Infection and Drug Resistance

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REVIEW

Type II Toxin-Antitoxin Systems in *Escherichia coli*

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Abstract: The toxin-antitoxin (TA) system is widespread in prokaryotes and archaea, comprising toxins and antitoxins that counterbalance each other. Based on the nature and mode of action of antitoxins, they are classified into eight groups (type I to VIII). Both the toxins and the antitoxins are proteins in type II TA systems, and the antitoxin gene is usually upstream of the toxin gene. Both genes are organized in an operon and expression of which is regulated at the transcriptional level by the antitoxin-toxin complex, which binds the operon DNA through the DNA-binding domain of the antitoxin. The TA system plays a crucial role in various cellular processes, such as programmed cell death, cell growth, persistence, and virulence. Currently, Type II TA systems have been used as a target for developing new antibacterial agents for treatment. Therefore, the focus of this review is to understand the unique response of Type II TA in *Escherichia coli* to stress and its contribution to the maintenance of resistant strains. Here, we review the Type II TA system in *E. coli* and describe their regulatory mechanisms and biological functions. Understanding how TA promotes phenotypic heterogeneity and pathogenesis mechanisms may help to develop new treatments for infections caused by pathogens rationally.

Keywords: *E. coli*, type II toxin-antitoxin, bacterial persistence, biofilm formation, phage infection

Introduction

TA systems are small genetic modules widely distributed in bacterial and archaeal genomes. Typically, the TA system is composed of toxins and antitoxins, and toxins are in most cases proteins that can influence DNA replication, transcription, protein synthesis, et al. Antitoxins may be RNA or proteins,[1] and under appropriate conditions, toxins can be prevented from performing their functions. Generally, toxins are relatively stable in structure, and antitoxins are relatively structurally unstable and are either rapidly degraded by intracellular proteases under stress or plasmid loss or down-regulated.[2] Initially, TA systems were identified in plasmids called plasmid maintenance systems. The first TA system identified was the plasmid-borne type II system, which plays a role in plasmid maintenance known as post-segregation killing.[3,4] Once the plasmid encoding TA system is lost from a cell, it cannot produce the unstable antitoxin to neutralize the remaining stable toxin, and the growth of that cell is stunted, eventually leading to cell death. Later, many TA systems were also found on chromosomes.[5–7] The toxins in the TA systems are all proteins (except the newly discovered type VIII system). Based on the nature of the antitoxin and its mode of action, the current types of antitoxins are as follows: type I antitoxins are sRNAs. Antitoxin sRNAs bind to the mRNA of the toxin to promote its degradation, impede the translation of the toxin, and inhibit the transcripts of its cognate toxins;[8–13] Type II antitoxins exist under normal growth conditions as TA complexes, which are proteins that bind to homologous toxins and form neutralizing complexes;[14–18] Type III antitoxins are also sRNAs, which bind homologous toxins and sequester them by forming neutralizing protein-RNA complexes;[1,19–21] Type IV antitoxins are proteins. Acts on the cellular target of its toxin analog and protects or detoxifies the target rather instead of blocking the toxin itself;[22,23] Type V RNase antitoxin prevents toxin accumulation

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by specific degradation of its mRNAs;[24,25] Type VI antitoxins are proteins that act as junctions, targeting their cognate toxins for degradation by ATP-dependent proteases;[26] Type VII antitoxins are proteins that are inactivated by post-translational modifications to their cognate toxins;[27–29] Type VIII antitoxin is RNAs which inhibit the expression of their cognate RNA toxins and inhibit the transcription of the toxin or interact with the toxin RNA. Degradation of the toxin RNA or the recruitment of Cas proteins as transcriptional repressors by mimicking CRISPR RNAs.[30,31] In addition to these well-defined types, some special cases have been described. For example, some modified toxins or antitoxins acting on cellular targets (types VII and IV, respectively) can form antitoxin-toxin protein complexes similar to type II systems,[23,27] and some toxins can be associated with different types of antitoxins (type II and type IV) within the same maneuver.[22]

Of these, type II TA systems are the most abundant and extensively studied, and each type II TA system is encoded by two small genes, which usually overlap by a few bases.[32,33] The toxin and its cognate antitoxin form a stable TA complex that blocks the function of the toxin. Under stress conditions, the antitoxin can be degraded by proteases (for example, Lon, ClpAP, and ClpXP), and the toxin is released from the complex to exert a bacteriostatic effect. Because the antitoxin is not as stable as the toxin within the cell, the antitoxin has to be continuously produced to inhibit the toxin. In most cases, the type II antitoxin genes are located upstream of the toxin genes, so that antitoxins are produced before their cognate toxins, or they form small genomic islands of their own.[34–36] However, upstream genes encode toxins in some special cases, such as *HigA*/*HigB, MqsR*/*YgiT*, and *MqsR*/*MqsA*.[37] In this review, we aim to provide an up-to-date overview of type II TA systems in *E. coli*, describe recent advances in these systems, and discuss research perspectives in this area.

