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Bacterial type II toxin-antitoxin systems acting through post-translational modifications

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ABSTRACT

The post-translational modification (PTM) serves as an important molecular switch mechanism to modulate diverse biological functions in response to specific cues. Though more commonly found in eukaryotic cells, many PTMs have been identified and characterized in bacteria over the past decade, highlighting the importance of PTMs in regulating bacterial physiology. Several bacterial PTM enzymes have been characterized to function as the toxin component of type II TA systems, which consist of a toxin that inhibits cell growth and an antitoxin that protects the cell from poisoning by the toxin. While TA systems can be classified into seven types based on nature of the antitoxin and its activity, type II TA systems are perhaps the most studied among the different TA types and widely distributed in eubacteria and archaea. The type II toxins possessing PTM activities typically modify various cellular targets mostly associated with protein translation and DNA replication. This review mainly focuses on the enzymatic activities, target specificities, antitoxin neutralizing mechanisms of the different families of PTM toxins. We also proposed that TA systems can be conceptually viewed as molecular switches where the 'on' and 'off' state of the system is tightly controlled by antitoxins and discussed the perspective on toxins having other physiologically roles apart from growth inhibition by acting on the nonessential cellular targets.

Contents

1. Introduction

Post-translational modifications (PTMs) are an important strategy for living organisms to increase the functional diversity of pro-

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teins, enabling the fulfillment of complex tasks. More than 300 PTMs have been reported and shown to influence diverse aspects of proteins including localization, stability, conformation and binding properties [1,2]. PTMs include the addition of chemical groups (e.g. phosphorylation, acetylation, hydroxylation, methylation, glycosylation, AMPylation and lipidation), the covalent linkage of polypeptides (e.g. ubiquitination), the cleavage of the peptide bond between two amino acids (e.g. proteolysis), or the modification of residues with specifc side chain (e.g. deamidation) [1,3,4]. In general, PTMs provide a means to control protein activities in response to specific cues, thereby playing an important role in regulating signaling pathways and gene expression among eukaryotes and prokaryotes [4]. Although it is generally thought that PTMs are more common in eukaryotic cells, many PTMs have been identified and characterized in bacteria cells over the past decade, thus highlighting the importance of PTMs in regulating bacterial physiology. Presently, phosphorylation [5], acetylation [6], carboxylation [7], methylation [8], proteolysis [9], lipidation [10], adenylation [11] and deamidation [12] make up the majority of characterized PTMs in bacteria.

Toxin-antitoxin (TA) systems are bacterial gene modules that consist of a toxin that has a deleterious effect on cells caused by the disruption of essential biological processes and an antitoxin (protein or non-coding RNA) that alleviates the toxic effect of the toxin [13]. TA systems can be encoded on plasmids or bacterial genomes and are mainly involved in post-segregational killing [14], phage abortive infection [15] and persistence [16]. Seven different types of TA systems have been identified based on the activity of the antitoxins and interaction modes between toxins and antitoxins [17–19]. The focus of this review will be type II TA systems, which have drawn much attention due to their wide distribution and diverse modes of action. For type II TA systems, neutralization of the toxin is achieved through the formation of a stable antitoxin-toxin protein complex. RNases make up a large proportion of type II toxins that impede protein translation through the cleavage of mRNAs, rRNAs or tRNAs [20]. However, a number of enzymes carrying out PTM modifications such as phosphorylation [21–25], AMPylation [26–28], ADP-ribosylation [29–31] and acetylation [32–38] were also found to function as type II toxins (Table 1). Here, we focus on these TA systems and discuss modes of action for the PTM toxins, the molecular mechanism of neutralization of toxins by its cognate antitoxin, and their functional diversity in different bacterial species.

Table 1
Examples of PTM enzymes functioning as type II TA systems.

