Wake me when it's over – Bacterial toxin–antitoxin proteins and induced dormancy

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Abstract

Toxin–antitoxin systems are encoded by bacteria and archaea to enable an immediate response to environmental stresses, including antibiotics and the host immune response. During normal conditions, the antitoxin components prevent toxins from interfering with metabolism and arresting growth; however, toxin activation enables microbes to remain dormant through unfavorable conditions that might continue over millions of years. Intense investigations have revealed a multitude of mechanisms for both regulation and activation of toxin–antitoxin systems, which are abundant in pathogenic microorganisms. This minireview provides an overview of the current knowledge regarding type II toxin–antitoxin systems along with their clinical and environmental implications.

Keywords: Bacteria, biomedical, microbiology, protein–protein interactions, proteolysis, enzymes

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Introduction

Toxin–antitoxin (TA) modules are bacterial operons that are part of the mobilome, moving from one organism to another via horizontal gene transfer,[1,2] and these loci are important for facilitating a state of dormancy in bacteria under stressful conditions. Multiple TA loci have been found in hundreds of sequenced bacterial genomes to date, including clinical isolates of *Mycobacterium tuberculosis*, *Pseudomonas* spp., methicillin-resistant *Staphylococcus aureus*, uropathogenic *Escherichia coli*, *Vibrio cholerae*, and *Yersinia pestis*.[3–6] These systems consist of a co-transcribed gene pair which encodes both a toxin that acts within the bacterium itself to arrest growth, and an antitoxin that interferes with the toxin activity. Five families of TA modules have been identified to date and are characterized by the nature of the associated antitoxins. Type I systems consist of an antisense RNA antitoxin that binds to the toxin mRNA, preventing ribosome binding and likely targeting the resulting RNA duplex for degradation by RNase III.[7] Type II TA modules encode a protein antitoxin and toxin that form a tight non-toxic complex upon translation.[8,9] Type III systems consist of a protein toxin bound by an RNA pseudoknot antitoxin formed from a tandem array of repeats.[10–12] Type IV TA systems are characterized by a protein antitoxin that does not bind the toxin, but instead interferes with the binding between the toxin and its target.[13,14] One representative of a type V toxin–antitoxin system has been described, in which an antitoxin protein specifically cleaves the mRNA of the toxin.[15] In this minireview, we will focus on the type II TA systems, which have been the most extensively studied among the five families.

Toxin–antitoxin loci were first identified on plasmids and thought to be merely addiction systems that ensured plasmid stability.[16] However, with advances in DNA sequencing came the ability to sequence and analyze numerous bacterial chromosomes, and investigators discovered that these gene pairs were nearly ubiquitous and found in the genomes of archaea, as well as Gram-negative and Gram-positive bacteria, often in multiple copies. Because bacterial chromosomes had no need for an addiction system, the principle of parsimony suggested that maintaining these operons must confer some sort of benefit to the organism. Although researchers were not certain why these modules were so highly conserved, the evidence strongly favored the notion that the maintenance of TA loci in bacterial genomes supported important biological functions. It was an exciting time for scientists, as the number of reports of new and heretofore unknown types of TA systems (and TA-like genes) started to increase rapidly.

The first goal was to determine the mechanisms of these modules, which would suggest how the systems might benefit the bacterial host. These early investigations were initially performed *in vitro*, because it was much easier to control conditions and there were numerous validated

protocols and reagents available. However, it was imperative to eventually employ *in vivo* models to determine the biological relevance of the *in vitro* findings to clinical isolates. This led to the discovery that TA loci were important for bacterial survival in a mammalian host, including *Salmonella enterica* serovar Typhimurium, *E. coli*, *M. tuberculosis*, and *Haemophilus influenzae* infections.[5,17–20] In addition, some loci have since been shown to be active in bacteria during infections of plant hosts.[21,22]

Clinical importance of toxin–antitoxin modules

One of the roles of TA systems is to increase bacterial survival under stressful environmental conditions, which includes antibiotic, oxidative, nutrient, pH or heat stress, and attack by bacteriophages or the host immune response.[8,17,19,23–26] For type II TA modules, the microbial general stress response induces bacterial cellular proteases, such as Lon and ClpXP, that degrade the labile antitoxin portion of the TA protein complex, freeing the more stable toxin protein to exert its effects (Figure 1).[27] Many type II TA toxins are ribonucleases that degrade bacterial mRNA in a sequence-specific or non-specific fashion, causing the organism to enter a state of bacteriostasis.[3,28–34] This is beneficial to a pathogenic bacterium, particularly during an infection, as growth arrest significantly decreases its metabolic burden, accumulation of DNA damage from reactive oxygen species, as well as minimizing the production of molecular patterns that signal receptors of the host's immune response. Further, because TA complexes are preformed, activation of this mechanism does not require transcription and translation of effectors, allowing the organism to mount an immediate response to its microenvironment.[35] Also, because most antimicrobials disrupt an essential function for replicating cells (i.e. DNA, RNA, and protein synthesis), treatment with these compounds is not effective when the bacterium is in a non-replicative state.[36] However, if stress is removed from the microenvironment, surviving cells can resume growth and again display their natural antibiotic susceptibilities.[37] These findings highlight the importance of TA modules in facilitating this rapid, effective and highly conserved survival mechanism.

