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Review

# Toxins, Targets, and Triggers: An Overview of Toxin-Antitoxin Biology

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**Bacterial toxin-antitoxin (TA) modules are abundant genetic elements that encode a toxin protein capable of inhibiting cell growth and an antitoxin that counteracts the toxin. The majority of toxins are enzymes that interfere with translation or DNA replication, but a wide variety of molecular activities and cellular targets have been described. Antitoxins are proteins or RNAs that often control their cognate toxins through direct interactions and, in conjunction with other signaling elements, through transcriptional and translational regulation of TA module expression. Three major biological functions of TA modules have been discovered, post-segregational killing (“plasmid addiction”), abortive infection (bacteriophage immunity through altruistic suicide), and persister formation (antibiotic tolerance through dormancy). In this review, we summarize the current state of the field and highlight how multiple levels of regulation shape the conditions of toxin activation to achieve the different biological functions of TA modules.**

Bacterial toxin-antitoxin (TA) modules encode a toxin that inhibits cell growth by interfering with vital processes and an antitoxin that protects the cell from the toxin (Page and Peti, 2016; Unterholzner et al., 2013). Four major types of these genetic elements have been described based on the nature of the antitoxin and how it inhibits the activity of the toxin protein (Figure 1). Antitoxins of type I and type III are RNA molecules that regulate the cellular levels of active toxin protein either by inhibiting translation of the toxin mRNA (type I) or through direct inhibition of the toxin protein (type III). In the other TA classes, the antitoxins are proteins that directly bind and inhibit the toxin protein (type II) or counteract it without direct interaction, e.g., by reversing its effect on the targets (type IV; for other recent reviews, see Page and Peti, 2016; Unterholzner et al., 2013). Two single instances of TA modules with regulatory principles different from types I–IV have been described as a type V and type VI TA module, respectively (see below).

Although TA modules are abundant in bacterial genomes and their molecular activities have been studied for decades, the biological function of most of them and its rooting in molecular mechanisms are poorly understood (Leplae et al., 2011; Van Melderen, 2010). Three major biological functions have so far been shown for TA modules: post-segregational killing (PSK; Gerdes et al., 1986b), abortive infection (Dy et al., 2014b), and persister formation/antibiotic tolerance (Harms et al., 2016). Although PSK and abortive infection are based on cell death through toxin activation upon loss of a mobile element or following infection by a bacteriophage, respectively, graded and controlled activation of toxins can protect bacteria from unfavorable environmental conditions and induce a transient state of dormancy that is the basis of the notorious antibiotic tolerance of bacterial persister cells. It is evident that the biology of TA modules has broad implications for public health and technology. PSK greatly contributes to the stability of bacterial plasmids, prophages, and other mobile elements that mediate the spread of antibiotic resistance and virulence genes in bacterial populations (Jensen and Gerdes, 1995). Furthermore, abortive infection not only protects bacteria from bacteriophage infection in the context of cultures grown for biotechnology but also interferes with the use of bacteriophages as therapeutic agents (“phage therapy”; Dy et al., 2014b). Finally, the recalcitrance of chronic infections such as urinary tract infections and tuberculosis has been linked to bacterial persistence controlled by TA modules (Harms et al., 2016; Page and Peti, 2016). It is thus important to understand the molecular mechanisms underlying the biological functions of TA modules to gain new options for intervention with clinical infections and develop new opportunities for the application of TA modules in health and technology.

In this review, we summarize the current state of the TA field and highlight the diversity and importance of regulatory features for TA module function. More specifically, we argue that regulatory features at the level of the toxin (target selection and specificity), the antitoxin (different modes of counteracting the toxin), and TA module expression (transcription/translation) all are key factors that translate the diverse molecular activities of TA module toxins into biological function.

## Molecular Biology and Evolution of TA Modules

### *Distribution and Abundance of TA Modules*

TA loci were initially discovered on bacterial plasmids and are now well known as an integral part of the mobilome, i.e., the pool of genetic elements that are frequently transferred horizontally (Gerdes et al., 1986b; Ogura and Hiraga, 1983). Comprehensive phylogenetic studies have shown that type II and type III TA loci are frequently associated with mobile elements and seem to be particularly prone to horizontal gene transfer (Blower et al., 2012; Goeders et al., 2016; Leplae et al., 2011). Conversely, type I TA loci appear to be mostly vertically

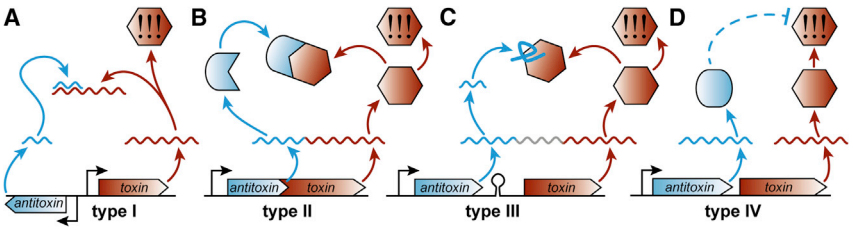


**Figure 1. The Four Main Types of TA Modules**
The illustration summarizes different modes of how toxins (red) are controlled by cognate antitoxins (blue) in type I–IV TA modules. Genetic loci and the positions of promoters are shown with colored and black arrows, respectively. RNAs are drawn as curly lines. Active toxin molecules that have been freed from antitoxin control are highlighted by exclamation marks.
(A) Type I TA module.
(B) Type II TA module.
(C) Type III TA module.
(D) Type IV TA module.
See also a more detailed illustration incorporating various levels of regulation in Figure 7.

inherited, but their seemingly narrow distribution in model organisms such as *Escherichia coli* or *Bacillus subtilis* may be biased because of the difficulty in reliably predicting these genetic elements due to their small size (Coray et al., 2017; Fozo et al., 2010). Recent work has indeed uncovered new families of type I TA modules in a wide range of bacterial genomes (Arnion et al., 2017; Coray et al., 2017; Fozo et al., 2010; Wen and Fozo, 2014).

TA loci of all types are also abundant on bacterial chromosomes (Coray et al., 2017; Fozo et al., 2010; Goeders et al., 2016; Leplae et al., 2011; Pandey and Gerdes, 2005). However, the number and composition of chromosomal TA loci vary considerably between different bacteria and even between closely related organisms. For example, *E. coli* K-12 MG1655, the main model organism of most studies on TA modules, encodes at least 19 type I TA loci, 13 type II TA loci, and three type IV TA loci (Figure 2). Conversely, a recent study counted 6 type I TA loci and 21 type II TA loci in the genome of its close relative *Salmonella enterica* Typhimurium (Lobato-Márquez et al., 2015). No clear links between the TA module repertoire and the biology of a given organism have been established. However, it appears that high numbers of TA loci often coincide with adaptation to hostile or dynamic environments and with frequent opportunities for horizontal gene transfer (Leplae et al., 2011; Pandey and Gerdes, 2005).

### Molecular Activities of Toxin Families and Important Examples

The toxins expressed from TA loci use a wide variety of molecular activities to interfere with cellular function to inhibit bacterial growth (Figure 3). Many toxins are able to cleave, degrade, or modify their cellular targets enzymatically and can thus obstruct bacterial physiology even at low protein concentrations. A wide variety of toxins are nucleases, comprising DNases like RalR (Guo et al., 2014), ribosome-dependent mRNA endonucleases like the RelE superfamily (Christensen and Gerdes, 2003; Pedersen et al., 2003), ribosome-independent mRNA endonucleases of the MazF/Kid, HicA, and SymE families (Jørgensen et al., 2009; Kawano et al., 2007; Zhang et al., 2003), and PilT N terminus (PIN) domain toxins of the VapC family that cleave tRNAs or rRNAs (Winther and Gerdes, 2011). Other toxins modify their cellular targets post-translationally, as exemplified by Doc, a kinase that targets the elongation factor EF-Tu (Castro-Roa et al., 2013), and HipA, which phosphorylates and inhibits the glutamyl-tRNA synthetase GltX (Germain et al., 2013). Further examples include toxins of the ζ/PezT family that phosphorylate and concomitantly inactivate a precursor of peptidoglycan synthesis (Mutschler et al., 2011), AMP transferases of the FicT family that inhibit DNA gyrase and topoisomerase IV (Harms et al., 2015), Gcn5-related N-acetyltransferase (GNAT) domain toxins with acetyltransferase activity targeting tRNA (Cheverton et al., 2016), and ADP-ribosyltransferases of the DarT family that modify single-stranded DNA (Jankevicius et al., 2016). Conversely, topoisomerase poisons of the CcdB and ParE families or the CbtA family toxins, which inhibit FtsZ and MreB polymerization, act by direct stoichiometric interaction with their targets at Achilles heels of bacterial physiology so that strong effects are achieved without enzymatic activity (Bernard and Couturier, 1992; Jiang et al., 2002; Masuda et al., 2012). A variety of small toxins, like those of the Hok family, function by depolarizing the bacterial membrane to abrogate the proton-motive force and shut down ATP synthesis (Gerdes et al., 1986a; Verstraeten et al., 2015). Interestingly, several major toxin superfamilies comprise subfamilies that exhibit major functional differences. Most prominently, the RelE family of mRNA endonucleases share the same fold with the ParE family of gyrase poisons, and the MazF family of ribosome-independent mRNA endonucleases is evolutionarily related to the CcdB gyrase poison (Anantharaman and Aravind, 2003; Dalton and Crosson, 2010; Hargreaves et al., 2002).

The various toxin families are not evenly distributed between TA module types, but, instead, some functional classes appear to prevail among certain types of TA modules. For example, small membrane-targeting peptide toxins have repeatedly evolved for type I TA modules, where they constitute the most abundant type of toxin, whereas they are not found in type II/III/IV TA modules (Arnion et al., 2017; Wen and Fozo, 2014). Well known type I TA modules with membrane-targeting toxins in *Escherichia coli* K-12 are *hok/sok* or *tisB/istR-1*, and RalR of *ralRA* (a DNase) or SymE of *symER* (an mRNA endonuclease) are examples for other forms of type I toxins that have been studied in this organism (Figure 2; Berghoff and Wagner, 2017; Guo et al., 2014; Kawano et al., 2007). Many different types of toxins are found among type II TA modules, but enzymes, and particularly RNases, dominate (Masuda and Inouye, 2017; Page and Peti, 2016). As an example, 10 of the 13 type II TA module toxins found in *E. coli* K-12 are mRNA endonucleases, with seven having a RelE family toxin (*relBE*, *yefM/yoeB*, *yafNO*, *dinJ/yafQ*, *higBA*, *prlF/yhaV*, and *mqsRA*), two having a MazF/Kid family toxin (*mazEF*, *chpSB*), and one

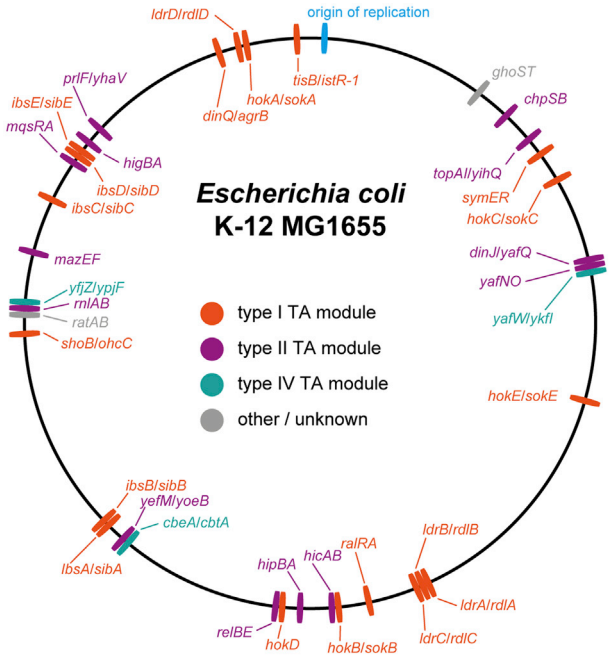


**Figure 2. TA Modules of *Escherichia coli* K-12**

The chromosomal loci of all known TA modules encoded by the model organism *E. coli* K-12 MG1655 are shown with a color code highlighting type I (orange), type II (purple), and type IV (cyan) TA modules. Most TA loci have been annotated in the current version of the MG1655 genome sequence (GenBank: U00096.3), and others were added based on the literature. Note that the majority of known TA modules belong to type I (19 loci), although those of type II (13 loci) have been studied in more detail. No type III TA module and three paralogous type IV TA modules have been described in *E. coli* K-12.

being a *hicAB* module encoding a HicB family toxin (Leplae et al., 2011; Figures 2 and 3). Furthermore, the three known families of type III TA modules (*toxIN*, *cptIN*, and *tenpIN*; all absent in *E. coli* K-12; Figure 2) were described to encode toxins with a fold related to that of MazF/Kid family mRNA endonucleases (Blower et al., 2012; Goeders et al., 2016). Only two families of type IV TA modules are known, represented by *cbeA/cbtA* of *E. coli* K-12 (targeting FtsZ and MreB polymerization) and *abiEi/ii* of *Streptococcus*, with a predicted nucleotidyltransferase toxin (Dy et al., 2014a; Masuda et al., 2012). In summary, toxins encoded by TA modules use a wide variety of molecular mechanisms to inhibit cell growth, and most of these have been well characterized.

