Type II toxin–antitoxin system in bacteria: activation, function, and mode of action

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Abstract The toxin–antitoxin (TA) system is composed of a stable toxin and an unstable antitoxin that neutralizes the toxin. Being perhaps the most studied among the different TA types, type II TA systems are widely distributed and often exist in multiple copies within chromosomes of eubacteria and archaea. Exhibiting diverse molecular activities such as RNases, kinases, and acetyltransferases, type II TA systems have been confirmed to be involved in diverse biological processes including plasmid maintenance, phage inhibition, persistence, stress response, and biofilm formation. In this review, we summarize the current state of the research in the type II TA field, emphasizing the activation mechanism, structure–function relationship, and biological functions of type II TA systems.

Keywords Toxin, Antitoxin, Persister, Horizontal gene transfer

INTRODUCTION

Toxin–antitoxin (TA) systems are small genetical modules encoding two components: a stable toxin protein and its cognate labile antitoxin (Harms *et al.* 2018). They were firstly discovered in the 1980s as plasmid-borne “addiction modules” that played a role in plasmid maintenance (Gerdes *et al.* 1986). Subsequently, homologues of TA systems were also discovered in chromosomes of bacteria and archaea, involving in diverse physiological processes including phage abortive infection (Dy *et al.* 2014), persistence (Moyed and Bertrand 1983), biofilm formation (Wang *et al.* 2011), and virulence (Zhu *et al.* 2009). TA systems were generally divided into seven types based on the nature of the antitoxins and its mode of action on toxins (Fig. 1). Antitoxins of type I and type III TA systems are small

RNAs that neutralize their cognate toxins by either inhibiting translation of toxins (type I) or directly interfering with the toxin activities (type III). For the other types of TA systems, antitoxins are exclusive proteins but display different mechanisms of antagonizing the toxin activities. For type II TA systems, antitoxins can form stable complexes with the cognate toxins to inhibit the catalytic activities of the toxins. Antitoxins of type IV TA systems can disturb the activities of toxins by protecting their molecular targets. Three single instances of TA modules with regulatory principles different from type I–IV TA systems have been described as type V, type VI, and type VII TA modules, respectively (Marimon *et al.* 2016; Page and Peti 2016; Wang *et al.* 2019). The type V antitoxin GhoS specifically cleaves the mRNA of its cognate toxin GhoT to block its translation (Wang *et al.* 2012), while the type VI antitoxin SocA acts as an adaptor protein to direct the toxin SocB to proteolysis, leading to SocB inactivation (Aakre *et al.* 2013). The type VII antitoxin

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Fig. 1 Modes of toxin neutralization by cognate antitoxins in the four major types of TA systems (Type I–IV). Toxins and antitoxins are shown in *purple* and *green*, respectively. Genetic loci are shown in *colored arrows* and RNA transcripts are drawn as *curly lines*. **A** Type I antitoxins encode antisense RNAs pairing with the toxin mRNA and result in translation inhibition of the cognate toxins. **B** Type II antitoxins are proteins which form stable complexes with the cognate toxins, leading the inhibition of the toxin activities. **C** Type III antitoxins are RNAs (not antisense RNAs) which directly bind to cognate toxins to neutralize the toxicity. **D** Type IV antitoxins are proteins that bind to the toxin targets (rather than the toxins) to prevent toxic effects

TomB enhances the spontaneous oxidation of a conserved cysteine residue of its cognate toxin Hha, resulting in the destabilization and inactivation of Hha (Marimon *et al.* 2016).

Among the different TA types, type II TA systems are mostly studied and undergo frequent horizontal gene transfers (Ramisetty and Santhosh 2016; Rocker and Meinhardt 2016). They are widely distributed and often exist in multiple copies within chromosomes of eubacteria and archaea. Toxins of type II TA systems have diverse molecular activities such as RNases, kinases, and acetyltransferases and play important roles in plasmid maintenance, phage inhibition, persistence, stress response, and biofilm formation. In this review, we summarize the current state of the research in the type II TA field, emphasizing the activation mechanism, structure–function relationship, and biological functions of type II TA systems. Our current knowledge on type II TA systems is just a tip of the iceberg. More understanding are needed of the molecular mechanisms of

diverse type II TA systems which would facilitate their application in biotechnology industry and clinical settings.