Type II TA Systems in E. coli

Thousands of type II TA sites have been found in *E. coli*.[6] In Type II TA systems, both the toxin and the antitoxins are small proteins. Antitoxins usually have two structural domains, one that binds DNA,[14,38,39] and the other that binds and inhibits the activity of the cognate protein toxin[14] (Figure 1). Antitoxins also often bind the promoters of their operons to repress transcription. In most cases, the toxins act as co-repressors. In some cases, they bind promoters of other genes. The type II TA system is regulated by marked differences in the cellular lifespan of antitoxins and toxins. That is, antitoxins are very sensitive to protein hydrolysis, whereas their cognate toxins are relatively stable. Thus, in response to

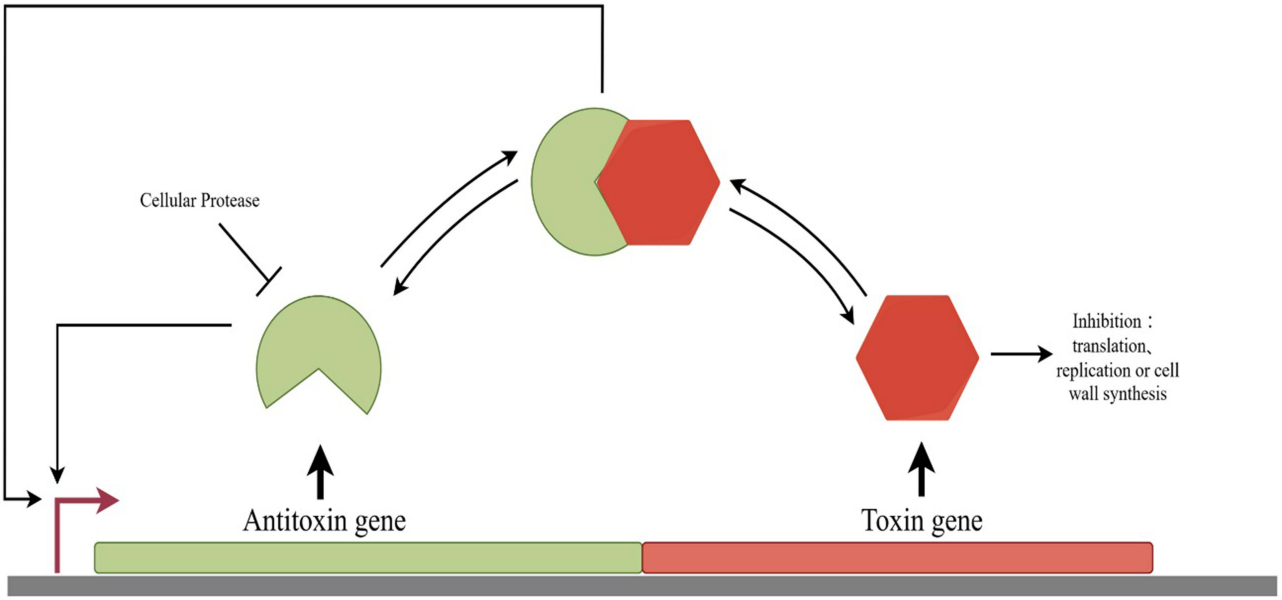

Figure 1 Type II toxin-antitoxin (TA) locus of *E. coli* K-12. Diagram of the genes and control loops of a typical type II TA locus. The red arrow to the right indicates the TA operon promoter. When the free toxin concentration is low, the promoter is repressed by the antitoxin during rapid growth, especially by the TA complex that binds tightly to the promoter region. In contrast, promoter activity is inhibited by free toxins, a regulatory phenomenon known as conditional synergy.

stress, antitoxins are selectively degraded. This leads to growth stagnation due to the effects of the toxins released on the cells. Many type II toxins are mRNA-disrupting enzymes (ribonucleic acid endonucleases) and are either ribosome-dependent (for example, *RelE*,[40–42] *YoeB*,[43] *YafQ*,[18,44] *YafO*,[18,45] *HigB*[46,47]) or ribosome-independent (for example, *YhaV, MazF, MqsR*,[48–50] *ChpBK, HicA, PemK*).[51,52] Another type II toxins inhibits DNA replication through interactions with DNA cleavage enzymes (for example, *CcdB, ParE, Doc*, and *HipA*) toxins act by phosphorylating elongation factors Tu and Glu-tRNA synthetase, respectively.[39,53]

Target of Toxin Action

Currently, type II toxins are classified into nine superfamilies based on their structural features: *ParE*/*RelE, MazF, HicA, VapC, HipA, FicT*/*Doc, AtaT*/*TacT, Zeta* and *MbcT*. We summarize the toxin targets and mechanisms of action in Table 1. Toxins within a family may have various modes of action. For example, ParE toxin targets DNA gyrase, blocks the replication of DNA, and induces genome instability leading to cell death,[54,55] while RelE has ribosome-dependent mRNA endonuclease activity. The toxin CcdB targets the GyrA subunit of the DNA gyrase, inhibiting DNA replication and causing DNA damage (Figure 2a). By inhibiting the DNA gyrase from catalyzing the rejoining of DNA in the cycle, CcdB locks the enzyme in the DNA into what is known as the cleavage complex, which works in a manner very similar to that of the quinolone antibiotics.[56] CcdB activity is thought to cause replication of the fork collapse, resulting in double-strand breaks, activation of the SOS response, and cell death. Fic, a type II toxin, targets DNA promoter and topoisomerase IV. It inhibits the ATP-hydrolyzing activity of DNA promoters, causing DNA damage and replication inhibition[57] (Figure 2a). Type II toxins, many of which are RNases with variable degrees of specificity, appear to have one major goal: to inhibit protein synthesis. For example, most toxins from the MazF family degrade free RNA with limited specificity, targeting mRNA and ribosomal RNA precursors[58] (Figure 2b). However, some MazF toxins are