## Biological Activities of TA Modules

Conversely, the biological function of most specific TA loci is not known, even in well-studied model organisms like *E. coli* K-12 where, e.g., the molecular basics of the 19 type I TA modules have been studied in considerable detail, but the biology of all except two or three has remained elusive (see below and Figure 2; Berghoff and Wagner, 2017; Wen and Fozo, 2014). Multiple biological functions of TA modules have been proposed and discussed in the literature (Magnuson, 2007; Van Melderen, 2010), but only three major ones are well supported by experimental data. These are the stabilization of mobile elements through PSK, the abrogation of bacteriophage infections through altruistic suicide ("abortive infection"), and the formation of dormant, antibiotic-tolerant cells known as persisters (Figure 4).

### *Post-segregational Killing*

Type I and type II TA loci were initially discovered as encoding "addiction modules" that could prevent the loss of plasmids from bacterial cultures through a mechanism known as PSK (Figure 4A). Well characterized examples include *hok/sok* of plasmid R1 (type I) and *ccdAB* of plasmid F (type II), which are both found in *E. coli* (Gerdes et al., 1986b; Ogura and Hiraga, 1983). PSK relies on the differential stability of short-lived antitoxin and stable toxin molecules that strongly reduce the appearance of plasmid-free cells by causing the death of plasmid-free offspring that are unable to continuously express the labile antitoxins (Jensen and Gerdes, 1995; Van Melderen et al., 1994). Apart from *hok/sok* and *ccdAB*, well studied PSK loci include the type II TA modules *parDE* of plasmid RK2 and *kis/kid* (also known as *pemIK*, homologous to *mazEF*) of plasmid R1 (Roberts et al., 1994; Tsuchimoto et al., 1988). Similarly, several type III TA loci and the *abiEi/ii* type IV TA locus were shown to stabilize model replicons (Dy et al., 2014a; Rao et al., 2015; Short et al., 2013). PSK is not restricted to plasmids but also acts in the same way to stabilize mobile elements and other genetically unstable regions in bacterial chromosomes (Szekeres et al., 2007; Wozniak and Waldor, 2009).

### *Abortive Infection*

Abortive infection is a mechanism of bacterial innate immunity that impairs bacteriophage propagation in a bacterial population through altruistic suicide of infected cells via, e.g., TA module activation prior to phage replication (Dy et al., 2014b; Figure 4B). For example, the *rnlAB* type II TA locus of *E. coli* K-12 was found to abort infections of bacteriophage T4 (Koga et al., 2011), and type II TA module *mazEF* as well as the *hok/sok* type I TA locus of plasmid R1 at least significantly obstructed phage T4 infections (Alawneh et al., 2016; Pecota and Wood, 1996). Similarly, the type IV TA module *abiEi/ii* and its relative *sanaTA* were also shown to be potent abortive infection systems (Dy et al., 2014a, 2014b; Sberro et al., 2013). Abortive infection also appears to be the primary biological function of type III TA modules, such as those of the *toxIN* and *tenpIN* families, which are known to abrogate productive infection of bacteriophages targeting a wide range of bacteria, including *Lactococcus lactis*, *Photorhabdus luminescens*, and *E. coli* (Goeders et al., 2016).

Interestingly, bacteriophages have developed a number of mechanisms to counter abortive infection by interfering with TA module induction or function. For example, bacteriophage T4 encodes both a "master key" antitoxin that can keep several RnlA family toxins inactive and the ADP-ribosyltransferase Alt inhibits the type II toxin MazF of *E. coli* (Alawneh et al., 2016; Otsuka and Yonesaki, 2012). Bacteriophages also often harbor specific protease inhibitors that can interfere with the degradation of protein antitoxins and, thus, impair abortive infection by type II or type IV TA modules in a more indirect fashion (Sberro et al., 2013).

### *Bacterial Persistence*

Persisters constitute a subpopulation of cells in a bacterial population that exhibit tolerance to antibiotics and other environmental stress conditions because of phenotypic transition into

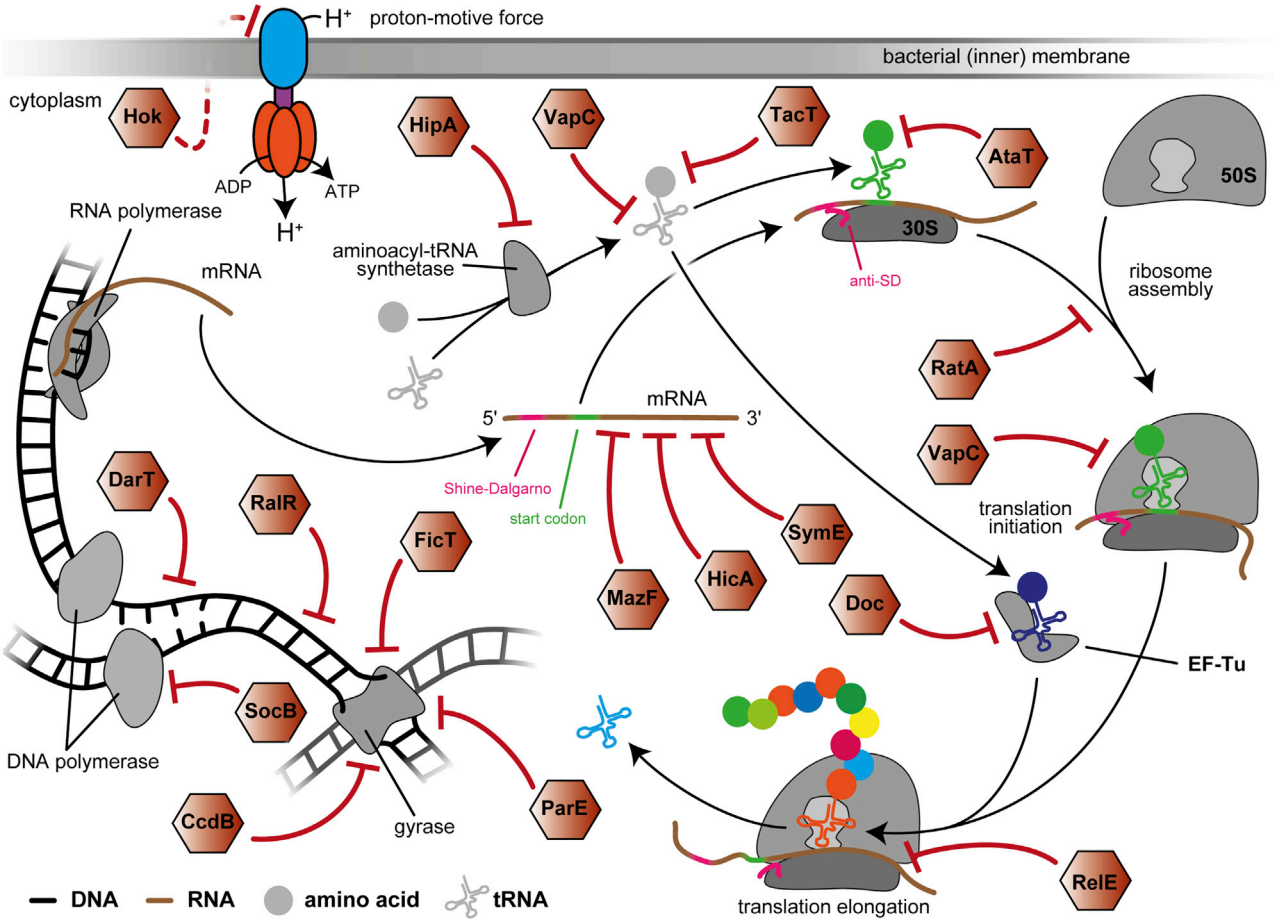


**Figure 3. Molecular Activities of TA-Encoded Toxins**
The illustration shows how the molecular activities of selected TA-encoded toxins interfere with vital processes of bacterial cells. Note that the majority of toxins inhibit translation (right), whereas other processes are targeted by a few families only (left).

a dormant state in which the cellular processes commonly poisoned by bactericidal antibiotics are inactive (Harms et al., 2016; Page and Peti, 2016; Figure 4C). The induction or modulation of this phenotypic conversion of a subpopulation in response to many types of stress, like nutritional starvation or exposure to sub-lethal concentrations of antibiotics, has been described as responsive diversification (Kotte et al., 2014). Furthermore, persister formation also contains a stochastic component that causes phenotypic heterogeneity in clonal populations as a risk-spreading strategy and, thus, promotes survival in dynamic environments, a phenomenon known as bet hedging (Veening et al., 2008).

Several genetic pathways govern the formation of persister cells by controlling the phenotypic transition into dormancy. It is intuitive that activation of TA toxins can serve as such a phenotypic switch by inhibition of vital cellular processes, and a persister-like state is readily reproduced in the laboratory upon ectopic expression of both type I (e.g., TisB and HokB) or type II (e.g., HipA or mRNA endonucleases like RelE and MazF) toxins (Dörr et al., 2009; Keren et al., 2004; Maisonneuve et al., 2011; Pedersen et al., 2002; Verstraeten et al., 2015). Consistently, several independent studies have found that the expression from TA loci is strongly induced in experimentally isolated persister cells of *E. coli* K-12 (Keren et al., 2004, 2011; Shah et al., 2006), and the first known *E. coli* mutant producing highly elevated persister levels, *hipA7*, carried two mutations in the HipA toxin that increase the basal level of toxin activity (Moyed and Bertrand, 1983; Schumacher et al., 2015). Recent work has also demonstrated that activation of *hokB/sokB* and *tisB/istR-1*, each in response to specific upstream signaling, causes the formation of *E. coli* persister cells through membrane depolarization by their small peptide toxins (see below; Dörr et al., 2010; Verstraeten et al., 2015). Furthermore, the inactivation of type II TA modules like *hipBA*, *relBE*, or *mqsRA* was shown to cause defects in persister formation or survival of *E. coli* K-12 under different conditions (Harrison et al., 2009; Keren et al., 2004; Wu et al., 2015). The important role of type II TA modules for the formation of antibiotic-tolerant persisters was confirmed *in vivo* using animal infection and treatment models of uropathogenic *E. coli* and *Salmonella enterica* Typhimurium (Helaine et al., 2014; Norton and Mulvey, 2012).

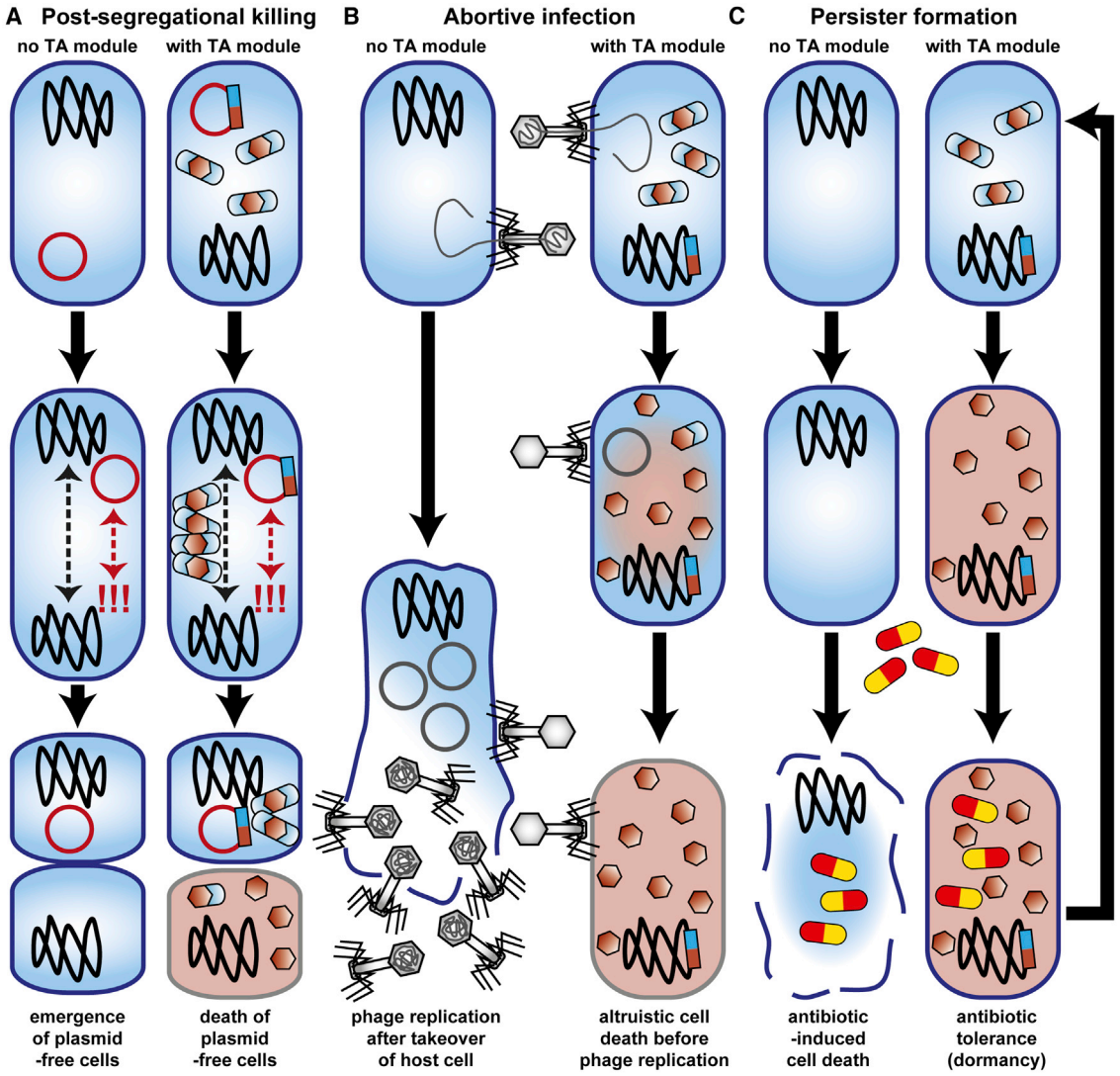


**Figure 4. Biological Functions of TA Modules**

Shown are the principles underlying the role of TA modules in their biological functions. Each schematic compares the fate of a bacterial cell without (left) or with (right) a TA module that acts in the respective biological context. Toxin genes/proteins are shown in red and antitoxins in blue. The obstruction of cell functioning by TA module toxins is highlighted by red coloring of the cytoplasm. Note that TA module activation causes cell death in the context of PSK and abortive infection but results in reversible dormancy during persister formation.

