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Toxin-antitoxin systems in bacterial growth arrest and persistence

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Bacterial persister cells constitute a subpopulation of genetically identical, metabolically slow-growing cells that are highly tolerant of antibiotics and other environmental stresses. Recent studies have demonstrated that gene loci known as toxin-antitoxin (TA) modules play a central role in the persister state. Under normal growth conditions, antitoxins potently inhibit the activities of the toxins. In contrast, under conditions of stress, the antitoxins are selectively degraded, freeing the toxins to inhibit essential cellular processes, such as DNA replication and protein translation. This inhibition results in rapid growth arrest. In this Review, we highlight recent discoveries of these multifaceted TA systems with a focus on the newly uncovered mechanisms, especially conditional cooperativity, that are used to regulate cell growth and persistence. We also discuss the potential for targeting TA systems for antimicrobial drug discovery.

The discovery of antibiotics in the early twentieth century revolutionized medicine[1]. However, it is now known that antibiotics are not the miracle drugs they once were thought to be. Rather, antibiotics can and do fail, sometimes with deadly results. One reason that this happens is because the bacteria they target acquire antibiotic resistance mutations, rendering the antibiotics ineffective. A second, increasingly appreciated reason that antibiotics fail is because the bacteria themselves use endogenous mechanisms to evade stress, including antibiotic exposure.

One of these mechanisms is persistence. Bacterial cells in the persister state are not antibiotic-resistant mutants. Rather, they have differentiated into a phenotype that has a high tolerance for antibiotics[2–4]. The resulting nondividing cells thus are able to survive until the environmental stress, such as nutrient starvation or antibiotic exposure, is removed. Once relieved, the persisters then revert back to the actively growing state and repopulate the original population. In wild-type *Escherichia coli*, the frequency of persisters in a planktonic bacterial population is only about 1 in a million. However, in stationary cultures and biofilms, complex multicellular bacterial communities that are highly resistant to antimicrobials, the frequency increases substantially[5], up to 1 in 100. The role of persisters in mediating antibiotic resistance has stimulated renewed efforts to understand the molecular mechanism(s) that underlie the persister phenotype.

In the last decade, it has become clear that gene pairs known as toxin-antitoxin (TA) systems act as effectors of dormancy and persistence[6–8]. TA gene loci[9,10] are highly abundant on bacterial plasmids[11,12], phages[13] and chromosomes[14]. They are composed of a toxin, which causes growth arrest by interfering with a vital cellular process, and a cognate antitoxin, which neutralizes the toxin activity during normal growth conditions[15]. Under conditions of stress the antitoxins are selectively degraded. This leaves the toxins to exert their toxic effects, which leads to growth arrest and dormancy[16,17]. In this Review, we highlight recent functional and structural studies of systems and discuss their increasingly expanding roles in bacterial physiology. We also describe new insights into the mechanisms, especially conditional cooperativity, by which TA systems regulate the transition between growth and persistence. Finally, we discuss the potential for targeting these systems for antimicrobial drug discovery. Given the pressing need for new antibiotics, novel approaches that target TA systems and the processes that regulate them are warranted.

Toxin-antitoxin systems

The first two TA gene loci were discovered more than three decades ago, when they were revealed to play a role in plasmid maintenance[11,12] through a then unknown mechanism. Namely, the products of the TA genes killed the progeny that did not retain the plasmid. These cells died because the 'antidote' antitoxins, which are highly labile compared to their cognate toxins, could not be regenerated in the absence of the plasmid. Thus, eventually, the plasmid-free cells contained only the stable toxins, whose activities ultimately resulted in cell death[16,17]. Because of the unique mechanism used to maintain the plasmid, this process was referred to as post-segregational killing (PSK)[12].

Since their initial discovery, thousands of TA operons have been identified not only in plasmids[11,12] and phages[13] but also, unexpectedly, on the chromosomes of most free-living bacteria, with some species (such as *Mycobacterium tuberculosis*) containing as many as 88 TA loci[18]. Unlike plasmid-based TA genes, chromosomal TA loci do not mediate PSK but instead function to ensure the survival of the population in response to stress[19,20]. Currently, there are six classes of TA systems, which are distinguished on the basis of the mechanisms used by the antitoxins to neutralize the activities of the toxins. While the product of the toxin gene is typically a protein, that of the antitoxin gene is either a noncoding RNA (in TA systems I and III) or a low-molecular-weight protein (in TA systems II, IV, V and VI). The six classes of TA systems in *E. coli* are shown in **Figure 1**.