DISTRIBUTION OF THE TYPE II TA SYSTEMS

Evolutionary analysis indicated that type II TA systems are prone to move between microbial genomes through horizontal gene transfer (HGT), which may account for the surprisingly wide distribution and great numbers of type II TA systems in chromosomes of archaea and eubacteria (Ramisetty and Santhosh 2016) (Table 1). The first genomic-scale survey of the type II TA system was performed by Pandey *et al.* in 2005 and demonstrated that although type II TA systems were abundant on bacterial chromosomes, the number and the location of TA loci varied greatly between unrelated bacteria. Of the 126 investigated prokaryotic genomes, a large fraction (~50%) had 1–5 TA loci whereas no TA loci

Table 1 Distribution of predicted type II TA systems in representative prokaryotic genomes

Strain	NCBI accession no.	Type II TA ^a pair number
<i>Bacillus subtilis</i>	NC_000964	8
<i>E. coli</i> plasmid R1	NG_034386	1
<i>E. coli</i> K-12 MG1655	NC_000913	31
<i>Mycobacterium bovis</i> AF2122/97	NC_002945	57
<i>Mycobacterium tuberculosis</i> H37Rv	NC_000962	76
<i>Nitrosomonas europaea</i> ATCC 19718	NC_004757	51
<i>S. Typhimurium</i> strain SL1344	NC_016810.1	27
<i>Staphylococcus aureus</i> N315	NC_002745	4
<i>Streptococcus pneumoniae</i> R6	NC_003098	6
<i>Pseudomonas aeruginosa</i> PAO1	NC_002516	4
<i>Pseudomonas fluorescens</i> Pf0-1	NC_007492	8
<i>Pseudomonas putida</i> KT2440	NC_002947	14
<i>Pseudomonas syringae</i> DC3000	NC_004578	15

^aThe type II TA systems were predicted by TAFinder tool (<https://bioinfo-mml.sjtu.edu.cn/TADB2/>)

were detected in ~25% of the organisms (Pandey and Gerdes 2005). A prediction algorithm RASTA-Bacteria was later developed by Sevin *et al.* to computationally identify the type II TAs and at least 65 TA systems were found in *Mycobacterium bovis* genome (Sevin and Barloy-Hubler 2007). Most recently, Xie *et al.* predicted type II TA systems out of 2786 completely sequenced prokaryotic genomes using the newly developed TA finder algorithm and showed that 66% of species harbored 1–20 type II TA loci in individual strains while 20% of species carried more than 20 loci (Xie *et al.* 2018). These results indicated that type II TA systems were not evenly distributed in prokaryotic genomes. However, no correlation was found between the number and nature of type II TA loci with the prokaryotic phylum or ecosystem (Sevin and Barloy-Hubler 2007).

ACTIVATION OF THE TYPE II TA SYSTEMS

In type II TA systems, the antitoxin genes are usually located in the upstream of the toxin genes, except for the *hipBA*, *higBA*, and *mqsRA* loci where the gene orders are exchanged. The general feature of the type II TA operon is that two genes are often separated by a short region (*e.g.*, *hipA/hipB*) or overlap (*e.g.*, *mqsR/mqsA*). The difference in gene order or intergenic spacing may affect the ratio of the toxin/antitoxin production rates, and thereby regulating the activity of the toxin (Harms *et al.* 2018). Despite the bactericidal or bacteriostatic effects of the toxin, the antitoxin is present at higher concentrations than the toxin under normal growth conditions, ensuring the bacterial growth is not affected by the toxin. Under the stressed conditions (*e.g.*, nutrient starvation, oxidative stress, antibiotic challenge), the antitoxins are more quickly degraded by protease