(A) PSK after loss of a plasmid (red circle).

(B) Abortive infection.

(C) Persister cell formation.

## Links between Molecular Activities and Biology of TA Modules

Despite significant efforts, it has largely remained mysterious how the molecular features and activities of TA modules are translated into biological function. Basically, there appear to exist no trivial links such that certain families of toxins or groups of TA modules would primarily act in particular biological contexts. On the contrary, it seems that a given TA module chassis can be differentially adapted to divergent biological functions. For example, representatives of both the *hok/sok* (type I) and the *phd/doc* (type II) families have been shown to act either in PSK or in bacterial persistence. Although *hok/sok* of plasmid R1 and *phd/doc* of the P1 prophage are known to act in PSK (Gerdes et al., 1986a; Lehnherr et al., 1993), *hokB/sokB* of *E. coli* K-12 and *phd/doc* of *Salmonella enterica* Typhimurium are involved in persister formation (Helaine et al., 2014; Verstraeten et al., 2015). Similarly, the *tacAT* type II TA module of *S. enterica* Typhimurium was shown to contribute to persister formation, whereas its homolog *gmvAT* on the virulence plasmid of *Shigella* is a potent PSK module (Helaine et al., 2014; McVicker and Tang, 2016).

Furthermore, it has been observed that plasmid-encoded TA loci of any type and family are often able to mediate PSK, whereas their direct homologs on bacterial chromosomes

usually perform poorly in plasmid stabilization experiments. For example, the *ccdAB* locus of the *E. coli* F-plasmid is known to be a potent PSK module (Ogura and Hiraga, 1983), whereas the chromosomal *ccdAB* locus of *E. coli* O157:H7 could not mediate PSK on a model replicon that was readily stabilized by its F-plasmid homolog (Wilbaux et al., 2007). Similarly, the chromosomal *hok/sok* loci of *E. coli* K-12 failed to mediate PSK, unlike their homolog of plasmid R1 (Gerdes et al., 1986b; Pedersen and Gerdes, 1999), but one of them, *hokB/sokB*, is instead known to have a function in persister formation (Verstraeten et al., 2015).

On a different level, another dividing line seems to run between TA loci mediating persister formation and those that act in PSK or abortive infection. Although functional overlap between persister formation and other functions is rare, a considerable number of TA loci have been shown to be proficient in both PSK and abortive infection, like *hok/sok* of plasmid R1, several type III TA loci, and the *abiEi/ii* type IV TA locus (Dy et al., 2014a; Pecota and Wood, 1996; Rao et al., 2015; Short et al., 2013). This discrepancy correlates well with the difference between inducing either a transient switch into dormancy (for persister formation) or cell death (for PSK and abortive infection), suggesting that it is linked to trade-offs in the tuning of TA module activities. However, the line between chromosomal and plasmid-encoded TA modules and the one between persister formation and PSK/abortive infection is not always sharp. As an example, the chromosomal *relBE* locus of *E. coli* K-12, known to act in persister formation (see above), readily stabilized a model replicon similar to a naturally plasmid-encoded homolog (Gotfredsen and Gerdes, 1998; Grønlund and Gerdes, 1999). However, *relBE* is encoded inside the cryptic prophage Qin within the *E. coli* K-12 genome and, thus, likely has a more recent mobile history than other chromosomal TA modules.

Taken together, the biological function of TA modules does not appear to be primarily determined by the molecular identity of its toxin and antitoxin components but, rather, controlled by additional regulatory features that manage the toxin's activity toward accomplishing one of the various biological functions. These regulatory features impose constrains on the biology of TA modules, and no TA locus is known to be proficient in all three major biological functions that have been described. One example of the biological background of such constrains is the varying autonomy of different TA modules from cellular signaling. On one hand, the common association of TA modules with mobile elements favors strong autoregulation, which enables a principle of "plug and play" in different host organisms. On the other hand, it is intuitive that the regulation of TA modules recruited for bacterial stress responses like persister formation needs to be wired to cellular signaling pathways. In the following sections, we summarize the knowledge of these two layers of TA system regulation and highlight how they link the molecular properties of toxin and antitoxin with biological functions.

## Toxin Target Specificities

Despite displaying a wide variety of molecular activities, the majority of TA module toxins target translation in one way or another (Figure 3). The reason for this preference over inhibition of other vital processes, like DNA replication or transcription, has not been clearly resolved. One appealing possibility is that toxins acting on the most downstream central dogma processes may be less prone to cause irreversible damage upon accidental activation. For example, the depletion of mRNA, tRNA, or rRNA is easily replenished by renewed transcription. In fact, it has been proposed that the abundance and diversity of toxins targeting translation are a result of this reversibility inherent to many translation inhibitors, which loosens the requirement for regulatory control and allows these TA modules to evolve more freely (Guglielmini and Van Melderen, 2011). Consistently, many TA modules are known to include additional direct or indirect mechanisms that ensure the reversibility of their toxins' activities (see below).

Experimental analyses of several representatives from a given toxin family have often revealed significant differences in target specificity. For example, two recent studies characterized GNAT domain toxins that inactivate tRNAs by acetylation. Although TacT of *Salmonella* Typhimurium targets a wide range of tRNAs, AtaT of *E. coli* O157:H7 was found to be specific for the initiator $tRNA^{fMet}$ (Cheverton et al., 2016; Jurėnas et al., 2017; Figure 3). Similarly, several representatives of the VapC RNase toxin family were shown to cleave unique tRNA and rRNA species at specific positions (Winther et al., 2016; Figure 3). Such selective modification of the tRNA pool would strongly affect codon usage and could potentially either broadly inhibit protein synthesis (if the initiator tRNA or rRNA are inactivated) or differentially suppress expression of parts of the proteome. Although the biological implications of these divergent substrate specificities have not been studied in detail, it is tempting to speculate that the startling diversity of around 50 VapC toxins in *M. tuberculosis* may contribute to the heterogeneity of persister cells formed by this notorious chronic pathogen (Ramage et al., 2009; Winther et al., 2016).

Similar to the VapC toxins that inactivate tRNAs, the endonuclease toxins that target mRNA exhibit considerable differences with respect to the consensus sequences that are cleaved (comprehensively reviewed by Masuda and Inouye, 2017). Toxins of the MazF/Kid family have been most broadly characterized, revealing that representatives of these ribosome-independent mRNA endonucleases recognize signatures with differing sequences and lengths varying from three to seven nucleotides, meaning that some MazF/Kid toxins would largely abrogate translation, whereas others only suppress a rather specific set of mRNAs (Masuda and Inouye, 2017). As an example of the latter, the Kis/Kid module of plasmid R1 appears to function as a plasmid rescue system that activates the Kid toxin upon plasmid destabilization to degrade specific mRNAs and inhibit cell division to promote plasmid replication, enforcing plasmid retention beyond classical PSK (Pimentel et al., 2014). A similar case of divergent recognition sequences is seen for various representatives of the RelE family of mostly ribosome-dependent mRNA endonucleases, but their cleavage specificities have been studied in much less detail (Masuda and Inouye, 2017).

In summary, we argue that the specificity of toxins for certain (subgroups of) targets provides a first level of regulation that can evolve to enable different biological activities by differentially interfering with cellular processes.

## Direct Control of Toxins by Cognate Antitoxins

The direct interaction of toxins and antitoxins is the central mechanism of toxin control for type II and type III TA modules that encode antitoxins acting as protein or RNA, respectively (Figure 1). Interestingly, although the RNA antitoxins of the different type I or type III TA modules form rather homogeneous groups (Goeders et al., 2016; Wen and Fozo, 2014), type II TA module antitoxins use a variety of unrelated protein folds to inhibit their cognate toxins. As an example, the antitoxins of the seven RelE family mRNA endonuclease toxins of *E. coli* K-12 belong to five different families (Anantharaman and Aravind, 2003; Brown et al., 2009; Leplae et al., 2011). Evolution thus appears to have resulted in a ''mix and match'' of toxin and antitoxin superfamilies so that representatives of a given toxin family are able to form functional type II TA modules with representatives of several antitoxin families and vice versa, but the effect of this phenomenon on TA module biology has not been studied (Arbing et al., 2010; Guglielmini and Van Melderen, 2011). In the following sections, we first summarize the key principles of direct regulation of toxins by their antitoxins before describing regulatory mechanisms based on TA module expression and post-translational mechanisms beyond direct inhibition of toxins.

### *Type II TA Modules*

Type II antitoxins are usually composed of two separate domains, an N-terminal DNA-binding domain that is critical for transcriptional autoregulation and a C-terminal domain that directly binds and inactivates the toxin (recently reviewed by Chan et al., 2016; Goeders and Van Melderen, 2014). Frequently observed key principles of this direct inactivation are interference with catalysis at the toxin's active site (e.g., for RelBE, MazEF, and VapBC complexes of *E. coli*, as shown in Figures 5A–5C; Bøggild et al., 2012; Dienemann et al., 2011; Kamada et al., 2003; Takagi et al., 2005) and steric obstruction of target binding (e.g., for CcdBA; De Jonge et al., 2009).

Activation of type II TA complexes is triggered by cellular proteases that degrade the antitoxin to physically liberate and, thus, activate the toxin as well as, in most cases, upregulate expression of the TA locus because of loss of negative transcriptional autoregulation (see below). In *E. coli*, most antitoxins are degraded by the Lon protease, whereas some are targeted by ClpP with the help of its adapters ClpA or ClpX, which can provide a first level of differential regulation (recently reviewed by Muthuramalingam et al., 2016). Antitoxins may be targeted to these proteases simply by intrinsic instability and unfolded regions so that, e.g., most type II antitoxins of *E. coli* K-12 have rather short half-lives of 15–20 min, which enables a fast response to changing cellular conditions (Goeders and Van Melderen, 2014). This differential stability of toxin and antitoxin is critical for the biological activity of all TA modules, but in particular for those that elicit cell death via PSK and abortive infection (Jensen and Gerdes, 1995; Van Melderen et al., 1994). Activation of type II TA modules involved in abortive infection has been mostly studied using the *E. coli* K-12 RnlAB model system, a type II TA module with an unspecific RNA endonuclease toxin that aborts infection of bacteriophage T4 by degrading phage mRNAs (Koga et al., 2011). Similar to the paradigm of PSK, the half-life of the RnlB antitoxin is only a few minutes and, thus, more than ten times shorter than the half-life of the RnlA toxin, so that phage T4 infection, characteristically shutting off host gene expression, results in RnlB degradation and, consequently, activation of RnlA (Koga et al., 2011).

Type II TA modules acting as effectors of bacterial persister formation are usually wired to specific cellular signaling that controls the activation of proteases for antitoxin degradation (Figures 1 and 7B). Several TA loci of *E. coli* K-12 have been shown to be individually important for persister formation under specific conditions. Although *yafQ* mutants displayed a substantial defect in persister formation only in bacterial biofilms, mutants in *hipBA* and *relBE* were both shown to be impaired in stationary phase persister formation (Harrison et al., 2009; Keren et al., 2004). Besides individual TA loci, the entire set of the type II mRNA endonuclease TA modules of *E. coli* K-12 was proposed to mediate persister formation in response to stochastic pulses of the second messenger guanosine tetra- and penta-phosphate ((p)ppGpp) and consequent Lon activation (reviewed by Maisonneuve and Gerdes, 2014). However, the contribution of these TA modules to *E. coli* K-12 persister formation under unstressed conditions has recently been questioned (Harms et al., 2017). In *Salmonella enterica* Typhimurium, persister formation via type II TA module activation is induced upon phagocytosis by macrophages through vacuole acidification and starvation-induced (p)ppGpp signaling (Helaine et al., 2014).

### *Type III TA Modules*

All known type III TA modules encode an mRNA endonuclease toxin with a fold related to that of the MazF/CcdB family and an RNA antitoxin in a bicistronic operon with a transcriptional terminator that insulates the downstream toxin gene from the high levels of upstream antitoxin expression (Figure 1; Goeders et al., 2016). Characteristically, type III antitoxin genes contain several repeats (three on average) that are post-transcriptionally processed into active single units through cleavage by their cognate RNase toxin (Blower et al., 2012). Several layers of antitoxin-mediated control have been unraveled in the *toxIN* model system of *Pectobacterium atrosepticum*. The *toxI* antitoxin RNA protects housekeeping RNAs from spurious toxin activity because it is a preferred substrate of the ToxN toxin, and each *toxI* transcript is cleaved into several single repeat units that inhibit ToxN in a 1:1 stoichiometry (Short et al., 2013). Structural studies have demonstrated that ToxIN forms a triangular complex composed of three ToxN units connected by three *toxI* RNA pseudoknots that cover the ToxN active sites (Figure 5D; Blower et al., 2011a). This overall arrangement was also found for the distantly related CptIN type III complex, suggesting that it is a common feature of this family of TA modules (Rao et al., 2015).