Table 1 Targets of Action of Toxins and Their Mechanisms of Action

Toxin	Target of toxin action	Mechanisms of action of toxins	Reference
CcdB	replication	Inhibition of DNA rejoining in the cycle catalyzed by DNA gyrase	[56]
Fic		Inhibition of the ATP-hydrolyzing activity of DNA promoter and topoisomerase IV leads to DNA degradation or inhibition of replication	[57]
SocB		Interacts with the beta-sliding clamp of DNA polymerase and reduces the persistence of DNA replication	[26]
ParE		Inhibition of DNA gyrase	[64]
RelE, HigB, YoeB, YafQ, MqsR, YafO	translation	Cleaves ribosome-dependent mRNA	[65–67]
MazF, YdcE, PemK, ChpBK		Cleaves mRNA, rRNA, tRNA	[1,52,58]
HicA, RelE		Cleaves mRNA	[42]
VapC		Cleaves tRNA	[68]
HipA		Specific phosphorylation of aminoacyl-tRNA synthetase inhibits binding of specific tRNAs to amino acids	[60]
Doc		Phosphorylation and inactivation of elongation factor Tu (ET-Tu)	[53]
MbcT	Metabolic stress	Hydrolyze and deplete NAD+	[61]
ζ, PezT	Cell wall synthesis	Phosphorylation of the peptidoglycan precursor uridine diphosphate-N-acetylglucosamine	[63]

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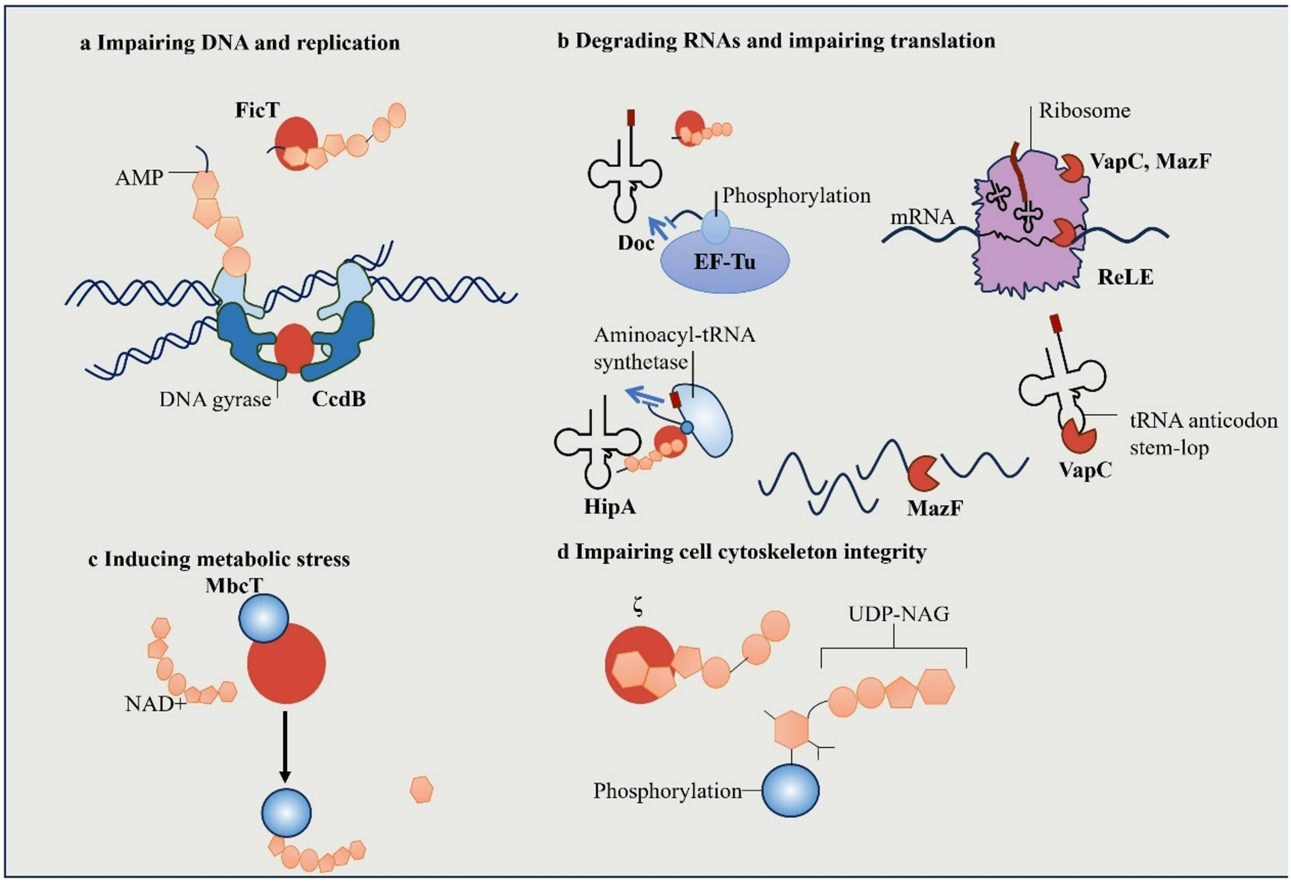

