Biology and evolution of bacterial toxin–antitoxin systems

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Abstract | Toxin–antitoxin systems are widespread in bacterial genomes. They are usually composed of two elements: a toxin that inhibits an essential cellular process and an antitoxin that counteracts its cognate toxin. In the past decade, a number of new toxin–antitoxin systems have been described, bringing new growth inhibition mechanisms to light as well as novel modes of antitoxicity. However, recent advances in the field profoundly questioned the role of these systems in bacterial physiology, stress response and antimicrobial persistence. This shifted the paradigm of the functions of toxin–antitoxin systems to roles related to interactions between hosts and their mobile genetic elements, such as viral defence or plasmid stability. In this Review, we summarize the recent progress in understanding the biology and evolution of these small genetic elements, and discuss how genomic conflicts could shape the diversification of toxin–antitoxin systems.

Toxin–antitoxin systems were discovered in the 1980s as two-gene modules that promote the maintenance of conjugative plasmids[1,2]. Thorough characterization of two such modules encoded on the R1 and F plasmids led to the establishment of the 'postsegregational killing' (PSK) model, referring to the ability of these toxin–antitoxin modules to kill cells that fail to inherit a plasmid copy[1,2]. As the survival of the host cells depends on the presence of the toxin–antitoxin genes, this phenomenon became widely known as 'plasmid addiction'[3]. Although these two modules promote the same PSK phenotype, the regulation and mode of action of these analogous systems seemed to involve different molecular mechanisms. In the case of the *ccd* system of the F plasmid, both components were proteins and expression of the *ccdB* toxic gene alone led to cell filamentation, induction of the SOS response and λ prophage induction[4–6]. It was later shown that the CcdA antitoxin protein interacts with the CcdB toxin, thereby inhibiting its toxic activity[7]. By contrast, expression of the *hok* toxin from the *hok–sok* system of plasmid R1 led to the formation of so-called ghost cells, indicative of cell membrane permeation[2,8]. In contrast with the *ccd* system, rescue of Hok-induced toxicity relied on an antisense RNA complementary to the *hok* mRNA, inhibiting translation of the Hok toxin[9]. These two discoveries paved the way for the field and defined the first two types of toxin–antitoxin system: type I (*hok–sok*) and type II (*ccd*). The PSK phenomenon relies in both cases on a differential stability of the antitoxin and toxin components: the toxic proteins are stable, whereas the antitoxin protein or RNA is unstable[9–11]. Upon plasmid loss, these antitoxins are degraded and toxins are produced (type I) or freed from the antitoxin–toxin complex (type II), leading to the death of plasmid-free cells.

Throughout the years, other plasmid-encoded toxin–antitoxin systems were discovered and characterized. Surprisingly, homologues of these systems were found in the *Escherichia coli* chromosome[12–14]. With the expansion of bacterial genome sequencing, it was found that chromosomal toxin–antitoxin systems were far from uncommon, with often multiple copies found in a given replicon[15–18]. In chromosomes, toxin–antitoxin systems are located mostly in genomic islands (prophages, integrative and conjugative elements or transposons) or constitute small genomic islets by themselves[16,19,20]. With time, novel types of toxin–antitoxin system with different modes of regulation and novel toxicity mechanisms were uncovered[21–29]. Although the functions of plasmid-encoded systems remained unambiguous, those proposed for their chromosomal counterparts were sometimes contradictory. They included stress-induced programmed cell death (PCD), stress response-promoting fitness and antibiotic treatment survival. Recent advances in the field suggest alternative views for the roles and functions of these highly abundant and mobile elements in the light of genome evolution. Despite the controversies regarding the roles of toxin–antitoxin systems, the past few decades led to substantial advances in terms of discovery and thorough characterization of novel toxic activities and mechanisms of antitoxicity, greatly expanding the number of toxin and antitoxin families and their possible combinations[21–29]. Structural studies have shed light on the evolutionary links between toxins with different activities[30–33].

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Inteins
Internal segments of proteins that self-excise and ligate the remaining segments (exteins) during protein splicing.

Furthermore, diversity in structural properties, such as conformational behaviour and stability of antitoxins even within the same family, has been uncovered[34–36]. Ultimately, fold sharing between some families of toxins and antitoxins as well as non-toxin–antitoxin systems, such as secretion or defence systems, was found[37,38].

In this Review, we provide a comprehensive survey of the progress achieved in the toxin–antitoxin field. We describe the basis of toxin–antitoxin classification, regulation at different levels and an exhaustive list of molecular modes of action for toxins. We recapitulate the past paradigms and discrepancies regarding the roles of toxin–antitoxin systems and, in the light of recent developments, discuss the possibility that these modules could be major players of genome evolution by favouring the selection of replicons (chromosomes or mobile genetic elements (MGEs)) that carry them. We also provide examples of how natural properties of toxin–antitoxin systems are successfully used in biotechnology and as potential medical tools (BOX 1). Finally, we propose a series of unanswered questions that could shape future research in the toxin–antitoxin field.

Classifying toxin–antitoxin systems

The minimal composition of a toxin–antitoxin system consists of a toxin that poisons the producing cell and its cognate antitoxin. Genes encoding both components are always genetically linked, typically organized as operons (except for type I and type VIII systems). The vast majority of the described toxins are proteins (except for the newly discovered type VIII systems, in which case they are RNAs). Antitoxins are either RNAs or proteins that inhibit toxicity at different levels: translation, activity or even stability of the toxin. Classification of toxin–antitoxin systems is based on the nature and mode of action of antitoxins. Numbered in the order of their discovery, the current types are as follows (FIG. 1): type I antitoxins are small RNAs that silence the transcript of their cognate toxins[9,39–42] (FIG. 1a); type II antitoxins are proteins that bind and form a neutralizing complex with their cognate toxins[7,43–45] (FIG. 1b); type III antitoxins are small pseudoknot RNAs that bind their cognate toxins and sequester them by forming neutralizing protein–RNA complexes[21,46,47] (FIG. 1c); type IV antitoxins are proteins that act on the cellular targets of their cognate toxins and either protect or detoxify the target rather than blocking the toxin itself[26,48,49] (FIG. 1d); type V RNase antitoxins prevent the accumulation of the toxin by specifically degrading its mRNA[22] (FIG. 1e); type VI antitoxins are proteins that act as adaptors targeting their cognate toxin for degradation by ATP-dependent proteases[23] (FIG. 1f); type VII antitoxins are proteins that inactivate their cognate toxins through post-translational modifications[27,50,51] (FIG. 1g); and type VIII antitoxins are RNAs that repress the expression of their cognate RNA toxins either by acting as an antisense RNA or by

Box 1 | **Toxin–antitoxin systems as tools in human health and biotechnology**

Toxin–antitoxin system-based antimicrobials

Several studies exploited toxin–antitoxin systems as targets of antimicrobial compounds, for example, through the conception of small peptides that can disrupt toxin–antitoxin complexes[207–211]. One major concern is that typically such toxin-binding peptides are largely disordered, are unstable and do not easily cross cell membranes. Another strategy relies on synthetic stable antisense molecules that silence antitoxin expression but not toxin expression[212]. However, as toxin–antitoxin systems are very narrowly distributed, a similar strategy could be used to silence the toxin targets, which are, in general, much more conserved than toxin–antitoxin systems. Another approach would be to derive peptides based on toxin structure. For instance, DNA gyrase was successfully inhibited by peptides based on the CcdB structure[213]. Finally, an antimicrobial strategy was elaborated by splitting type II toxins with inteins. These fusions are programmed to re-ligate into a functional toxin under specific conditions, for example, when delivered in a specific host. Specific killing of antimicrobial-resistant *Vibrio cholerae* was achieved with use of this system in mixed populations[214].

Toxin-based antivirals and cancer therapies

Some of the toxins of toxin–antitoxin systems, such as endonucleases, are active when delivered to eukaryotic cells and were thus considered for gene therapies eliminating infected cells. For instance, fusion between the MazF toxin and its antitoxin using viral protease cleavage sites as linkers was shown to be cleaved efficiently by specific viral proteases, leading to the death of virus-infected cells[215]. Another strategy relies on the expression of MazF under the control of viral transactivators, leading to MazF expression in infected cells and viral replication inhibition[216]. Similarly, toxins were placed under tumour-specific regulation that would drive their expression in cancer cells to selectively eliminate malign cells[217,218].

