CellPress

Trends in Microbiology

Opinion

Type VII Toxin/Antitoxin Classification System for Antitoxins that Enzymatically Neutralize Toxins

Xiaoxue Wang,[1,2,*] Jianyun Yao,[1,2] Yi-Cheng Sun,[3] and Thomas K. Wood [4,*]

Toxin/antitoxin (TA) systems are present in nearly all bacterial and archaeal strains and consist of a toxin that reduces growth and an antitoxin that masks toxin activity. Currently there are six primary classes for TA systems based on the nature of the antitoxin and the way that the antitoxin inactivates the toxin. Here we show that there now are at least three additional and distinct TA systems in which the antitoxin is an enzyme and the cognate toxin is the direct target of the antitoxin: Hha/TomB (antitoxin oxidizes Cys18 of the toxin), TglT/TakA (antitoxin phosphorylates Ser78 of the toxin), and HepT/MntA (antitoxin adds three AMPs to Tyr104 of the toxin). Thus, we suggest the type VII TA system should be used to designate those TA systems in which the enzyme antitoxin chemically modifies the toxin post-translationally to neutralize it. Defining the type VII TA system using this specific criterion will aid researchers in classifying newly discovered TA systems as well as refine the framework for recognizing the diverse biochemical functions in TA systems.

Highlights

The new classification type VII is for antitoxins that enzymatically modify toxins through transient interactions, rather than primarily through binding (cf. type II).

The improved classification system will simplify toxin/antitoxin (TA) classifications when new types of post-translational modification of toxins by antitoxins are found.

The new classification system reserves type V for antitoxins that enzymatically modify substrates other than the toxin.

Studying the neutralization mechanisms of antitoxins provides a valuable means to explore the conditions that lead to toxin activation.

Prediction of TA systems via bioinformatic searches will be more accurate using the conserved active enzymatic motifs in antitoxin components [e.g., GSX10DXD in minimal NTase (MNT)-domain proteins].

Current TA Classifications

Two basic features that nearly all **toxin/antitoxin (TA)** (see Glossary) systems share are that they consist of two neighboring genes and that one gene product is toxic while the other gene product masks its toxicity. Currently, TA systems are classified into six main categories [1]. All toxins are proteins, and the antitoxins are either noncoding RNAs or proteins. The antitoxins of type I and type III TA systems are noncoding RNAs. The antisense RNA antitoxin binds to the toxin mRNA to arrest translation of the toxin (type I), and the RNA antitoxin neutralizes the toxin protein directly via a protein–RNA interaction (type III). For the rest, the antitoxins are proteins but they differ in the way they block the toxicity of the toxin (Figure 1). The type II TA systems are the most extensively studied TA modules. The neutralization mechanism of the type II TA system relies on direct protein–protein interactions, and the antitoxin forms a TA complex with the toxin, resulting in its neutralization. The type IV TA system is characterized by the lack of direct interaction between the protein toxin and the protein antitoxin, both of which act on the same target. The type V TA system, GhoT/GhoS, is distinguished by the enzymatic activity of the antitoxin GhoS that cleaves specifically the toxin mRNA [2]. In the type VI TA system, SocB/SocA, the antitoxin SocA acts as a proteolytic adapter, promoting the degradation of the toxin protein [3]. In the following examples, we discuss the features of the three recently reported TA systems in which the antitoxin is an enzyme and modifies the cognate toxin to neutralize it, and we propose to categorize them as type VII TA systems.

Hha/TomB

The first TA system found in which the antitoxin is an enzyme which modifies the toxin protein directly is the Hha/TomB system of *Escherichia coli* [4] and *Yersinia enterocolitica* [5]. The

[1]Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Key Laboratory of Marine Materia Medica, Innovation Academy of South China Sea Ecology and Environmental Engineering, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 511458, China
[2]Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), Guangzhou 511458, China
[3]MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, and Center for Tuberculosis Research, Chinese Academy of Medical Sciences and

Peking Union Medical College, Beijing 100730, China
[4]Department of Chemical Engineering, Pennsylvania State University, University Park, PA 16802-4400, USA