Given that RNA antitoxins of type III TA modules seem to have a shorter half-life than their cognate toxins even in the absence of a stress-induced signal (Fineran et al., 2009), the PSK observed for several type III TA modules may simply be a result of the differential stability of toxin and antitoxin, similar to the setup of PSK by type I and type II TA modules (Gerdes et al., 1988; Van Melderen et al., 1994). Conversely, it is unknown how bacteriophage infection activates type III TA modules to trigger abortive infection. The fact that type III TA antitoxins are RNAs (and not proteins) was proposed to allow for increased sensitivity to disruption of cellular homeostasis by efficiently sensing phage

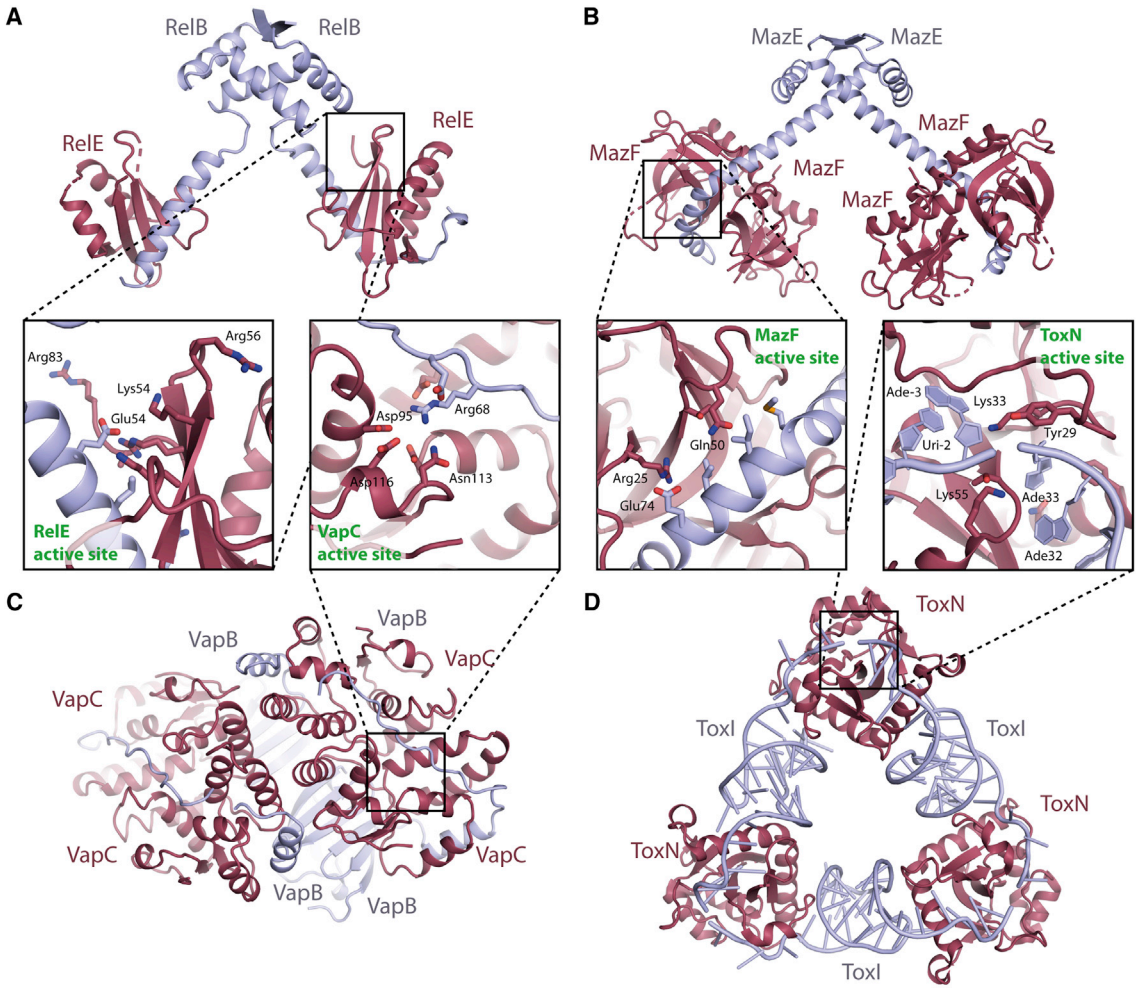


**Figure 5. Direct Control of Type II and Type III Toxins by Their Antitoxins**
The molecular basis of toxin inhibition by selected type II and type III antitoxins is shown, based on crystal structures of RelBE (type II), MazEF (type II), VapBC (type II), and ToxIN (type III). For each TA complex, the toxin is shown in red and the antitoxin in blue. The boxes show details of the TA interactions around the toxin active site, with relevant amino acids highlighted.
(A) RelBE (4FXE of Bøggild et al., 2012).
(B) VapBC (5K8J of Bendtsen et al., 2017).
(C) MazEF (4ME7 of Simanshu et al., 2013).
(D) ToxIN (2XDD of Blower et al., 2011a).

infection through a general shutdown of host gene expression (Short et al., 2013). Alternatively, it has been suggested that dissociation of the complex and activation of the type III TA module could be induced by a still unknown trigger like, e.g., small molecules that may be indicators of phage infection (Blower et al., 2011a, 2011b).

In summary, type II and type III TA module antitoxins inhibit their toxins in tight complexes through direct interactions that usually interfere with catalysis at the active site of (enzymatic) toxins and/or block target binding.

## Transcriptional Regulation of TA Modules

### *Integration into Cellular Signaling Pathways*

A prime example of the integration of TA modules into cellular signaling pathways is the SOS response, a transcriptional program induced by single-stranded DNA based on the gradual inactivation of the LexA repressor with increasing levels of DNA damage (Baharoglu and Mazel, 2014). Six TA loci of *E. coli* K-12 have LexA boxes in their promoters; namely, the *tisB/istR-1*, *dinQ/agrB*, *symE/symR*, and *hokE/sokE* type I TA modules and the *dinJ/yafQ* and *yafNO* type II TA modules (Figure 2; see Dörr et al., 2010, and Berghoff and Wagner, 2017, for a summary). For the type II TA loci, the LexA boxes are positioned in front of the operon formed by the toxin and antitoxin gene so that both would be de-repressed upon DNA damage and additional mechanisms to enforce antitoxin degradation would thus be required for TA module activation. However, the control of type II TA modules by SOS signaling and the possible biological implications have not been extensively studied. Instead, several reports have shown that a wide range of stress

conditions, like exposure to acidic environments and starvation for glucose or amino acids, can specifically induce the transcription of different type II mRNA endonuclease TA loci in *E. coli* K-12 (Christensen-Dalsgaard et al., 2010; Shan et al., 2017). Given that the transcriptional induction of these loci is often a consequence of antitoxin degradation (see below), these results may suggest stress-dependent activation of cellular proteases to target specific sets of antitoxins. As an example, the MqsA antitoxin is usually a stable protein but is quickly degraded by Lon under conditions of oxidative stress (Wang et al., 2011).

For the type I TA loci under SOS control, LexA controls transcription of the toxin gene only, and a series of studies elegantly showed that sub-lethal levels of DNA damage induce persister formation via the SOS response by selectively inducing transcription of the TisB toxin (Dörr et al., 2009, 2010). Similarly, the DinQ toxin is also activated by SOS signaling and may contribute to persister formation under some circumstances, but it seems to primarily control nucleoid compaction and DNA repair (Berghoff and Wagner, 2017).

Apart from the SOS response, cellular signaling downstream of the second messenger (p)ppGpp in *E. coli* K-12 was shown to control not only the activation of mRNA endonuclease type II TA modules (see above) but also the *hokB/sokB* type I TA module. A seminal study recently found that, similar to the induction of *tisB* by the SOS response, transcription of *hokB* is induced by (p)ppGpp signaling in a way that is dependent on the ribosome-associated guanosine triphosphate hydrolase (GTPase) Obg (Verstraeten et al., 2015). This induction of *hokB* transcription tips the balance of type I TA translational control toward toxin production (see below) and, consequently, results in the formation of persister cells through membrane depolarization (Verstraeten et al., 2015).

### *Transcriptional Autoregulation of Type II TA Modules*

Transcriptional autoregulation of TA modules is particularly prevalent among type II TA modules and has been extensively studied, revealing several regulatory principles that support the biological functions of TA modules by controlling when, how intensively, and how long they are induced. Type II TA loci are typically expressed from a single promoter upstream of the two genes that is transcriptionally repressed by the binding of antitoxin and, in many cases, TA complexes to an operator element (comprehensively reviewed by Page and Peti, 2016).

The most prevalent mode of autoregulation among well studied type II TA modules is known as "conditional cooperativity" (Afif et al., 2001; Garcia-Pino et al., 2010; Overgaard et al., 2008), and we chose this phenomenon as an example of how biological functions can be linked to transcriptional autoregulation on the molecular level. It is the core of conditional cooperativity that the level of transcriptional auto-repression depends on the ratio of toxin and antitoxin in TA complexes because the toxin acts at a co-repressor at low T:A ratios (by promoting cooperative auto-repression) and as a de-repressor at high T:A ratios (by interfering with auto-repression; Figure 6A). At the molecular level, conditional cooperativity is achieved by the formation of TA complexes with different stoichiometries that exhibit different affinities for their corresponding operator sequences. Figure 6B illustrates the principles of conditional cooperativity based on the example of *E. coli relBE* (Bøggild et al., 2012; Overgaard et al., 2008), but transcriptional autoregulation via conditional cooperativity has also been observed for several other type II TA modules, such as *ccdAB*, *kis/kid*, or *phd/doc* (Afif et al., 2001; Garcia-Pino et al., 2010; Monti et al., 2007). However, the underlying molecular mechanisms exhibit significant differences, indicating that conditional cooperativity has evolved independently multiple times (Loris and Garcia-Pino, 2014; Page and Peti, 2016).

It is difficult to directly address the biological functions of conditional cooperativity, but mathematical modeling based on biochemical studies and structures of TA complexes supports proposed links to the biology of TA modules. For example, the strong repression observed at a low T:A ratios guarantees a low level of TA locus expression under regular growth conditions and saves metabolic energy (Gelens et al., 2013) while sequestering toxins in TA complexes in an inactive form (Figure 6A; Cataudella et al., 2013). A significant amount of toxin can therefore be released immediately upon TA module activation, which may be important for PSK or in response to sudden starvation. This feature is not limited to the conditional cooperativity scheme, however, but shared with other TA modules that are auto-repressed by TA complexes independent of the T:A ratio (Feng et al., 2014; Lou et al., 2008). Conditional cooperativity additionally has the ability to buffer the repression of TA locus expression against cellular noise. When the T:A ratio of a TA module rises, transcription of TA mRNA is de-repressed. Because translation is usually biased toward producing an excess of antitoxin molecules (Li et al., 2014), elevated transcription decreases the T:A ratio and restores repression so that conditional cooperativity reduces the fortuitous activation of TA modules (Figure 6A; Cataudella et al., 2012). The switch between repression and de-repression of TA module expression controlled by conditional cooperativity is very sharp, especially when transcriptional repression is exerted cooperatively through several operator sites (Vandervelde et al., 2017). Furthermore, conditional cooperativity creates a barrier between states of low and high T:A ratios that may cause bistability between a growing and a dormant state of bacterial cells and can explain the characteristic switch-like behavior of TA module activation (Figure 6A; Cataudella et al., 2013). Switching into the toxin-dominated state with very high T:A ratios can be caused by antitoxin degradation in response to specific cellular signaling induced by certain triggers or randomly as part of a bet hedging strategy (Cataudella et al., 2013; Gelens et al., 2013; Tian et al., 2017). Antitoxin degradation robustly transforms the cell into a toxin-dominated, non-growing state because it raises the T:A ratio, increases TA locus expression, and, thus, amplifies the amount of free toxin. When antitoxin degradation ceases, strong expression of the TA locus results in a rapid increase in antitoxin molecules that inactivate the free toxins and restore transcriptional repression (Figure 6A; Cataudella et al., 2012). Consequently, conditional cooperativity may also support the resuscitation of persister cells.