Toxin–antitoxin systems as selection–counterselection systems

The *ccd* system was largely exploited in commercial applications by generation of positive selection vectors for the cloning of recombinant DNA. The first commercial application of a toxin–antitoxin system used the *ccdB* toxic gene to generate a cloning vector that enables positive selection[219,220]. A series of vectors containing a multiple cloning site embedded in the *ccdB* gene were developed. Cloning of a foreign DNA fragment would disrupt the *ccdB* sequence, rendering the plasmid no longer toxic, whereas the empty cloning vectors carrying the intact *ccdB* sequence would be counterselected. A similar strategy was adopted with the use of the type I toxin IbsC[221]. The *ccdB* gene was also used in a Gateway cloning system, where recombination of the desired DNA insert eliminates the *ccdB* gene and provides a powerful way of selecting only successful recombinants[219]. Further strategies used in recombinant protein production engineered strains that encode a toxin on the chromosome with the cognate antitoxin encoded on an expression vector, thus providing stability to expression vectors over prolonged periods without the need for antibiotics[222]. Similar strategies have also been applied in eukaryotes, where cell lines conditionally expressing the type II Kid toxin become dependent on transgene co-expression of the Kis antitoxin[223]. Toxin–antitoxin systems were also repurposed to enhance recombinant protein production. MazF expression was used to eliminate the majority of transcripts except the one encoding the recombinant protein of interest, which is engineered to be devoid of MazF-cleavage sequences[224,225].

Toxin–antitoxin system-based containment systems

The use of genetically modified organisms is limited owing to the risk of their uncontrolled spread. Toxin–antitoxin systems can be adopted as kill-switch modules, providing an attractive solution to contain various genetically modified organisms[217,226]. Gene-containment systems usually comprise toxin and antitoxin genes under the control of different promoters[217,227]. For example, the GeneGuard system encodes the toxin on the plasmid and the antitoxin on the chromosome, therefore coupling the plasmid to a specific host[228]. Other technologies were developed in plants to control the spread of transfer DNA from genetically modified agrobacteria or the development of specific plant tissues, notably tissue essential for pollen development[227,229].

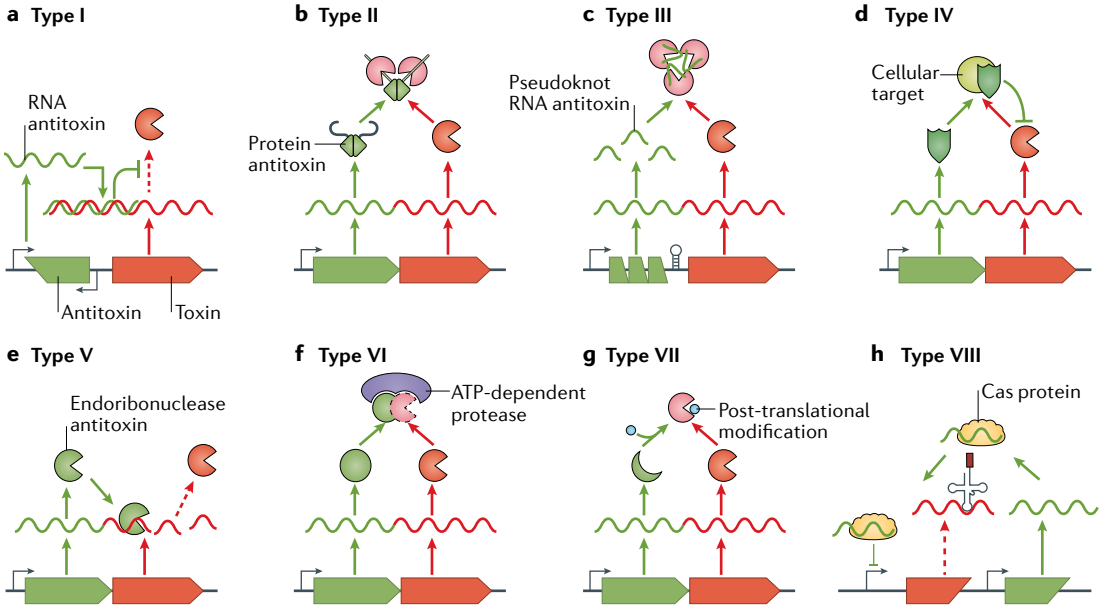

Fig. 1 | **Currently proposed types of toxin–antitoxin system. a** | Type I systems encode RNA antitoxins (green) that silence toxin expression (red). **b** | Type II systems encode protein antitoxins that interact with and sequester the toxins away from their targets. **c** | Type III systems encode pseudoknot RNA antitoxins that interact with and sequester the toxins away from their targets. **d** | Type IV systems encode protein antitoxins that protect the toxin targets from toxin activity. **e** | The type V system encodes an endoribonuclease antitoxin that specifically degrades the transcript encoding the toxin. **f** | The type VI system encodes an antitoxin protein that acts as an adaptor targeting the toxin for degradation. **g** | Type VII systems encode protein antitoxins that inactivate the toxin by post-translational modification. **h** | Type VIII systems encode RNA antitoxins that participate in the transcriptional repression of the RNA toxin expression. The example shown in this figure is based on the *creT–creA* system.

mimicking a CRISPR RNA that recruits a Cas protein acting as a transcriptional repressor[29,52] (FIG. 1h). In addition to these well-defined types, particular cases have been described. For instance, some antitoxins that modify the toxin or act on the cellular target (types VII and IV, respectively) can form antitoxin–toxin protein complexes reminiscent to those of type II systems[27,49], and some toxins can be associated with antitoxins of different types (II and IV) within the same operon[26].

Type II toxin–antitoxin systems are probably the most abundant and diverse, being represented in the genomes of most bacteria and some archaea[15,16,18,53]. Type I, III, IV, VII and VIII seem to be less represented[29,54–56], whereas types V and VI are constituted of only one single representative system[22,23].

Regulation of toxin–antitoxin systems

Toxin–antitoxin systems are often tightly regulated to maintain a homeostatic 'neutralized' state. Most of the time this regulation ensures stoichiometric excess of antitoxins over their cognate toxins in steady-state conditions. Various regulation mechanisms at every level of their expression have been described.

Transcriptional autoregulation

A large number of toxin–antitoxin systems, especially type II, are encoded in an antitoxin-first, toxin-second organization with overlapping open reading frames or separated by very short intergenic regions[16]. Type III toxin–antitoxin operons follow a similar organization, where antitoxin RNAs are encoded first; however, they are separated from the toxin open reading frames by Rho-independent terminators[47,55]. Such organization might limit the production of toxin and enable preferential synthesis of short-lived antitoxins. However, several type II toxin–antitoxin systems possess an inverse organization in which the toxin-encoding gene precedes the gene encoding the antitoxin. In some of these systems, constitutive promoters that specifically transcribe the antitoxin have been described[57–59].

Expression of type II toxin–antitoxin operons is generally autoregulated at the transcriptional level by toxin–antitoxin complexes (FIG. 2a). Antitoxins of these systems are composed of a structured DNA-binding domain that recognizes and binds to specific operator regions located within the toxin–antitoxin promoter and an intrinsically disordered domain that folds upon interaction with the cognate toxin[60–62]. In many type II systems, transcriptional repression is intimately linked to toxin neutralization. In such systems, depending on the antitoxin-to-toxin ratio, hetero-oligomeric complexes showing different affinities for the operator region are formed, with toxin-saturated complexes having generally low affinity for their promoter[63–65]. When the toxin level exceeds the antitoxin level, repression is alleviated, thereby enabling de novo transcription and translation of antitoxins[63–65] (FIG. 2a). This negative feedback loop, known as conditional cooperativity, elicits a tight homeostatic regulation, which is likely to prevent accidental toxin liberation due to transcriptional fluctuations of the toxin–antitoxin operon. However, the disorder-to-order recognition model is not universal to all type II systems