Figure 2 The activity of toxins in the TA system. (**a**) Topoisomerase is inactivated by the amylase *FicT*. the DNA cleavage enzyme is poisoned by direct binding of *CcdB*. (**b**) Translation is the target of numerous toxins that act at every level of protein synthesis. *VapC* toxin cleaves the tRNA anticodon stem-loop or the stem-loop toxin loop of 23s ribosomal RNA. *MazF* toxin degrades free mRNA and ribosomal RNA, and *RelE* toxin cleaves translational mRNAs at the ribosomal A site. *HipA* toxin phosphorylates aminoacyl- tRNA synthetase phosphorylates and prevents tRNA charging. doc phosphorylates elongation factors and prevents tRNA delivery to the ribosome. (**c**) *MbcT* toxin degrades NAD+. (**d**) ζ toxin phosphorylates precursors of peptidoglycan synthesis. Adapted with permission from Springer Nature from Jurėnas D, Fraikin N, Goormaghtigh F, Van Melderen L. Biology and evolution of bacterial toxin-antitoxin systems. *Nat Rev Microbiol*. 2022;20(6):335–350.[1]

specific to a single species of tRNAs. In contrast, toxins of the VapC family specifically cleave the anticodon stem-loop[59] (Figure 2b), of different target tRNAs, and the sarcin-ricin loop of 23S rRNAs, which has structural similarity to the anticodon stem-loop. RelE and related toxins cleave mRNA in a co-translational way, entering the A site of the translating ribosome. mRNA cleavage usually occurs between positions 2 and 3 of the target codon. A variety of type II toxins affect tRNA function by modifying rRNAs or tRNA cofactors after they have been translated. HipA toxin selectively phosphorylates aminoacyl-tRNA synthetase and prevents the binding of particular tRNAs to amino acids[60] (Figure 2b). Doc toxin phosphorylates and inactivates the elongation factor Tu (EF-Tu), inhibiting tRNA presentation to the translating ribosome (Figure 2b). The type II MbcT toxin hydrolyzes and depletes NAD+, the primary electron carrier necessary for redox reactions.[61] (Figure 2c). Type II ζ-toxin phosphorylates and depletes UDP-activated sugars, inhibiting the production of peptidoglycan and lipopolysaccharide, resulting in loss of cell wall integrity (Figure 2d). In a nutshell, toxins are ribonucleases, kinases, and acetyltransferases that, when overexpressed, prevent cell growth. The antitoxin binds to the active site of the homologous toxin and inhibits its activity. MazE binds to the active site of the homologous toxin and neutralizes its RNase activity. The binding of RelB to its cognate toxin results in the displacement of the c-terminal region necessary for toxin activity. ε and PezA spatially site-block ATP/GTP binding sites and inhibit the activity of their respective homologous toxin.[62,63] However, few antitoxins inhibit the activity of their cognate toxin. For example, HipB inhibits homologous toxins by confirming that the toxin is in an inactive state. In conclusion, antitoxins inhibit toxin activity directly by binding to the active site and indirectly by binding to other sites.

Regulation of the Type II TA System

The TA system is normally tightly regulated to maintain a balanced "neutralized" state in the body. Under normal growth conditions, this regulation ensures that under stable conditions, the antitoxin exceeds the amount of its cognate toxin to inhibit its action.

Antitoxins of the type II TA system commonly contain DNA-binding and toxin-neutralizing domains.[69] The antitoxin-toxin complex binds to the operon and participates in the autoregulation of operon transcription. However, in some type II TA systems where the antitoxin lacks a DNA-binding domain and no transcriptional regulating mechanism is apparent, other factors outside the TA operon may influence the expression of toxin and antitoxin.

In a few cases, the antitoxin of the type II TA system is the only factor affecting the transcription of the operon. For example, in the *E. coli* HigBA system,[65] both the antitoxin HigA and the HigB-HigA complex bind the HigBA system operon with similar binding strength, indicating that antitoxin HigA binding to the operon is not affected by the toxin HigB. More complex is the regulatory mechanism of the DinJ/yafQ system associated with the SOS response in *E. coli*.[70] There are two promoters in the system, and the antitoxin DinJ binds the first promoter containing the LexA box, which regulates the transcription of the second promoter. LexA is a transcriptional repressor that plays a role in the SOS response.[18] The SOS and TA-mediated stress responses and the link between them may represent a more comprehensive regulatory network when bacteria are under stress.

Typically, antitoxin alone binds the operon with low affinity, and the toxin acts as a co-deterrent to enhance the binding affinity of the antitoxin to the operon to form a more stable complex. In the *E. coli* VapBC system, the addition of toxin VapB resulted in tighter binding of antitoxin VapC to the operon.[71]

The toxin can affect the binding of the antitoxin to the promoter DNA of the TA system.[72] In the MqsRA system of *E. coli*, the antitoxin MqsA contains two folded structural domains: an HTM-XRE structural domain that binds to the promoter DNA and a Zn^{2+}-stabilizing structural domain that is used to neutralize the toxin MqsR. Due to the partial overlap of the binding sites, MqsA binding to the promoter and binding to MqsR are mutually exclusive, resulting in operon derepression when there is more MqsR than MqsA.