Interestingly, several antitoxins of type II TA modules have also been shown to serve as transcriptional regulators of genes beyond their cognate TA loci, the transcription of which would thus also be induced upon antitoxin degradation. Examples of this phenomenon include *E. coli* K-12 HipB and MqsA, which

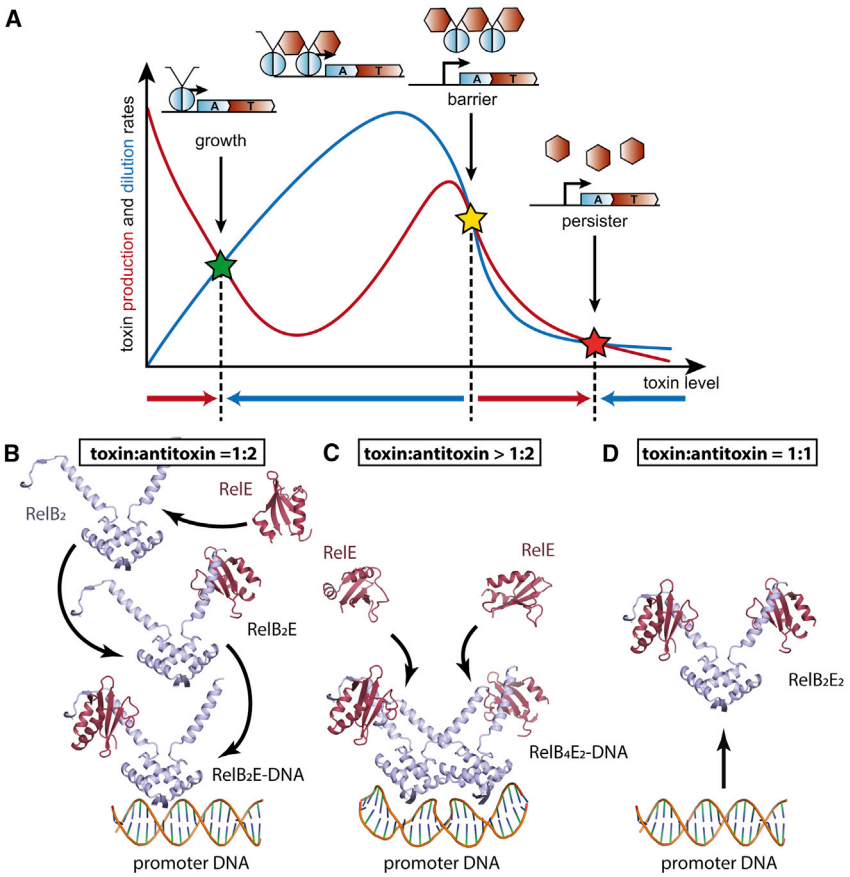


**Figure 6. Transcriptional Autoregulation of Type II TA Modules through Conditional Cooperativity**

(A) This illustration summarizes how conditional cooperativity controls the level of active toxin through regulation of TA module expression. The horizontal axis represents toxin protein levels, and the vertical axis shows the toxin production rate (red line) and its dilution rate (blue line) for a given toxin level. Above, the typical forms of TA complexes in each region are represented. Depending on whether the production rate exceeds the dilution rate or falls below it, the toxin either accumulates (bottom red arrows) or is depleted (bottom blue arrows). Three stars mark toxin levels where production and dilution rate are balanced, resulting in a stable state. The green star represents regular bacterial growth, where TA module transcription is repressed and the toxin is stored in TA complexes. In this state, toxin accumulation needs to overcome the barrier of conditional cooperativity (yellow star) to switch into the toxin-dominated state (red star). Note how the switch-like behavior of TA module activation and repression is apparent from the diverging blue and red arrows around the barrier of conditional cooperativity (yellow star).

(B–D) Illustrations of the structural basis of conditional cooperativity as proposed for the RelBE TA system. (B and C) At low toxin:antitoxin ratios, transcriptional repression of the *relBE* locus by $RelB_2$ dimers (blue) is enhanced by binding to RelE (red), which serves as a co-repressor in $RelB_2E$ (B) and $RelB_4E_2$ (C) complexes (Overgaard et al., 2008).

(D) When the toxin:antitoxin ratio is raised (e.g., because antitoxin degradation), $RelB_2E_2$ complexes form, and transcriptional repression is lost. The crystal structures suggest that DNA binding of RelB occurs in adjacent major grooves, similar to the Arc repressor-operator complex (Bøggild et al., 2012; Raumann et al., 1994). However, steric clashes caused by RelE molecules interfere with adjacent DNA binding of $RelB_2E_2$ complexes and, thus, abolish transcriptional repression.

were shown to control regulons including the (p)ppGpp synthetase gene *relA* and the general stress response sigma factor gene *rpoS*, respectively (Lin et al., 2013; Soo and Wood, 2013). The biological implications of this phenomenon have not been deeply studied, but it is clear that broader transcriptional regulation by type II TA antitoxins could closely couple TA module activation to other branches of cellular stress signaling.

## Translational Regulation of TA Modules

### *Translational Control of Type I TA Modules and Others*

Type I TA modules are defined by antitoxins that function as antisense RNAs to abolish toxin translation and comprise the most well-studied examples of translational control of toxin activation (see Wen and Fozo, 2014 for a general review). Two type I TA modules, *hok/sok* of plasmid R1 and *tisB/istR-1* of *E. coli* K-12, have been studied in detail and revealed an overall similar mechanism of antitoxin control (Figure 7A; recently reviewed by Berghoff and Wagner, 2017). In both cases, the toxin gene is initially expressed as a primary transcript that cannot be translated because of secondary structures in the 5′ UTR. Processing of this primary transcript at either the 5′ end (*tisB/istR-1*) or 3′ end (*hok/sok*) generates the active toxin mRNA. In case of *tisB*, the *istR-1* antitoxin directly competes with ribosome binding (Darfeuille et al., 2007), whereas the *sok* antitoxin prevents expression of the *hok* gene by blocking translation of its *mok* leader peptide (Thisted and Gerdes, 1992). In addition to a direct inhibition of toxin translation, antitoxin binding results in the formation of an RNA heteroduplex that is cleaved by RNase III and then degraded. Other families of type I TA modules in *E. coli* K-12, like *ldr/rdl*, *ibs/sib*, or *dinQ/agrB*, as well as evolutionarily unrelated type I TA loci in *Helicobacter* and *Bacillus* exhibit mechanisms of antitoxin control that are very similar to *hok/sok* or *tisB/istR-1* (Figure 2; Arnion et al., 2017; Berghoff and Wagner, 2017; Wen and Fozo, 2014).

Beyond the regulation of type I toxin translation, it is well known that the activation of mRNA endonuclease toxins can *trans*-activate other TA modules by suppressing the translation of their antitoxins (reviewed by Goeders and Van Melderen, 2014). A special case is *ghoST* of *E. coli* K-12, a TA module encoding a membrane-targeting peptide toxin, GhoT, that is the only example for such a toxin outside of the type I TA class (Wang et al., 2012). Rather than functioning as an antisense RNA, the antitoxin GhoS is an RNase that prevents toxin expression by cleaving its mRNA, a setup that was classified as a novel type V TA module (Wang et al., 2012). Interestingly, the activation of *ghoST* appears to depend on another TA system, MqsRA, in that MqsR degrades the mRNA of GhoS to allow GhoT translation (Wang et al., 2012). The biological implications of TA module

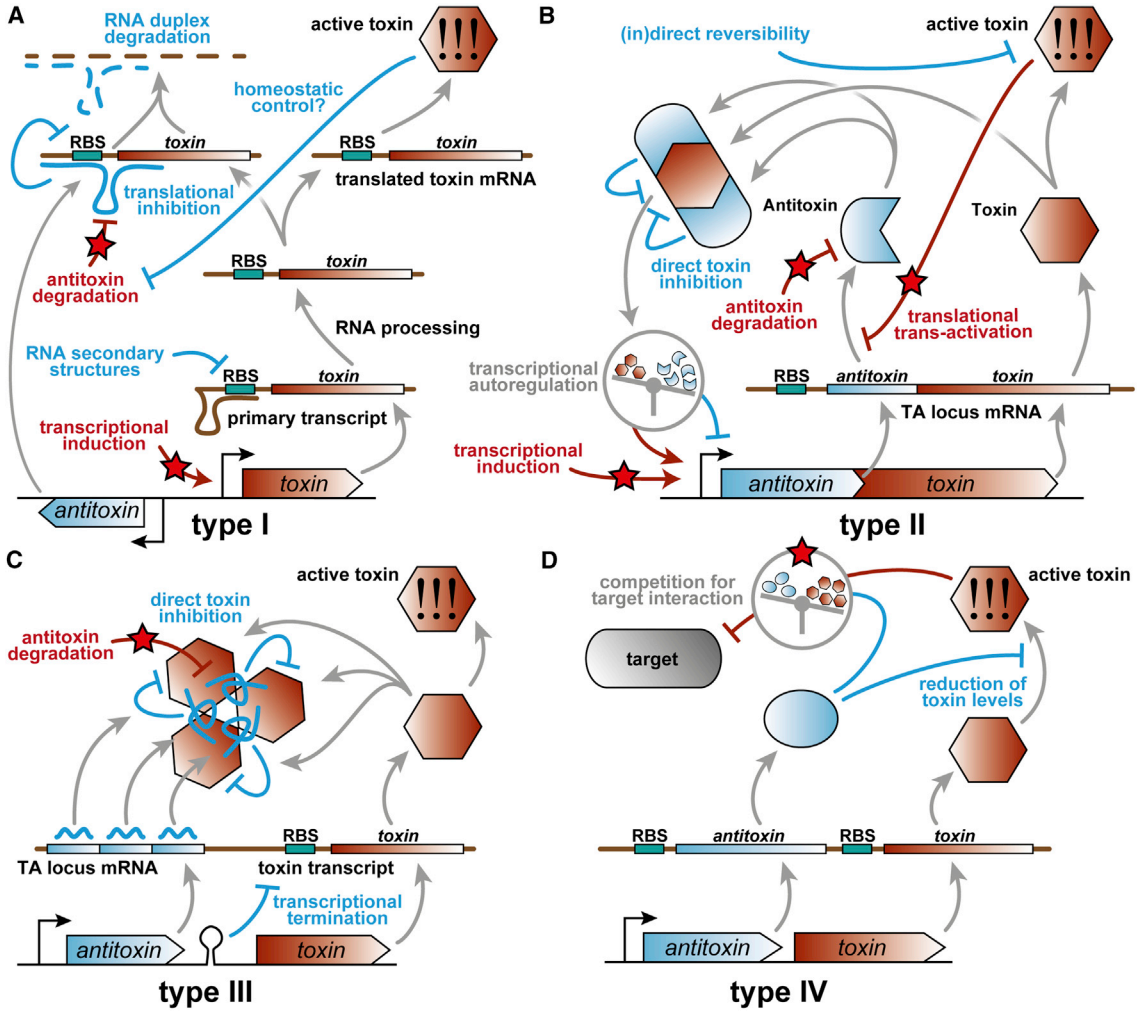


**Figure 7. Overview of Regulatory Mechanisms that Control the Activation of TA Modules**

(A–D) The illustration schematically highlights how regulatory input shapes the activity of type I–IV TA modules (details explained in the main text). Mechanisms promoting/inhibiting toxin activation are shown in red and blue, respectively. Processes that cause TA module activation in response to biological triggers are highlighted with a star.

(A) Depending on their genetic architecture, the activation of type I TA modules requires biological triggers to cause transcriptional induction (e.g., SOS signaling for *tisB/istR-1* or (p)ppGpp signaling for *hokB/sokB*) and/or antitoxin degradation (e.g., loss of the DNA template for PSK or host genome degradation by some phages for abortive infection).

(B) Activation of type II TA modules requires degradation of the antitoxin (e.g., because of activation of the protease Lon in response to (p)ppGpp signaling) and can be enhanced by transcriptional induction or TA module cross-activation.

(C) It is not known how type III TA modules are activated, but it seems clear that degradation of the antitoxin is critical (e.g., after loss of the DNA template for PSK or upon abrogation of host transcription by bacteriophages, causing abortive infection).

(D) Type IV TA modules are represented based on *cbeA/cbtA*, where toxin and antitoxin compete for the inhibition or promotion of target functioning, respectively. Any regulatory input needs to tip the balance of this competition; e.g., by affecting toxin or antitoxin protein levels.

*trans*-activation have not been extensively studied, but it may provide an additional layer of regulation by synchronizing the activation of different TA modules.

### *Biological Adaptation of Translationally Controlled Type I TA Modules*

It is intuitive that type I TA modules may mediate PSK and abortive infection similar to type II and type III TA modules, given the intrinsic instability of their RNA antitoxins (Gerdes et al., 1988; Jensen and Gerdes, 1995). However, unlike for the other families, antitoxin degradation does not directly result in the liberation of active toxin proteins. Type I toxins are not translated in the presence of their cognate antitoxins and cannot be expressed in the absence of a suitable DNA template. It has therefore remained a conundrum for many years how type I TA modules can mediate PSK; e.g., after plasmid loss. The key to this riddle is the primary transcript of type I toxin genes that decouples toxin transcription and translation (Gerdes et al., 1988). This primary transcript is protected from RNase degradation by the lack of antitoxin binding, and it is continuously processed into its translationally active form (Franch and Gerdes, 1996). Type I toxin translation is therefore initiated with a short delay after loss of the DNA template and/or antitoxin degradation,

enabling efficient PSK and abortive infection (Figure 7A; recently reviewed by Berghoff and Wagner, 2017).

A critical role of the primary transcript in the context of bacterial persistence was worked out by a recent study that highlighted the importance of the secondary structure element preventing toxin translation in the *tisB/istR-1* model system (Berghoff et al., 2017). The deletion of this RNA element alone facilitated TisB translation from the primary transcript and, thus, greatly promoted persister formation in response to transcriptional induction caused by SOS signaling. Interestingly, the additional removal of *istR-1* caused prolonged dormancy and very high rates of persister formation even without SOS signaling because TisB expression now depended solely on stochastic bursts of transcription. This study therefore showed that the two RNA elements enable the "primed" induction of *tisB/istR-1* by a specific trigger. Inactivation of these control elements results in a largely stochastic mode of persister formation similar to the activation of type II TA modules in response to bursts of (p)ppGpp (Berghoff et al., 2017; Maisonneuve and Gerdes, 2014). Evolution may therefore shape the RNA control elements of type I TA modules in response to environments that would favor either a stochastic (bet hedging) or primed ("responsive") mode of persister formation (Berghoff and Wagner, 2017).