Toxin superfamily	Toxin (organism)	Molecular activities	References
HipA	HipA (*E. coli* K12) HipT (*E. coli* O127: H6)	Phosphorylation of GltX Phosphorylation of TrpS	[21,2224]
FicT/Doc	Doc (*E. coli*) FicT (*P. aeruginosa*, *E. coli* and *Yersinia enterocolitica*) Fic-1 (*P. fluorescens* 2P24) VbhT (*Bartonella schoenbuchensis*)	Phosphorylation of EF-Tu elongation factor DNA-gyrase and TopoIV adenylylation AMPylation of DNA gyrase GyrB Functions as a T4SS effector	[25272826]
ARTs	ParT (*Sphingobium* sp. YBL2) MbcT (*M. tuberculosis*) DarT (*M. tuberculosis*)	ADP-Ribosylation of Prs Catalyzes NAD^+ degradation ADP-Ribosylation of DNA	[656629,30]
GNAT	TacT (*S.* Typhimurium) AtaT (*E. coli* O157: H7) AtaT2 (*E. coli* O157: H7) GmvT (*Shigella flexneri*) KacT (*K. pneumoniae*) ItaT (*E. coli* HS)	Acetylation of elongator tRNAs Acetylation of initiator tRNA ($tRNA^{fMet}$) Acetylation of the aminoacyl moiety of gly-$tRNA^{Gly}$ Acetyl-CoA-dependent inhibition of translation Possible acetylation of tRNA Acetylation of isoleucyl-$tRNA^{Ile}$	[323336383734]

2. Protein kinases as type II toxins

Protein kinases catalyze phosphorylation of substrate proteins and play important roles in modulating cellular processes ranging from signaling transduction to energy metabolism [39,40]. Phosphorylation mostly occurs on Ser, Thr, and Tyr residues in eukaryotes [41]. The enzymes that transfer phosphor groups to these residues, namely Ser/Thr and Tyr kinases, belong to a large protein superfamily, the members of which typically exhibit catalytic core structure comprised of two lobes. The N-terminal lobe is primarily involved in ATP binding while the C-terminal lobe is larger and responsible for substrate binding and transferring of the ATP derived phosphate group. Although members of the Ser/Thr and Tyr kinase superfamily can share little sequence homology, the catalytic core of the protein kinases is well conserved, including nearly 12 invariant residues that are involved in achieving the correct position of the ATP molecule and protein substrate [42].

In bacteria, phosphorylation was previously thought to only occur on histidine and aspartic acid residues, as observed in two-component signaling systems (TCS) [43]. The isocitrate dehydrogenase (IDH) from *E. coli* was the first bacterial protein characterized to have Ser/Thr phosphorylation sites and numerous Ser/Thr protein kinases have been identified in many different bacterial species ever since [44]. These protein kinases participate in diverse bacterial physiological processes including central metabolism, cell division and differentiation, cell wall synthesis, secondary metabolism, virulence, and antibiotic resistance [45]. Among these, HipA from *E. coli* was the first characterized bacterial Ser/Thr kinase that constituted a toxin of the type II TA system [46].

The gene encoding HipA (high persister gene A) was the first gene identified to be linked with bacterial persistence and therefore, was the focus of considerable research efforts [16,21,24,46–48]. HipA in *E. coli* is composed of 440 residues and phosphorylates the glutamyl-tRNA synthetase (GltX). The conserved Ser239 residue is phosphorylated by HipA thus inhibiting the aminoacylation activity of GltX, preventing it from transferring glutamate to $tRNA^{Glu}$. Therefore, more uncharged $tRNA^{Glu}$ occupies the ribosomal A site which stimulates the ribosome-associated (p)ppGpp (guanosine tetra- and pentaphosphate) synthease RelA, triggering the stringent response and inducing persistence [21,22]. The antitoxin gene *hipB* is located upstream of the *hipA* toxin gene and together form a type II TA module (Fig. 1A). The antitoxin HipB forms a stable protein complex, but the interface of HipA/HipB is perplexingly far from the HipA active site [48]. The crystal structures of HipA/HipB-DNA complex revealed binding of HipB promote the HipA dimerization via its N-terminal domain, resulting in the occlusion of HipA's active-sites (Fig. 2A) [49]. The HipA/HipB-DNA complex also confirmed that the HipA/HipB complex could act as a repressor that autoregulates transcription of the *hipBA* operon through binding to the promoter region. Additionally, autophosphorylation of HipA residue Ser150 provides another layer of regulation over HipA activation. Structural studies indicate