Bigger[38] was the first to provide empirical evidence that bacterial pathogens (in this case, *S. aureus*) formed tolerant cells which he termed ''persisters'' that survived treatment with penicillin. Following this ground-breaking study, McDermott[39] reported that persister cells could also be formed upon starvation, and that at least some species displayed differences in morphology upon entering a persister state. Further, he hypothesized that the ability of a bacterium to ''play dead'' was important for its survival in the host, and that dormant infections resulted from alterations in the host environment, whereas latent infections required a change in the pathogen itself. Finally, he coined the term ''drug indifference'' to describe the ability of persisters to survive antibiotic treatment *in vivo*, but display susceptibility to the same drug when grown *in vitro*.[39]

Most clinically relevant bacteria cannot form endospores, which are tough seed-like structures that allow the organism to exist in a suspended animation state for many years, decades, or even longer. These constructs are resistant to environmental conditions that are normally lethal, such as nutrient deprivation, desiccation, and the presence of noxious chemicals and ionizing radiation. A few medically important Gram-positive human pathogens can form spores, including *Bacillus anthracis* (anthrax), *Clostridium tetani* (tetanus), *Clostridium botulinum* (botulism), and *Clostridium difficile* (antibiotic treatment-induced colitis).[40,41]

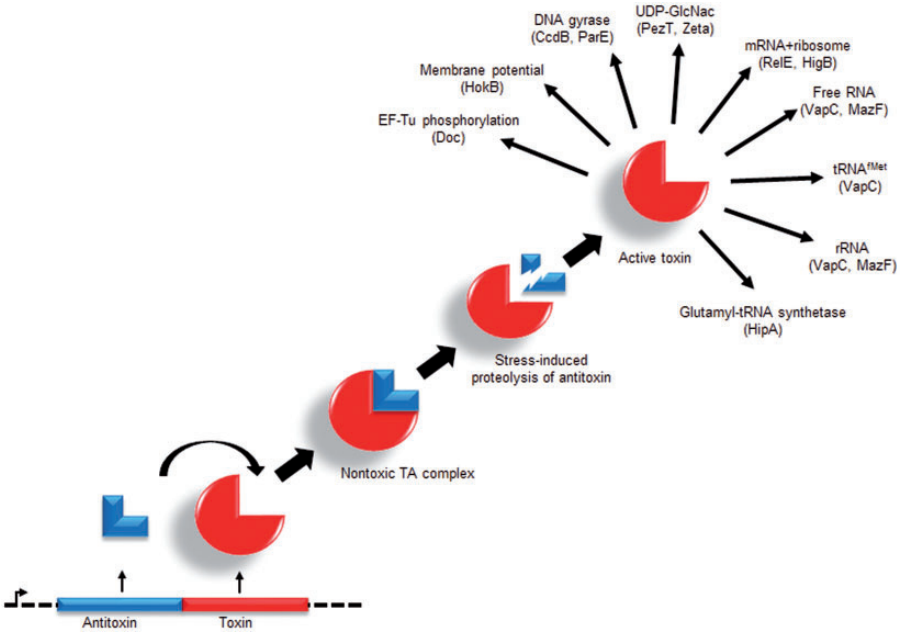

Figure 1 Canonical type II TA module expression and stress-induced degradation of the antitoxin, leading to an active toxin. A number of targets are shown (arrows), along with the names of representative toxins displaying similar activities. See text for details and references

In non-spore-forming bacteria, TA loci facilitate entry into a bacteriostatic state upon stressful conditions.[37] However, a recent study has implicated TA systems in the possible facilitation of recurrent infections with *C. difficile*, a spore-forming pathogen,[40] so these modules might be functional and affect virulence in both groups. Holberger *et al.*[42] provided evidence that the PF04740 proteins found in both pathogenic and non-pathogenic *Bacillus* species represented a new class of type II TA toxins, in which the N-terminal regions defined the protein family, whereas the C-terminal portion (CT) of each toxin assayed was responsible for its ribonuclease activity. The organization of these modules is reversed compared to canonical TA modules, such that the toxin preceded its antitoxin, reminiscent of the *higBA* locus. Although the CT portions were highly divergent, the N-terminal regions were conserved and contained an ESAT-6/WXG100 motif, a putative secretion signal in *Bacillus*. Two purposes were proposed for these modules: that the RNase toxins could be released into the environment to scavenge nucleosides prior to sporulation, or that release of the toxins could enhance competition for environmental niches.