Given that disruption of membrane integrity by Hok family toxins is lethal (Gerdes et al., 1986a), the level of membrane depolarization needs to be carefully controlled to enable reversible dormancy for bacterial persistence. It was recently hypothesized that a homeostatic control mechanism may regulate HokB levels in such a way that membrane depolarization does not result in irreversible loss of cell viability (Gerdes, 2016). This model is based on the previous finding that the activity of RNase E, the enzyme that continuously degrades the SokB antitoxin RNA, correlates with membrane association. Assuming that membrane depolarization may detach and, thus, inactivate RNase E, the activity of HokB itself would correspondingly abrogate SokB antitoxin degradation and, thereby, inhibit *hokB* translation (Figure 7A). A feedback mechanism following this principle would thus provide a safeguard that limits the membrane depolarization by HokB to bacteriostatic levels, but this remains to be experimentally demonstrated.

## Post-translational Regulation and Type IV TA Modules

Type IV TA modules are fundamentally different from the other types because, by definition, the antitoxin does not directly inhibit its cognate toxin but, rather, counteracts the activity of the toxin indirectly; e.g., by acting on the target (Figures 1 and 7D). Compared with at least type I and type II TA modules, type IV TA modules have been poorly studied with regard to regulatory features, and only *cbeA/cbtA* of *E. coli* K-12 and its two paralogs have been studied mechanistically (Masuda et al., 2012; Wen et al., 2017; Figure 2). Both the CbtA toxin and its CbeA antitoxin interact directly and independently with the cytoskeletal proteins MreB and FtsZ, but although CbtA inhibits their polymerization, CbeA promotes the bundling of MreB and FtsZ filaments (Heller et al., 2017; Masuda et al., 2012). Remarkably, the CbeA antitoxin also prevents the inhibition of FtsZ or MreB polymerization caused by several other proteins or pharmacological inhibitors, demonstrating that it functions fully independently of its toxin (Masuda et al., 2012).

The lack of direct interactions between toxin and antitoxin implies that the balance of their activities in the cell can only be adjusted by controlling their level of expression or their stability (Brown and Shaw, 2003). Dedicated studies failed to detect any effect of toxin and/or antitoxin proteins on the transcriptional regulation of *cbeA/cbtA* and its paralogs in *E. coli* K-12, suggesting that they may not exert transcriptional autoregulation (Wen et al., 2017). However, one study reported that ectopic expression of *cbeA* or paralogous antitoxins dramatically reduced the protein levels of their cognate toxins (Brown and Shaw, 2003). These antitoxins might thus control the activity of cognate toxins by more direct translational, or post-translational mechanisms beyond the competition for interactions with their cytoskeletal targets. No biological function of *cbeA/cbtA* and its paralogs has been determined with certainty, but the deletion of their toxin genes significantly increased the sensitivity of *E. coli* to hydrogen peroxide treatment (Wen et al., 2017). It is therefore tempting to speculate that these TA modules might respond to oxidative stress and slow down bacterial growth to enable effective repair of cellular damage.

Post-translational regulation is also a common feature of many TA modules beyond those classified as type IV and usually provides additional mechanisms to counteract the activities of the toxins to promote cellular resuscitation. For example, the DarG antitoxin does not only directly inhibit its cognate DarT toxin but also strips off the ADP ribosylation from single-stranded DNA modified by DarT (Jankevicius et al., 2016). Similarly, the CcdA antitoxin can extract CcdB from poisoned complexes with its gyrase target through a two-step mechanism of consecutive interactions via its intrinsically unfolded C terminus (De Jonge et al., 2009). This mechanism results not only in toxin inactivation but also causes "rejuvenation" of the poisoned topoisomerase and, thus, protects bacterial cells from severe DNA damage after fortuitous activation of CcdB. Although such a sophisticated control seems to be unnecessary for the biological function of *ccdAB* as a PSK locus of the F-plasmid, it is likely important to protect the cell from toxin molecules that have inadvertently escaped the multilayered control of TA locus expression and antitoxin inhibition (Figure 7B). Consistently, the post-translational control of CcdBA is directly linked to the transcriptional autoregulation of *ccdAB* via conditional cooperativity because both phenomena critically depend on the different low- and high-affinity interactions of toxin and antitoxin that are enabled by the intrinsically unfolded nature of the CcdA C terminus (De Jonge et al., 2009).

Beyond the direct post-translational regulation by cognate antitoxins, the activities of toxins can also be reversed by extrinsic detoxification, like the processing of stalled ribosomes by *trans*-translation after activation of mRNA endonucleases or de-acetylation of tRNAs modified by TacT through the action of housekeeping hydrolases (recently reviewed by Hall et al., 2017). Another possibility of post-translationally regulating TA module toxins is their targeted degradation by cellular proteases. SocB, a *Caulobacter* toxin that inhibits DNA replication by interfering with the assembly of the replication elongation complex, is counteracted by its protein antitoxin SocA (Aakre et al., 2013).

SocA serves as an adaptor for the ClpXP protease and, thereby, promotes the continuous degradation of SocB, an arrangement that was proposed as a novel type VI TA module (Aakre et al., 2013). Similarly, the SymE mRNA endonuclease toxin of *E. coli* K-12 is continuously degraded by Lon (Kawano et al., 2007). It is tempting to speculate that this setup may promote bacterial resuscitation after activation of the *symER* type I TA module because the SymE toxin does not cause membrane depolarization and, thus, cannot be regulated by the homeostatic control mechanism that has been proposed for *hokB*/*sokB* and related type I TA modules (see above; Gerdes, 2016).

## Tripartite TA Modules

In addition to the classical paradigm of TA pairs, a number of three-component TA modules have been identified that mostly appear to be derivatives of classical type II TA modules. The *paaRAE2* module of *E. coli* O157:H7 is composed not only of a ParE family toxin and a PaaA family antitoxin but also the protein PaaR, which serves as an additional regulator (Hallez et al., 2010). Like classical type II TA modules, the PaaAE2 complex represses transcription of the TA locus promoter, but full repression additionally requires PaaR (Hallez et al., 2010). Similarly, the ω-ε-ζ TA module of *Streptococcus pyogenes* is composed of an ε-ζ TA with an additional transcriptional regulator, ω, but, in this case, ε and ζ do not contribute to transcriptional regulation (Volante et al., 2014). Other examples of tripartite TA modules have been described in, e.g., *Bacillus* (comprehensively reviewed by Chan et al., 2016) or *M. tuberculosis*, where a chaperone is included in a classical *higBA* TA module and is critical to prevent premature antitoxin degradation (Bordes et al., 2016).

Tripartite TA loci are most often encoded on mobile elements, and both the prophage-encoded *paaRAE2* locus of *E. coli* O157:H7 as well as plasmid-encoded ω-ε-ζ of *Streptococcus* were shown to be active as PSK modules (Hallez et al., 2010; Volante et al., 2014). It is therefore tempting to speculate that the tripartite setup may be somehow advantageous for PSK. This hypothesis is supported by studies on *pasAB*, a plasmid-encoded relative of RelBE that exists both as a classical bipartite TA module and as a three-component variant, *pasABC*. Plasmid stabilization assays and direct competition experiments showed that *pasABC* is more potent as a PSK module than *pasAB*, although the biological role of the PasC component, possibly promoting TA or toxin-target interactions, has remained elusive (Deane and Rawlings, 2004).

## Concluding Remarks

In this review, we summarized the astonishing molecular diversity of TA modules as well as the wide range of biological functions they maintain in bacterial cells and highlight the links that have been identified between these two important aspects of TA biology. Based on multiple examples, we argue that the diverse molecular characteristics of TA modules can be seen as the "hardware" that, despite their captivating diversity, is only weakly coupled to certain biological activities. Instead, it seems that biological function is largely dependent on a number of less obvious, primarily regulatory features that control when and for how long a given TA locus responds to changes in cellular physiology and how intensively the molecular activity of the toxins is unleashed. For example, it appears that TA module activation for PSK and abortive infection primarily relies on transcriptional and translational autoregulation, where disruption of the TA balance causes toxin activation. Conversely, TA modules acting as effectors of persister cell formation are usually deeply integrated into cellular signaling pathways that tightly control their activation and use their characteristic auto-regulatory features to tune the induction, duration, and intensity of the phenotypic switch into dormancy. Mechanisms to support the reversibility of TA activation are prevalent among TA loci regardless of biological function, both to support resuscitation after transient dormancy and to buffer against accidental induction of toxin activities. We have summarized the most important principles and mechanisms of regulatory control of TA module activation in Figure 7.

Although the biological functions of many TA modules still remain elusive, the molecular characteristics of their toxins and how they are regulated are often known in higher detail (Figures 2 and 3). Future work in the field should therefore focus strongly on elucidating the biological functions of various TA modules to unravel how the biology of TA modules is shaped by the interplay of molecular features and regulatory principles. Furthermore, it would be interesting to compare homologous pairs of TA modules that are known to have different biological functions and examine in detail how they have been shaped by differential adaptation. A deeper understanding of TA module biology and the underlying molecular mechanisms would greatly facilitate their use in biotechnology and may inspire new approaches to interfere with the various harmful effects of TA modules in clinical infections.

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## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES

Aakre, C.D., Phung, T.N., Huang, D., and Laub, M.T. (2013). A bacterial toxin inhibits DNA replication elongation through a direct interaction with the β sliding clamp. Mol. Cell *52*, 617–628.

Afif, H., Allali, N., Couturier, M., and Van Melderen, L. (2001). The ratio between CcdA and CcdB modulates the transcriptional repression of the ccd poison-antidote system. Mol. Microbiol. *41*, 73–82.

Alawneh, A.M., Qi, D., Yonesaki, T., and Otsuka, Y. (2016). An ADP-ribosyltransferase Alt of bacteriophage T4 negatively regulates the Escherichia coli MazF toxin of a toxin-antitoxin module. Mol. Microbiol. *99*, 188–198.

Anantharaman, V., and Aravind, L. (2003). New connections in the prokaryotic toxin-antitoxin network: relationship with the eukaryotic nonsense-mediated RNA decay system. Genome Biol. *4*, R81.

Arbing, M.A., Handelman, S.K., Kuzin, A.P., Verdon, G., Wang, C., Su, M., Rothenbacher, F.P., Abashidze, M., Liu, M., Hurley, J.M., et al. (2010). Crystal structures of Phd-Doc, HigA, and YeeU establish multiple evolutionary links

between microbial growth-regulating toxin-antitoxin systems. Structure *18*, 996–1010.

Arnion, H., Korkut, D.N., Masachis Gelo, S., Chabas, S., Reignier, J., Iost, I., and Darfeuille, F. (2017). Mechanistic insights into type I toxin antitoxin systems in Helicobacter pylori: the importance of mRNA folding in controlling toxin expression. Nucleic Acids Res. *45*, 4782–4795.

Baharoglu, Z., and Mazel, D. (2014). SOS, the formidable strategy of bacteria against aggressions. FEMS Microbiol. Rev. *38*, 1126–1145.

Bendtsen, K.L., Xu, K., Luckmann, M., Winther, K.S., Shah, S.A., Pedersen, C.N.S., and Brodersen, D.E. (2017). Toxin inhibition in C. crescentus VapBC1 is mediated by a flexible pseudo-palindromic protein motif and modulated by DNA binding. Nucleic Acids Res. *45*, 2875–2886.

Berghoff, B.A., and Wagner, E.G.H. (2017). RNA-based regulation in type I toxin-antitoxin systems and its implication for bacterial persistence. Curr. Genet. *63*, 1011–1016.

Berghoff, B.A., Hoekzema, M., Aulbach, L., and Wagner, E.G. (2017). Two regulatory RNA elements affect TisB-dependent depolarization and persister formation. Mol. Microbiol. *103*, 1020–1033.

Bernard, P., and Couturier, M. (1992). Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. J. Mol. Biol. *226*, 735–745.

Blower, T.R., Pei, X.Y., Short, F.L., Fineran, P.C., Humphreys, D.P., Luisi, B.F., and Salmond, G.P. (2011a). A processed noncoding RNA regulates an altruistic bacterial antiviral system. Nat. Struct. Mol. Biol. *18*, 185–190.

Blower, T.R., Salmond, G.P., and Luisi, B.F. (2011b). Balancing at survival's edge: the structure and adaptive benefits of prokaryotic toxin-antitoxin partners. Curr. Opin. Struct. Biol. *21*, 109–118.

Blower, T.R., Short, F.L., Rao, F., Mizuguchi, K., Pei, X.Y., Fineran, P.C., Luisi, B.F., and Salmond, G.P. (2012). Identification and classification of bacterial type III toxin-antitoxin systems encoded in chromosomal and plasmid genomes. Nucleic Acids Res. *40*, 6158–6173.

Bøggild, A., Sofos, N., Andersen, K.R., Feddersen, A., Easter, A.D., Passmore, L.A., and Brodersen, D.E. (2012). The crystal structure of the intact E. coli RelBE toxin-antitoxin complex provides the structural basis for conditional cooperativity. Structure *20*, 1641–1648.

Bordes, P., Sala, A.J., Ayala, S., Texier, P., Slama, N., Cirinesi, A.M., Guillet, V., Mourey, L., and Genevaux, P. (2016). Chaperone addiction of toxin-antitoxin systems. Nat. Commun. *7*, 13339.