Well-established TA systems: types I–III. Type I TA systems have a noncoding RNA antitoxin and a protein toxin. In these systems, small regulatory antisense RNAs (sRNAs) base-pair to the mRNA of the toxin to inhibit its translation[21]. Under normal growth conditions, this duplexing inhibits ribosome binding and the RNA duplex is rapidly degraded by RNase III[22]. However, under conditions of stress, the pool of antitoxin sRNA is reduced, resulting in the translation of the now non-duplexed toxin mRNA[23]. The toxins

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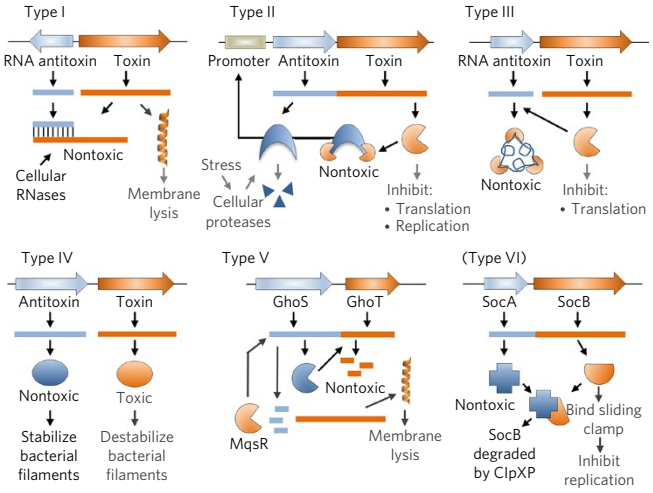

Figure 1 | Toxin-antitoxin systems. Toxins are shown in orange and antitoxins in blue; activities that are nontoxic are in black font, whereas those that are toxic are in gray. Type I: the sRNA antitoxin base pairs with toxin mRNA to inhibit translation; the membrane lytic toxins function to depolarize the cell membrane and disrupt ATP synthesis. Type II: the antitoxin and toxin are proteins; under growth conditions, the toxin is bound to the antitoxin, which inhibits its activity. Both the antitoxin and, in most cases, the TA complex bind the TA promoter to repress transcription. Under stress conditions, cellular proteases such as Lon and ClpXP are activated that preferentially cleave the antitoxins, freeing the toxins to inhibit growth by inhibiting translation or replication. Type III: the antitoxin sRNA is processed by the endoribonuclease (RNase) toxin, resulting in the formation of RNA pseudoknot–toxin complexes, which inhibit toxin activity. Type IV: the protein antitoxin stabilizes bacterial filaments, while the protein toxin destabilizes them; in the absence of the antitoxin, this toxin-mediated destabilization inhibits cell division. Type V: the antitoxin GhoS is an RNase specific for the toxin *ghoT* mRNA; under conditions of stress, the mRNA of the antitoxin is degraded by the MqsR toxin, resulting in GhoT translation and membrane lysis. Type VI: the SocA antitoxin is an adaptor protein that binds the SocB toxin to promote its degradation by ClpXP. When not degraded, the toxin binds the sliding clamp to inhibit DNA replication.

themselves are short hydrophobic peptides that insert and disrupt membranes, leading to a loss of membrane potential and, in turn, growth arrest (**Fig. 1**). The *hok*/*sok* (*parB* locus) gene pair on plasmid R1 was the first type I TA system discovered[12]. Multiple *hok*/*sok* homologs have since been identified on chromosomes throughout the bacterial kingdom, including that of *E. coli*[24].

Type II TA systems are the largest and best-studied TA system class, with thousands of type II TA loci identified in most free-living bacteria, such as *E. coli* (**Fig. 2a**)[25]. Unlike the type I antitoxins, the type II antitoxins are proteins. They typically have two domains, one that binds DNA[26] and a second that binds and inhibits the activity of the cognate protein toxin (**Fig. 1**)[14,27]. The antitoxins also often bind the promoters of their own operon in order to repress transcription; in most[14,26] but not all[28] cases, the toxins function as co-repressors. In some cases, they bind the promoters of other genes[29]. Type II TA systems are regulated by distinct differences in the cellular lifetimes of the antitoxins and toxins[16,17]. Namely, the antitoxins are highly susceptible to proteolysis, whereas their cognate toxins are comparatively stable. Thus, in response to stress, the antitoxins are selectively degraded. This leads to growth arrest due to the cellular effects of the now free toxins. Type II toxins function by inhibiting replication (i.e., by inhibiting DNA gyrase[27,30]) or translation (i.e., by cleaving mRNA[31–34], inactivating ribosome elongation factors[35,36] or inactivating glutamyl-tRNA synthetase (GltX)[37,38], among other processes).