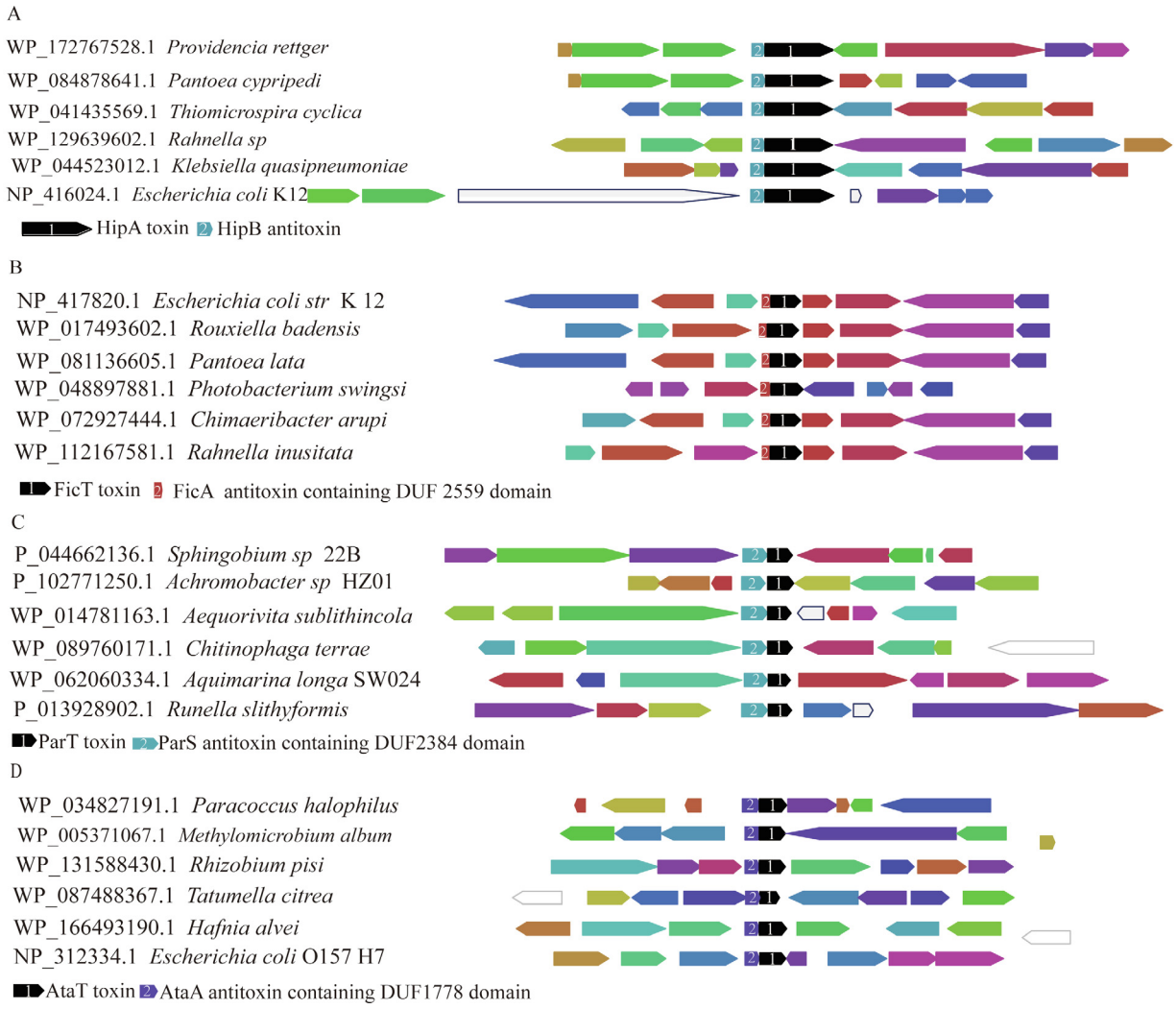

Fig. 1. Gene neighborhood conservation of HipA/HipB (A), FicT/FicA (B), ParT/ParS (C), and AtaT/AtaA (D) TA systems from different bacterial species. The accession numbers of protein sequences were retrieved from the RefSeq database (https://www.ncbi.nlm.nih.gov/) through a BlastP search using toxin sequences as the query, and used as input for the webFlaGs server [80,81] to generate the gene neighborhood. The toxins genes are shown in black and the antitoxin genes are numbered.