Structural and mechanistic characterizations of type II TA systems

Type II toxins have been shown to target ribonucleic acid by: (a) cleaving free RNA (VapC from *Haemophilus* strains, MazF, Kid/PemK, HicA, and MqsR),[5,28,43–45] (b) cleaving RNA in the context of a ribosome during translation (YafO, RelE, HigB, and YoeB),[34,46–48] and (c) cleaving $tRNA^{fMet}$ (VapC from *S. enterica* serovar Typhimurium).[49] Other targets include DNA gyrase (CcdB and ParE);[16] uridine diphosphate-*N*-acetylglucosamine, a peptidoglycan precursor (PezT and Zeta toxins);[25] decreasing membrane potential (HokB);[50] and phosphorylating glutamyl-tRNA synthetase (HipA)[51,52] or EF-Tu (Doc).[53] The *obg* gene, which encodes a conserved P-loop GTPase, has been implicated in persistence using antisense RNA (asRNA), because the gene is essential for viability in *E. coli*. The Obg protein was shown to increase persistence in both *E. coli* and *P. aeruginosa* via the mechanism of inducing expression of the HokB toxin, which results in membrane depolarization leading to both dormancy and antibiotic tolerance.[50] While a substantial number of type II TA systems have been characterized by structural and mechanistic studies,[54–56] several selected examples are highlighted below and organized according to the mechanism of toxicity.

Ribosome-independent ribonuclease TA systems

Toxins of the VapBC (virulence associated proteins) TA family contain a PIN (PilT N-terminus) domain, which is associated with Mg^{2+} or Mn^{2+}-dependent ribonuclease activity.[30] Indeed, purified VapC from *Mycobacterium smegmatis* was shown to cleave RNA preferentially at AUAU and AUAA sites *in vitro* and *in vivo*.[57] The VapC-1 toxin of non-typeable *H. influenzae* (NTHi) was also shown to function as a ribonuclease with no activity against DNA.[28] Similarly, *in vitro* studies with VapC-5 from *M. tuberculosis* suggested that the toxin acts on free RNA as both an endo- and exo-ribonuclease.[58] While VapC toxin homologues from *Shigella flexneri* and *S. enterica* serovar Typhimurium were found to cleave initiator tRNA between the anticodon stem and loop,[49] VapC20 of *M. tuberculosis* cleaves the sarcin-ricin loop of 23S ribosomal RNA, based on secondary structural recognition rather than sequence recognition.[59] Although VapC-mt4 from *M. tuberculosis* demonstrated sequence-specific endo-ribonuclease activity against ACGC or AC(A/U)GC motifs, the activity was relatively weak and translational inhibition and growth arrest preceded RNA cleavage, suggesting that toxin activity might be mediated primarily by RNA binding rather than cleavage.[60] Recent crystallographic studies of the VapC30 toxin from *M. tuberculosis* reveal a homodimer with a canonical $\alpha/\beta/\alpha$ sandwich fold containing four parallel β-strands flanked on both sides by six α-helices.[61] Overall, the structure shared similar architecture with VapC from *S. flexneri*, VapC2 from *Rickettsia felis*, VapC15 from *M. tuberculosis*, and VapC3 from *P. aerophilum* (Table 1). The structure of the VapB30 antitoxin is distinct from other VapB family members, aside from the N-terminal α-helix. Interestingly, the C-terminal region of VapB30 blocks the activity of the distal dimeric toxin through a swapped inactivation process,[61] whereas the structures of *M. tuberculosis* VapBC3[62] and VapBC5[58] show individual antitoxins bound with a cognate toxin to directly block the active site.[63]

The *mazEF* TA family, encoding the MazE antitoxin and MazF toxin, has been studied extensively and MazF homologues have been identified in archaea and bacteria, with the majority of studies focusing on the *E. coli* system.[64] The first crystallographic structures reported were of the *E. coli* chromosomal MazEF complex, which form a linear heterohexamer of alternating toxin and antitoxin homodimers ($MazF_2$-$MazE_2$-$MazF_2$).[65] The MazF toxin is an endoribonuclease that cleaves single-stranded mRNA, with specificity for ACA sequences, to reduce overall protein synthesis through rapid degradation of bulk mRNA.[65] MazF also targets the 5′ untranslated region among a distinct subset of transcripts, sometimes removing the Shine-Dalgarno sequence to generate leaderless mRNA. Additionally, MazF cleaves 16S rRNA to remove the anti-Shine-Dalgarno sequence, thereby generating specific ribosomes that allow translation of MazF-processed mRNAs.[66,67] Operons for *mazEF* in *M. tuberculosis* were shown to encode endo-ribonucleases that target sequence-specific regions of the *era* mRNA, with MazF-mt1 cleaving U*AC triplet sequences (* indicates the cleavage site) and MazF-mt6 cleaving U-rich regions.[68] Similarly, MazF-mt3 demonstrates specific cleavage of RNA at UU*CCU or CU*CCU and MazF-mt7 cleaves at U*CGCU. It was proposed that MazF toxins might alter protein expression via differential cleavage of mRNA.[69] Recently, MazF-mt6 was reported to cleave 23S rRNA at a single UU*CCU sequence in the ribosomal aminoacyl (A) site which contacts tRNA and ribosome recycling factor. MazF-mt6-mediated cleavage of rRNA was demonstrated to inhibit protein synthesis even in the absence of mRNA cleavage. Additionally, MazF-mt6 was found to destabilize the 50S-30S ribosomal subunit association.[70] Similarly, another mechanism of toxicity was