The majority of type II toxins are endoribonucleases (RNases), which often adopt a microbial RNase fold (similar to those of RNase T1 and RNase SA)[39,40]. The *ribosome-dependent* RNase toxins bind directly to the A site of the ribosome, where they cleave ribosome-associated mRNA (**Fig. 2b**; RelE[41,42], YoeB[43], YafO[44], YafQ[45], HigB[46–48] in *E. coli*). Other RNase toxins are *ribosome independent*, including MqsR[31,34], which also adopts a microbial RNase fold[39], and MazF, a functional dimer with a unique fold (**Fig. 2c**)[40,49]. Unlike those of their highly similar RNase toxin counterparts, the structures of both the cognate antitoxins and the oligomeric toxin–antitoxin complexes differ considerably from one another (**Fig. 2d**,**e**)[39,50–52].

In type III systems, as in type I systems, the antitoxin is a small RNA[23]. However, instead of duplexing with toxin mRNA to prevent toxin synthesis, the antitoxin forms pseudoknots that bind directly to the toxin (**Fig. 1** and **Fig. 2e**, right). The founding and best-studied member of the type III class is *toxIN*[53,54]. The *toxN* toxin gene is preceded by a short palindromic repeat, which is itself preceded by a tandem array of nucleotide repeats. ToxN, an RNase, cleaves not only the *toxIN* transcript—into the active 36-nucleotide antitoxin sRNAs—but also other mRNAs. Its activity is inhibited when it associates with *toxI* sRNA, which blocks its active site.

Newly discovered TA systems: types IV–VI. The most recently identified TA systems are types IV–VI. In type IV, as in type II, both the antitoxin and toxin are proteins[55]. However, whereas in type II systems antitoxins and toxins bind to form a tight complex, the antitoxin and toxin of the type IV system never interact. Instead, the toxin prevents growth by binding and inhibiting the polymerization of the bacterial cytoskeletal proteins MreB and FtsZ, thereby blocking cell division. The antitoxin antagonizes toxin activity by promoting and stabilizing MreB and FtsZ cytoskeletal filament bundling (**Fig. 1**)[56]. In the only known type V system, the antitoxin (GhoS) is an RNase that, under growth conditions, cleaves the toxin (GhoT) mRNA[57]. However, under conditions of stress, the mRNA of GhoS is degraded by the type II toxin MqsR. This results in the translation of GhoT, a small hydrophobic peptide that, like the toxins from type I systems, damages the cell membrane (**Fig. 1**). Thus, this is an example of one TA system (*ghoST*) that is directly regulated by that of another (*mqsRA*)[58]. The most recently discovered TA system (type VI) is composed of a protein toxin, SocB, and a protein antitoxin, SocA. The toxin blocks replication elongation by binding directly to the β sliding clamp DnaN and outcompeting other clamp-binding proteins. The antitoxin SocA is a proteolytic adaptor protein that neutralizes SocB toxicity by promoting its degradation by ClpXP (**Fig. 1**)[59].

TA systems and their role in persistence

It is now well established that TA systems, especially type II TA systems, play a central role in persistence[2,7,60–62]. The first gene identified as directly affecting persistence was the *E. coli* gene *hipA* (high persister protein A), encoding a kinase that inactivates glutamyl-tRNA synthetase[37,38]. Its role in persistence was discovered when a variant of *hipA* (*hipA7*, which contains two mutations, G22S and D291A) was isolated that exhibits a ~100- to 1,000-fold increase in persistence[60]. Although this mutant was identified more than three decades ago, the mechanism by which it increased persistence remained elusive until this year. First, structural and functional studies showed that this mutant increases persistence because it destabilizes the higher-order oligomers that form when the HipAB complexes bind the *hipAB* operator. This destabilization exposes the HipA active site, rendering the toxin more active[63]. A second study then revealed that HipA activity leads to the ppGpp-mediated activation of the type II RNase toxins[64], whose activities ultimately lead to persistence.