that Ser150 is located in the ATP-binding "Gly-rich loop", which displays an "in-out" conformational equilibrium. Phosphorylated Ser150 shifts the Gly-rich loop to the "out conformation", which disrupts the ATP-binding pocket and inactivates the kinase activity [48]. Since overexpression of HipA is linked with persistence, negative regulation, or inactivation of HipA, kinase activity could be essential for the dormant cells to become active again.

In uropathogenic *E. coli* isolates, a gain-of-function allele named *hipA7* (G22S and D291A) showed a 1000-fold increase in persistence [46,50]. Additionally, *hipA* (D88N) and *hipA* (P86L), which were isolated from either patients with urinary tract infections or laboratory screens, also resulted in a high persistence phenotype [49]. Notably, most HipA mutations that led to increased persistence mapped to its N-terminal lobe, which is responsible for dimerization of HipA and HipB. Formation of HipA dimers blocks the active sites and inactivates HipA. Therefore, the G22S, P86L, and D88N mutations in HipA could disturb HipA dimerization and activate HipA, leading to higher persistence [49]. Most recently, it was demonstrated that when HipA7 and HipA were moderately overexpressed from plasmids, HipA7 only phosphorylates GltX while the wild-type HipA phosphorylates several additional substrates involved in translation and replication, such as ribosomal protein L11 (RplK) and the negative modulator of replication initiation SeqA [23]. This suggests HipA targets a pool of cellular substrates, the phosphorylation of which is likely to be responsible for growth inhibition. When overexpressed, HipA7 was found to have reduced kinase activity compared to HipA, consistent with its less toxic phenotype. However, when endogenously expressed from the chromosome, HipA was neutralized by the HipB, whereas the HipA7 activity was not completely inhibited by HipB due to the impaired dimerization interface, leading to the phosphorylation of GltX and much greater persistence [23].

Recently, a HipA homologue named HipT was characterized in *E. coli* O127 [24]. HipT comprises 335 residues and exhibits sequence similarity (25% sequence identity over 193 residues) with the C-terminal part of HipA. Upstream from *hipT* is a *hipB* homologue (107 residues), followed by a gene named *hipS* (103 residues). The HipS protein shares sequence homology (27% sequence identity over 98 residues) with the N-terminal part of HipA that is missing from HipT. Through a gene library screening it was revealed that HipT phosphorylates tryptophanyl-tRNA synthetase (TrpS) rather than GltX at S197 and S199, which is within the Gly-rich loop motif highly similar with that of GltX. Phosphorylation of TrpS could potentially prevent $tRNA^{Trp}$ from charging,