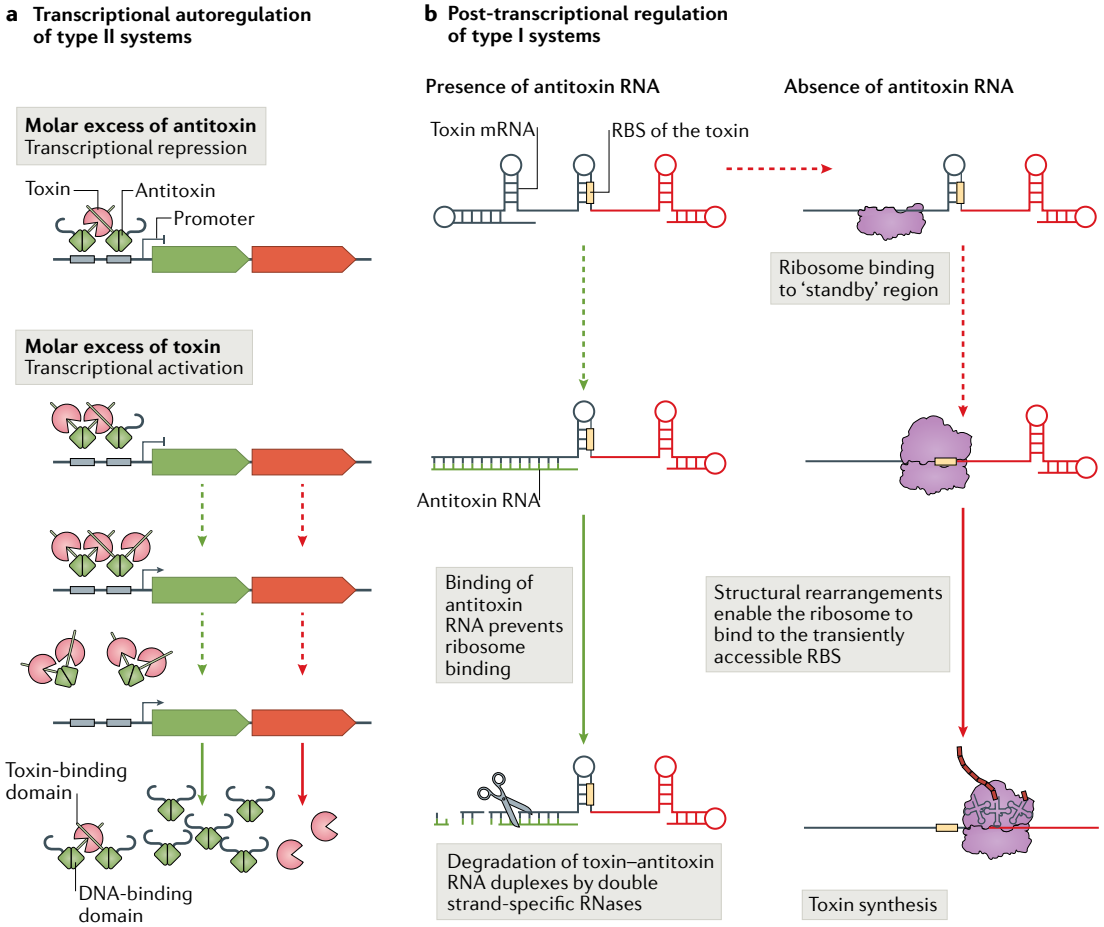

Fig. 2 | **Major types of regulation of toxin–antitoxin systems. a** | Transcriptional autoregulation of type II systems. Antitoxins (in green) are composed of two domains: a DNA-binding domain that recognizes palindromic sequences, overlapping with the promoter sequence of their cognate toxin–antitoxin operon, and a toxin-binding domain. In general, toxins (red) act as co-regulators, depending on the antitoxin-to-toxin ratio. Molar excess of antitoxin favours the formation of the repression complex. Molar excess of toxin reshapes the autoregulatory complex as antitoxic domains binding to extra toxins yield complexes unable to bind the operator sequences, leading to transcriptional activation of the toxin–antitoxin operon. **b** | Post-transcriptional regulation of type I systems. The toxin-encoding transcript forms secondary structures in which the ribosome-binding site (RBS) of the toxin (yellow box) is occluded in a hairpin structure. The antitoxin RNA (green) binds to the 5′ complementary sequence of the toxin mRNA (black) and outcompetes the ribosomes for binding to this region (left panel). The duplex RNA is degraded by double-strand-specific RNases (scissors). In the absence of the RNA antitoxin, the 30S ribosome subunit is able to bind to this 'standby' region (right panel). This leads to major structural rearrangements, and the ribosome relocates to the transiently accessible RBS and initiates synthesis of the toxin (red).

as some neutralizing domains of antitoxins form stably folded structures even in the absence of the cognate toxin[34–36,58,66]. In addition, there are several examples of type II systems where transcriptional regulation is exerted by a separate, third component, and the antitoxin has solely the neutralizing function[67,68]. In a newly described type VIII RNA–RNA toxin–antitoxin pair, the antitoxin RNA repurposes a CRISPR effector protein as a transcriptional repressor of the expression of the toxin RNA[29]. In this case, the antitoxicity mechanism principally relies on transcriptional repression.

Post-transcriptional regulation

The mode of regulation of type I toxin–antitoxin systems relies on the inhibition of toxin expression by complementary RNA antitoxins, which are often encoded on the strand opposite to the toxin-encoding gene[9,69,70]. In the case of the *E. coli* K-12 *hok–sok* and *tisB–istR* systems, complex modes of regulation that involve RNA processing, secondary structures and relocation of the ribosomes from a standby site located upstream of the temporarily accessible ribosome-binding site have been discovered (FIG. 2b). In the absence of the antitoxin, the 30S ribosome subunit binds upstream of the toxin ribosome-binding site sequence, which unfolds the hairpin masking the ribosome-binding site and makes it accessible to initiate toxin translation[40,71–73]. Type I RNA antitoxins compete with ribosomes for this region, and the resulting toxin–antitoxin RNA duplexes can further be cleaved by RNase III to eliminate the toxin transcript[40,74,75]. These RNA antitoxins were also shown to be less stable than the transcripts of their cognate toxins, thus enabling preferential retention of toxin transcripts and toxin translation when antitoxin RNAs are

not replenished[9]. In type II toxin–antitoxin systems, it has been reported that translation of toxins is much less efficient than that of antitoxins, which promotes a molar excess of antitoxin to ensure complete neutralization[76,77]. More specifically, the transcript coding for the Kid toxin of the *parD* type II system is specifically cleaved within the coding sequence, leading to lower toxin translation[77]. Whether this regulation applies to other type II systems remains to be investigated.

In type III systems, post-transcriptional maturation of antitoxin RNAs is required for the formation of the neutralizing toxin–antitoxin complex. The RNA antitoxin is composed of repeated sequences and transcribed as such. The endoribonuclease toxin then cleaves the precursor antitoxin RNA transcript into single repeats which autonomously adopt a pseudoknot structure that is able to neutralize the toxin[21,47,78].

Post-translational regulation

Once synthesized, type II and type III toxins are neutralized by forming tight complexes with their cognate antitoxins. Complexed toxins are inactivated through various mechanisms; for example, occlusion of the catalytic site of the toxin[47], steric hindrance that prevents toxins from accessing their substrate[79], disruption of secondary structures[44] or inhibition of the formation of catalytically active toxin dimers[45].

It has long been documented that many type II antitoxins are less stable than their cognate toxins, which is likely to be due to the recognition of their intrinsically disordered domains by ATP-dependent proteases such as Lon[10], ClpXP[80] or ClpAP[81]. However, degradation of the type II antitoxins engaged in the toxin–antitoxin complexes and subsequent liberation of the toxins from these complexes is much less documented. Antitoxins show increased stability when bound to their cognate toxins both in vivo and in vitro[10,82–84]. The long-lasting paradigm in the field suggested that stressful conditions would stimulate degradation of the antitoxin and promote liberation of the toxin (see later). However, in the case of several chromosomal *E. coli* toxin–antitoxin systems, it was recently shown that stress has a minimal impact on antitoxin stability and cannot account for toxin liberation[85]. A subgroup of type II toxin–antitoxin systems in which the third gene encodes a SecB-like chaperone (toxin–antitoxin–chaperone (TAC) systems) has been described[86–88]. In these cases, the chaperone is required for proper folding of the antitoxin, preventing degradation by ATP-dependent proteases. In the absence of the chaperone, the antitoxin undergoes aggregation and fails to neutralize the toxin[87]. Interestingly, antitoxins from different families are addicted to chaperone folding by acquisition of carboxy-terminal chaperone-addiction motifs[87]. Strikingly, these motifs comprise a protease recognition region for eventual degradation[88]. By contrast, the SocA type VI antitoxin functions as an adaptor that promotes the degradation of its cognate toxin by the ClpXP protease as a means of neutralization[23].