The central role of type II RNase toxins in persistence was also demonstrated in landmark microarray experiments on isolated

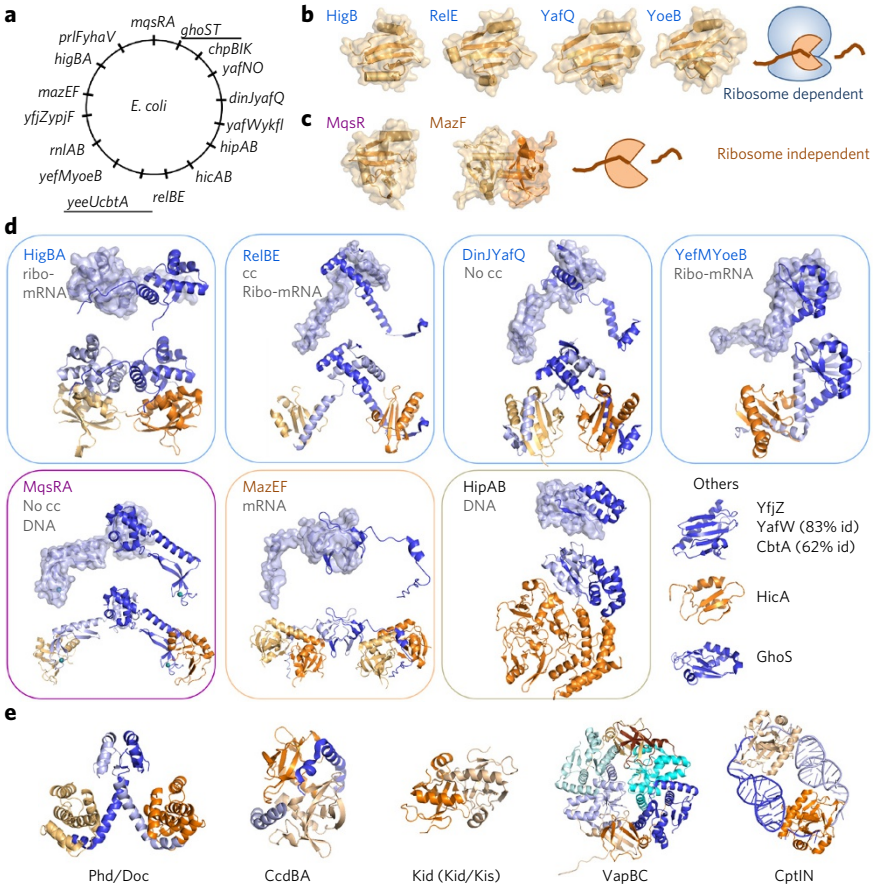

Figure 2 | TA systems in *E. coli*. (**a**) TA loci in the *E. coli* genome (type I systems not shown). Types IV and V loci are underlined; the rest are type II. (**b**) Ribosome-dependent RNase toxins (ribbons with transparent surfaces); all adopt the microbial RNase fold. (**c**) Ribosome-independent RNase toxins; although MqsR adopts a microbial RNase fold, it does not require the ribosome for activity. MazF is a dimer. (**d**) TA structures. Toxins in orange/gold; antitoxins in blue/light blue. TA systems with a ribosome-dependent toxin have a blue border (PDB IDs: HigBA, 4MCX[47]; RelBE, 4FXE[51]; DinJYafQ, 4Q2U[90]; YefMYoeB, 2A6Q[95]). MqsRA, which is an RNase with structural characteristics of ribosome-dependent toxins but functional characteristics of ribosome-independent toxins, is bordered in magenta (3HI2[39]). The MazEF system is bordered in orange (4MDX[96]). The HipAB system, in which the toxin is a kinase, is bordered in brown (4YG7[63]). Structures of antitoxins only are in blue (YfjZ, 2EA9, the sequences of YafW and CbtA are highly similar to YfjZ ('% id' is the percentage of sequence identity to YfjZ); GhoS, 2LLZ[57]), while toxins are in orange (HicA, 4C26[97]). cc, regulated by conditional cooperativity; no cc, not regulated by conditional cooperativity; ribo-mRNA, structure of the ribosome–mRNA–toxin complex is known (RelBE[41], YoeB[43], HigB[48]); mRNA, the toxin–mRNA complex is known (MazF[96]); DNA, structure of the antitoxin–promoter (MqsA[91,98]) or the TA complex–promoter (HipAB[63]) is known. (**e**) Structures of well-studied TA systems not present in *E. coli*: Phd/Doc (3K33, ref. 70; left; type II TA system), CcdBA (3G7Z[75]; left middle, type II TA system), Kid/Kis (1M1F[99]; middle, type II TA system), VapBC (3TND[73]; right middle, type II TA system) and CptIN (4RMO[100]; right, type III TA system). Antitoxins and toxins are colored as in **d**.

E. coli persisters. Namely, multiple type II toxins, especially RNase toxins, were highly upregulated in persister as compared to non-persister cells[7,8]. A direct role for RNase toxins in persistence was then confirmed using both ectopic expression experiments (ectopic expression of RNase toxins increases persistence) and TA gene deletion experiments (the simultaneous deletion of ten TA systems in *E. coli* resulted in a 100-fold reduction in persistence)[6,7,61,62]. Most recently, it was shown that the systematic deletion of individual type II TA systems from *Salmonella* also reduced macrophage-induced persisters, demonstrating that 14 distinct type II TA modules in *Salmonella* are activated once they are phagocytosed[65].