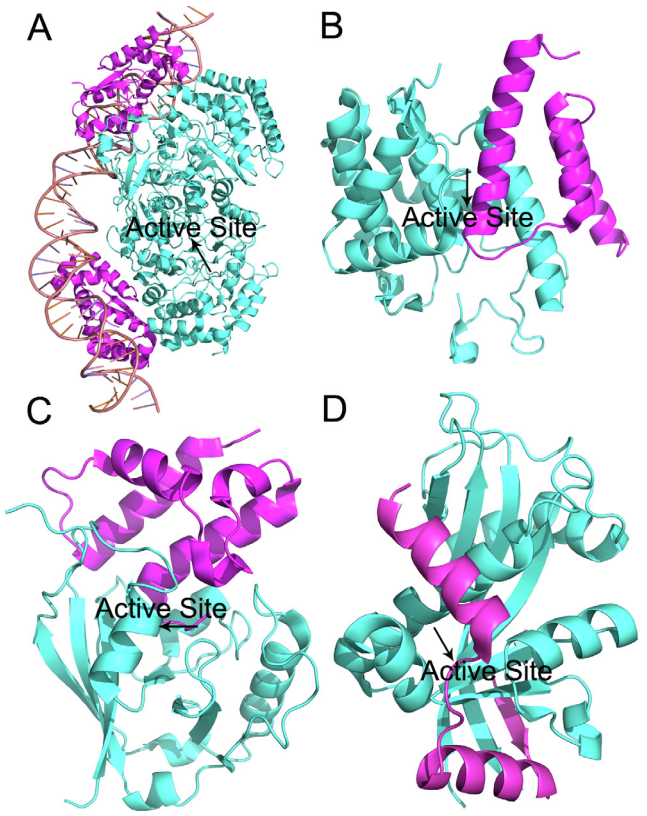

Fig. 2. Reprehensive toxin/antitoxin complex structures of the four type II TA systems using PTM enzymes as toxins. The toxins are colored in cyan, antitoxins are colored in magenta, and the active sites of toxins are marked with arrows. A The structure of HipA/HipB-DNA complex (PDB ID: 4YG7) from *E. coli* [49]. B The structure of the FicT/FicA complex (PDB ID: 5JFF) from *E. coli* [55]. C The structure of the ParT/ParS complex (PDB ID: 6D0H) from *Sphingobium sp* [65]. D The structure of the AtaT/AtaA complex (PDB ID: 6GTQ) from *E. coli* [72]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which is corroborated by the notion that ectopic overexpression of HipT stimulates (p)ppGpp accumulation in *E. coli* O127. Oddly, the HipB homologue alone does not neutralize the activity of HipT whereas the HipS protein exhibiting sequence similarity with the N-terminal domain of HipA is sufficient to inhibit the HipT toxicity [24]. However, HipB can augment the ability of HipS to neutralize HipT, suggesting the toxin-antagonizing mechanism of HipBST could be profoundly different from that of HipBA.

In addition to canonical protein kinases acting as Type II toxins, there is currently a single case of a type II TA system in which the Doc toxin is a non-canonical protein kinase belonging to the Fic protein superfamily [25]. Fic proteins are ubiquitous in all kingdoms of life and usually catalyze AMPylation of target proteins [26]. Although Doc exhibits a structural similarity to the Fic superfamily, it acts as a novel type of protein kinase and phosphorylates the conserved threonine 382 of EF-Tu, the translation elongation factor, due to a slight alteration in the catalytic motif. Interestingly, the phosphorylation of EF-Tu can be reverted in the presence of ADP or GDP by the Doc toxin [25]. In contrast, its cognate antitoxin PhD does not dephosphorylate the target EF-Tu, but rather occupies the NTP binding site on Doc to inhibit its phosphorylation activity [25,51]. Phosphorylated EF-Tu can no longer bind aminoacylated tRNAs and this causes halting of the translation process, but it has not been tested if overexpression of the Doc toxin promotes bacterial persistence.

3. Ampylation enzymes as type II toxins

In contrast to toxins that exhibit kinase activity, there are a number of type II toxins that possess the canonical signature motif HxFx (D/E) GNGRxxR associated with AMPylation, *i.e.*, the covalent addition of an adenosine 5′-monophosphate (AMP) onto a target protein [52]. The antitoxins are located upstream these toxin-encoding genes, containing a conserved DUF2559 protein domain (Fig. 1B). In *Pseudomonas aeruginosa*, *E. coli,* and *Yersinia enterocolitica*, the FIC-domain-containing FicT toxins adenylylate the DNA gyrase and topoisomerase IV at their ATP-binding sites. Modifications of these two enzymes block ATPase activity, cause rapid halting of DNA replication, and consequently, lead to bacterial growth inhibition [27]. Lu et al. independently demonstrated that a FicT homologue named Fic-1 in *Pseudomonas fluorescens* 2P24 strain catalyzed AMPylation of the DNA gyrase GyrB at Tyr109 and inhibited DNA replication and bacterial growth [28]. Interestingly, FicT homologues were shown to induce cell filamentation, a growth characteristic commonly linked with inactivation of GyrB [28]. Since induced bacterial filamentation typically allows cells to subvert predation [53], it is possible that induction of FicT toxins by biotic or abiotic factors may help bacteria evade phagocytosis by host immune cells or predation by protozoa and rotifers in natural environments.