In type VII systems, antitoxins are enzymes that inhibit their cognate toxins by post-translational modifications at specific amino acid residues. Neutralization by phosphorylation[51], AMPylation[27] or oxidation[50] of the toxin has been described so far. Additionally, some type II toxins that exert their activity through post-translational modifications of their targets were also shown to self-modify at conserved residues, which leads to their inactivation in some cases. However, the physiological relevance of this process has not been properly assessed[89,90].

Toxin activities and targets

Typically, toxins are small single-domain proteins or peptides that are active in the cytoplasm or inner membrane of their host bacterium, although RNA toxins have also been described recently [29,52] (FIG. 3).

Toxins impairing DNA and replication

The CcdB type II toxin targets the GyrA subunit of DNA gyrase (FIG. 3a). By inhibiting the DNA re-ligation step of the DNA gyrase catalytic cycle, CcdB locks the enzyme on the DNA into a so-called cleaved complex, using a mode of action very similar to that of quinolone antibiotics. CcdB activity is thought to induce the collapse of replication forks, resulting in double-strand breaks, induction of the SOS response and cell death[91–93]. Expression of ParE type II toxins induces the SOS response as well, although these toxins are structurally unrelated to CcdB[67,94,95]. It remains unclear whether the molecular mechanism underlying ParE toxicity is similar to that of CcdB as a $GyrA_{462}$ mutant that is resistant to CcdB is still sensitive to ParE[92,96]. The type II Fic (filamentation induced by cAMP)-fold toxins AMPylate DNA gyrase and topoisomerase IV, which inhibits their ATP hydrolysis activity and results in the inhibition of DNA decatenation and replication[33] (FIG. 3a). In contrast to the aforementioned toxins acting on DNA gyrase, some toxins were shown to directly target DNA (FIG. 3a). The type IV DarT toxin is an ADP-ribosyltransferase that transfers an ADP-ribose moiety from NAD^+ onto single-stranded DNA, causing DNA damage and eliciting the SOS response[49]. The type I RalR toxin is a non-specific DNase that cleaves both methylated and unmethylated DNA[97]. Finally, the type VI SocB toxin was shown to impair the processivity of DNA replication by directly interacting with the β-sliding clamp of the DNA polymerase[23] (FIG. 3a).

Toxins degrading RNAs and impairing translation

Protein synthesis seems to be the primary target of type II toxins[98] (FIG. 3b). Many of these toxins are RNases with differing degree of specificity. For example, toxins from the MazF family degrade free RNAs with low specificity, targeting mRNAs as well as ribosomal RNA (rRNA) precursors[99–101]. However, some MazF toxins were shown to be specific to single species of tRNAs[102]. Interestingly, the ToxN type III toxins adopt the same fold as MazF toxins, and were also shown to degrade free mRNAs[46,47,78]. By contrast, toxins from the VapC family specifically cleave anticodon stem-loops of different target tRNAs[103–106], as well as the sarcin–ricin loop of the 23S rRNA, which shows structural similarities to anticodon stem-loops[105,107]. RelE and related toxins cleave mRNAs co-translationally by entering the A site of

Chaperone-addiction motifs
Specific sequences that promote antitoxin destabilization unless recognized by the chaperone.

DNA gyrase
Type II topoisomerase enzyme that relieves positive supercoiling in front of the replication forks.

Aminoacylation
Ligation of an amino acid to its cognate tRNA, also known as tRNA charging.

AMPylase
Enzyme that ligates AMP to an amino acid side chain of a target protein.

translating ribosomes. Cleavage usually occurs between the second position and the third position of the targeted codons[108–110]. HicA toxins show RNase activity, although their substrate specificities remain to be investigated[111]. The rather unusual type I RNase toxin SymE adopts an AbrB fold, which is found in DNA-binding proteins, including MazE antitoxins[41].

A variety of type II toxins affect tRNA functions through post-translational modifications of tRNAs or factors that service the tRNAs. The HipA toxin specifically phosphorylates aminoacyl-tRNA synthetases and therefore inhibits charging of specific tRNAs[112,113]. By contrast, the Doc toxin phosphorylates and inactivates elongation factor Tu (EF-Tu), thus inhibiting tRNA presentation to the translating ribosome[32]. Interestingly, Doc adopts a Fic fold similarly to the AMPylating enzymes that target topoisomerases but uses an inverted substrate to transfer a phosphate group instead of the AMP moiety[32]. GNAT-fold acetyltransferase toxins inactivate tRNAs by acetylating the amino acid charged on specific tRNAs[114–116]. The recently characterized ToxSAS (toxic small alarmone synthetase) family FaRel2 toxin transfers a pyrophosphate moiety from ATP to the CCA end of tRNA acceptor stems, thereby impairing aminoacylation[28]. Type VII toxins also compromise the functions of tRNAs either by ligation of pyrimidines to their acceptor stems, which prevents their charging in the case of the MenT toxin[25], or by cleaving the acceptor stem of a subset of tRNAs in the case of HEPN toxins[27]. The newly described CreT type VIII toxin sequesters rare tRNAs and therefore causes growth arrest[29].

Toxins impairing cell envelope and cytoskeleton integrity

The smallest toxins are single-helix transmembrane peptides typically encoded by type I systems, such as Hok, TisB, Lsr, DinQ and Fst. Although most of them were shown to cause membrane depolarization and disrupt the proton motive force upon insertion in the inner membrane[2,8,42,117,118], some were also reported to impair cell division by triggering nucleoid condensation[39,118,119] (FIG. 3c). The type V GhoT toxin is predicted to have two membrane-piercing helices, and its overexpression results in ghost cells, similarly to type I toxin–antitoxin systems[22]. Type II ζ-toxins inhibit peptidoglycan and lipopolysaccharide biosynthesis by depleting UDP-activated sugars through phosphorylation, which results in a loss of cell wall integrity[120,121]. Finally, the