The role of TA systems in persistence was recently expanded in a breakthrough study in which it was shown that not only type II but also type I toxins are directly linked to persistence[66]. In this work, the authors demonstrated that Obg, a universally conserved GTPase, induces persistence by activating the transcription of the type I *hokB* toxin. The HokB membrane-associated peptide ultimately results in membrane depolarization, which leads to persistence. Unexpectedly, the authors also showed that this function of Obg depends on ppGpp, to which it may bind directly. Although the molecular basis of *hokB* activation by Obg is not yet known, this work nevertheless demonstrates a functional convergence through (p)ppGpp of both type I and type II toxins in persistence. Collectively, these data demonstrate that entry to and exit from the persister state appear to be intricately linked to the expression of toxins and antitoxins.

Indeed, mathematical modeling studies have demonstrated that the unique features of TA systems readily give rise to two stable populations of bacteria, a small dormant population and a rapidly growing population (**Fig. 3**)[67]. Stated most simply, cells enter the persister state when the toxin concentration exceeds a threshold set by that of its antitoxin. How is this achieved within a population of growing cells? One possibility is that some cells, owing to local accessibility to nutrients, experience micro-starvation conditions. This results in an increase in the levels of the (p)ppGpp alarmone, which activates the Lon protease and results in antitoxin degradation[64,68,69]. The net result is a switch from a low to high TA ratio, allowing the now free toxin to exert its toxic effects. However, although this provides a molecular description of how cells enter the persister state, the molecular mechanisms that allow cells to exit the persister state have proven more elusive. Critically, a number of recent studies have demonstrated that one mechanism potentially used by type II TA systems to exit persistence is a phenomenon known as conditional cooperativity[26,70,71].

Conditional cooperativity

Most TA loci, especially type II TA loci, are transcriptionally autoregulated[72]. This is because the antitoxins contain two domains, one that binds DNA and a second that binds their cognate toxins. The antitoxins, via their DNA binding domains, bind directly to one or more operators located in the promoters of their respective TA loci, thereby repressing transcription. In most, but not all, cases, the cognate toxins bind the antitoxins to enhance DNA binding. This increased affinity results in a more potent repression of transcription than is observed with the antitoxins alone[14,26,70,71].

The toxin-binding domains of antitoxins are often intrinsically disordered (IDPs, intrinsically disordered proteins) in the absence of toxin[47,51,73–75]. Although IDPs are present throughout the eukaryotic proteome, where they play essential roles in signaling, among other processes[76–79], they are less prevalent in the prokaryotic genome.

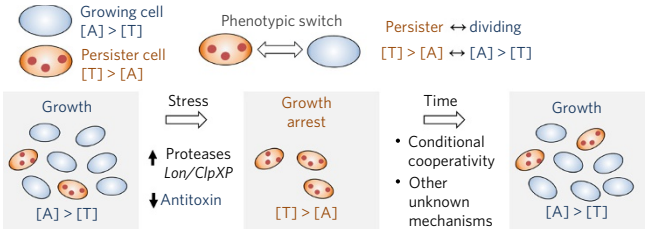

Figure 3 | TA systems and persistence. In growing cells (blue), the concentration of antitoxin exceeds that of the toxin. In persister cells (orange), the situation is reversed, with toxin concentration exceeding antitoxin concentration. Switching from one phenotypic state to the other requires the ratio of toxin to antitoxin to change. In a growing bacterial population, a small fraction of cells are in the persister state. Exposure to stress, such as nutrient deprivation or antibiotics, leads to the activation of bacterial proteases, especially *Lon* and *ClpXP*, which preferentially cleave antitoxins, resulting in an excess of toxin. This results is the rapid killing of the majority of actively growing cells; the exposure to stress may also activate a very small fraction of cells to switch into the persister state. Less is known about how cells emerge from the persister state, but one potential mechanism observed in multiple type II TA systems is conditional cooperativity.