*Correspondence:
xxwang@scsio.ac.cn (X. Wang) and
twood@engr.psu.edu (T.K. Wood).

genes encoding Hha/TomB were identified as active in biofilms [6], and toxin Hha reduces biofilm formation by decreasing fimbriae production [4]. Antitoxin TomB in this system transiently interacts with the toxin Hha to form a stable complex and inactivates Hha by oxidation of Hha Cys18 to SO_xH species (sulfenic, sulfinic, and sulfonic acid) [5]; this oxidation destabilizes Hha (Figure 2A). In effect, the Hha/TomB TA system acts as an oxygen sensor in that antitoxin TomB is active in the presence of oxygen; this concept is proposed to have relevance for biofilms so that cells in anoxic zones would have reduced growth due to Hha activity [5]. We previously proposed that Hha/TomB constitute a new type VII system [7,8].

TglT/TakA (MenT3/MenA3)

Another example of an antitoxin that functions as an enzyme is the Rv1045/Rv1044 TA system in *Mycobacterium tuberculosis*. Toxin Rv1045 shares homology with AbiEii that belongs to a **bacterial abortive infection (Abi)** system from *Streptococcus agalactiae*, and was predicted to be a type IV TA system as no interaction could be detected between the toxin and the antitoxin proteins [9]. Two recent studies nicely demonstrated the biochemical activity of the toxin and the antitoxin and identified a novel neutralization mechanism of this TA pair. In regard to the antitoxin activity, we demonstrated that Rv1044 (proposed to be renamed as TakA) functions as a novel