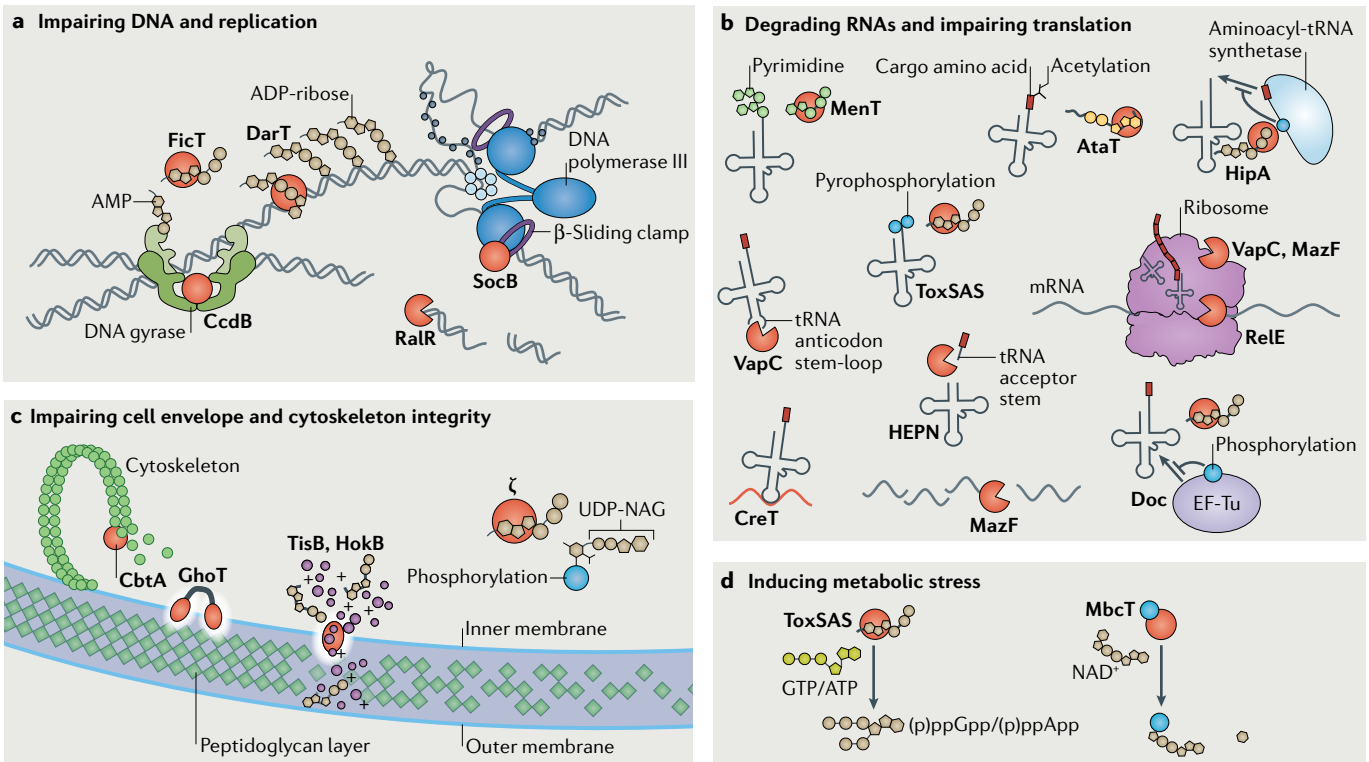

Fig. 3 | **Cellular activities of toxins of toxin–antitoxin systems. a** | DNA is directly targeted by the DNase RalR and the ADP-ribosyltransferase DarT. The replication machinery is targeted by SocB, which binds to the β-sliding clamp subunit of DNA polymerase III. Topoisomerases are inactivated by the AMPylase FicT. DNA gyrase is poisoned by direct binding of CcdB. **b** | Translation is targeted by a multitude of toxins acting at every level of protein synthesis. VapC toxins cleave tRNA anticodon stem-loops or the sarcin–ricin loop of the 23S ribosomal RNA. HEPN toxins cleave tRNA acceptor stems, whereas the MenT toxin blocks them by adding pyrimidines and toxic small alarmone synthetase (ToxSAS) by pyrophosphorylation. AtaT-like toxins acetylate the cargo amino acids of tRNAs. The CreT toxin sequesters rare tRNAs. HipA toxins phosphorylate aminoacyl-tRNA synthetases and prevent tRNA charging. Doc phosphorylates elongation factor Tu (EF-Tu) and prevents delivery of tRNAs to the ribosome. MazF toxins degrade free mRNAs and ribosomal RNAs, whereas RelE toxins cleave translated mRNA in the ribosomal A site. **c** | TisB, HokB and GhoT are small peptides that form pores in the inner membrane and disturb its integrity. ζ toxins phosphorylate precursors of peptidoglycan synthesis. The CbtA toxin binds to the septum protein FtsZ and the cytoskeleton protein MreB and inhibits their polymerization. **d** | ToxSAS toxins exhaust cellular GTP and ATP pools by synthesizing the alarmone (p)ppGpp. The MbcT toxin degrades NAD^+. NAG, *N*-acetylglucosamine.

type IV CbtA toxin was shown to inhibit cell division by preventing polymerization of both MreB and FtsZ through direct interactions[48,122].

Toxins inducing metabolic stress

Some recently described toxins degrade or synthesize small metabolites, which induces metabolic stress and inhibits cellular functions. For example, the type II MbcT toxin hydrolyses and depletes NAD^+, a central electron carrier essential for redox reactions[24]. A subfamily of ToxSAS toxins (FaRel) from type II and type IV systems feature a Rel-like (p)ppGpp synthase domain and deplete GTP–GDP and ATP–ADP pools by pyrophosphorylation, which leads to the accumulation of (p)ppGpp and (p)ppApp, respectively[26] (FIG. 3d).

Roles of toxin–antitoxin systems

Regulation of bacterial physiology and fitness

Following their discovery on bacterial chromosomes, type I and type II toxin–antitoxin systems were considered as beneficial elements for adaptation, increasing the fitness of bacterial populations in the face of stress conditions, as effectors of PCD, stress responses or antibiotic persistence[123–127]. These major models stem mainly from studies performed with *E. coli* K-12 laboratory strains (unless otherwise indicated), which contain 19 type I systems and 12 type II systems, respectively. Among those, particular interest was shown in ten type II RNase toxins and two type I pore-forming toxins. The proposed mechanism behind most of these phenotypes suggests that type II toxin–antitoxin systems are 'activated' under stressful conditions; that is, that the toxin is liberated from the toxin–antitoxin complexes owing to antitoxin degradation and can thus exert its toxic activity. This so-called activation would lead to different outcomes, depending on the model: in the PCD model, MazF activation would lead to cell death[124], whereas in the stress response and persistence models, activation of toxins (including MazF) would lead to reversible cell growth inhibition[13]. However, the relevance and validity of these models were strongly questioned and are controversial, as discussed next.

MazEF as a suicide module governing programmed cell death under many stress conditions. The MazF type II toxin was shown to be the central effector of a complex pathway triggered by several stresses, notably amino acid starvation, thymineless death, DNA damage, oxidative stress and antibiotic treatments. These conditions were proposed to cause depletion of the MazE antitoxin in a (p)ppGpp-dependent manner. The release of free MazF would lead to translation inhibition in a selective manner: expression of supposed survival proteins would be shut down, while mRNAs encoding cell death proteins devoid of specific codons cleaved by MazF (ACA) would be resistant and translated in these conditions. This selective translation inhibition would ultimately result in the death of the vast majority of the population, thereby releasing nutrients for surviving cells as a form of altruistic behavior[123,124,128–130]. Activation of MazF is thought to occur via the EDF (extracellular death factor) quorum sensing-like peptide, which results from the proteolytic degradation of the enzyme glucose 6-phosphate dehydrogenase by the ClpXP ATP-dependent protease[128]. EDF is thought to activate the MazF endoribonuclease activity by competing with MazE antitoxin binding[129]. The PCD model was further expanded by introduction of the notion of translation reprogramming: a MazF regulon was proposed to be composed of genes containing the ACA MazF cleavage site at their 5′ untranslated regions. Upon MazF activation, cleavage would occur preferentially at these sites, thereby generating leaderless transcripts. Concomitantly, MazF would cleave an ACA site at the 3′ end of 16S rRNAs, eliminating the anti-Shine–Dalgarno sequence and thereby generating specialized ribosomes that would preferentially translate the pool of leaderless mRNAs that constitute the MazF regulon[131].

A first study questioned the PCD hypothesis by showing that MazF toxicity was bacteriostatic and fully reversible upon overexpression of its cognate antitoxin MazE[132]. Subsequent studies revealed that some of the strains used in the original work[130] were actually mutated for the *relA* gene encoding the main enzyme involved in (p)ppGpp synthesis[133,134], although the study authors claimed that the PCD phenotype was dependent on (p)ppGpp synthesis. In addition, these studies failed to reproduce the PCD phenotype either with the original strains or with *relA*$^+$ strains[133,134]. Regarding MazF-mediated translation reprogramming, RNA-sequencing data revealed that MazF cleaves most transcripts within their coding region, showing no preference for 5′ untranslated regions. MazF-generated leaderless mRNAs were also cleaved in their coding sequences and were not preferentially translated[99,101,135]. Moreover, MazF was shown to be unable to cleave rRNAs in assembled ribosomes and instead cleaves pre-rRNAs, preventing ribosome maturation and translation activity[99,100].

Toxin–antitoxin systems as bacteriostatic stress modules and effectors of dormancy involved in antibiotic persistence. As mentioned already, although the PCD model assumes that activation of the *mazEF* system is irreversible, independent studies reported that ectopic induction of MazF and RelE, another type II toxin, is bacteriostatic and can be rescued by later co-expression of their cognate antitoxins[132]. Ectopic overexpression of these toxins was shown to reduce translation by cleaving mRNAs[13,136]. In addition, transcription of the *mazEF* and *relBE* systems was shown to be upregulated upon amino acid starvation in a (p)ppGpp-independent manner and interpreted as activation of these systems. These data led to a model in which chromosomally encoded toxin–antitoxin systems would constitute a new type of stress response element reducing the global level of translation during nutritional stress in a (p)ppGpp-independent manner[13,136,137]. Later, the type II *mqsRA* system, in which the toxin gene precedes the antitoxin gene, was associated with multiple phenotypes, including motility, biofilm formation[138] and resistance to bile salts[139] and oxidative stress[127]. In addition to its autoregulatory activity, the MqsA antitoxin was strikingly shown to act as a global transcriptional regulator by repressing

Thymineless death
Rapid loss of viability occurring as a result of thymine deprivation.