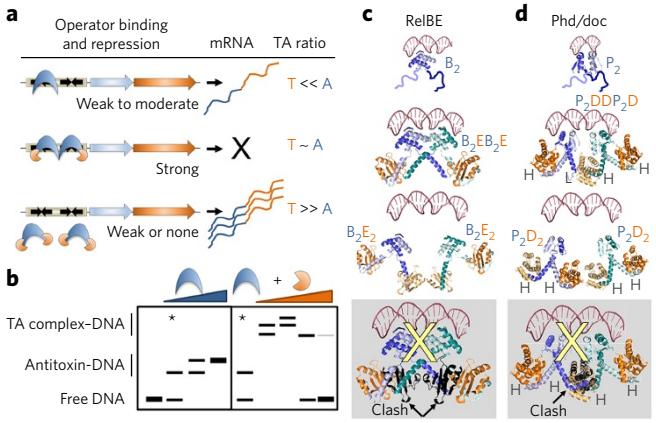

Figure 4 | The molecular basis of conditional cooperativity. (**a**) Cartoon illustrating the distinct TA oligomerization states, their ability to bind their promoters and the resulting transcription levels. Gene loci are shown as arrows; promoters are in beige, antitoxins in blue and toxins in orange. Antitoxins bind their promoters weakly, resulting in moderate transcriptional repression. When the TA ratio is similar, toxin binding to the antitoxin enhances its affinity for DNA, resulting in robust repression. Increasing concentrations of toxin destabilize DNA binding, resulting in robust transcription. (**b**) Schematic of conditional cooperativity using electrophoretic mobility shift assays. Left, increasing concentrations of antitoxin results in enhanced DNA binding; most promoters have multiple operators, leading to multiply shifted bands. Right, in the presence of antitoxin (at concentration indicated by *), increasing concentrations of toxin results in robust DNA binding until a certain TA ratio is reached, after which binding is reduced. (**c**) In the absence of RelE (orange/gold), RelB (blue/light blue) binds its promoter, which has two adjacent operators, with weak affinity (only one shown in top panel). At a 2:1 RelB:RelE ratio (B_2EB_2E), both operators are bound by the RelB dimers while only one RelB within each dimer is bound to RelE toxin (middle top panel). However, increasing concentrations of RelE lead to the formation of a RelB–RelE complex at a 1:1 ratio (B_2E_2). This oligomerization state is incompatible with DNA binding (middle bottom panel) because the newly bound RelE toxins (black in bottom panel; indicated by arrows) would clash with the adjacent B_2E_2 dimer (PDB ID 4XFE[51]; interaction with DNA modeled). (**d**) In the absence of Doc (orange/gold), Phd (blue/light blue) binds its promoter, which has two adjacent operators, with weak affinity (only one shown in top panel). At Phd:Doc ratios of 4:1, 4:2 or 4:3, DNA binding is robust (middle top panel; the 4:3 ratio is shown: P_2DDP_2D). In these oligomers, the central Doc toxin interacts with adjacent Phd dimers via a high-affinity (H) and a low-affinity site (L). Further increases in the concentration of Doc lead to a Phd:Doc ratio of 1:1 (P_2D_2). In this state, all the Doc toxins bind the PhD antitoxins at only the high-affinity sites. This leads to derepression because the newly bound Doc toxin would clash with the toxin in an adjacent complex (newly bound toxin at the high-affinity site in black, indicated by arrows; PDB ID 3K33 (ref. 70); interaction with DNA modeled).

Antitoxins are the notable exception, in that most antitoxin binding domains are IDPs in the absence of toxin[51,75,80,81]. What is the advantage of the disordered state? One hypothesis is that the IDP nature of the toxin-binding domain may make antitoxins more susceptible to proteolytic degradation than their toxin counterparts[82]. A second is that they can mediate high-affinity interactions with only a limited number of residues: that is, IDPs are able to bind to extended surfaces that would require four- to five-fold more residues if the domain were folded[83]. A third is that they are important for TA system regulation[70,75]. The latter has been experimentally demonstrated in a number of TA systems in which the IDPs function to couple transcription with toxin activity and inhibition[84]. They also appear to be especially important for systems regulated by conditional cooperativity, as described below.

In 1998, it was discovered that in the Doc/Phd TA system, large excesses of toxin derepressed rather than repressed transcription[85], a phenomenon that has since been named conditional cooperativity[70]. A similar observation was made shortly thereafter (**Fig. 4a**): namely, that the switch from a repression to a derepression complex was accompanied by a change in the oligomerization state of the TA complex itself. This group studied the Type II TA system CcdB/CcdA (comprising control of cell death protein B, the toxin that inhibits DNA gyrase, and protein A, the antitoxin). Maximal DNA binding and repression was observed when the ratio of CcdB to CcdA was 1:1 ($CcdB_2$–$CcdA_2$). However, CcdB:CcdA ratios greater than 1 abolished DNA binding (**Fig. 4b**). This resulted in enhanced, rather than repressed, transcription[26]. Thus, the authors hypothesized that under conditions of excess toxin, the transcription and translation of both CcdA and CcdB would increase, eventually leading to a ratio that permitted cells to exit the persister state and resume robust growth. Since then, additional TA systems have also been shown to be regulated by conditional cooperativity, including *parDE*[86], *relBE*[71] *kid/kis*[87], *vapBC*[58] and *phd/doc*[85]. Critically, incorporating conditional cooperativity into mathematical models of TA regulation have shown that it can provide the bistability necessary to support two populations within a single culture, one actively growing and one dormant[88,89]. Thus, the prevalence of conditional cooperativity in the type II TA family has led to the suggestion that it is a mechanism by which TA transcription is dynamically adjusted to the toxin concentration in the cell and, furthermore, that it might provide a mechanism for exiting the persister state. In spite of the insights provided by these recent experimental and modeling studies, the detailed mechanisms by which cells emerge from the persister state to repopulate the original population remain to be elucidated.

