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 genetic 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
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 Meinhart 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

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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.
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

| Strain                   | NCBI accession no. | Type II TA* pair number |
|-------------------------|--------------------|-------------------------|
| *Bacillus subtilis*     | NC_000964          | 8                       |
| *E. coli* plasmid R1    | NC_034386          | 1                       |
| *E. coli* K-12 MG1655   | NC_000913          | 31                      |
| *Mycobacterium bovis* AF2122/97 | NC_002945 | 57                      |
| *Mycobacterium tuberculosis* H37Rv | NC_000962 | 76                      |
| *Nitrosomonas europaea* ATCC 19718 | NC_004757 | 51                      |
| *S. Typhimurium strain* SL1344 | NC_016810.1 | 27                      |
| *Staphylococcus aureus* N315 | NC_002745 | 4                       |
| *Streptococcus pneumoniae* R6 | NC_003098 | 6                       |
| *Pseudomonas aeruginosa* PA01 | NC_002516 | 4                       |
| *Pseudomonas fluorescens* Pf0-1 | NC_007492 | 8                       |
| *Pseudomonas putida* KT2440 | NC_002947 | 14                      |
| *Pseudomonas syringae* DC3000 | NC_004578 | 15                      |

*The type II TA systems were predicted by TAfinder tool (https://bioinfo-mml.sjtu.edu.cn/TADB2/)*
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
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 tRNAfMet in the anticodon region and contains a typical PIN domain structure (Fig. 3D, CATH ID: 3.40.50.1010) (Kamada et al. 2005). Although members of the MazF superfamily exhibit great divergence, they share a similar fold with the RelE superfamily, the ParE superfamily (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 α3 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-acetylgalactosamine 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; Skjerning 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

### 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 | 3.30.30.110 |
| MazF              | MazF  | Ribosome-dependent mRNA cleavage | 3.30.920.30 |
|                   | CcdB  | DNA gyrase inhibition | 3.30.2310.20; 3.30.2310.40   |
| 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 | N/A |
| AtaT/TacT         | AtaT  | Acetylation of initiator tRNA | N/A |
|                   | TacT  | Acetylation of elongation tRNA | N/A |
| Zeta              | Zeta  | Phosphorylation of UDP-Glc-Nac | 3.40.50.30 |
| MbcT              | MbcT  | Phosphorylation of NAD+ | N/A |
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<sub>Met</sub> (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<sub>Ile</sub> 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 Meinhart 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. 2006; 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 Melderden, 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 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.

**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).

**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 *hipA*7 mutant, which bears two substitutions in the HipA toxin (*G22S* and *D291S*) and exhibits increased persistence by 100–10,000-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-translationale Gln (tRNA$^{\text{Glu}}$) synthetase GltX, leading to the accumulation of uncharged tRNA$^{\text{Glu}}$. The RelA protein was then activated, triggering the stringent response with increased levels of the (p)ppGpp alarmon (Germain *et al.* 2013; Kasp y *et al.* 2013). Stringent response had been demonstrated to be involved in persister formation as mutants devoid of the (p)ppGpp alarmon 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 tRNA$^{\text{Glu}}$ synthetase alone is sufficient to stimulate high proportions of persisters (Semanjksi *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 ΔTA10 *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*prlF/yhaV*, Δ*yafNO*, Δ*mqsRA*, Δ*hicAB*) deleted (Maisonuneve *et al.* 2013). However, this result was recently challenged by Ramisetty *et al.*, who independently constructed the ΔTA10 *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). Melder group further constructed a new ΔTA10 *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
Type II toxin-antitoxin systems in bacteria

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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Type II toxin-antitoxin systems in bacteria

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June 2020 | Volume 6 | Issues 2–3

79 | June 2020 | Volume 6 | Issues 2–3