Anti-Shine–Dalgarno sequence
Sequence in the prokaryotic ribosome that helps to align the ribosome for translation initiation at the ATG start codon.

expression of the *rpoS* and *csgD* genes, which encode pleiotropic regulators of stress responses and biofilm formation[127,140].

In the past decade, chromosomal toxin–antitoxin systems were widely associated with the generation of antibiotic persister cells. The combination of multiple toxin–antitoxin deletions in the so-called Δ10 strain (including *mazEF* and *relBE*) in *E. coli* led to a gradual decrease of persistence upon exposure to ampicillin and ciprofloxacin, indicating that these ten toxin–antitoxin systems are redundant and act synergistically[126]. Further single-cell experiments based on the use of fluorescent reporters revealed the signalling cascade leading to toxin–antitoxin activation: stochastic accumulation of (p)ppGpp in persister cells would result in the activation of the Lon protease through the synthesis of polyphosphate, resulting in the proteolysis of antitoxins, the release of toxins and global translation arrest. This (p)ppGpp-dependent toxin–antitoxin-induced 'dormancy' would enable a few intoxicated cells to transiently tolerate antibiotic treatments and resume growth upon antibiotic removal[126]. A similar model was proposed for *Salmonella enterica* subsp. *enterica* serovar Typhimurium grown in macrophages in vitro: toxin–antitoxin systems would be activated in the *Salmonella*-containing vacuole upon phagocytosis by macrophages, inducing a dormant state that might promote tolerance to antibiotic treatment and regrowth upon antibiotic removal[125]. In this case, multiple toxin–antitoxin systems were also shown to be involved, however in a non-redundant manner as single toxin–antitoxin deletions showed for most of them a strong decrease in antibiotic persistence frequency. As for *E. coli*, (p)ppGpp and the Lon protease were shown to participate in the activation of these toxin–antitoxin systems, and thus to be pivotal for persistence[126]. Implications of type I toxin–antitoxin systems in antibiotic persistence have been less documented, with two major examples involving the HokB and TisB pore-forming toxins in antibiotic persister cell formation[141–143]. Their expression leads to membrane depolarization through their pore-forming toxic activity, thereby depleting ATP levels and inducing a dormant state. For TisB, ciprofloxacin triggers the SOS response and *tisB* expression as a result[141]. In the case of HokB, activation of the toxin results from a signalling cascade initiated by (p)ppGpp as for type II toxins, but involving the protein ObgE, a versatile GTPase considered to be a cell cycle checkpoint regulator[142].

A first study questioned the signalling cascade involved in the activation of a particular type II toxin–antitoxin system of *E. coli* (*yefM–yoeB*, deleted in the Δ10 strain). It was shown that Lon-dependent degradation of the YefM antitoxin and transcriptional activation of the toxin–antitoxin system upon amino acid starvation do not rely on (p)ppGpp and polyphosphate[144]. Two later studies refuted the involvement of type II toxin–antitoxin systems in *E. coli* persistence by showing that the major strains used in these studies were contaminated by Φ80 and λ phages[145–148]. In addition, major experimental flaws concerning the use of fluorescent reporters monitoring (p)ppGpp synthesis and toxin–antitoxin system activation were evidenced, ruling out the entire regulatory cascade potentially leading to the activation of toxin–antitoxin systems in persisters at the single-cell level[148]. Toxin–antitoxin systems were also shown to be dispensable for antibiotic persistence of *Salmonella enterica* and *Pseudomonas putida* grown in vitro[149,150], thereby further questioning the link between toxin–antitoxin systems and persistence upon exposure to antibiotics.

More generally, the concept of toxin–antitoxin system activation under stress conditions was recently revisited for the ten RNase toxins characterized in *E. coli*[85]. Various abiotic stresses (for example, antibiotics or heat shock) were shown to induce transcription of toxin–antitoxin operons. However, this transcriptional upregulation did not result in toxin-specific RNA cleavage, indicating that toxins are not liberated from toxin–antitoxin complexes under these stress conditions. Transcriptional upregulation of these toxin–antitoxin operons is likely to be a secondary effect resulting from the alleviation of autoregulation due to antitoxin degradation[85,144,151]. Indeed, antitoxin mutants that lacked DNA-binding activity, as well as mutants devoid of the Lon protease, no longer displayed transcriptional activation under stress conditions[85]. In the case of type I systems, although high ObgE and HokB levels correlate with high persistence at the single-cell level, deletion of *hokB* had no impact on survival in the presence of ofloxacin[142]. The involvement of TisB in persistence upon exposure to fluoroquinolones could not be reproduced by independent studies and might be dependent on the experimental conditions used[141,152,153]. In conclusion, the role of chromosomally encoded toxin–antitoxin systems in stress responses and antibiotic persistence seems to be tenuous. However, there are reports of point mutations in the genes encoding toxin–antitoxin systems that increase persistence[154–156], the most documented example being the *hipA7* gain-of-function allele, where a dysregulation of the system induces growth latency and tolerance in a small population of cells[157]. Therefore, although toxin–antitoxin systems do not seem to regulate persistence per se, they constitute malleable frameworks on which high-persistence mutants can be selected.

Toxin–antitoxin systems and genomic conflicts

A parsimonious view is to consider toxin–antitoxin systems as selfish genes. They show a non-uniform distribution among genomes and are prone to horizontal gene transfer. They promote their own vertical transmission and thereby benefit the replicon that carries them, sometimes at the expense of the host cell[5,43,71]. Another hallmark of selfish elements is their involvement in genomic conflicts[21,158–160], which becomes increasingly described for toxin–antitoxin systems and might constitute the basis for their functions.

Addiction: ensuring vertical transmission and horizontal transmission. As stated already, plasmid-encoded toxin–antitoxin systems promote vertical inheritance of their replicon by a mechanism known as PSK (or addiction)[2]. Owing to the intrinsic instability of antitoxins, failure to inherit a toxin–antitoxin-encoding plasmid leads to antitoxin depletion and killing of plasmid-free