The AMPylation ability of Fic proteins is usually controlled by a conserved mechanism, in which an inhibitory α-helix with a consensus sequence of (S/T) XXXEG blocks the ATP binding site and inactivates the AMPylation activity [54]. For the FicT toxin, the cognate antitoxin FicA harbors the α-helix motif that uses an invariable glutamate residue to compete with ATP γ-phosphate binding [52]. For the FicT toxin, the cognate antitoxin FicA harbors the α-helix motif that uses an invariable glutamate residue to compete with ATP γ-phosphate binding (Fig. 2B) [55]. Comparative sequence analysis has revealed that many Fic proteins contain the α-helix motif fused to either the N- terminus or the C- terminus and disruption of the interface between the inhibitory α-helix and Fic proteins releases the AMPylation activities and leads to substantial toxicity and bacterial filamentation [52]. Therefore, despite the diverse domain organizations of Fic proteins, *i.e.*, the inhibitory α-helix motif could be either within the Fic protein themselves or on a separate antitoxin yet the regulatory mechanism of the AMPylation activities has been evolutionary conserved.

Aside from the Fic proteins as type II toxins modulating bacterial physiology, several Fic proteins were found to be associated with bacterial virulence and function as effector proteins delivered into host cells by secretion systems. For example, the effector of type III secretion system (T3SS), named VopS from *Vibrio parahaemolyticus* [56], AMPylates the small GTPases of the Rho family, such as RhoA, Rac and Cdc42 [57], thereby inhibiting actin assembly in infected cells. It appears that Fic proteins involved in bacterial virulence are constitutively active and act without antitoxins neutralizing their toxicity. Interestingly, the recently identified FicT/FicA-family toxin-antitoxin module VbhT/VbhA from *Bartonella schoenbuchensis* was shown to be associated with a T4SS functioning as a classical conjugation system [26]. Unlike the classic FicT toxins, the VbhT toxin is a fusion protein composed of an N-terminal FicT-like FIC domain and a C-terminal BID (Bep intracellular delivery) domain which serves as the type IV secretion signal. It was demonstrated that VbhT is an interbacterial effector protein delivered into recipient cells by the T4SS [26]. Although the biological function of VbhT as a T4SS effector is currently unknown, the VbhT/VbhA module provides an important missing evolutionary link between TA systems and effector of secretion systems, suggesting that toxins of TA systems may be recurrently acquired as secreted effectors or virulence factors, expanding the

effector arsenal and facilitating bacterial infection of a broader range of hosts or interspecies competition.

4. ADP-ribosylation enzymes as type II toxins

ADP-ribosylation is a ubiquitous PTM that transfers the ADP-ribose (ADPr) moiety of nicotinamide adenine dinucleotide (NAD^+) to a substrate protein and releases nicotinamide [58,59]. The ADPr moiety can be transferred onto protein side-chains with a nucleophilic oxygen, nitrogen, or sulfur, resulting in ADP-ribosylation modifications on the serine, threonine, cysteine, histidine, arginine, and lysine residues [60]. In bacterial pathogens, ADP-ribosyltransferases (ARTs) typically mediate host-pathogen interactions, promoting pathogenesis, intracellular replication of pathogens, and modulating the host immune response [31]. These are exemplified by the diphtheria toxin of *Corynebacterium diphtheriae* and cholera toxin of *Vibrio cholerae*. These two toxins have three key residues at the active sites, featuring the conserved H-Y-E and R-S-E residues for diphtheria toxin and cholera toxin, respectively [61]. The diphtheria toxin catalyzes ADP-ribosylation of the eukaryotic translation elongation factor 2 (eEF2) at histidine 715, which is a modified histidine referred to as a diphthamide residue that is critical for protein synthesis [62]. Conversely, the cholera toxin modifies an arginine residue on the A subunit of G-proteins, thereby inducing cytotoxicity by continuously activating G-proteins [63]. It appears that ART enzymes show great sequence diversity, which explains the distinct substrate preferences of different sub-class ART enzymes.