In some cases, inhibition of the TA system depends on the molar ratio between toxin and antitoxin. When the molar ratio of toxin to antitoxin is in the right proportion, for example in the Phd/Doc system the ratio of toxin to antitoxin is about 1:1, the antitoxin-toxin complex binds tightly to the DNA. Excessive toxins can induce the formation of saturated complexes and release inhibition of the TA system. When toxin levels exceed antitoxin levels, inhibition is relieved, allowing the antitoxin to be transcribed and translated from scratch. This negative feedback loop is called "conditional synergy.[73] Similarly, in the CcdAB system, the low binding affinity between the antitoxin and the operon alone does not effectively inhibit transcription in the TA system, and the addition of the toxin enhances the binding of the antitoxin to the operon and the inhibitory effect. When the toxin is re-increased, it leads to structural changes in the TA complex and introduces spatial site resistance thereby activating transcription of the TA system. In the RelBE system, two RelB dimers bind synergistically to adjacent sites on the operon, and two RelB dimers flanking each other can each bind a RelE monomer to form a complex that further stabilizes RelB binding to the operon. Excessive amounts of RelE disrupt the contact between neighboring RelB dimers and spatially impede the binding of RelB to the DNA, thereby activating transcription.[74]

Typically, the antitoxin gene is located upstream of the toxin gene, but in some TA systems the antitoxin gene is located downstream of the toxin gene, and such TA systems with reversed gene order include the HicAB system, where the HicAB system consists of two promoters that turn on transcription.[75] The upstream promoter allows expression of toxin and antitoxin genes, contains a Sxy-dependent cyclic adenosine receptor protein binding site, and is activated by Sxy, and the downstream promoter is repressed by HicB, and this repression can be lifted when HicA is in excess. This mechanism permits the production of more antitoxin when the ratio of toxin to antitoxin is too high.

This complex regulatory network demonstrates the multiple mechanisms employed by the TA system in maintaining the delicate balance between toxins and antitoxins, highlighting the adaptability and complexity of this genetic module.

Biological Role of the Type II TA System in E. coli

In *E. coli*, most TAs are located on the chromosome including (*PrlFyhaV, MazEF, MqsRA, ChpBIK, DinJyafQ, HicAB, YafNO, RelBrelE, yefMyoeB*, and *HipAB*) and five plasmid loci (*PemKI, CcdAB, VagCD, Phd/Doc*, and *ParDE*). The toxin activity leads to plasmid addiction to the cell; hence, the plasmid-encoded TA system is known as the plasmid addiction module (Figure 3a). The chromosomal TA system of bacteria is involved in various biological processes such as stress response,[76] biofilm formation,[77] phage inhibition,[78] virulence[79] and persistence.[80]

TA Systems and Their Role in Persistence

Bacterial persistence refers to the presence of rare cells insensitive to antibiotics even in fast-growing bacterial populations. It is a phenomenon that causes bacterial cells to resist multiple antibiotics and other environmental damage[81] (Figure 3b). This phenomenon was first discovered by Bigger in 1944 when he was researching how penicillin kills *Staphylococcus aureus*, and he discovered that penicillin often fails to sterilize exponentially growing cells. He correctly hypothesized that persistent bacteria escape killing by antibiotics because they are in a slow-growing or dormant state.[82] The first link between persistence and the TA module was established when Moyed & Bertrand[83] discovered the presence of highly persistent mutations in HipAB. HipA, a gene encoding a protein of 440 amino acids, is located in the terminal region of the *E. coli* chromosome at 34.3.[84,85] HipA is preceded by hib (88 codons), and HipB is a dimer that represses transcription through its helix-turn-helix structural domain that binds synergistically to the four operators of the HipAB promoter region,[86] and toxin HipA is a serine/threonine kinase that is partially phosphorylated in vivo and autophosphorylated in vitro.[87,88] It phosphorylates and inhibits the activity of GltX (glutamine- tRNA