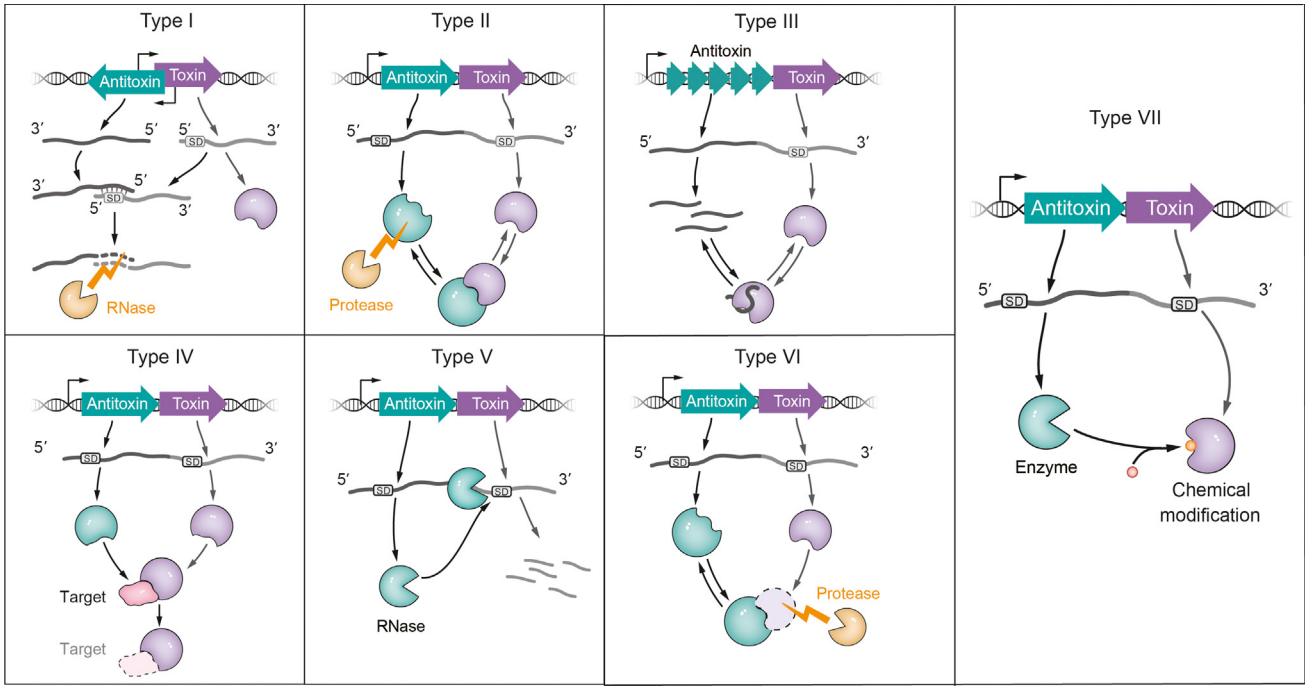

Figure 1. Summary of the Seven Different Types of Toxin/Antitoxin (TA) Systems. In the type I TA system, the antisense RNA antitoxin binds to the toxin mRNA to arrest translation of the toxin. In the type II TA system, the antitoxin protein neutralizes the toxin via a direct protein–protein interaction. In the type III TA system, the RNA antitoxin neutralizes the toxin protein via a direct protein–RNA interaction. In the type IV TA system, there is no direct interaction between the toxin and antitoxin; instead, the antitoxin counteracts the toxic effect of the toxin by interfering with the interaction of the toxin and its target. In the type V TA system, the protein antitoxin inhibits the toxin by cleaving its mRNA. In the type VI TA system, the binding of antitoxin to the toxin triggers toxin degradation. In the type VII TA system, the antitoxin neutralizes the toxin protein by chemically modifying the toxin post-translationally. Double-stranded DNA at the top shows the antitoxin gene, shown in green, and the toxin gene, shown in purple; arrowheads indicate the transcription direction, promoters are indicated by arrows with 90° right turns, and the RNA produced is indicated as a thick gray line at the 5′ and 3′ positions. SD indicates the Shine–Dalgarno region of the mRNA, and the proteins produced are indicated as purple notched spheres for toxins (all seven types produce protein toxins), green notched spheres for antitoxins (for protein antitoxins of the type II, IV, V, VI, and VII systems), and orange notched spheres for cellular proteases/RNases. The target of the type IV system is indicated by a pink cloud shape, and chemicals used to modify the toxin by the antitoxin are indicated as orange spheres.

atypical serine protein **kinase** that inactivates toxin Rv1045 (proposed to be renamed as TglT) by phosphorylating it at Ser78 [10] (Figure 2B). Furthermore, we found that TakA is a direct target of TglT and that there is a transient interaction between TglT and TakA [10], which makes it distinctive compared with other type IV TA systems. In regard to the toxin activity, Cai *et al*. demonstrated that toxin Rv1045 (named MenT3) functions as a tRNA **nucleotidyltransferase (NTase)** which added pyrimidines (C or U) to the 3′-CCA acceptor stems of uncharged tRNAs to prevent tRNA charging [11].

HepT/MntA

The two-gene module encoding the HEPN (higher eukaryotes and prokaryotes nucleotide-binding) domain protein and cognate MNT (minimal NTase) domain protein has been predicted to represent one of the most abundant TA systems in archaea and bacteria [12]. The HEPN domain protein as the toxin and the neighboring MNT domain protein as the antitoxin have been confirmed in the halophilic bacterium *Halorhodospira halophila* SL1 [13] and in the model psychrotrophic bacterium *Shewanella oneidensis* [14]. Our subsequent work demonstrated that the HEPN domain toxin functions as an RNase with a $RX_{4-6}H$ active **motif** and cleaves mRNA *in vitro* [14,15]. Recently, we discovered that the MntA antitoxin (MNT-domain protein) acts as an NTase and chemically modifies the HepT toxin (HEPN-domain protein) in both bacteria and archaea [16] (Figure 2C). In *in vitro* enzymatic assays, it was shown that three AMPs are transferred to HepT by MntA in a consecutive fashion with ATP serving as the substrate. More importantly, structural analysis revealed that the three AMPs were transferred to the Tyr104 next to the active RNase domain RX_4H in HepT and that **polyadenylylating** HepT by MntA is crucial for reducing HepT toxicity. We therefore proposed to classify the HepT/MntA TA system as a type VII TA system [16]. Furthermore, we found that the $GSX_{10}DXD$ motif of MntA is the key active motif for polyadenylylating and neutralizing HepT. Our bioinformatics analysis of HEPN/MNT modules in both bacteria and archaea revealed that HEPNs with the RX_4HXY motif, and neighboring MNTs with the $GSX_{10}DXD$ motif, represent a large family of type VII TA systems. The presence of these