systems than the toxins (Chan *et al.* 2016) (Fig. 2). This is because, in many cases, antitoxins are intrinsically disordered proteins or have disordered terminal regions which make them more vulnerable to degradation by intracellular proteases than toxins (Cherny *et al.* 2005). Interestingly, in *Mycobacterium tuberculosis*, a SecB-like chaperone was found to be adjacent to the HigBA TA module and protect the antitoxin HigA from degradation and aggregation by interacting with its C-terminal fragment, which is known as a chaperone addiction sequence and makes the antitoxin aggregation-prone (Bordes *et al.* 2011, 2016; Guillet *et al.* 2019). Degradation of antitoxins by proteases finally leads to activation of toxins (Fig. 2), which inhibit the bacterial growth by targeting multiple physiological processes including DNA replication, gene transcription or protein translation (Chan *et al.* 2016).

As activation of the toxin depends on proteolytic degradation of the antitoxin, it is critical to understand the regulation mechanism of the antitoxin stability. Most of the well-characterized antitoxins were demonstrated to be degraded by the Lon protease (Muthuramalingam *et al.* 2016), which belongs to the AAA+ protease family that couples the ATP hydrolysis to drive substrate translocation and degradation. There were also reports showing that the two-component protease ClpP, cooperating with the ATP-dependent chaperones ClpA, ClpC or ClpX, carries out degradation of antitoxins in certain type II TA systems (Flynn *et al.* 2003; Loris and Garcia-Pino 2014; Page and Peti 2016; Smith *et al.* 1999; Wu *et al.* 1999). Moreover, multiple proteases can be involved in degradation of one antitoxin, implying that controlling intracellular stability of the antitoxin is a complex process (Flynn *et al.* 2003; Wu *et al.* 1999). Notably, for a newly identified TA module GraTA, with its toxin GraT targeting ribosome biogenesis (Tamman

Fig. 2 Activation pathways of the type II TA systems. Genetic loci and promoters of type II TA modules are shown in colored arrows. The antitoxins usually negatively regulate the TA promoter. Upon exposure to environmental stress, the intracellular proteases (e.g., Lon, ClpAP, ClpCP and ClpXP) are activated and degrade antitoxins, resulting in overexpression and release of toxins, which subsequently act on the cellular targets and cause cell growth arrest

et al. 2014), the antitoxin GraA is degraded by neither Lon nor Clp protease but an unidentified ATP-independent protease (Tamman *et al.* 2015). Oddly, an intrinsically disordered region was found in the toxin GraT rather than the antitoxin, raising an intriguing possibility of the toxin GraT being under proteolytic control (Talavera *et al.* 2019). This result significantly deviates from the current model that toxins are always more stable than antitoxins, suggesting alternative models should be considered when investigating proteolytic activation of novel families of type II TA systems.

Although antitoxins are generally considered to be intrinsically disordered proteins or possess unstructured terminal regions, there are still many characterized antitoxins forming rigid and stable structures in the absence of their cognate toxins (e.g., MqsA and GraA) (Brown *et al.* 2011; Talavera *et al.* 2019). Questions remain how these antitoxins are specifically recognized and degraded by the Lon or ClpP family proteases under stressed conditions. One possible mechanism ensuring the specific degradation of antitoxins is through adaptor proteins that target the substrates for degradation by interacting simultaneously with substrates and proteases (Kirstein *et al.* 2009). In *Staphylococcus aureus*,

three antitoxins of Axe1, Axe2 and MazE were delivered by the adaptor protein TrfA to the ClpCP protease for degradation (Donegan *et al.* 2010, 2014). Recently, an adaptor protein for the Lon protease, named HspQ, was characterized to enhance the Lon substrate selectivity (Puri and Karzai 2017), but it was not clear whether this adaptor protein could mediate the Lon-dependent degradation of antitoxins. Another important question still awaiting an answer is whether degradation of antitoxins under different stress conditions is dependent on the elevated expression or allosteric activation of the proteases.