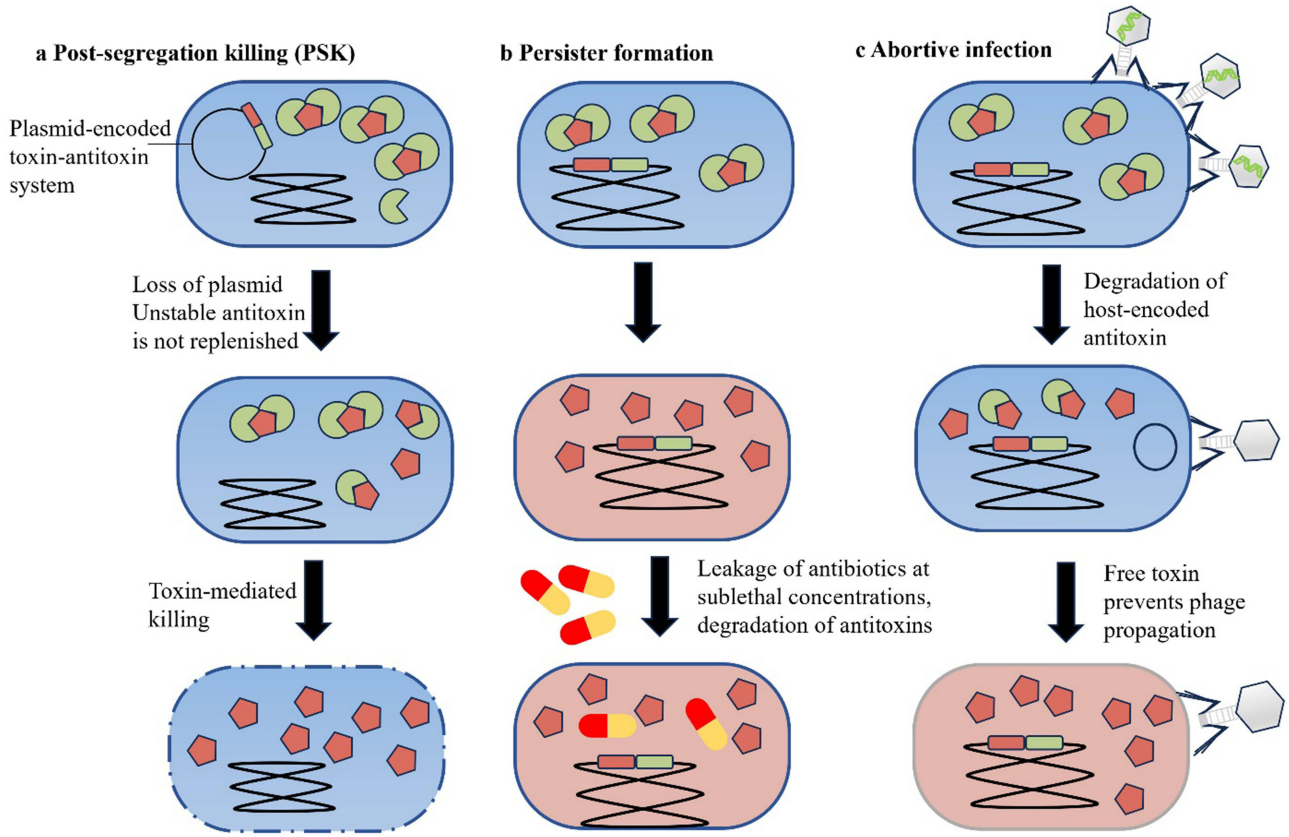

Figure 3 Shows the rationale for the role of TA modules in their biological functions. (**a**) Post-isolation killing mechanism, type II system-mediated plasmid addiction relies on differential stabilization between toxin (red) and antitoxin (green) proteins. When the unstable antitoxin is no longer replenished, the toxin will be released from the antitoxin-toxin complex. It will be able to kill these cells, thus contributing to plasmid maintenance. (**b**) In abortive infection, in phage-infected cells, transcription of host genes is repressed, the unstable antitoxin is not replenished, and the toxin will be released from the antitoxin-toxin complex and be able to prevent phage multiplication. (**c**) Persister formation, where persisters constitute a subpopulation of cells in the bacterial population that exhibit tolerance to antibiotics and other environmental stress conditions due to a phenotypic shift to a dormant state.

synthetase),[89,90] thereby reducing the amount of glutamine tRNA in the cell. The lack of glutamine-tRNA in cells triggers an increase in ppGpp (p), leading to the activation of more toxin-antitoxin modules, which are required for the HipA-induced persistence phenotype.[81] Mild overproduction of HipA at levels that do not impair cell growth results in increased levels of persistent protein.[91,92] HipA mutations have been shown to accumulate over time in clinical isolates of *E. coli* from patients with recurrent urinary tract infections.[93] In addition to these gain-of-function mutations, in many cases, artificial overexpression of the toxin results in elevated levels of persistence.[94]

Another related to persistence is the RelBE family, with the RelBE locus in *E. coli* encoding the antitoxin RelB and the toxin RelE. RelE belongs to a well-described superfamily of toxins with many homologs in both bacteria and archaea.[95] RelE is a ribonuclease that cleaves mRNA located at the ribosomal a-site between the second and third bases of the codon at the a-site.[40] RelB automatically regulates the transcription of the RelBE operon by combining the two operator sequences of the RelBE promoter region.[96,97] *E. coli* K-12 encodes six RelBE-like loci.[48,49,98–100] Several RelBE-like genes (for example, *mqsR* and *yoeB*) have been used to define the RelBE subfamily, and MqsRA and YafQ are shown to be associated with persistence.[101–103] Under non-stress conditions, the antitoxin MqsA regulates the general stress response by repressing the rpoS promoter. As MqsA is degraded by Lon, environmental stresses lead to decreased levels of MqsA, resulting in increased levels of RpoS, which increases biofilm formation and persistence.[104] In addition, it has been shown that the deletion of five or more type II toxin-antitoxin modules in *E. coli* results in reduced formation of holdout bacteria during exponential growth in enriched media.[105,106]

In addition, mathematical modeling indicated that the TA system is uniquely characterized by two distinct bacterial populations: a dormant population and a fast-growing type.[107] To put it simply, when the concentration of a toxin exceeds a certain threshold, the cell enters a state of continuous survival. How is this state achieved in a growing population of cells? One possibility is that certain cells, where nutrients are locally available, experience a state of micro starvation. This causes an increase in (p)ppGpp levels, which activates Lon protease and leads to antitoxin degradation. The result is a shift from low to high TA ratios, allowing the currently free toxins to begin to exert their toxic effects. However, while we have explained how cells enter the holding state at the molecular level, the molecular mechanisms that allow them to leave the holding state are still unclear. More importantly, recent studies have suggested that the type II TA system may utilize a phenomenological retention mechanism known as "conditional synergism".[73]