Table 1 Three-dimensional structures of the type II TA systems described in this minireview

TA Family	Toxin	Antitoxin	Mechanism of Toxicity	PDB IDs	Method	Organisms
vapBC	VapC	VapB	Ribosome-independent mRNA cleavage	1W8I, 2FE1, 3TND, 4CHG, 4XGQ, 4XGR, 3ZVK, 3H87, 3DBO, 1Y82	X-ray diffraction	*Archaeoglobus fulgidus, Pyrobaculum aerophilum, Shigella flexneri, Mycobacterium tuberculosis, Rickettsia felis, Pyrococcus furiosus*
mazEF	MazF	MazE	Ribosome-independent mRNA cleavage	3NFC, 1UB4, 4MDX, 4ME7, 4MZP, 4MZT, 2MF2, 4MZM, 4OF1	X-ray diffraction and solution NMR	*Escherichia coli, Bacillus subtilis, Staphylococcus aureus*
relBE	RelE	RelB	Ribosome-dependent mRNA cleavage	1WMI, 2K29, 3BPQ, 2KC9, 2KC8, 3G5O, 2KHE, 3OEI, 4FXI, 4FXH, 4FXE, 4V7J, 4V7K	X-ray diffraction and solution NMR	*Escherichia coli, Pyrococcus horikoshii, Methanocaldococcus jannaschii, Mycobacterium tuberculosis, Thermus thermophilus*
higBA	HigB	HigA	Ribosome-dependent mRNA cleavage	4YPB, 4YZV, 4MCX, 4MCT, 2ICT, 4PX8	X-ray diffraction	*Escherichia coli, Proteus vulgaris*
ccd	CcdB	CcdA	Inhibits DNA gyrase	2H3A, 2H3C, 3TCJ, 2ADL, 2H3C, 2ADN, 3G7Z, 3HPW, 3VUB, 2VUB, 2KMT, 3JRZ, 3JSC, 1X75, 4VUB, 1VUB, 4ELZ, 4ELY	X-ray diffraction and solution NMR	*Escherichia coli, Aliivibrio fischeri, Shigella flexneri*
parDE	ParE	ParD	Inhibits DNA gyrase	3KXE, 2AN7	X-ray diffraction and solution NMR	*Escherichia coli, Caulobacter crescentus*
hipBA	HipA	HipB	Phosphorylates glutamyl-transfer RNA synthase	4YG7, 4YG4, 4YG1, 3DNV, 3HZI, 3FBR, 3DNU, 3DNT, 2WIU, 3TPB, 3TPD, 3TPT, 3TPV, 4PU3, 4PU4, 4PU5, 4PU7, 4PU8	X-ray diffraction	*Escherichia coli, Shewanella oneidensis*
phd-doc	Doc	PhD	Inhibits the 30S ribosome and Phosphorylates EF-Tu	3K33, 3KH2, 3HS2, 3HRY, 3DD7, 3DD9	X-ray diffraction	*Escherichia coli*, Enterobacteria phage P1, Enterobacteria phage P2

Note: The information was collected from the Protein Data Bank (www.rcsb.org).[63]