TYPE II TOXINS: CLASSIFICATION AND MODE OF ACTION

Based on structural similarities, the currently characterized toxins of type II TA systems can be classified into nine superfamilies, namely ParE/RelE, MazF, HicA, VapC, HipA, FicT/Doc, AtaT/TacT, Zeta and MbcT (Table 2). The RelE superfamily toxins adopt the RelE-like structural fold (Fig. 3A, CATH ID: 3.30.2310.20 and 3.30.2310.40) which is composed of three α -helices and

Table 2 Nine superfamilies of type II toxins: structural features and action mechanisms

Toxin superfamily	Toxin	Molecular activities	Structural features (CATH ID)
ParE/RelE	ParE	DNA gyrase inhibition	3.30.2310.20; 3.30.2310.40
	RelE	Ribosome-dependent mRNA cleavage	
MazF	MazF	Ribosome-dependent mRNA cleavage	2.30.30.110
	CcdB	DNA gyrase inhibition	
HicA	HicA	Ribosome-dependent mRNA cleavage	3.30.920.30
VapC	VapC	Anticodon region of initiator tRNA cleavage	3.40.50.1010
HipA	HipA	Phosphorylation of GltX	N/A
FicT/Doc	FicT	TopoIV adenylation and DNA gyrase	N/A
	Doc	Phosphorylation of EF-Tu elongation factor	
AtaT/TacT	AtaT	Acetylation of initiator tRNA	N/A
	TacT	Acetylation of elongation tRNA	
Zeta	Zeta	Phosphorylation of UDP-Glc-NAC	3.40.50.30
MbcT	MbcT	Phosphorylation of NAD ⁺	N/A

a five-strand β -sheet (Takagi *et al.* 2005). Although sharing similar structures, the molecular functions of the RelE superfamily (RelE, ParE, HicA, MqsA, YafQ, YafO, *etc.*) are heavily diverged. The MazF superfamily toxins share a core structure consisting of a seven-stranded, twisted antiparallel β -sheet surrounded by three α -helices (Fig. 3B, CATH ID: 2.30.30.110) (Kamada *et al.* 2003). Members of the MazF superfamily include MazF and CcdB but their mode of action can also be very different. The HicA superfamily forms a dsRNA-binding domain-like fold (Fig. 3C, CATH ID: 3.30.920.30) composed of a triple-stranded β -sheet, with two helices packed against one face (Butt *et al.* 2014). Members of the HicA superfamily mediate ribosome-independent mRNA cleavage, which results in inhibition of bacterial growth (Turnbull and Gerdes 2017). The VapC superfamily generally exhibits a nuclease activity that leads to growth arrest by cleaving tRNA^{fMet} in the anticodon region and contains a typical PIN domain structure (Fig. 3D, CATH ID: 3.40.50.1010) consisting of a small and central five-stranded β -sheet (β 1– β 5) surrounded by seven α -helices (Dienemann *et al.* 2011). The HipA superfamily has a structure similar to human CDK2/cyclin A kinase (Fig. 3E), consisting of an N-terminal α/β domain and a C-terminal all α -helical domain (Schumacher *et al.* 2009). HipA possesses the Ser/Thr kinase activity and phosphorylates the glutamyl-tRNA-synthetase GltX *in vivo* to promote antibiotic persistence (Germain *et al.* 2013; Kaspy *et al.* 2013). The FicT/Doc toxins adopt the Fic domains that are defined by their conserved active-site motif [HXFX(D/E)(A/G)N(G/K)R] consisting of six α -helices with helices α 3 and α 4 supporting the catalytic loop

(Fig. 3F) (Stanger *et al.* 2016). The currently characterized members of FicT/Doc toxins can function as either adenylation enzymes that add AMP moieties to target proteins (FicT) or kinases that phosphorylate other proteins (Doc) (Castro-Roa *et al.* 2013; Harms *et al.* 2015). Interestingly, FicT toxins can also be linked to effectors of secretion systems as a Fic-domain-containing protein VbhT which was recently identified to be fused with a type IV secretion signal (T4SS) domain BID, and secreted into competing bacterial cells as a bactericidal effector protein (Engel *et al.* 2012; Harms *et al.* 2017). The AtaT toxins act as acetyltransferases and adopt a structural fold comprising a central seven-stranded β -sheet bounded by four α -helices (Fig. 3G), with the α 3 helix containing the (Q/R-x-x-G-x-G/A) signature motif (Jurenas *et al.* 2019). The Zeta toxins function as a UDP-N-acetylglucosamine kinases to inhibit cell wall biosynthesis and exhibit an α/β structure (Fig. 3H, CATH ID: 3.40.50.30), with a six β -stranded central β -sheet surrounded by several α -helices (Meinhart *et al.* 2003). Most recently, type II toxins with ADP-ribosyltransferase activities were characterized (Freire *et al.* 2019; Piscotta *et al.* 2019; Skjærning *et al.* 2019) and the representative toxin MbcT exhibits a β -sandwich fold formed by six β -strands flanked by nine α -helices (Fig. 3I) and triggers bacterial cell death by depleting the intracellular NAD⁺ pool.