Recently, the RES domain-DUF2384 TA family was found to be widely distributed in diverse bacterial genomes (Fig. 1C). Crystal structure of the RES-domain containing toxin from *Pseudomonas putida* revealed that it shares high structural similarity to the catalytic domain of diphtheria toxin. Subsequent investigation indicated this toxin inhibited the bacterial cell growth by rapidly degrading NAD^+ [64]. In *Sphingobium* sp. YBL2, a RES-domain containing toxin named ParT, was identified a mono-ADP-ribosyltransferase (mART) that specifically modifies phosphoribosyl pyrophosphate synthetase (Prs), a vital enzyme involved in nucleotide biosynthesis [65]. The antitoxin ParS inserts its C-terminal carboxyl group into the active site of ParT, possibly leading to the blockage of the ParT active site (Fig. 2C) [65]. Consistently, while overexpression of the ParT toxin exerts a bacteriostatic effect in *E. coli*, co-expression of the ParS antitoxin restores the normal growth phenotype, confirming that ParT/ParS constitutes a *bona fide* toxin-antitoxin [65].

In the human pathogen *Mycobacterium tuberculosis*, a TA module belonging to the RES domain-DUF2384 TA family was also identified [66]. The toxin named MbcT was characterized to be an NAD^+ phosphorylase that catalyzes NAD^+ degradation in the presence of inorganic phosphate, triggering rapid cell death of *M. tuberculosis*. The antitoxin MbcA binds to MbcT and neutralizes its toxicity by sterically blocking the active site of the toxin using the C-terminus of MbcA, an inhibition mechanism conceptionally similar with that of ParS neutralizing ParT [66]. However, it is currently unknown whether MbcT modifies protein substrates. Interestingly, in *M. tuberculosis* there is an additional characterized ART as the type II toxins named DarT, which shares the similar structural folds with the 2′-phosphotransferase and specifically catalyzes ADP-ribosylation of thymidines on single-stranded DNA in a sequence-specific manner, inducing the bacterial SOS response and inhibiting DNA replication [29]. DarT does not target protein substrates and its cognate antitoxin DarG not only binds and inhibits the DarT toxin, but also acts on the targets of DarT by de-ADP-ribosylation as well [30]. The precise mechanism on how DarG neutralizes the toxicity of DarT is currently unclear and warrants further investigation.

5. N-acetyltransferases As type II toxins

Acetylation is an important PTM that is widespread among prokaryotes and eukaryotes [67]. The GCN5-related *N*-acetyltransferases (GNAT) catalyze the transfer of an acetyl group from acetyl-CoA to diverse substrates, from small molecules such as aminoglycoside antibiotics to macromolecules [67]. Recently, novel type II TA systems were discovered in certain bacterial species with the toxins adopting the GNAT-fold and their cognate antitoxins exhibiting a ribbon-helix-helix (RHH) fold belonging to the DUF1778 protein family (Fig. 1D) [68,69]. To date, the molecular mechanism of action was studied extensively for four of these GNAT-toxins, including AtaT and AtaT2 from *E. coli* O157:H7 [33,36] and TacT from *Salmonella enterica* serovar Typhimurium [32] and ItaT from *E. coli* HS [34], all of which target aminoacyl-tRNAs rather than protein substrates.

TacT was the first reported GNAT toxin that targeted aminoacyl-tRNAs [32]. It catalyzes acetylation of the free amine group of charged elongator tRNAs, which was postulated to impede the interaction between tRNAs and EF-Tu, therefore resulting in translation inhibition [32]. Compared with TacT, the AtaT toxin showed more stringent substrate specificity by targeting only the methionine-charged initiator tRNA (Met-$tRNA^{fMet}$) [33]. It seems that AtaT is also capable of discriminating between initiator and elongator tRNAs, but the structural basis for this specificity is currently not clear. Acetylation of Met-$tRNA^{fMet}$ blocks its interaction with initiation factor 2 (IF2), thus impairing the assembly of the 30S initiation complex and preventing translation initiation [33]. AtaT2, which is a paralogue of AtaT, specifically targets glycine-charged glycyl-tRNA instead, inducing ribosome stalling at all four glycyl codons [36]. Moreover, the ItaT toxin from the *E. coli* HS strain, also showed rather different substrate specificity. It exclusively acetylates Ile-$tRNA^{Ile}$, leading to translation halting and cell growth inhibition [34]. Since all these four characterized GNAT toxins target different aminoacyl tRNAs and GNAT toxins generally show high sequence diversity among different bacterial species, it is tempting to speculate that various GNAT toxins may target other types of aminoacyl-tRNAs or even protein components of protein translation machinery.