Stress Response and Biofilm Formation

Biofilms are formed in aquatic environments through bacterial attachment to underwater surfaces, air–liquid interfaces, and each other. Biofilms attach via appendages such as hyphae,[108,109] flagella,[110] and microcolonies are formed from microbial products including polysaccharides,[110] glycoproteins, and DNA.[111,112] *E. coli* biofilms form in the gastrointestinal tract and bladder of the human host. The pathogenic *E. coli* cause urinary tract infections, including cystitis (bladder infection) and pyelonephritis (kidney infection). The first TA system found to be associated with biofilm formation was the MqsRA system of *E. coli*, consisting of the MqsR toxin with the MqsA antitoxin. MqsR toxin is an RNase[49] that cleaves mRNA at the GUC site and requires the proteases Lon and ClpXP for its toxicity,[113] and the MqsA antitoxin binds to the toxin through its N-terminal structural domain and to DNA through the helix-turn-helix (HTH) motif in its C-terminal structural domain.[98] MqsR was induced in a transcriptomic study that identified differentially regulated genes in biofilm cells. In *E. coli*, it is predominantly curli,[114–116] which promotes biofilm formation, and the gene *csgD* is required for the secretion of curli fibers to the bacterial membrane surface. It has been shown that when well nourished, MqsA increases motility by increasing FlhD (a major regulator of motility) partly through rpoS31 inhibition and partly through *csgD* inhibition and that under stressful conditions, MqsA is degraded by proteolytic enzymes,[104] and MqsR is activated.[117] Degradation of MqsA leads to the de-suppression of RPO and *csgD*, as well as inhibition of FlhD, which subsequently increases biofilm.

The RelBE locus of *E. coli* encodes the RelE toxin and the RelB antitoxin. RelE is an mRNA enzyme that cleaves mRNA,[118] located at the ribosomal A site, including its mRNA, while RelB inactivates RelE by forming a tight complex with it. RelB is a metabolically unstable protein, whereas RelE is stable.[119] RelB can counteract RelE activity through protein–protein interactions. RelB also inhibits RelBE transcription, and RelE serves as a co-inhibitor of RelBE transcription.[120] It was shown that RelE encodes an integral translational repressor that is activated during amino acid

(aa) starvation. RelBE promoter is strongly activated during aa starvation, RelBE reduces the rate of post-starvation translation in amino acid-deficient *E. coli* wild-type cells, and glucose starvation induces RelBE transcription, suggesting a possible generalized role for RelBE during nutrient limitation. Induction of transcription occurs independently of RelA and SpoT and is instead dependent on Lon protease.[119]

The well-studied MazEF system is the first system described to regulate and be responsible for bacterial programmed cell death. MazF encodes the stabilizing toxin, MazF, and MazE encodes the unstable antitoxin, MazE. ATP-dependent ClpPA serine proteases can degrade MazE. MazF is a ribonucleic acid endonuclease that cleaves the mRNAs on the ACA sequence in a ribosome-independent manner.[7,121] When MazE is co-expressed with MazF, MazE counteracts the toxic activity of MazF, and since MazE is an unstable protein, constant production of MazE is necessary to prevent MazF from exerting itself. Therefore, in some stressful situations, if the expression of the MazEF module on the chromosome is impeded, the amount of MazE in the cell is reduced thus allowing the MazF toxin to be released. Examples include antibiotics that inhibit transcription and translation such as rifampicin, chloramphenicol, and macrolide and DNA damage induced by methotrexate or nalidixic acid. It was found these antibiotics and other stress conditions known to cause bacterial cell death act through the MazEF module.[122]

Defense Against Phage Infection

Bacteria are often infected by many phages, and host bacteria have evolved diverse molecular strategies in the race with phages, one of which is abortive infection (Abi). In TA-mediated abortive infection, phage infection triggers loss of antitoxin or release of toxin from the toxin-antitoxin complex, which inhibits phage multiplication (Figure 3c) by inhibiting basic cellular processes leading to toxin-mediated bacterial growth arrest.[123]

In 2004, Hazan and Engelberg-Kulka et al demonstrated that the type II chromosome MazF/MazE system inhibits phage P1.[124] MazEF TA is located on the relA operon and has all the characteristics of an "addiction module", including the ability to cause cell death. It was demonstrated that the *E. coli* MazEF system prevents phage P1 from entering its lysogenic phase. This phage exclusion is due to the activation of an altruistic cell death program that prevents phage propagation and thus protects the bacterial culture, additionally, the absence of MazEF produces more P1 phages; thus, the phage exclusion phenotype was validated without overproduction of this TA system. In a study by Abdulraheem M. Alawneh et al, it was demonstrated that the growth of T4 phage was significantly increased by disruption of MazE-MazF. Moreover, MazF was ribosylated by the T4 Alt protein ADP immediately after infection, and this modification led to a decrease in MazF RNA cleavage activity, which is the first example of chemical modification of a toxin to regulate its activity.[125] It has been reported that, in *E. coli* MC4100 and JM109, MazEF-containing cells produce 10–15-fold fewer phages after induction or infection with the mild phage p1. However, no clear data is reporting the mechanism of MazF activation, and a recent study was unable to reproduce these preliminary findings in *E. coli* K12.[126]