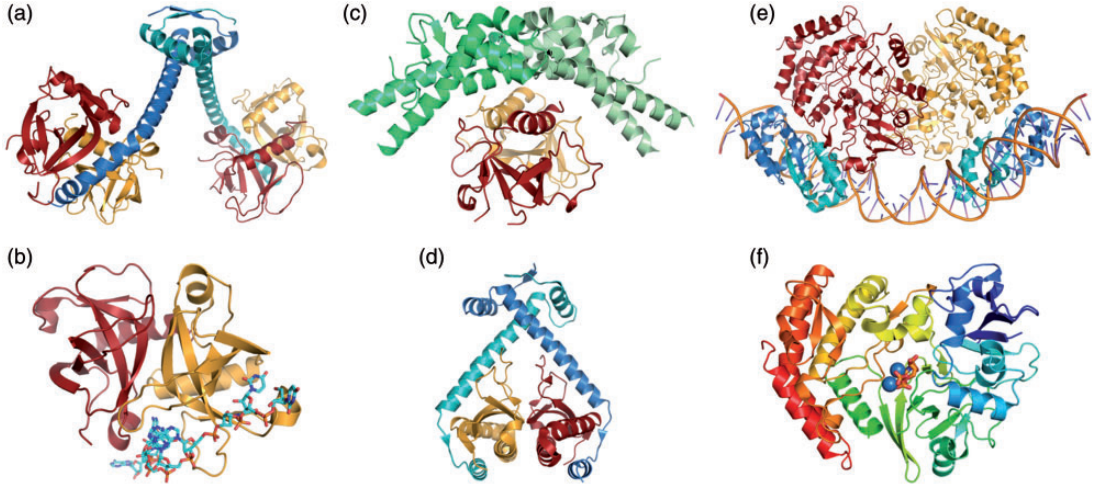

Figure 2 Cartoon representations of selected toxin and antitoxin structures deposited in the PDB database.[63] The selection includes toxins with different mechanisms, including ribonuclease, DNA gyrase inhibitor and kinase (from left). (a) MazF toxin homodimers from *B. subtilis* (red and gold) are inhibited when bound to the C-terminal region of MazE antitoxin proteins (blue and cyan) (PDB ID: 4ME7).[73] (b) A dimeric structure of MazF from *B. subtilis* (red and gold) is in complex with RNA (cyan sticks) (PDB ID: 4MDX).[73] (c) A homodimer of the DNA gyrase subunit A dimerization domain (green and light green) is bound to a CcdB homodimer (red and gold) from *E. coli* (PDB ID: 1X75).[74] (d) A ParE toxin homodimer from *Caulobacter vibrioides* (red and gold) is inhibited by a ParD antitoxin homodimer (blue and cyan) (PDB ID: 3KXE).[75] (e) A multi-molecular complex of *E. coli* HipA toxin homodimers (red and gold) and HipB antitoxin homodimers (blue and cyan) bound to DNA operator sites O1 and O2 (orange) (PDB ID: 4YG7).[76] (f) The ribbon diagram of a HipA toxin structure is colored as a rainbow from blue (N-terminus) to red (C-terminus) and shows two bound Mg^{2+} ions (blue spheres) and ATP (orange sticks) (PDB ID: 3DNT).[77] All cartoon diagrams were generated with the program PYMOL.[78]

discovered for MazF-mt7, based on interactions with topoisomerase I that inhibit the activity of both enzymes.[71] Collectively, these results suggest a variety of ways that MazF proteins might mediate toxicity. Studies with MazF from *Bacillus subtilis* demonstrated sequence-specific cleavage at U*ACAU.[72] Crystal structures were determined of *B. subtilis* MazF, including complexes of MazF with MazE (Figure 2(a)) or mRNA containing the uncleavable dUACAU sequence (Figure 2(b)).[73] The MazF-mRNA complex showed dUACAU bound to MazF with the bases projecting towards the dimer interface of the toxin and the phosphate backbone moieties projecting away from the surface. Residues R25 and T48, which are highly conserved among MazF homologues, form hydrogen bonds with the oxygen atoms of the scissile phosphate between dU3 and A4 of the mRNA and either R25A or T48A mutations resulted in a loss of toxicity.[73] The mRNA binding and cleavage by MazF are blocked through the positioning of the MazE C-terminal helical region within the RNA binding channel of the MazF dimer (Figure 2(a)).[73]

Ribosome-dependent ribonuclease TA systems

The *relBE* TA family toxin, RelE, has been extensively studied both *in vitro* and *in vivo* and has been shown to function as a ribosome-dependent endonuclease.[79] Under normal conditions, RelE is inhibited by forming a complex with the antitoxin, RelB, which has been revealed at the atomic level by structural studies of RelBE from *E. coli*, *M. tuberculosis*, *Methanocaldococcus jannaschii*, and *Pyrococcus horikoshii* (Table 1). Both *in vitro* and *in vivo* studies showed that the overall RelB:RelE ratio regulates transcriptional repression. Binding to the *relO* operator is enhanced by RelE up to a RelB:RelE ratio of 2:1, beyond which the affinity for DNA is reduced, which has been described as ''conditional cooperativity.''[80–82] Following degradation of RelB by Lon protease, one of the bacterial stress-induced proteases, RelE binds to the ribosome and cleaves mRNA in the A site.[79,83] Crystal structures were determined of *E. coli* RelE bound to *Thermus thermophilus* 70S ribosomes in complex with mRNA and $tRNA^{fMet}$ before and after mRNA cleavage.[84] The structures show that binding of RelE to the A site on the 30S subunit significantly reorganizes the mRNA, leading to 2′-OH-induced hydrolysis between the second and third codon positions.[84] Although RelE was shown to cleave codons preferentially *in vitro*,[79] recent studies suggest that RelE specificity might be broader than originally thought.[85,86]