Glossary

Abortive infection (Abi) system: systems that are activated by phage infection and limit viral replication by reducing cell metabolism, thereby providing protection to the bacterial population.
Kinase: an enzyme that catalyzes the transfer of a phosphate group from ATP to a specified molecule (e.g., protein phosphorylation).
Motif: a salient recurring feature.
Nucleotidyltransferase (NTase): an enzyme that adds nucleotides to substrates such as nucleic acids, proteins, and antibiotics.
Polyadenylylation: post-translational modification by the covalent addition of more than one AMP molecule to a hydroxyl side chain of a protein or RNA.
Toxin/antitoxin (TA): TA loci comprise two genes, one coding for a stable toxin whose overexpression reduces growth or causes growth stasis, and the other coding for an unstable (usually) antitoxin that counteracts the action of the toxin.

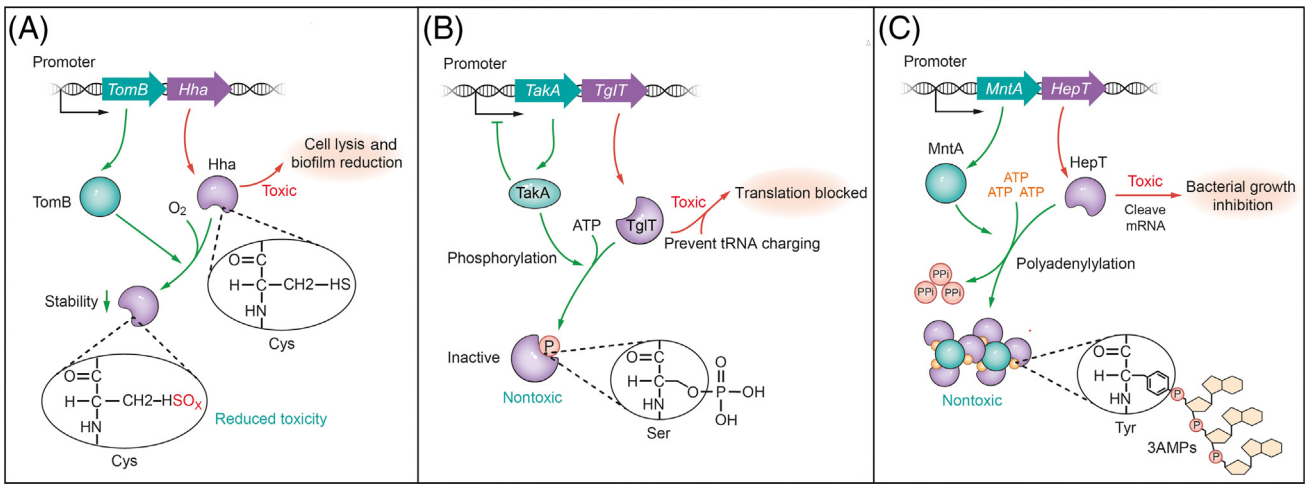

Figure 2. Summary of the Type VII Toxin/Antitoxin (TA) Systems in Which the Enzyme Antitoxin Chemically Modifies the Toxin to Neutralize It. (A) Toxin Hha causes cell lysis and reduces biofilm formation. Antitoxin TomB inactivates Hha by oxidation. The neutralized toxin Hha is shown with oxidized Cys18 after it is modified by TomB ('SOx' in red indicates sulfenic, sulfinic, or sulfonic acid). (B) Toxin TglT (MenT3 or Rv1045) blocks translation by preventing tRNA charging. Antitoxin TakA (MenA3 or Rv1044) inactivates TglT by phosphorylating the toxin with ATP serving as the substrate. The neutralized toxin TglT is shown with phosphorylated Ser78 after modification by TakA. (C) Toxin HepT inhibits cell growth by cleaving mRNAs. Antitoxin MntA neutralizes HepT by polyadenylylation with ATP serving as the substrate. Neutralized toxin HepT is shown with three AMPs added to Tyr104 after modification by MntA. Symbols as defined in the Figure 1 caption.

key active motifs also suggests that the adenylylation of the toxin by the antitoxin represents a common mechanism for these TA pairs.