Brown, J.M., and Shaw, K.J. (2003). A novel family of Escherichia coli toxin-antitoxin gene pairs. J. Bacteriol. *185*, 6600–6608.

Brown, B.L., Grigoriu, S., Kim, Y., Arruda, J.M., Davenport, A., Wood, T.K., Peti, W., and Page, R. (2009). Three dimensional structure of the MqsR:MqsA complex: a novel TA pair comprised of a toxin homologous to RelE and an antitoxin with unique properties. PLoS Pathog. *5*, e1000706.

Castro-Roa, D., Garcia-Pino, A., De Gieter, S., van Nuland, N.A.J., Loris, R., and Zenkin, N. (2013). The Fic protein Doc uses an inverted substrate to phosphorylate and inactivate EF-Tu. Nat. Chem. Biol. *9*, 811–817.

Cataudella, I., Trusina, A., Sneppen, K., Gerdes, K., and Mitarai, N. (2012). Conditional cooperativity in toxin-antitoxin regulation prevents random toxin activation and promotes fast translational recovery. Nucleic Acids Res. *40*, 6424–6434.

Cataudella, I., Sneppen, K., Gerdes, K., and Mitarai, N. (2013). Conditional cooperativity of toxin - antitoxin regulation can mediate bistability between growth and dormancy. PLoS Comput. Biol. *9*, e1003174.

Chan, W.T., Espinosa, M., and Yeo, C.C. (2016). Keeping the wolves at bay: antitoxins of prokaryotic type II toxin-antitoxin systems. Front. Mol. Biosci. *3*, 9.

Cheverton, A.M., Gollan, B., Przydacz, M., Wong, C.T., Mylona, A., Hare, S.A., and Helaine, S. (2016). A Salmonella toxin promotes persister formation through acetylation of tRNA. Mol. Cell *63*, 86–96.

Christensen, S.K., and Gerdes, K. (2003). RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. Mol. Microbiol. *48*, 1389–1400.

Christensen-Dalsgaard, M., Jørgensen, M.G., and Gerdes, K. (2010). Three new RelE-homologous mRNA interferases of Escherichia coli differentially induced by environmental stresses. Mol. Microbiol. *75*, 333–348.

Coray, D.S., Wheeler, N.E., Heinemann, J.A., and Gardner, P.P. (2017). Why so narrow: distribution of anti-sense regulated, type I toxin-antitoxin systems compared with type II and type III systems. RNA Biol. *14*, 275–280.

Dalton, K.M., and Crosson, S. (2010). A conserved mode of protein recognition and binding in a ParD-ParE toxin-antitoxin complex. Biochemistry *49*, 2205–2215.

Darfeuille, F., Unoson, C., Vogel, J., and Wagner, E.G. (2007). An antisense RNA inhibits translation by competing with standby ribosomes. Mol. Cell *26*, 381–392.

De Jonge, N., Garcia-Pino, A., Buts, L., Haesaerts, S., Charlier, D., Zangger, K., Wyns, L., De Greve, H., and Loris, R. (2009). Rejuvenation of CcdB-poisoned gyrase by an intrinsically disordered protein domain. Mol. Cell *35*, 154–163.

Deane, S.M., and Rawlings, D.E. (2004). Plasmid evolution and interaction between the plasmid addiction stability systems of two related broad-host-range IncQ-like plasmids. J. Bacteriol. *186*, 2123–2133.

Dienemann, C., Bøggild, A., Winther, K.S., Gerdes, K., and Brodersen, D.E. (2011). Crystal structure of the VapBC toxin-antitoxin complex from Shigella flexneri reveals a hetero-octameric DNA-binding assembly. J. Mol. Biol. *414*, 713–722.

Dörr, T., Lewis, K., and Vulić, M. (2009). SOS response induces persistence to fluoroquinolones in Escherichia coli. PLoS Genet. *5*, e1000760.

Dörr, T., Vulić, M., and Lewis, K. (2010). Ciprofloxacin causes persister formation by inducing the TisB toxin in Escherichia coli. PLoS Biol. *8*, e1000317.

Dy, R.L., Przybilski, R., Semeijn, K., Salmond, G.P., and Fineran, P.C. (2014a). A widespread bacteriophage abortive infection system functions through a Type IV toxin-antitoxin mechanism. Nucleic Acids Res. *42*, 4590–4605.

Dy, R.L., Richter, C., Salmond, G.P., and Fineran, P.C. (2014b). Remarkable mechanisms in microbes to resist phage infections. Annu. Rev. Virol. *1*, 307–331.

Feng, J., Kessler, D.A., Ben-Jacob, E., and Levine, H. (2014). Growth feedback as a basis for persister bistability. Proc. Natl. Acad. Sci. USA *111*, 544–549.

Fineran, P.C., Blower, T.R., Foulds, I.J., Humphreys, D.P., Lilley, K.S., and Salmond, G.P. (2009). The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. Proc. Natl. Acad. Sci. USA *106*, 894–899.

Fozo, E.M., Makarova, K.S., Shabalina, S.A., Yutin, N., Koonin, E.V., and Storz, G. (2010). Abundance of type I toxin-antitoxin systems in bacteria: searches for new candidates and discovery of novel families. Nucleic Acids Res. *38*, 3743–3759.

Franch, T., and Gerdes, K. (1996). Programmed cell death in bacteria: translational repression by mRNA end-pairing. Mol. Microbiol. *21*, 1049–1060.

Garcia-Pino, A., Balasubramanian, S., Wyns, L., Gazit, E., De Greve, H., Magnuson, R.D., Charlier, D., van Nuland, N.A., and Loris, R. (2010). Allostery and intrinsic disorder mediate transcription regulation by conditional cooperativity. Cell *142*, 101–111.

Gelens, L., Hill, L., Vandervelde, A., Danckaert, J., and Loris, R. (2013). A general model for toxin-antitoxin module dynamics can explain persister cell formation in E. coli. PLoS Comput. Biol. *9*, e1003190.

Gerdes, K. (2016). Hypothesis: type I toxin-antitoxin genes eneter the persistence field–a feedback mechanism explaining membrane homeostasis. Philos. Trans. R. Soc. Lond. B Biol. Sci. *371*, 20160189.

Gerdes, K., Bech, F.W., Jørgensen, S.T., Løbner-Olesen, A., Rasmussen, P.B., Atlung, T., Boe, L., Karlstrom, O., Molin, S., and von Meyenburg, K. (1986a). Mechanism of postsegregational killing by the hok gene product of the

parB system of plasmid R1 and its homology with the relF gene product of the E. coli relB operon. EMBO J. *5*, 2023–2029.

Gerdes, K., Rasmussen, P.B., and Molin, S. (1986b). Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. Proc. Natl. Acad. Sci. USA *83*, 3116–3120.

Gerdes, K., Helin, K., Christensen, O.W., and Løbner-Olesen, A. (1988). Translational control and differential RNA decay are key elements regulating postsegregational expression of the killer protein encoded by the parB locus of plasmid R1. J. Mol. Biol. *203*, 119–129.

Germain, E., Castro-Roa, D., Zenkin, N., and Gerdes, K. (2013). Molecular mechanism of bacterial persistence by HipA. Mol. Cell *52*, 248–254.

Goeders, N., and Van Melderen, L. (2014). Toxin-antitoxin systems as multilevel interaction systems. Toxins (Basel) *6*, 304–324.

Goeders, N., Chai, R., Chen, B., Day, A., and Salmond, G.P. (2016). Structure, evolution, and functions of bacterial type III toxin-antitoxin systems. Toxins (Basel) *8*, E282.

Gotfredsen, M., and Gerdes, K. (1998). The Escherichia coli relBE genes belong to a new toxin-antitoxin gene family. Mol. Microbiol. *29*, 1065–1076.

Grønlund, H., and Gerdes, K. (1999). Toxin-antitoxin systems homologous with relBE of Escherichia coli plasmid P307 are ubiquitous in prokaryotes. J. Mol. Biol. *285*, 1401–1415.

Guglielmini, J., and Van Melderen, L. (2011). Bacterial toxin-antitoxin systems: translation inhibitors everywhere. Mob. Genet. Elements *1*, 283–290.

Guo, Y., Quiroga, C., Chen, Q., McAnulty, M.J., Benedik, M.J., Wood, T.K., and Wang, X. (2014). RalR (a DNase) and RalA (a small RNA) form a type I toxin-antitoxin system in Escherichia coli. Nucleic Acids Res. *42*, 6448–6462.

Hall, A.M., Gollan, B., and Helaine, S. (2017). Toxin-antitoxin systems: reversible toxicity. Curr. Opin. Microbiol. *36*, 102–110.

Hallez, R., Geeraerts, D., Sterckx, Y., Mine, N., Loris, R., and Van Melderen, L. (2010). New toxins homologous to ParE belonging to three-component toxin-antitoxin systems in Escherichia coli O157:H7. Mol. Microbiol. *76*, 719–732.

Hargreaves, D., Santos-Sierra, S., Giraldo, R., Sabariegos-Jareño, R., de la Cueva-Méndez, G., Boelens, R., Díaz-Orejas, R., and Rafferty, J.B. (2002). Structural and functional analysis of the kid toxin protein from E. coli plasmid R1. Structure *10*, 1425–1433.

Harms, A., Stanger, F.V., Scheu, P.D., de Jong, I.G., Goepfert, A., Glatter, T., Gerdes, K., Schirmer, T., and Dehio, C. (2015). Adenylylation of gyrase and Topo IV by FicT toxins disrupts bacterial DNA topology. Cell Rep. *12*, 1497–1507.

Harms, A., Maisonneuve, E., and Gerdes, K. (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. Science *354*, aaf4268-1–aaf4628-9.

Harms, A., Fino, C., Sorensen, M.A., Semsey, S., and Gerdes, K. (2017). Prophages and growth dynamics confound experimental results with antibiotic-tolerant persister cells. MBio *8*, e01964-17.

Harrison, J.J., Wade, W.D., Akierman, S., Vacchi-Suzzi, C., Stremick, C.A., Turner, R.J., and Ceri, H. (2009). The chromosomal toxin gene yafQ is a determinant of multidrug tolerance for Escherichia coli growing in a biofilm. Antimicrob. Agents Chemother. *53*, 2253–2258.

Helaine, S., Cheverton, A.M., Watson, K.G., Faure, L.M., Matthews, S.A., and Holden, D.W. (2014). Internalization of Salmonella by macrophages induces formation of nonreplicating persisters. Science *343*, 204–208.

Heller, D.M., Tavag, M., and Hochschild, A. (2017). CbtA toxin of Escherichia coli inhibits cell division and cell elongation via direct and independent interactions with FtsZ and MreB. PLoS Genet. *13*, e1007007.

Jankevicius, G., Ariza, A., Ahel, M., and Ahel, I. (2016). The toxin-antitoxin system DarTG catalyzes reversible ADP-ribosylation of DNA. Mol. Cell *64*, 1109–1116.

Jensen, R.B., and Gerdes, K. (1995). Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol. Microbiol. *17*, 205–210.

Jiang, Y., Pogliano, J., Helinski, D.R., and Konieczny, I. (2002). ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of Escherichia coli gyrase. Mol. Microbiol. *44*, 971–979.

Jørgensen, M.G., Pandey, D.P., Jaskolska, M., and Gerdes, K. (2009). HicA of Escherichia coli defines a novel family of translation-independent mRNA interferases in bacteria and archaea. J. Bacteriol. *191*, 1191–1199.

Jurėnas, D., Chatterjee, S., Konijnenberg, A., Sobott, F., Droogmans, L., Garcia-Pino, A., and Van Melderen, L. (2017). AtaT blocks translation initiation by N-acetylation of the initiator tRNAfMet. Nat. Chem. Biol. *13*, 640–646.

Kamada, K., Hanaoka, F., and Burley, S.K. (2003). Crystal structure of the MazE/MazF complex: molecular bases of antidote-toxin recognition. Mol. Cell *11*, 875–884.

Kawano, M., Aravind, L., and Storz, G. (2007). An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. Mol. Microbiol. *64*, 738–754.

Keren, I., Shah, D., Spoering, A., Kaldalu, N., and Lewis, K. (2004). Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli. J. Bacteriol. *186*, 8172–8180.

Keren, I., Minami, S., Rubin, E., and Lewis, K. (2011). Characterization and transcriptome analysis of Mycobacterium tuberculosis persisters. MBio *2*, e00100–e00111.

Koga, M., Otsuka, Y., Lemire, S., and Yonesaki, T. (2011). Escherichia coli rnlA and rnlB compose a novel toxin-antitoxin system. Genetics *187*, 123–130.

Kotte, O., Volkmer, B., Radzikowski, J.L., and Heinemann, M. (2014). Phenotypic bistability in Escherichia coli's central carbon metabolism. Mol. Syst. Biol. *10*, 736.

Lehnherr, H., Maguin, E., Jafri, S., and Yarmolinsky, M.B. (1993). Plasmid addiction genes of bacteriophage P1: doc, which causes cell death on curing of prophage, and phd, which prevents host death when prophage is retained. J. Mol. Biol. *233*, 414–428.

Leplae, R., Geeraerts, D., Hallez, R., Guglielmini, J., Drèze, P., and Van Melderen, L. (2011). Diversity of bacterial type II toxin-antitoxin systems: a comprehensive search and functional analysis of novel families. Nucleic Acids Res. *39*, 5513–5525.