Another well-studied ribosome-dependent ribonuclease TA system is the *higBA* (host inhibition of growth) family, which is related to the *relBE* family.[34] In contrast to many other TA families, including *relBE*, the *higBA* operon has an inverted gene structure with the toxin gene preceding the antitoxin gene.[87] Crystallographic structures of a tetrameric HigB-$(HigA)_2$-HigB TA complex from *Proteus vulgaris* have been reported.[88] The overall tertiary fold of the HigB toxin is similar to the RelE family; however, the structures revealed some unusual molecular details about the HigBA TA system. Interestingly, the HigA antitoxin makes minimal interactions with HigB and does not block the active site, which is solvent-exposed.[88] Additionally, HigA monomers contain a DNA-binding helix-turn-helix motif that binds an individual operator sequence, which is unlike antitoxins of other superfamilies that require dimerization to form DNA-binding motifs.[88] The tetrameric complex was critical for binding the DNA operator, as HigBA heterodimers were unable to bind.[88] Recently, crystal structures were

determined of *P. vulgaris* HigB bound to the *T. thermophilus* 70S ribosome containing either an AAA or ACA codon in the A site.[89] The structures revealed that a microbial RNase-like nucleotide loop of HigB is able to recognize either cytosine or adenosine at the second A-site position. The residue N71 of HigB along with nucleotide C1054 of 16S rRNA contributes to a pocket at the third A-site nucleotide that is specific for adenosine at the +6 position.[89] Toxin recognition of mRNA substrates through the third nucleotide of the codon is in contrast to the way tRNAs utilize an anticodon stem loop to recognize A site codons.[89] Together, these studies provide a mechanistic explanation for the way ribosomes enable HigB and possibly other ribosome-dependent ribonucleases to achieve substrate specificity.

TA systems that target DNA gyrase

DNA gyrase is an essential bacterial topoisomerase that hydrolyzes ATP to introduce negative supercoils into DNA and is also a target of the quinolone family of antibiotics. The active enzyme is a heterotetramer of two subunits, GyrA, which cleaves DNA, and GyrB that hydrolyzes ATP.[74] The *ccd* (coupled cell division) system of the *E. coli* F plasmid, comprises a CcdB toxin and a CcdA antitoxin.[16] The CcdB toxin is a 101 amino acid residue protein that binds to GyrA with strong affinity[90] and inhibits DNA gyrase by either poisoning a covalent DNA-gyrase complex or binding free gyrase, both of which involve identical interactions between CcdB and GyrA (Figure 2(c)).[91] Inhibition of gyrase by CcdB is reversible, as the CcdA antitoxin can compete with gyrase to bind and sequester CcdB, thereby allowing DNA gyrase to regain function.[92] The CcdA antitoxin is a protein of 72 amino acid residues and two independent domains. The N-terminal domain contains a ribbon-helix-helix fold that facilitates DNA binding and dimerization, whereas the C-terminal domain, which is intrinsically unstructured, binds and inhibits the CcdB toxin.[93] The high flexibility of the C-terminal region is thought to increase the susceptibility of CcdA to cleavage by the Lon protease.[93] Structural studies of the CcdB toxin and the CcdA antitoxin proteins by both nuclear magnetic resonance (NMR) and X-ray crystallography have been reported (Table 1).

Similar to CcdB, the ParE toxin also blocks DNA replication through inhibition of DNA gyrase.[94] A crystal structure of the ParD-ParE complex from *Caulobacter crescentus* was reported and reveals an $\alpha_2\beta_2$ heterotetrameric complex (Figure 2(d)), which is supported by solution studies.[75] The ParD antitoxin contains an N-terminal ribbon-helix-helix DNA binding motif, which facilitates dimerization, as well as an extended α2 helix and an α3 helix towards the C-terminus that both interact with ParE.[75] Interestingly, structural comparisons between toxins revealed hydrophobic grooves for antitoxin recognition and binding that are conserved between the *parDE* and *relBE* families.[75] Although ParE shares both primary and tertiary structural homology with the RelE toxin, ParE lacks the three catalytic residues required for mRNA cleavage on the ribosome.[75,84]