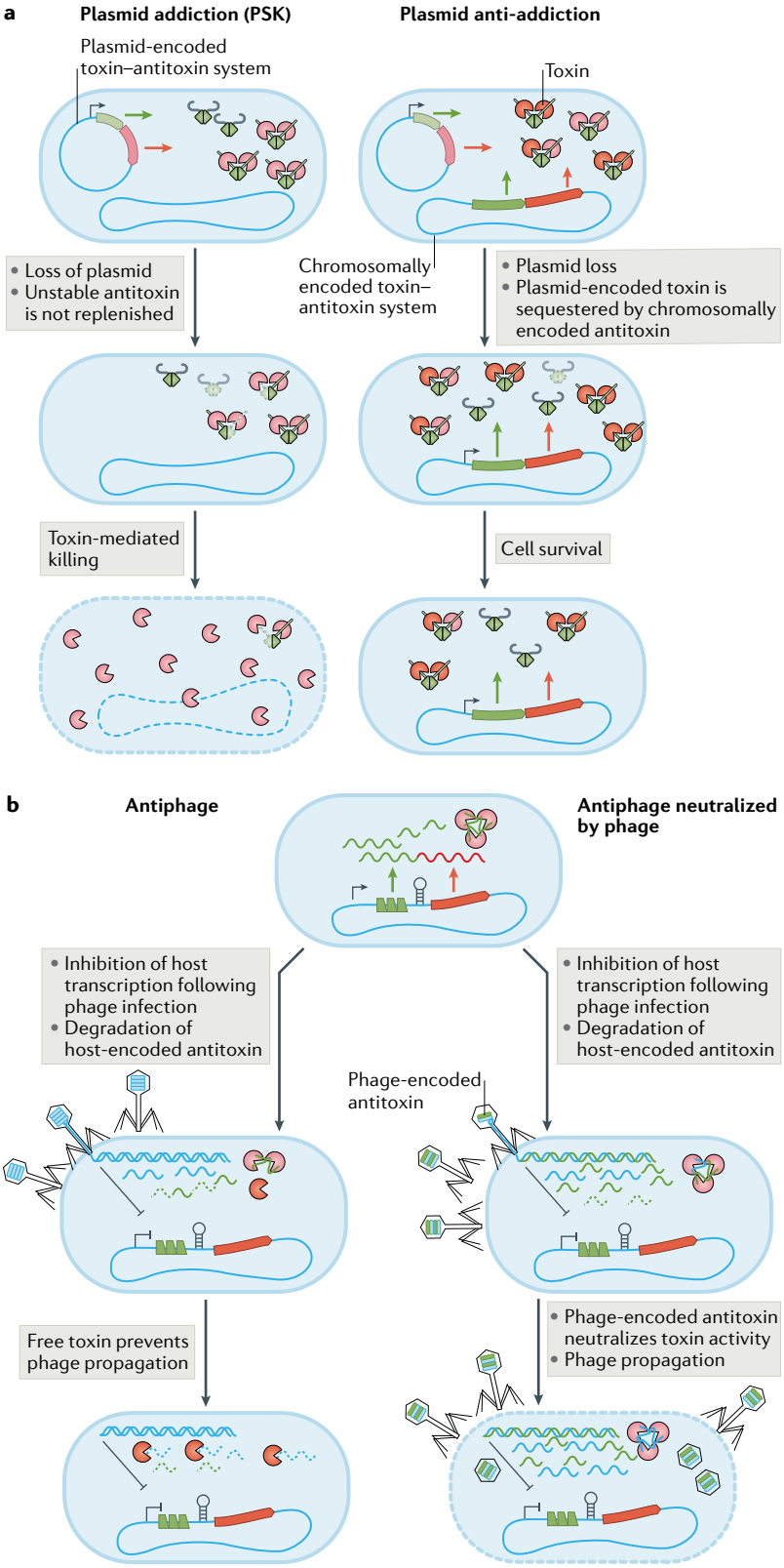

Fig. 4 | **Roles of toxin–antitoxin systems in genomic conflicts. a** | Plasmid addiction and anti-addiction. Plasmid addiction mediated by type II systems relies on the differential stability between the toxin (red) and antitoxin (green) proteins. In daughter cells that fail to inherit a plasmid copy, the unstable antitoxin is no longer replenished. Toxins will be freed from the antitoxin–toxin complex and able to kill these cells, thereby contributing to plasmid maintenance (postsegregational killing (PSK) mechanism). 'Anti-addiction' refers to the ability of a chromosomally encoded toxin–antitoxin system to interfere with PSK. The antitoxin can sequester the plasmid-encoded toxin and protect the cell against killing upon plasmid loss. **b** | Antiphage and neutralization by phage. The antiphage mechanism mediated by type III systems relies on the differential stability between the toxin (red) and the RNA antitoxin (green). In cells infected by a phage, the transcription of the host genes is inhibited, and the unstable antitoxin is not replenished. Toxins will be released from the antitoxin–toxin complex and are able to degrade the phage transcripts, which will prevent phage particle formation and therefore phage propagation. The neutralization of antiphage mechanism relies on the ability of antitoxins encoded by phages to neutralize the toxin activity. Upon infection and inhibition of host transcription, neutralization of the toxin is maintained, allowing phage propagation.

cells (FIG. 4a). Notorious examples include the type I *hok–sok* system from plasmid R1 and type II *ccd* from the F plasmid, *kis–kid* from the R1 plasmid, *parDE* from plasmid RK2, *phd–doc* from P1, an extrachromosomal prophage, and *ω–ε–ζ*, a three-component system from the pSM19035 plasmid[1,2,43,96,161,162]. Toxin–antitoxin systems from type III (*toxIN*) and type VII (*abiE*) families were also detected on plasmids[21,163]. In the case of *ω–ε–ζ*, the ω transcriptional regulator was also shown to repress the expression of genes regulating replication initiation, thus directly participating in plasmid maintenance by regulating plasmid copy number[164]. A similar mechanism was later proposed for a plasmid-encoded type II *prpAT* system in which the PrpA antitoxin competes with the Rep replication initiator for a portion of the iteron sequences in the replication origin[165]. Whether this property is more widely distributed remains to be shown.

Reminiscent of the PSK function, toxin–antitoxin systems were also suggested to contribute to the maintenance of integrative MGEs. For example, the *paaR2–paaA2–parE2* system from the CP933P prophage of *E. coli* O157:H7 was proposed to stabilize this prophage[67]. A toxin–antitoxin system with strong similarity to the type VII AbiE family, *mosAT*, is found in the SXT integrative and conjugative element carried by some *Vibrio cholerae* strains, and was shown to enhance the stability of this genetic element[166]. This toxin–antitoxin system was shown to be upregulated during circularization of SXT, a process necessary for the conjugative transfer of this element but which renders it vulnerable to curing[166]. Most strains of *V. cholerae* also encode a superintegron, which consists of a platform for the capture and shuffling of mobile cassettes[167]. These superintegron arrays often encode dozens of type II toxin–antitoxin systems, which are thought to stabilize cassette arrays that are not being expressed but could provide a potential fitness gain upon cassette shuffling[168–170].

Whereas PSK ensures vertical transmission of toxin–antitoxin systems, an extension of this model later proposed that the evolutionary success of toxin–antitoxin-encoding plasmids did not stem from

Iteron sequences
DNA sequences recognized by replication initiation proteins that are involved in the control of the copy number of plasmids.

plasmid–host relationships but rather from competition between incompatible plasmids for a host. Toxin–antitoxin-encoding plasmids were shown to exclude plasmids from the same incompatibility group devoid of toxin–antitoxin systems by killing daughter cells that inherit the plasmid devoid of the toxin–antitoxin system[159,171,172]. Therefore, toxin–antitoxin systems can be seen as elements that enhance the fitness of plasmids that compete for the same host.

Toxin–antitoxin systems as anti-addiction modules. Chromosomal toxin–antitoxin systems were hypothesized to function as a reservoir of neutralizing activities against plasmid-encoded toxin–antitoxin systems, thus preventing plasmid addiction[160] (FIG. 4a). A study showed that the antitoxin of a chromosomally encoded *ccd* system from *Dickeya dadantii* neutralized the toxin of the F plasmid-encoded *ccd* system, protecting it from PSK. Moreover, chromosomal toxin–antitoxin systems can readily evolve anti-addictive potential in laboratory conditions, as exemplified by the selection of mutants in the *mazE* and *chpBI* antitoxin genes that are able to neutralize the toxin Kid, which is encoded by the R1 plasmid[173,174]. Although the anti-addiction phenomenon should be studied at a larger scale, these results suggest that the acquisition of chromosomal toxin–antitoxin systems or evolution of antitoxins can provide a competitive advantage by protecting against PSK due to the loss of toxin–antitoxin-carrying mobile elements[160].

Toxin–antitoxin systems as a phage defence mechanism. Toxin–antitoxin systems can also function as abortive infection systems; that is, genes that are able to kill infected cells to prevent phage spreading[21,175–177] (FIG. 4b). As for plasmid stabilization, the phage defence phenomenon has been observed for several types of system: *hok–sok* (type I)[176], *rnlAB* (type II)[178], *toxIN* (type III)[21] and *abiE* (type VII)[163]. Although the details on how phage infections trigger the activation of toxin–antitoxin systems remain largely untested, it is accepted that downregulation of the host processes that promote phage replication prevents the replenishment of antitoxins and liberates the toxin. A recently published article demonstrated that the ToxIN type III toxin–antitoxin system is activated upon infection of *E. coli* by phage T4 (REF.[158]). During late infection, T4 stringently blocks host transcription, leading to the degradation of the intrinsically unstable ToxI antitoxin RNA, and thus to the release of the ToxN toxin[158].

Unsurprisingly, phages have developed mechanisms that circumvent abortive infection. Examples of ToxIN escape mutants have been described, notably the acquisition of a *toxI* antitoxin gene, enabling the phage to escape ToxN activity[179–181]. In another example, phage T4 was shown to encode Dmd, a promiscuous antitoxin that can neutralize the RnlA toxin and prevent abortive infection by this toxin[177,178]. Additionally, T4 and T7 phages encode Lon protease inhibitors that could circumvent the activation of a type II toxin–antitoxin system by inhibiting antitoxin degradation[182,183]. These examples illustrate how phages and their hosts are engaged in a large-scale arms race that involves toxin–antitoxin systems in addition to restriction-modification and CRISPR systems.

Evolution of toxin–antitoxin systems

Although a wealth of information is available regarding the activities and the mechanistic aspects of the different types of toxin, knowledge of the evolution of toxin–antitoxin systems is less well documented and remains fragmentary.