Notably, toxins from the same superfamily can have low sequence similarities and exhibit great divergence in their molecular activities. For example, within the ParE/RelE superfamily, although ParE superfamily shares a similar fold with the RelE superfamily, the ParE superfamily is a gyrase poison (Jiang *et al.* 2002) that

Fig. 3 Reprehensive structural folds of the nine superfamilies of the type II toxins. **A** The structure of the RelE toxin (PDB ID: 3BPQ) from *Methanococcus jannaschii* (Francuski and Saenger 2009). **B** The structure of the MazF (PDB ID: 4MDX) toxin from *Bacillus subtilis*. **C** The structure of the HicA toxin (PDB ID: 4C26) from *Burkholderia pseudomallei* (Butt *et al.* 2014). **D** The structure of the VapC toxin (PDB ID: 5ECW) from *Shigella flexneri* (Xu *et al.* 2016). **E** The structure of the HipA toxin (PDB ID: 3FBR) from *E. coli* (Schumacher *et al.* 2009). **F** The structure of the FicT toxin (PDB ID: 5JFF) from *E. coli* (Stanger *et al.* 2016). **G** The structure of the AtaT toxin (PDB ID: 6GTP) from *E. coli* (Jurenas *et al.* 2019). **H** The structure of zeta toxin from *Streptococcus pyogenes* (PDB ID: 1GVN) (Meinhart *et al.* 2003). **I** The structure of MbcT toxin from *Mycobacterium tuberculosis* (PDB ID: 6FKG) (Freire *et al.* 2019)

leads to the accumulation of DNA breaks whereas the RelE superfamily exhibits ribosome-dependent mRNA endonuclease activity (Pedersen *et al.* 2003). Functional divergence was also observed within the AtaT/TacT superfamily. While the AtaT toxin inhibits translation initiation by acetylating the amino acid moiety of the

initiator tRNA^{Met} (Jurenas *et al.* 2019), the TacT toxin inhibits translation elongation by targeting aminoacylated elongation tRNA (Cheverton *et al.* 2016). Most recently, an acetyltransferase toxin ItaT that belongs to the AtaT/TacT superfamily was shown to specifically and exclusively acetylate Ile-tRNA^{Ile} thereby blocking

translation and inhibiting cell growth (Wilcox *et al.* 2018). Moreover, toxins from the CcdB/MazF superfamily also display distinct activities and targets. The MazF toxins are ribosome-independent mRNA endoribonucleases (Zhang *et al.* 2003), whereas the CcdB toxins function as DNA gyrase inhibitors (Dao-Thi *et al.* 2005). For the FicT/Doc superfamily, while the FicT superfamily toxins adenylylate DNA gyrase and/or topoisomerase IV at their ATP-binding sites (Harms *et al.* 2015; Lu *et al.* 2016), the Doc toxins function as protein kinases to phosphorylate the translation elongation factor EF-Tu (Castro-Roa *et al.* 2013). Since the biological functions of TA systems are closely related to the target selection and specificity of toxins, it would be possible to unveil novel biological roles of type II TA systems if new targets or molecular activities could be identified and characterized for certain toxins within the type II toxin superfamilies.