TA systems that encode kinases

Within the *hipBA* (high persistence) family of *E. coli*, *hipA* encodes the 440-residue toxin protein, HipA, which is co-transcribed with a smaller upstream gene, *hipB*, that encodes an 88-residue antitoxin protein, HipB. The HipA toxin is a member of the phosphatidylinositol 3/4-kinase superfamily[95] and structural studies by multiple groups have confirmed that HipA has a eukaryotic serine/threonine kinase-like fold that is most similar to human CDK2/cyclin A kinase.[77,96] Initially, HipA was reported to phosphorylate the transcription factor, EF-Tu;[77,97] however, more recent results indicate that the target is S239 of glutamyl-tRNA synthetase, which is located near the enzyme active site.[51,52] The kinase activity of HipA is regulated by phosphorylation of the residue S150, which forms part of the ATP-binding P loop motif and inactivates the kinase by disrupting the ATP binding pocket.[97] Previously, mutations in the *E. coli* HipA protein had been associated with a substantial increase in persister cells.[98] Recent structural studies showed that these mutations localize to the HipA N-subdomain-1, which is distal to the toxin active site and HipB binding site; however, this region facilitates HipA dimerization within higher order complexes of HipA-HipB and multiple operators in *hipBA* promoters (Figure 2(e)).[76] The HipA–HipA interface blocks the kinase active sites (Figure 2(f)), so persister-associated mutations in the N-subdomain-1 are thought to release HipA from the inactive state.[76]

The *phd-doc* TA module encodes the 126-residue toxin, Doc (death on curing), and 73-residue antitoxin, Phd (prevent host death), to maintain the plasmid-prophage P1 in *E. coli*.[99] Regulation of the *phd/doc* operon involves conditional cooperativity that is regulated by complexes of Phd and Doc.[100] Doc is a member of the Fic (filamentation induced by cyclic AMP) family of AMPylation enzymes, which are found in all kingdoms of life.[101] The toxicity of Doc was initially reported to be inhibition of translation elongation through interactions with the 30S ribosomal subunit, similar to the aminoglycoside antibiotic hygromycin B.[53] More recently, it was discovered that Doc is a new type of kinase that phosphorylates the translation elongation factor EF-Tu on the conserved threonine, T382, thereby preventing EF-Tu from binding aminoacylated tRNAs.[102]

Microbial dormancy

Dormancy is the state of most of the prokaryotes on Earth, whose total numbers are estimated to be in the range of 4.0–6.0×10^{30} cells in three enormous habitats: soil, seawater, and the marine sediment or terrestrial subsurface.[103] Microbes in these environments display extremely low metabolic activity, but are not completely inactive due to the need to maintain their DNA integrity.[104,105] Price and Sowers[105] estimated that the energy demands of a cell in a dormant state were three orders of magnitude lower than that of a replicating organism. However, a bacterium in a dormant state must maintain not only an intact genome, but also an energized membrane to allow ATP synthesis, both of which are requirements for successful regrowth upon improved conditions. Resuscitation triggers, such as

nutrient upshift or specific biomolecules,[106,107] as well as stochastic processes of transcriptional regulation and the ''microbial scout'' hypothesis,[108–110] have been implicated in the ability of these dormant bacteria to awaken. For example, this might entail a mechanism of the spontaneous resuscitation of random cells in a population, which subsequently produce a protein known as ''resuscitation promoting factor'' (Rpf) if conditions are conducive for growth. This molecule then binds to and signals the remaining dormant population to resuscitate.[107]

Geomicrobiologists have found that conditions in Siberian permafrost soil, which are hundreds of thousands of years old,[111] and in deep sub-seafloor sediments, which are millions of years old,[112–114] harbor a diverse community of microorganisms at 10^3 to 10^8 cells/cm^3.[115–117] D'Hondt *et al.*[115] found that the sediments in the South Pacific Gyre were characterized by extremely low biomass and metabolic activity. Sub-seafloor sediments harbor between 55% and 85% of all the microbes on the planet,[103,112] and Schippers *et al.*[118] provided direct evidence that many of these microbes are alive and capable of metabolism (i.e. not in endospores) by determining the presence of ribosomes using catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH). Given the amount of carbon availability and usage, the estimated average turnover rate of organisms in such sediments is on the order of 1000 years, and might be as long as several thousand years.[103,119]

These surprising findings have been shown to be characteristic of microbial life on Earth. Inagaki *et al.*[120] hypothesized the existence of a ''paleome,'' microbial fossil biomolecules encased in black shale identified in deep subsurface sediments. In an intriguing study, these investigators utilized molecular genetic culture-independent analyses to demonstrate that the preserved DNA from deep sections of terrestrial core sediment (250 to 300 cm below the surface) corresponding to the Cretaceous lower Albian period (~100 million years old) contained phylotypes that were consistent with those found in extant marine environments.[120] Interestingly, another careful study found that the bacterial community in deep marine sediment east of Japan (>2 km below the seafloor) harbored a group of microorganisms that resembled a terrestrial community, while those in the shallow sediment at the same sample site reflected a marine population.[121] This suggests that at least some microbes have survived for tens of millions of years after burial in the sediment, which challenges our notions of the longevity of prokaryotes and the stability of nucleic acid in such an environment.