Li, G.W., Burkhardt, D., Gross, C., and Weissman, J.S. (2014). Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. Cell *157*, 624–635.

Lin, C.Y., Awano, N., Masuda, H., Park, J.H., and Inouye, M. (2013). Transcriptional repressor HipB regulates the multiple promoters in Escherichia coli. J. Mol. Microbiol. Biotechnol. *23*, 440–447.

Lobato-Márquez, D., Moreno-Córdoba, I., Figueroa, V., Díaz-Orejas, R., and García-del Portillo, F. (2015). Distinct type I and type II toxin-antitoxin modules control Salmonella lifestyle inside eukaryotic cells. Sci. Rep. *5*, 9374.

Loris, R., and Garcia-Pino, A. (2014). Disorder- and dynamics-based regulatory mechanisms in toxin-antitoxin modules. Chem. Rev. *114*, 6933–6947.

Lou, C., Li, Z., and Ouyang, Q. (2008). A molecular model for persister in E. coli. J. Theor. Biol. *255*, 205–209.

Magnuson, R.D. (2007). Hypothetical functions of toxin-antitoxin systems. J. Bacteriol. *189*, 6089–6092.

Maisonneuve, E., and Gerdes, K. (2014). Molecular mechanisms underlying bacterial persisters. Cell *157*, 539–548.

Maisonneuve, E., Shakespeare, L.J., Jørgensen, M.G., and Gerdes, K. (2011). Bacterial persistence by RNA endonucleases. Proc. Natl. Acad. Sci. USA *108*, 13206–13211.

Masuda, H., and Inouye, M. (2017). Toxins of prokaryotic toxin-antitoxin systems with sequence-specific endoribonuclease activity. Toxins (Basel) *9*, E140.

Masuda, H., Tan, Q., Awano, N., Wu, K.P., and Inouye, M. (2012). YeeU enhances the bundling of cytoskeletal polymers of MreB and FtsZ, antagonizing the CbtA (YeeV) toxicity in Escherichia coli. Mol. Microbiol. *84*, 979–989.

McVicker, G., and Tang, C.M. (2016). Deletion of toxin-antitoxin systems in the evolution of Shigella sonnei as a host-adapted pathogen. Nat. Microbiol. *2*, 16204.

Monti, M.C., Hernández-Arriaga, A.M., Kamphuis, M.B., López-Villarejo, J., Heck, A.J., Boelens, R., Díaz-Orejas, R., and van den Heuvel, R.H. (2007). Interactions of Kid-Kis toxin-antitoxin complexes with the parD operator-promoter region of plasmid R1 are piloted by the Kis antitoxin and tuned by the stoichiometry of Kid-Kis oligomers. Nucleic Acids Res. *35*, 1737–1749.

Moyed, H.S., and Bertrand, K.P. (1983). hipA, a newly recognized gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis. J. Bacteriol. *155*, 768–775.

Muthuramalingam, M., White, J.C., and Bourne, C.R. (2016). Toxin-antitoxin modules are pliable switches activated by multiple protease pathways. Toxins (Basel) *8*, E214.

Mutschler, H., Gebhardt, M., Shoeman, R.L., and Meinhart, A. (2011). A novel mechanism of programmed cell death in bacteria by toxin-antitoxin systems corrupts peptidoglycan synthesis. PLoS Biol. *9*, e1001033.

Norton, J.P., and Mulvey, M.A. (2012). Toxin-antitoxin systems are important for niche-specific colonization and stress resistance of uropathogenic Escherichia coli. PLoS Pathog. *8*, e1002954.

Ogura, T., and Hiraga, S. (1983). Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci. USA *80*, 4784–4788.

Otsuka, Y., and Yonesaki, T. (2012). Dmd of bacteriophage T4 functions as an antitoxin against Escherichia coli LsoA and RnlA toxins. Mol. Microbiol. *83*, 669–681.

Overgaard, M., Borch, J., Jørgensen, M.G., and Gerdes, K. (2008). Messenger RNA interferase RelE controls relBE transcription by conditional cooperativity. Mol. Microbiol. *69*, 841–857.

Page, R., and Peti, W. (2016). Toxin-antitoxin systems in bacterial growth arrest and persistence. Nat. Chem. Biol. *12*, 208–214.

Pandey, D.P., and Gerdes, K. (2005). Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. Nucleic Acids Res. *33*, 966–976.

Pecota, D.C., and Wood, T.K. (1996). Exclusion of T4 phage by the hok/sok killer locus from plasmid R1. J. Bacteriol. *178*, 2044–2050.

Pedersen, K., and Gerdes, K. (1999). Multiple hok genes on the chromosome of Escherichia coli. Mol. Microbiol. *32*, 1090–1102.

Pedersen, K., Christensen, S.K., and Gerdes, K. (2002). Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. Mol. Microbiol. *45*, 501–510.

Pedersen, K., Zavialov, A.V., Pavlov, M.Y., Elf, J., Gerdes, K., and Ehrenberg, M. (2003). The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. Cell *112*, 131–140.

Pimentel, B., Nair, R., Bermejo-Rodríguez, C., Preston, M.A., Agu, C.A., Wang, X., Bernal, J.A., Sherratt, D.J., and de la Cueva-Méndez, G. (2014). Toxin Kid uncouples DNA replication and cell division to enforce retention of plasmid R1 in Escherichia coli cells. Proc. Natl. Acad. Sci. USA *111*, 2734–2739.

Ramage, H.R., Connolly, L.E., and Cox, J.S. (2009). Comprehensive functional analysis of Mycobacterium tuberculosis toxin-antitoxin systems: implications for pathogenesis, stress responses, and evolution. PLoS Genet. *5*, e1000767.

Rao, F., Short, F.L., Voss, J.E., Blower, T.R., Orme, A.L., Whittaker, T.E., Luisi, B.F., and Salmond, G.P. (2015). Co-evolution of quaternary organization and novel RNA tertiary interactions revealed in the crystal structure of a bacterial protein-RNA toxin-antitoxin system. Nucleic Acids Res. *43*, 9529–9540.

Raumann, B.E., Rould, M.A., Pabo, C.O., and Sauer, R.T. (1994). DNA recognition by beta-sheets in the Arc repressor-operator crystal structure. Nature *367*, 754–757.

Roberts, R.C., Ström, A.R., and Helinski, D.R. (1994). The parDE operon of the broad-host-range plasmid RK2 specifies growth inhibition associated with plasmid loss. J. Mol. Biol. *237*, 35–51.

Sberro, H., Leavitt, A., Kiro, R., Koh, E., Peleg, Y., Qimron, U., and Sorek, R. (2013). Discovery of functional toxin/antitoxin systems in bacteria by shotgun cloning. Mol. Cell *50*, 136–148.

Schumacher, M.A., Balani, P., Min, J., Chinnam, N.B., Hansen, S., Vulić, M., Lewis, K., and Brennan, R.G. (2015). HipBA-promoter structures reveal the basis of heritable multidrug tolerance. Nature *524*, 59–64.

Shah, D., Zhang, Z., Khodursky, A., Kaldalu, N., Kurg, K., and Lewis, K. (2006). Persisters: a distinct physiological state of E. coli. BMC Microbiol. *6*, 53.

Shan, Y., Brown Gandt, A., Rowe, S.E., Deisinger, J.P., Conlon, B.P., and Lewis, K. (2017). ATP-dependent persister formation in *Escherichia coli*. MBio *8*, e02267-16.

Short, F.L., Pei, X.Y., Blower, T.R., Ong, S.L., Fineran, P.C., Luisi, B.F., and Salmond, G.P. (2013). Selectivity and self-assembly in the control of a bacterial toxin by an antitoxic noncoding RNA pseudoknot. Proc. Natl. Acad. Sci. USA *110*, E241–E249.

Simanshu, D.K., Yamaguchi, Y., Park, J.H., Inouye, M., and Patel, D.J. (2013). Structural basis of mRNA recognition and cleavage by toxin MazF and its regulation by antitoxin MazE in Bacillus subtilis. Mol. Cell *52*, 447–458.

Soo, V.W., and Wood, T.K. (2013). Antitoxin MqsA represses curli formation through the master biofilm regulator CsgD. Sci. Rep. *3*, 3186.

Szekeres, S., Dauti, M., Wilde, C., Mazel, D., and Rowe-Magnus, D.A. (2007). Chromosomal toxin-antitoxin loci can diminish large-scale genome reductions in the absence of selection. Mol. Microbiol. *63*, 1588–1605.

Takagi, H., Kakuta, Y., Okada, T., Yao, M., Tanaka, I., and Kimura, M. (2005). Crystal structure of archaeal toxin-antitoxin RelE-RelB complex with implications for toxin activity and antitoxin effects. Nat. Struct. Mol. Biol. *12*, 327–331.

Thisted, T., and Gerdes, K. (1992). Mechanism of post-segregational killing by the hok/sok system of plasmid R1. Sok antisense RNA regulates hok gene expression indirectly through the overlapping mok gene. J. Mol. Biol. *223*, 41–54.

Tian, C., Semsey, S., and Mitarai, N. (2017). Synchronized switching of multiple toxin-antitoxin modules by (p)ppGpp fluctuation. Nucleic Acids Res. *45*, 8180–8189.

Tsuchimoto, S., Ohtsubo, H., and Ohtsubo, E. (1988). Two genes, pemK and pemI, responsible for stable maintenance of resistance plasmid R100. J. Bacteriol. *170*, 1461–1466.

Unterholzner, S.J., Poppenberger, B., and Rozhon, W. (2013). Toxin-antitoxin systems: biology, identification, and application. Mob. Genet. Elements *3*, e26219.

Van Melderen, L. (2010). Toxin-antitoxin systems: why so many, what for? Curr. Opin. Microbiol. *13*, 781–785.

Van Melderen, L., Bernard, P., and Couturier, M. (1994). Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria. Mol. Microbiol. *11*, 1151–1157.

Vandervelde, A., Drobnak, I., Hadži, S., Sterckx, Y.G., Welte, T., De Greve, H., Charlier, D., Efremov, R., Loris, R., and Lah, J. (2017). Molecular mechanism governing ratio-dependent transcription regulation in the ccdAB operon. Nucleic Acids Res. *45*, 2937–2950.

Veening, J.W., Smits, W.K., and Kuipers, O.P. (2008). Bistability, epigenetics, and bet-hedging in bacteria. Annu. Rev. Microbiol. *62*, 193–210.

Verstraeten, N., Knapen, W.J., Kint, C.I., Liebens, V., Van den Bergh, B., Dewachter, L., Michiels, J.E., Fu, Q., David, C.C., Fierro, A.C., et al. (2015). Obg and membrane depolarization are part of a microbial bet-hedging strategy that leads to antibiotic tolerance. Mol. Cell *59*, 9–21.

Volante, A., Soberon, N.E., Ayora, S., and Alonso, J.C. (2014). The interplay between different stability systems contributes to faithful segregation: *Streptococcus pyogenes* pSM19035 as a model. Microbiol. Spectr. *2*, PLAS-0007-2013.

Wang, X., Kim, Y., Hong, S.H., Ma, Q., Brown, B.L., Pu, M., Tarone, A.M., Benedik, M.J., Peti, W., Page, R., and Wood, T.K. (2011). Antitoxin MqsA helps mediate the bacterial general stress response. Nat. Chem. Biol. *7*, 359–366.

Wang, X., Lord, D.M., Cheng, H.Y., Osbourne, D.O., Hong, S.H., Sanchez-Torres, V., Quiroga, C., Zheng, K., Herrmann, T., Peti, W., et al. (2012). A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. Nat. Chem. Biol. *8*, 855–861.

Wen, J., and Fozo, E.M. (2014). sRNA antitoxins: more than one way to repress a toxin. Toxins (Basel) *6*, 2310–2335.

Wen, Z., Wang, P., Sun, C., Guo, Y., and Wang, X. (2017). Interaction of type IV toxin/antitoxin systems in cryptic prophages of *Escherichia coli* K-12. Toxins (Basel) *9*, E77.

Wilbaux, M., Mine, N., Guérout, A.M., Mazel, D., and Van Melderen, L. (2007). Functional interactions between coexisting toxin-antitoxin systems of the ccd family in Escherichia coli O157:H7. J. Bacteriol. *189*, 2712–2719.

Winther, K.S., and Gerdes, K. (2011). Enteric virulence associated protein VapC inhibits translation by cleavage of initiator tRNA. Proc. Natl. Acad. Sci. USA *108*, 7403–7407.

Winther, K., Tree, J.J., Tollervey, D., and Gerdes, K. (2016). VapCs of Mycobacterium tuberculosis cleave RNAs essential for translation. Nucleic Acids Res. *44*, 9860–9871.

Wozniak, R.A., and Waldor, M.K. (2009). A toxin-antitoxin system promotes the maintenance of an integrative conjugative element. PLoS Genet. *5*, e1000439.

Wu, N., He, L., Cui, P., Wang, W., Yuan, Y., Liu, S., Xu, T., Zhang, S., Wu, J., Zhang, W., and Zhang, Y. (2015). Ranking of persister genes in the same Escherichia coli genetic background demonstrates varying importance of individual persister genes in tolerance to different antibiotics. Front. Microbiol. *6*, 1003.

Zhang, Y., Zhang, J., Hoeflich, K.P., Ikura, M., Qing, G., and Inouye, M. (2003). MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in Escherichia coli. Mol. Cell *12*, 913–923.