Although conventional type II toxins are known as small proteins with a single domain, bioinformatics analysis also revealed elongated type II toxins-like proteins containing multiple domains in the bacterial genomes. Two instances of these types of toxins were characterized, either having negative effects on the cell growth or disturbing the peptidoglycan synthesis. For example, the toxin protein PhoH2 consisted of an N-terminal PIN domain with RNase activity and a C-terminal RNA helicase domain (Andrews and Arcus 2015). It was proposed that the coupled RNA helicase domain could unwind RNA and facilitate the RNA cleavage by the PIN domain in a sequence specific manner. Another example is the EzeT multi-domain toxin encoded in genomes of many different bacterial species. EzeT contains a C-terminal domain homologous to the Zeta toxins and an N-terminal *cis*-acting antitoxin domain. The C-terminal domain of EzeT catalyzes the phosphorylation UDP-N-acetylglucosamine, leading to cell lysis upon overexpression while the N-terminal domain strongly attenuates kinase activity and keeps EzeT in an autoinhibited state (Rocker and Meinhardt 2015). Although activation mechanism of EzeT has not been demonstrated by experiments, it was hypothesized partial unfolding of the N-terminal domain mediated by certain chaperones or other protein factors could possibly lead to EzeT activation. These data imply that the organization and architecture of type II TA systems are much more complicated than previous thought and many novel functionalities of type II TA systems are worth deeper investigation.

TYPE II ANTITOXINS: CLASSIFICATION AND MODE OF ACTION

Most of the type II antitoxins have an N-terminal toxin-neutralizing domain and a C-terminal DNA-binding domain. Based on sequence similarities, type II antitoxins have been classified into 20 superfamilies (Leplae *et al.* 2011). However, the structures of the DNA-binding domains of the type II antitoxins generally fall in only four different categories, namely helix-turn-helix (HTH), ribbon-helix helix (RHH), YefM/Phd-type, and VapB-type (Fig. 4). The HTH-motif is a classical DNA-binding module that can be found in many prokaryotic transcription regulators and it usually binds to the major groove of DNA via the second α -helix (Wintjens and Rooman 1996). Examples of HTH-motif containing antitoxins include PezA (Khoo *et al.* 2007), HigA (Schureck *et al.* 2014) and MqsA (Brown *et al.* 2011). The RHH-motif has the topology of β -strand- α -helix- α -helix and the β -strands mediate dimerization by forming an antiparallel β -sheet, which recognizes specific DNA sequences (Schreiter and Drennan 2007). The RHH-motif contained proteins are ubiquitous in prokaryotic transcription factors that involve in diverse physiological processes. The antitoxins containing RHH-motifs include CcdA (Madl *et al.* 2006), ParD (Oberer *et al.* 2007), RelB (Boggild *et al.* 2012), DinJ (Liang *et al.* 2014) and FitA (Mattison *et al.* 2006). The YefM/Phd-type domain has three β -strands and two α -helices, with the three β -strands mediating dimerization by forming a six-stranded antiparallel β -sheet (Garcia-Pino *et al.* 2016). The bottom surface of the YefM/Phd-type domain has a patch of positive charged residues involved in DNA recognition. Currently, the YefM/Phd-type domains are only found in the YefM and Phd antitoxins. The VapB-type domain dimerizes to form a so-called layered swapped-hairpin β -barrel structure (Bendtsen *et al.* 2017a), with the first β -hairpins from each antitoxin binding to the DNA major groove. Antitoxins containing the VapB-type domains include VapB (Bendtsen *et al.* 2017b; Mate *et al.* 2012), MazE (Bobay *et al.* 2005; Kamada *et al.* 2003) and Kis (Kamphuis *et al.* 2007).

Although antitoxins specifically interact with their cognate toxins, toxins and antitoxins from different superfamilies can associate to form novel TA pairs (Guglielmini and van Melderren, 2011). For example, the Phd antitoxin homologues can neutralize toxins from at least three superfamilies of toxins including Fic/Doc, RelE/ParE and VapC (Arbing *et al.* 2010). Toxins from the RelE/ParE superfamily can also associate with RelB, Phd, HigA and even antitoxins that do not belong to any well-characterized antitoxin superfamilies (Guglielmini