Criteria for the Type VII TA System

Six different types of TA systems have been reported and are well recognized. With increasing identification of TA systems with novel features, it could be confusing for many researchers to classify these new TA pairs. One of the challenges is the lack of a general but specific antitoxin neutralization-mechanism-dependent criterion for classifying different types of TA systems. Therefore, using a more specific criterion that includes other TA systems in which the antitoxin is an enzyme that inactivates the toxin protein by chemically modifying the toxin will simplify the type VII classification system. In the proposed type VII TA system, the antitoxin functions as an enzyme, and the cognate toxin protein is a direct target of the enzymatic activity of the antitoxin. Critically, the enzymatic reaction results in the neutralization of the toxin. Using this enzyme-based criterion, type VII TA systems can be easily distinguished from other types of TA systems with protein antitoxins.

Notably, the type VII TA system is conceptually different from the type II TA system where the toxin is an enzyme, but the antitoxin binds the toxin. The typical type II antitoxin adopts a modular organization consisting of a DNA-binding domain and a protein-binding domain, but it lacks an enzymatic-activity domain. The neutralization mechanism of type II TA systems relies on a direct protein–protein interaction between the toxin protein and the toxin-binding domain of the antitoxin. For example, the binding of type II antitoxin MazE to toxin MazF induces a conformational change in MazF, thus blocking substate binding to the toxin [17,18]. Similarly, for the type II TA systems AtaT/AtaR and KacT/KacA, the binding of the antitoxin blocks the functional hotspots of the Gcn5-related *N*-acetyltransferase (GNAT) toxin and prevents the formation of an active toxin dimer which is needed to inhibit translation by acetylation of aminoacyl-tRNAs [19–21]. Therefore, a stable interaction between the toxin and antitoxin is critical for toxin neutralization in type II TA systems. Indeed, the typical type II antitoxin binds the toxin with high affinity (~ nM binding constants) to form a stable TA complex [22,23] that precludes antitoxin-specific degradation by proteases [8]. By contrast, for the type VII TAs Hha/TomB, TglT/TakA, and HepT/MntA, toxin neutralization relies on a direct post-translational modification of the toxin by the antitoxin, even though transient or weaker interactions may be observed between the toxin and antitoxin proteins in these TA pairs. Specifically, the polyadenylylated Tyr104 residue of the HepT toxin is next to the active RNase RX_4H domain of HepT in the HepT/MntA TA pair [16], and the phosphorylated serine residue of TglT toxin is also close to the active NTase domain of TglT in the TglT/TakA TA pair [10]. Reactivation of a toxin in a type VII TA system thus requires the reversal of the modification of the toxin by a separate enzyme (such as a phosphodiesterase to remove phosphates added by the antitoxin), while reactivation of a toxin in a type II TA system requires degradation of the antitoxin bound to the toxin by proteases (but this is not likely [8]). Therefore, there are major differences in the neutralization mechanisms for type II and type VII TA systems.

Similarly, for type III TA systems, the RNA antitoxin binds the toxin in a nontransient manner and does not chemically change the toxin. Moreover, for type IV TA systems, the toxin is not the direct target of the antitoxin and there is another cellular target upon which both toxin and antitoxin act, thus making the type IV system distinct from the new type VII TA system. Additionally, in the type V system, the antitoxin is an enzyme, but it does not interact with the toxin; instead, it acts on the toxin mRNA. The neutralization mechanism of the type VI TA system relies on the binding of the antitoxin to trigger the degradation of the toxin instead of chemically modifying it. Therefore, the type VII TA system, in which the toxin is directly modified post-translationally by the antitoxin enzyme , makes it distinct from the other six types of TA systems.