The dormant state induced by TA systems can be achieved by spontaneous switching under stable environmental conditions, creating a subpopulation of persister cells that can respond quickly to a changing environment.[122,123] Lennon and Jones[113] have described the benefit of these persisters as being microbial ''seed banks'' that ensure the long-term viability of the bacterial population by providing for genetic diversity. Indeed, Ayrapetyan *et al.*[37] suggested that the viable but non-culturable (VBNC) state identified in non-sporulating bacteria and the drug-tolerant bacterial persister cell are both part of a shared ''dormancy continuum.'' Their hypothesis was that the persister state is a gateway into the VBNC state, and this is why persisters both (a) occur in lower numbers, and (b) resume growth more rapidly upon antibiotic removal, because the so-called VBNC cells can resuscitate, but require more time to do so than persister cells.[24] These studies have therefore raised concerns about the validity of measuring viability via CFU counts. Type II TA loci have been linked to both states in various organisms.

Future outlook

It is likely that new types of TA modules will continue to be discovered as the genomes of novel organisms are sequenced. It is also possible that coordinate regulation of various loci and groups of modules might be identified. Currently, unanswered questions of importance to the field include (but are not limited to) the following: are there specific triggers for various TA loci, and can groups be identified? How do the different TA types interact with one another (e.g. type II ribonucleases are activated by protein antitoxin degradation, which might then degrade the RNA antitoxins of type III toxins), and does the threshold level of response to various stresses differ when the organism is infecting a host? Does the specific stressor correlate with the activation of certain TA loci? Further, are all the loci activated simultaneously, or is there a hierarchy in the cascade of toxin activation across types in response to stress? When are these systems induced during the formation of a biofilm? Helaine and Kugelberg[124] noted a need for new methods that will allow the study of the multifactorial processes that result in dormant persister cells. However, a related issue is whether advanced techniques, such as the examination of the behavior of single cells in a microfluidics model, can be reflective of actual conditions within an infecting population.

The number of TA systems in the genome has been shown to correlate with the virulence capacity of the organism.[125] For example, *M. tuberculosis* strains encode at least 79 TA modules, whereas the non-pathogenic species *M. smegmatis* maintains only four.[126] Targeting bacterial TA systems might be an effective strategy for the development of novel antibiotics. Indeed, the deletion of a VapBC module in NTHi significantly decreased its virulence in primary human tissues and in an animal model of otitis media.[19] Wen *et al.*[127] recently highlighted the remarkable overlap between the targets of TA toxins and antibiotics. Likewise, many investigators have proposed that TA modules have therapeutic potential as novel antimicrobials.[55,128–130] In a comprehensive recent review, Chan *et al.*[130] discussed the ''druggability'' of various type II systems, with a view toward disrupting the protein–protein interactions of the toxin–antitoxin complex in order to free the toxin. However, this tactic also has the potential to induce persister cells that are difficult to treat.[131] Furthermore, the redundancy of TA systems and other pathways to persistence presents significant challenges that must be addressed.[132,133] In the final analysis, it is possible that targeting multiple TA modules using a range of approaches might result in the most successful antimicrobial strategy.

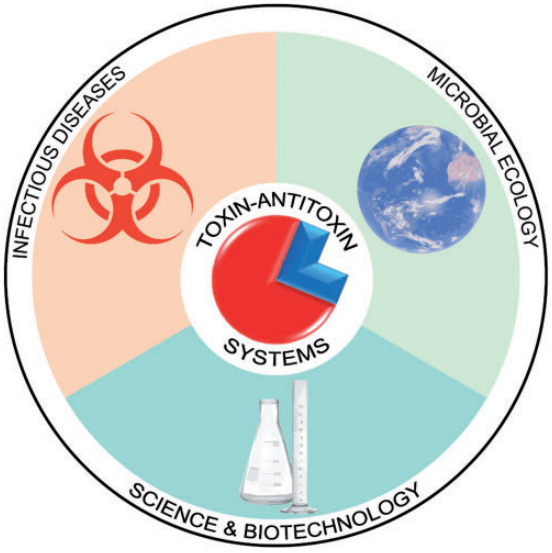

Figure 3 Toxin–antitoxin systems significantly impact a wide range of areas, from human health and enhancing microbial survival in the environment to enabling discoveries in molecular biology and medicine

Concluding remarks

Given the ubiquity of toxin–antitoxin systems among bacteria and their critical roles in adaptation to adverse conditions, we anticipate that future studies will reveal additional complexities in the way these systems operate and function together to sustain life and optimize survival within a range of environments (Figure 3). Knowledge obtained from the study of TA systems has already enabled the development of novel antiviral strategies and biotechnology applications, such as positive selection plasmids and biosensors.[130] Therefore, further studies to elucidate the molecular details underlying these important systems will likely continue to drive advancements in biotechnology, synthetic biology and medicine.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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