Fig. 4 Protein-DNA recognition of representative antitoxins. **A** The structure of the antitoxin MqsA in complex with its cognate operator sequence (PDB ID: 3O9X) from *E. coli* (Brown *et al.* 2011). **B** The structure of the antitoxin CcdA in complex with its operator sequence (PDB ID: 2H3C) from *E. coli* (Madl *et al.* 2006). **C** The crystal structure of the antitoxin Phd in complex with the DNA sequence (PDB ID: 4ZM0) from *Escherichia* phage P1 (Garcia-Pino *et al.* 2016). **D** The crystal structure of the antitoxin VapB in complex with the DNA sequence (PDB ID: 5L6L) from *Caulobacter crescentus* (Bendtsen *et al.* 2017a)

and van Melder *et al.* 2011). This “mix and match” phenomenon, which was useful to develop a “guilt by association” algorithm to predict novel toxins associated with known antitoxins and vice versa (Sevin and Barloy-Hubler 2007), suggests that classification of toxins and antitoxins should be considered independently.

In general, the type II antitoxins antagonize the activities of their cognate toxins by blocking or masking the toxin active sites (Blower *et al.* 2011; Bøggild *et al.* 2012; Schureck *et al.* 2014). For example, the Zeta and PezT toxins are inactivated due to the steric hindering of the ATP/GTP binding sites by their cognate antitoxins such as epsilon (Meinhart *et al.* 2003) and PezA (Khoo *et al.* 2007). The MazE wraps around the MazF toxin to occlude the enzymatic active site, leading to neutralization of MazF toxic effect in the cell. In the case of the RelE toxin, binding of the RelB antitoxin leads to displacement of a C-terminal α -helix essential for the RelE activity (Li *et al.* 2009). However, some antitoxins do not act upon their cognate toxins by blocking the active

sites. The HipB antitoxin allosterically regulates the activity of the HipA toxin by locking the toxin in an inactive conformation (Schumacher *et al.* 2009). For the MqsR/MqsA and HigB/HigA TA systems, the corresponding antitoxins bind to sites that are distant from the toxin active sites. It was suggested that both the MqsA and HigA antitoxins neutralize the toxin activities by competing its binding to ribosome or RNA (Brown *et al.* 2009; Schureck *et al.* 2014), but the precise mechanism needs further confirming or investigation.

BIOLOGICAL FUNCTIONS OF TYPE II TA SYSTEMS

The plasmid-borne TA systems are generally thought to stabilize the plasmids through a mechanism called post-segregational killing (PSK), which leads to death of the daughter cells that do not inherit the parental plasmids. This is because antitoxins were degraded much faster than toxins so that the plasmid-free daughter cells could

not survive the poisoning of the intracellular toxins. For example, Hok/Sok of plasmid R1, CcdA/CcdB of plasmid F, and ParD/ParE of plasmid RK2 were well-characterized TA systems involved in PSK in *E. coli* (Harms *et al.* 2018). For the chromosome-borne TA systems, it is believed that they have broader impacts to the host cells, especially participating in stress responses when living in unfavorable circumstances. For instance, TA systems can help the host bacteria to resist phage infection through the mechanism known as phage abortive infection, which leads to the activation of toxins by antitoxin degradation in the phage-infected cells. Two type II TA systems, namely MazE/MazF and RnlA/RnlB, have been shown to significantly block infection of phage P1 and phage T4, respectively (Alawneh *et al.* 2016; Koga *et al.* 2011).