Molecular basis of conditional cooperativity

How these distinct TA complex oligomerization states alter antitoxin affinity for DNA is also now being unraveled at a molecular level. RelB alone has only weak affinity for its operator (**Fig. 4c**). In contrast, the $RelB_2$–RelE (B_2E) complex binds extremely tightly and potently inhibits *relBE* transcription. However, further increases in RelE toxin concentration destabilize DNA binding, with DNA binding reaching affinity levels similar to those for RelB alone at

ratios of 1:1 ($RelB_2$–$RelE_2$, B_2E_2)[71]. The three-dimensional structure of the B_2E_2 complex revealed why it is not compatible with DNA binding[51]. As shown in **Figure 4c**, two B_2E (B_2E–B_2E) complexes bind the *relEB* operator without clashing. However, increasing concentrations of RelE leads to the formation of two B_2E_2 complexes. This destabilizes DNA binding because the additional RelE molecules (B_2E–B_2E → B_2E_2–B_2E_2; **Fig. 4c**, bottom) clash with the neighboring proteins. Thus, the B_2E_2 complexes are unable to bind both operators simultaneously, resulting in derepression of the operon and an increase in transcription.

Similarly, the Phd antitoxin of the Phd/Doc TA system also binds its promoter with moderate affinity compared to the Phd_2–Doc complex[85]. Increasing concentrations of the Doc toxin, like those of RelE, destabilize DNA binding, with very little DNA binding observed at 1:1 ratios and higher[70]. However, the structural mechanism is different than that for the RelB/RelE system. That is, PhD binds Doc at two sites, a high-affinity (H) binding site and a low-affinity (L) binding site (**Fig. 4d**). The affinity of one or two PhD molecules for the *phd/doc* promoter is weak. However, the addition of the Doc toxin increases the affinity considerably because Doc stabilizes the PhD molecules by binding one of the PhD proteins via the H interface and the second via the L interface. This stabilization, in addition to the increased avidity due to the bridging of two adjacent Phd dimers on the operator, enhances the affinity of the complex for DNA[70]. Up to two more Doc molecules can bind this complex without destabilizing binding, as they bind the available H sites of the free PhD molecules. However, further addition of Doc results in fully occupied H sites, which leads to steric clashing when bound to DNA. As a result, the repressor complex is destabilized and transcription increases. Although the molecular details differ, the *ccdAB* TA system uses a similar mechanism of autoregulation, and thus the two systems represent an interesting case of convergent evolution[75].

Importantly, while a number of type II TA systems are regulated by conditional cooperativity, some are not (**Fig. 5a**). This includes *dinJyafQ*[90] and *mqsRA*[28] (**Fig. 5b**). In the case of DinJ/YafQ, both the DinJ antitoxin and the YafQ TA complex bind and repress transcription to the same extent. In the case of MqsR/MqsA, MqsA binds its promoter with extremely tight affinity (K_d ~800 pM), resulting in a clamping of the antitoxin about its DNA operator[91]. Addition of the MqsR toxin, however, does not enhance binding but instead destabilizes it, as the binding sites of MqsR and DNA on MqsA overlap: that is, their binding is mutually exclusive. Thus, the mechanism(s) used by these systems to dynamically adjust antitoxin and toxin concentrations in the cell once they have entered the persister state are still unknown.