Table 1. Representative List of Antitoxins and Toxins from Different TA Systems That Share the Same Enzymatic Activity

Enzyme	*Enzymatic antitoxin*		*Enzymatic toxin*	
	Antitoxin	Target	Toxin	Target
RNase	GhoT (type V)	mRNA of toxin GhoS	Type II: MqsR, MazF, YafQ, ChpB	mRNA, tRNA, rRNA
NTase	MntA (type VII)	Y (RX_4HXY) of toxin HepT	Fic (type II), TglT (type VII)	DNA gyrase, Topo IV, tRNA
Kinase	TglA (type VII)	S (SXDXD) of toxin TglT	Type II: Doc, HipA	EF-Tu, GltX

Outstanding Questions

What physiological conditions activate type VII toxins (beyond increases in transcription during stress), and what are the physiological roles of these systems beyond links to phage inhibition for TglT/TgkA and links to biofilm formation for Hha/TomB?

Do type VII TA systems act as antiphage weapons?

Is the intracellular ATP level related to the activation of type VII toxins?

What is the origin of the type VII antitoxins; that is, how do they evolve from metabolic enzymes?

Do enzymes exist that will reverse the modifications of the inactivated type VII toxin to reactivate it?

Are there additional types of post-translational modification of toxins; that is, are there additional enzymatic activities to be discovered for type VII TA systems?

Concluding Remarks and Future Perspectives

Although the first report of TA systems was in the 1980s, for systems found in conjugative plasmids, the physiological functions of these small and ubiquitous modules are still controversial [24]. In recent years, many toxin and antitoxin components with novel biochemical functions and unique neutralization mechanisms have been discovered in various bacterial and archaeal strains [10,25,26]. Studying the neutralization mechanism of antitoxins in TA modules provides a valuable means to explore the conditions that lead to toxin activation. For example, inactivation of type VII toxins HepT and TglT by the antitoxins requires ATP as the substrate. Considering that the TglT/TgkA TA system belongs to the Abi system, and that the HEPN-domain-containing proteins are commonly found in phage defense systems [12], it is likely that the activation of toxins might be closely related to changing ATP levels during phage attack. Critically, phage inhibition is one of the few demonstrated physiological roles of TA systems [24,27,28].

Equally important, when TA systems are first identified via bioinformatics, the toxin component is usually identified as the one with enzymatic activity. However, as indicated in this perspective, increasing evidence shows that antitoxins can also function as enzymes (Table 1). Interestingly, several antitoxins share similar enzymatic activities with the toxins. For example, the type V antitoxin GhoS is an RNase [2] (albeit a highly specific one) and many type II toxins are RNases [29]. The type II toxin HipA is a kinase whose target is glutamyl-tRNA synthetase (GltX), and growth arrest by HipA requires the phosphorylation of the serine residue Ser239 of GltX [30,31]. The type VII antitoxin TglA also functions as a kinase, and it phosphorylates toxin TglT at residue Ser78 [10]. Toxins with NTases have been reported for FicT [32,33] and TglT, and, in particular, FicT toxin and MntA antitoxin both share a conserved active motif $GSX_{10}DXD$ in mediating adenylylating activities. Noticeably, MntA is the first NTase that can catalyze the transfer of three AMPs in a consecutive fashion [16]. These discoveries provide important insights about the origin and evolution of TA modules and suggest that toxins and antitoxins are interconvertible with a few amino acid changes, as we first demonstrated experimentally [34].

Since many different types of post-translational modification are present in prokaryotes, transferring other groups, such as methyl groups, acetyl groups, or even more complex polypeptide groups, by enzymatic antitoxins to their cognate toxins, is expected to be found in the future (see Outstanding Questions). Therefore, using this criterion for the type VII TA system will simplify TA classification and be helpful for other researchers to classify their TA systems in the future.

Acknowledgments

For X.W., this work was supported by the National Science Foundation of China (91951203, 31625001), by the National Key R&D Program of China (2018YFC1406500), and the Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong laboratory (Guangzhou) (GML2019ZD0407). For Y.S., this work was supported by the CAMS Initiative for Innovative Medicine (2016-I2M-1-013). For T.K.W., this work was supported by funds derived from the Biotechnology Endowed Professorship at the Pennsylvania State University.

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