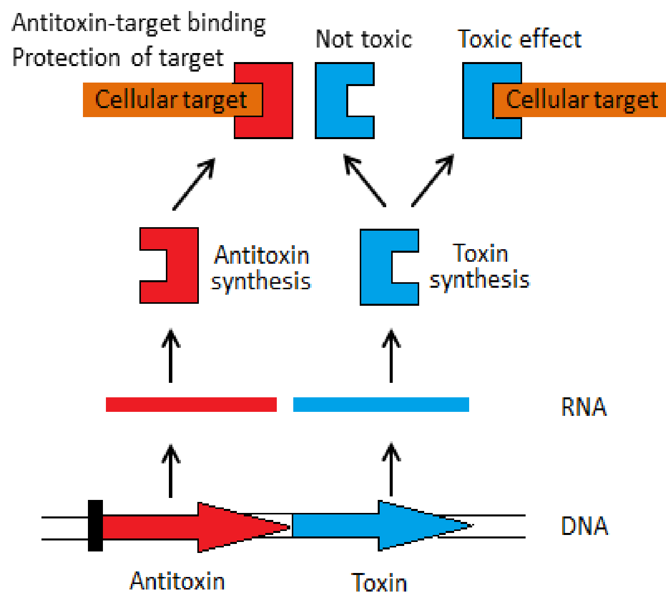

Figure 4. Regulatory mechanisms employed by type IV toxin-antitoxin systems. The antitoxin is a protein which neutralizes the toxin acting as an antagonist. Black rectangle denotes promoter.

Type IV toxin-antitoxin systems

In the type IV TA system, the antitoxin is a protein which neutralizes the toxicity of the toxin protein interacting with the target and protecting it from the action of the toxin (Masuda et al., 2012). Thus, in this case, the inhibition does not occur via direct binding between antitoxin and toxin (Figure 4).

The type IV was recently proposed based on the study of YeeU-YeeV (renamed CbeA- CbtA) TA system, from *Escherichia coli*. In this system, the toxin CbtA has been reported to inhibit the polymerization of bacterial cytoskeletal proteins, MreB and FtsZ. The antitoxin CbeA does not form a complex with CbtA but functions as an antagonist for CbtA toxicity (Masuda et al., 2012).

Type V toxin-antitoxin systems

Type V has a protein antitoxin, which cleaves the toxin-encoding mRNA (Figure 5) (Wang et al., 2012). This is another recent characterized TA system that was proposed based on the study of GhoT/GhoS system. The toxin GhoT is a membrane lytic peptide involved in ghost cell formation (lysed cells with damaged membranes) and tolerance to antibiotics exposition without undergoing genetic change (persistence). In this system, the monomeric form of antitoxin GhoS cleaves the GhoT-encoding mRNA in a specific site (rich in residues of uridine and adenosine) (Wang et al., 2012). Type II toxin-antitoxin MqsR/MqsA was implicated in the control of GhoT/GhoS system (Wang et al., 2013). This is the first evidence of a TA system regulated by another TA

system.

BIOTECHNOLOGICAL APPLICATIONS OF TA SYSTEMS

TA systems as new tools for molecular biology

The recombinant DNA technology and heterologous protein expression are the most common practices in molecular biology laboratories and TA systems have proven to be very useful in improving these key technologies. The company Delphi Genetics SA pioneered this field of biotechnological applications (Stieber et al., 2008).

One of the most common problems in DNA cloning is the low frequency of insertion of fragments into linearized vectors. Strategies based on TA systems can be used to solve this problem. One strategy is positive selection using plasmids containing a toxin gene. In this case, the insertion of the gene of interest destroys the toxin gene and allows the bacteria to growth. The principle of the Gateway system is other strategy in which site-specific recombination is used to insert a gene of interest into the vector and the recombinants are selected by the replacement of the toxin gene by the gene of interest. Another strategy is the StabyCloning™ system in which a truncated inactive version of the antitoxin gene is present in the linearized plasmid vector and the bacteria used contain the toxin gene in their chromosome. In this system, only cells containing a vector with an insert in the desired orientation can form colonies because the attachment of a 14 bp sequence to the 5´ end of the DNA fragment to be cloned, for instance by including it in the PCR primer, restores the active antitoxin, which is capable to counteract the toxin that has been introduced into the genome of the host cell (Unterholzner et al., 2013; Stieber et al., 2008).

Bacterial cells that had lost the expression plasmid during cell division can result in a population of bacteria lacking cloned gene. Antibiotic selection pressure must be maintained during the whole fermentation process in order to obtain high yields, solving the problem of plasmid instability in the use of bacteria for protein production. However several drawbacks are frequently associated with the use of antibiotics for this purpose such as high cost and special treatments. In this context, the use of TA system technologies allows stabilization of vector without necessity of antibiotics (Unterholzner et al., 2013; Stieber et al., 2008). Recently, new molecular platforms using TA system technology are being developed seeking more time-efficient and cost-effective (Mok and Li, 2013; Sevillano et al., 2013).

Pharmacological applications of TA systems

Since TA systems are able to repress growth or kill cells and are widely present in bacterial genomes, they are considered potential targets for the development of new antibacterial drugs (Engelberg-Kulka et al., 2004; Park et al., 2013). One strategy is the design of new ligands to promote artificial activation of the toxin compound. In this context, type II TA systems appears as good candidate, allowing several modes to perform the activation of TA module (Unterholzner et al., 2013): 1) Disruption of TA complexes; 2) prevention of complex formation; 3) activation of cellular proteases; 4) inhibition of TA transcription; 5) overexpression of the TA system and subsequent removal of the activating drug; 6) induction of plasmid loss.

Another strategy for the development of new antibacterial drugs is to design a specific inhibitor based on toxin-cellular target interaction. The mode as the toxin poisons the cell would be emulated by small molecules that could be developed as novel antibacterial agents. In this scenario, toxins that poison DNA gyrase, as CcdB and ParE, could be a useful start point for modelling the interaction of new ligands that inhibit this important target for antibacterial drug (Barbosa et al., 2012; Trovatti et al., 2008; Collin et al., 2011; Barbosa et al., 2010). Another potential use of TA systems may be in gene therapy. Recently, a retroviral vector containing the *E. coli* mazF gene showed a potential use of TA systems against viral infections (Chono et al., 2011).

CONCLUSIONS

TA systems are found on plasmids and chromosomes and are widely reported in bacteria and archaea. Although initially discovered on plasmids as mechanism of plasmid maintenance, TA systems have been implicated in several others events such as formation of persister cells, stress resistance, protection from bacteriophages and regulation of biofilm formation. These recent discoveries show the actual importance of TA systems in research. Thus, our knowledge about TA systems has greatly increased in the last years. Only in 2012, two novel types of TA systems were reported (Masuda et al., 2012; Wang et al., 2012). Additionally to the discovery of the various functions of TA modules, the biotechnological application of these systems is an attractive area to explore. TA systems are used to enhance cloning selection and protein expression in bacterial cells and novel platforms are being developed for this purpose. The promise of being new candidates for targets in the development of antibacterial drugs and their use in gene therapy are other attractive applications for research on TA modules.

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