In 2011, the type II RnlA/RnlB system was shown to inhibit T4 phages in *E. coli*.[127] In most TA systems, the antitoxin genes are located upstream of their cognate toxin genes. Unlike most type II TA systems where the toxin genes are in the reverse order of the antitoxin genes, the RnlA/RnlB system provides T4 phages deficient for the gene dmd A strong defense against the dmd phage, while the aggregation efficiency of the dmd phage was reduced 1000–10,000-fold on rnlAN-containing strains.[128,129] Yonesaki et al initially identified the T4 gene dmd as necessary to stabilize mRNAs expressed late in T4 development.[130] Infection with the T4 dmd mutant results in rapid degradation of the antitoxin RnlB, which may lead to the release of the RnlA ribonuclease toxin.[127] RnlB is also rapidly degraded in a Lon and ClpX-dependent manner in uninfected cells. Because RnlB is intrinsically unstable, T4 infection-induced shutdown of host transcription, including that of RnlAB, may allow the release of active RnlA because RnlB cannot be replenished. When activated upon infection by the T4 dmd mutant, the RnlA toxin cleaves phage RNA,[130,131] although the sequence specificity of *RnlA* remains uncertain. Another twist to the RnlA system is that RnlA activity is somewhat dependent on host-encoded RNaseHI, which degrades RNA-DNA hybrids.[132,133] Indeed, the finding that RNaseHI promotes T4 infection upon RnlA activity and inhibition of RnlA by RnlB, possibly through direct interactions, but how RNaseHI affects the RnlAB system remains to be elucidated.

Recent bioinformatics analyses indicate that DarTG family members of TA systems are frequently encoded in defense islands, leading to the prediction that these systems are phage defense elements. Although *E. coli* K-12 does not encode

any DarTG homologs, representatives of two different subfamilies DarTG1 and DarTG2 from environmental *E. coli* isolates strongly protect *E. coli* MG1655 against different phages.[134] During phage infection, infection with RB69 or T5 phage, respectively, triggers the release of DarT toxin from both the DarTG1 and DarTG2 systems, followed by ADP-ribosylation of phage DNA. This modification results in the inhibition of DNA synthesis and, to a lesser extent, RNA synthesis, which prevents phages from generating viable progeny. In addition, DarG antitoxins were shown to possess ADP-glycohydrolase activity, which allowed them to enzymatically reverse modifications made by their cognate DarT toxin.[135] In addition, phages that have evolved to overcome the DarTG defense by mutations in their DNA polymerase or the anti-DarT factor gp61.2 encoded by many T-even phages were isolated.[136]

Interestingly, phages have developed many mechanisms to combat abortive infections by interfering with TA module induction or function. For example, phage T4 encodes a "master key" antitoxin that inactivates several RnlA family toxins, and the ADP-ribosyl transferase Alt inhibits the type II toxin MazF in *E. coli*. phages that often contain specific protease inhibitors that interfere with the degradation of protein antitoxins, thereby attenuating abortive infections of the type II TA module more indirectly.[123,131]

While some studies have shown that TA systems can and do prevent phage predation, it is not clear whether most TA systems play such a role. Notably, the RnlAB system only protects phages lacking dmd T4 phage, so other *E. coli* phage defense systems likely overcome the existing TA system in *E. coli* k12. Alternatively, the TA system may only protect a very narrow phage spectrum and is used for the great diversity of phages. Some of the phages defended against by individual TA systems in *E. coli* k12 may not have been identified or tested yet. Studies are needed to further explore and characterize the role of TA systems in phage defense. How TA systems impede phage replication and development also requires further investigation.

Conclusion Remarks

This paper focuses on the modulation and biological functions of the type II toxin-antitoxin system in *E. coli*. Despite 40 years of research on the TA system, and many questions remain unanswered. For example, there is still some ambiguity regarding biological function; the model describing how the toxin-antitoxin module leads to persistence formation through (p)ppGpp signaling is an attractive one; however, there is evidence that the toxin-antitoxin module is not the only factor contributing to persistent cell formation. The deletion of multiple toxin-antitoxin modules or the deletion of (p)ppGpp leads to a reduction in the number of persister cells. Still, neither eliminates the presence of persister cells. Deletion of 10 TA motifs in *E. coli* suggested a role in persistent formation, but subsequent reassessment did not support this conclusion. This problem may be attributed to infection by hidden phages.[137] Similarly, in another study, persister cell formation was not directly related to the TA module.[138] Thus, there is still a lot of debate about the role of the TA module in bacterial physiology, and it is necessary to carry out more extensive and precise experiments that should be conducted to further reveal the biological function of TA.

With the development of toxin-antitoxin systems, exploring how these systems move between replicons and bacterial species and what drives their evolutionary success is necessary. A better understanding of the mechanisms behind bacterial toxin-antitoxins may lead to the design of drugs that can combat chronic and recurrent infections. In addition, combining TA systems with other biological systems to develop novel bioregulatory elements will also hold promise in the field of synthetic biology.

Data Sharing Statement

No data was used for the research described in the article.

Ethical Statement

This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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Disclosure

The authors declare no conflicts of interest.

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