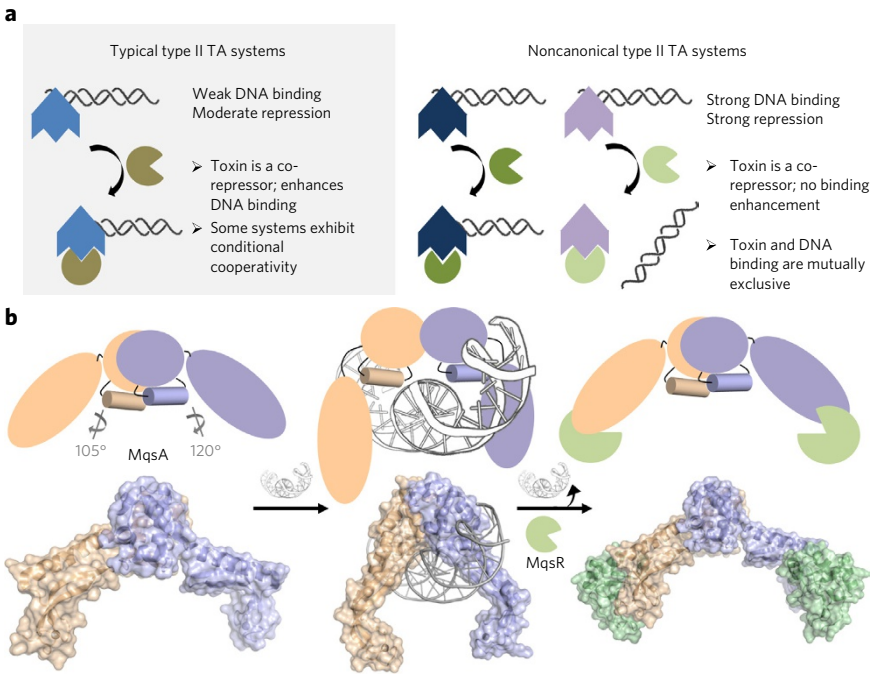

Figure 5 | Characteristics of type II TA systems that exhibit or do not exhibit conditional cooperativity. (**a**) Many TA systems exhibit conditional cooperativity. In these cases, the toxins function as co-repressors that enhance DNA binding. Two systems that do not exhibit conditional cooperativity are DinJ/YafQ (the YafQ toxin does not enhance DNA binding and increasing concentrations of YafQ do not result in increased transcription) and MqsRA (MqsR functions solely as a derepressor). (**b**) Cartoon and structural illustration of the MqsA dimer (left; PDB ID 3GN5; ref. 39), the MqsA–operator complex (middle; binds DNA with a subnanomolar K_d; PDB ID 3O9X[91]) and a model of the $MqsR_2$–$MqsA_2$ complex (right; modeled using PDB IDs 3GN5 and 3HI2; ref. 39). MqsR, which also binds MqsA with a subnanomolar K_d, destabilizes DNA binding[28].

Outlook: targeting persistence by targeting TA systems

Each year, 2 million people in the United States become infected with bacteria that are resistant to antibiotics, with an estimated 23,000 of those dying as a result of those infections[92]. It is now clear that persisters are likely to play a role in at least some of these deaths. This is because persisters underlie latent infections and post-treatment relapse. Furthermore, the repeated rounds of antibiotic treatments needed to treat these recurrent infections increase the likelihood that the bacteria will become resistant to the antibiotics used to eradicate them. Thus, new strategies for eliminating persisters not only will reduce the number of recurrent infections but also are expected to have the added benefit of reducing the number of antibiotic-resistant mutations.

The importance of TA systems in persistence make them natural targets for drugs that either prevent persistence or facilitate exit from the persister state. One possible approach is to develop drugs that inhibit the toxins, thereby inhibiting entry into growth arrest. Because most free-living bacteria contain multiple TA systems, it is still not clear whether inhibiting a single TA system (toxin) will be sufficient to inhibit persistence; rather, it might require a cocktail of inhibitors. Another possibility is to target biological processes that regulate TA levels. The feasibility of this approach was recently demonstrated by Lewis and colleagues, who showed that the compound acyldepsipeptide (ADEP4), which binds the ClpP protease, eliminates persisters[93]. It does so by causing ClpP to become a non-specific protease that targets both nascent and unfolded proteins for degradation. Remarkably, they showed that using both ADEP4 and rifampicin completely eradicated the chronic biofilms of both the astomyelitis-associated strain UAMS-1 and *Staphylococcus aureus*. Although it will be important to determine whether this intervention is applicable to other pathological organisms, these results are very exciting. Finally, another less direct but also intriguing strategy is the design of peptides that inhibit DNA gyrase. For example, the structure of the ParE toxin was used to design a series of peptides that completely inhibited bacterial DNA gyrase[94]. Clearly, current efforts to understand the molecular basis of persistence and to determine how these recalcitrant cells can be effectively targeted by novel therapeutics suggest that new effective antibiotic therapies may be on the immediate horizon.

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Competing financial interests

The authors declare no competing financial interests.

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