The AtaT/AtaR TA system was used as a model to illustrate the molecular mechanism of the neutralization of the GNAT toxins and their cognate antitoxins [70,71]. Crystal structure of AtaT/AtaR revealed that AtaR binds and neutralizes AtaT via its intrinsically disordered C-terminal region (Fig. 2D) [72]. This region impedes binding of Met-$tRNA^{fMet}$ and prevents the alignment of acetyl-CoA to a catalytically active orientation [70]. Moreover, increased stoichiometry of AtaR could further disrupt the AtaT dimerization interface, which is required for binding of Met-$tRNA^{fMet}$, thus providing an additional layer of regulation [71]. Since the C-terminal region is conserved in most antitoxins of GNAT toxins, it is possible that this neutralization mechanism of GNAT toxins is well-preserved in evolution.

6. TA systems as molecular switches having diverse physiological roles?

Being more common in eukaryotes, PTMs are generally thought to serve as a molecular switch mechanism, which modulates broad cellular biological functions in response to specific cues [73]. The

enzymes that carry out PTM modifications are usually tightly regulated since untimely or continuous activation of PTM enzymes can be toxic or detrimental to cells [73]. In this review, we discussed four types of PTM enzymes that act as type II toxins, and whose toxicity is tightly regulated by their cognate antitoxins. Intriguingly, the neutralizing mechanism of the FicA antitoxin against FicT toxin is essentially analogous to the eukaryotic Fic proteins, in which an inhibitory α-helix fused to either the N-terminal or C-terminal of the Fic protein blocks the ATP binding site and inactivates AMPylation activity [54]. For the HipA toxins, the HipB antitoxin binds HipA at sites far away from the active site, but locks HipA in an inactive conformation [48]. This is similar with the allosteric regulation of eukaryotic protein kinases such as Src and Abl [74,75]. Therefore, it seems that that TA systems can be conceptually viewed as molecular switches controlling bacterial growth states, which comprise the 'on' and 'off' states and are tightly controlled by antitoxins [76]. The signals triggering these switches are generally thought to be environmental stresses, which activate cellular proteases such as Lon and ClpP, and lead to degradation of antitoxins [77]. However, it is currently unknown if acting on the molecular targets by toxins could have other physiologically roles apart from growth inhibition. This perhaps requires the toxins to evolve to be less harmful to their host bacterium and target other molecular pathways not essential for the bacterial survival. Interestingly, in *Pseudomonas putida,* the GraT toxin showed unusually mild toxicity compared to other conventional toxins [78] whereas in *E. coli* the expression of the mutant *hipA7* allele which promoted high-persistence was less toxic compared to the wild-type *hipA* and caused only a mild growth delay [79]. Nonetheless, currently no evidence shows that toxins could control bacterial phenotypes unrelated to growth or survival by acting on nonessential cellular targets. It is therefore tempting to characterize more TA systems to reveal their physiological roles and better understand their functional diversity.

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CRediT authorship contribution statement

Si-Ping Zhang: Conceptualization, Data curation, Formal analysis, Validation, Writing-original draft. **Han-Zhong Feng:** Conceptualization, Data curation, Formal analysis, Software, Validation. **Qian Wang:** Data curation, Formal analysis, Software, Validation. **Megan L. Kempher:** Investigation, Methodology.Shuo-Wei Quan: Conceptualization, Formal analysis, Software, Validation. **Xuanyu Tao:** Conceptualization, Data curation, Software, Validation. **Shaomin Niu:** Data curation, Formal analysis, Software, Validation. Yong Wang: Conceptualization, Data curation, Formal analysis, Validation. **Hu-Yuan Feng:** Conceptualization, Data curation, Formal analysis, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **Yong-Xing He:** Conceptualization, Data curation, Formal analysis, Validation, Project administration, Supervision, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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