Another stress response phenomenon mediated by TA systems is persister formation upon antibiotic challenges, as observed in *E. coli* and other bacteria. Persister cells are a small portion of cells showing dormant and multidrug-tolerant characteristics when exposed to antibiotics (Lewis 2010). The link between persister formation and type II TA systems was first established in 1983 when Moyed and Bertrand isolated a *hipA7* mutant, which bears two substitutions in the HipA toxin (G22S and D291S) and exhibits increased persistence by 1000–10,000-fold versus the wildtype strain in *E. coli* K-12 upon ampicillin treatment (Moyed and Bertrand 1983). HipA is a serine or threonine kinase that phosphorylates the aminoacyl-tRNA-Glu (tRNA^{Glu}) synthetase GltX, leading to the accumulation of uncharged tRNA^{Glu}. The RelA protein was then activated, triggering the stringent response with increased levels of the (p)ppGpp alarmone (Germain *et al.* 2013; Kaspy *et al.* 2013). Stringent response had been demonstrated to be involved in persister formation as mutants devoid of the (p)ppGpp alarmone has significantly decreased persistence upon antibiotic exposure (Germain *et al.* 2015). Therefore, HipA may stimulate persister formation via triggering the stringent response. Recently, HipA was shown to phosphorylate additional targets such as ribosomal proteins, which could also contribute to persister formation. However, the HipA7 toxin mutant was found to exclusively phosphorylate GltX, suggesting targeting tRNA^{Glu} synthetase alone is sufficient to stimulate high proportions of persisters (Semanjski *et al.* 2018).

Besides the HipAB TA system, other type II TA systems were also shown to be related to bacterial persistence. Deletion of the *mqsRA* (Kim and Wood 2010; Luidalepp *et al.* 2011) or *yafQ* (Harrison *et al.* 2009) has been shown to reduce the survival of *E. coli* under antibiotic exposure, suggesting these TA loci contribute

to bacterial persistence. Overexpression of RelE or MazF toxins can drastically promote persister formation of *E. coli*, suggesting these toxins are involved in persistence in conditions when their cognate antitoxins are degraded (Keren *et al.* 2004; Tripathi *et al.* 2014). Moreover, spontaneous mutations to antitoxins YafN or VapB were demonstrated to result in extended lag phase and higher persistence, implying that activation of their cognate toxins YafO or VapC could play important roles in persister formation in the population (Levin-Reisman *et al.* 2017).

One of the most cited pieces of evidence supporting the role of type II TA modules in persister formation was published by Gerdes group, showing the decreased persistence of Δ TA10 *E. coli* strain with ten type II toxins as endoribonucleases or toxins/antitoxins (Δ mazF, Δ chpB, Δ relBE, Δ dinJ/yafQ, Δ yefM/yoeB, Δ higBA, Δ prfF/yhaV, Δ yafNO, Δ mqsRA, Δ hicAB) deleted (Maisonneuve *et al.* 2013). However, this result was recently challenged by Ramisetty *et al.*, who independently constructed the Δ TA10 *E. coli* strain and demonstrated that this mutant did not show significant differences in persistence with chloramphenicol, erythromycin, and kanamycin, compared with the wildtype strain (Ramisetty *et al.* 2016). Melderer group further constructed a new Δ TA10 *E. coli* strain and confirmed again that there is no direct link between induction of the ten TA systems and persistence to antibiotics, and the authors also suggested that proper and defined experimental conditions are of major importance when performing persistence assays (Goormaghtigh *et al.* 2018). These results provide strong evidence that the ten endoribonuclease TA modules do not play an essential role in spontaneous persister formation, however, due to the great numbers and diverse molecular activities of type II TA systems in the bacterial genomes, the role of other type II TA modules played in bacterial persistence needs re-evaluation.

CONCLUSION

As exemplified above, type II TA systems are widespread and involve in a broad range of physiological activities such as post-segregational killing, phage abortive infection, and persistence. After significant efforts of the scientific community, the molecular features and activities of many TA systems were characterized and reported in detail. Nevertheless, the biological function of a large proportion of type II TA systems remains mysterious. Intriguingly, several non-classical toxins, which have multi-domains or fusion domains, increase the complexity of the type II TA

systems. Moreover, the extent of functional divergence between the homologous type II TA systems in different bacteria is still elusive. Investigations of the predicted type II TA systems in diverse prokaryotic genomes are likely to have unanticipated findings as novel functionalities and regulatory rewiring can be developed through horizontal gene transfer, which could add to the complexity of type II TA systems.

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Compliance with Ethical Standards

Conflict of interest Si-Ping Zhang, Qian Wang, Shuo-Wei Quan, Xiao-Quan Yu, Yong Wang, Ding-Ding Guo, Liang Peng, Hu-Yuan Feng, and Yong-Xing He declare that they have no conflicts of interest.

Human and animal rights and informed consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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