Dynamics of toxin–antitoxin systems

Association of toxin–antitoxin systems with MGEs and their selfish nature are probably the major contributors to their evolution. Whereas toxin–antitoxin systems are thought to provide a fitness increase to their replicon, MGEs serve as vehicles to disseminate toxin–antitoxin systems horizontally. Although originally found on plasmids, toxin–antitoxin systems are passengers on virtually all kinds of MGEs, including superintegrons, transposons, integrative and conjugative elements, and prophages[67,77,166,168,169]. For example, different families of toxin–antitoxin systems are found associated with plasmids and transposons, which suggests multiple acquisition events[20,184]. MGEs are efficient vehicles for toxin–antitoxin systems due to their self-transposable and/or transmissible nature, as illustrated by an experimental evolution assay where a transposon encoding a HEPN–MNT system transposed into a plasmid of interest, thus increasing its stability[185].

Toxin–antitoxin systems are likely to settle in chromosomes through horizontal transfer[16–18]. Although large-scale up-to-date analyses are missing, this concept is notably supported by the observation that most of the type II toxin–antitoxin systems in the *E. coli* K-12 strain have close homologues in enterobacterial plasmids[17]. Toxin–antitoxin system-carrying plasmids or MGEs in general might be integrated in chromosomes, an event further stabilized as a consequence of the addictive nature of the toxin–antitoxin systems, even in the absence of selective pressures imposed on the entire element. MGEs could, in turn, decay and leave only toxin–antitoxin systems behind. This scenario is supported by the observation that toxin–antitoxin systems often constitute genomic islets and are highly variable from one strain to another, even within the same species[16,19,20] (FIG. 5).

The high mobility and addictive potential are likely to explain how multiple and sometimes homologous toxin–antitoxin systems can co-exist in a single host[20]. Recent studies indicate that toxin–antitoxin system pairs are evolutionarily selected to discriminate between cognate and non-cognate partners[168,186]. Although paralogous systems (at least some of them) seem to arise from duplications (especially type I systems)[69,187,188], and therefore might lead to promiscuous cross-interacting intermediates at that stage in evolution, toxin–antitoxin systems are thought to eventually diverge through purifying selection[186,187]. Nevertheless, toxin–antitoxin systems are not protected from genetic drift as they can eventually decay and disappear if mutations or rearrangements inactivating the toxin gene arise[17,20,167,168,189,190]. Although few studies have approached this question,

Genetic drift
Stochastic fluctuations in the frequency of alleles that occur randomly and can eventually lead to the loss or fixation of these alleles.

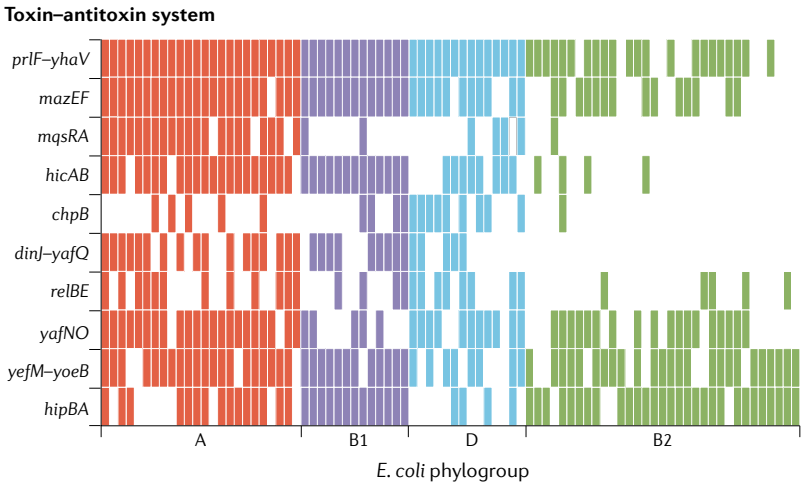

Fig. 5 | **Comparison of chromosomes and toxin–antitoxin locus between *Escherichia coli* strains.** This figure shows the PCR-mediated detection of 11 chromosomal toxin–antitoxin systems in 85 *Escherichia coli* strains distributed across four phylogroups (A, B1, B2 and D) and shows that toxin–antitoxin systems are seldom conserved from one strain to another, even within the same phylogroup. Some toxin–antitoxin systems show a quasi-universal conservation across one phylogroup (for example, *mqsRA*) or several phylogroups (for example, *prlF–yhaV* and *hipBA*), indicating a vertical transmission of these systems within phylogroups. Some systems are almost universally conserved (for example, *prlF–yhaV*). Others appear sporadically across the phylogeny with no clear patterns (for example, *chpB* or *relBE*), indicating multiple acquisition events through horizontal gene transfer[17]. Strikingly, some toxin–antitoxin systems with a very strong genetic linkage (for example, *dinJ–yafQ* and *yafNO*, located 6 kb apart) do not show a strong co-occurrence in these genomes, suggesting that these systems can be horizontally acquired independently of each other, and multiple times through evolution. The figure is based on data from REF.[20].

this is notably the case for the multiple *hok–sok* systems encoded in *E. coli* K-12 that are inactivated by insertion sequences, point mutations or major genetic rearrangements[190] and for type II systems in various *E. coli* strains or related *Shigella* strains[17]. The decay of the chromosomally encoded *ccd* system within natural strains of *E. coli* is another example illustrating that point mutations are selected and lead to toxin inactivation[189]. Cross-interactions between homologous systems might also potentially lead to degeneration of toxin–antitoxin systems, as observed for type II homologous systems encoded in the megaplasmid of *Sinorhizobium meliloti*, in which an antitoxin associated with an inactive toxin is able to counteract the toxic activity of a non-cognate toxin[191].

As toxin–antitoxin systems move by horizontal gene transfer, bacteria having access to foreign DNA should, in theory, contain high numbers of toxin–antitoxin systems. Nevertheless, previous studies indicated that there is no clear correlation between chromosome size and the number of toxin–antitoxin systems present[15,16,18], although there is a trend for typical species with reduced-size chromosomes such as obligate intracellular bacteria — which have undergone reductive evolution — to contain no or few toxin–antitoxin systems[15]. Some closely related species contain a high number of toxin–antitoxin systems, which correlates with their high number of insertion sequences and phage sequences[16]. This is indicative of an intermediary state of massive expansion of insertion sequences and pseudogenes in the process of reductive evolution of the genome[192], again linking toxin–antitoxin systems with MGEs and genome evolution. Moreover, a high number of toxin–antitoxin systems can be associated with genome plasticity and intense gene flux in particular species[16].

Phylogroups
Taxonomic groups of organisms related through their evolutionary history.

Diversity and modularity of toxin–antitoxin systems

Toxins of toxin–antitoxin systems belong to several families that adopt different structural folds, which suggests that no universal ancestor exists and that toxin–antitoxin systems have emerged multiple times during evolution[16,193]. Nevertheless, some toxins have different modes of action despite their structural similarities. This indicates that they are likely to have originated from a common ancestor and diverged during evolution. For example, the RelE mRNase toxins have a fold similar to the folds of the ParE topoisomerase poisons[31,193]. Similarly, the MazF mRNases share a common fold with the CcdB family, which poisons DNA gyrase[30,60,194]. The same applies for the Fic-fold toxins, where the Doc toxin is a kinase that phosphorylates EF-Tu and the FicT toxin is an adenylylase that transfers an AMP moiety to topoisomerases[32,33]. Divergence within most of the type II toxin families is seen at the level of substrate specificities. Preference for different target substrates has been well documented for mRNase toxins from the RelE, MazF and VapC families as well as for GNAT acetyltransferases and HipA kinases[105,109,113,115]. Some of the structural folds are found in different types of toxin–antitoxin system, such as the MazF fold, which has been identified in type II or type III systems[46,47]. More strikingly, the type I toxin SymE adopts an SpoVT–AbrB fold common in type II antitoxins[41], and nucleotidyltransferases can act as toxins modifying tRNAs or as antitoxins neutralizing the toxins by modification, as seen for AbiE and HEPN–MNT type VII systems, respectively[25,27]. Similarly, the CbeA antitoxin is structurally related to the RelE family of toxins[195].

Although within a toxin–antitoxin pair the antitoxin is specific to its cognate toxin because of the neutralization activity, type II antitoxins feature DNA-binding domains adopting various folds (HTH, RHH, SpoVT–AbrB and Phd–YefM) that regulate expression of the toxin–antitoxin operon. These families do not associate with one specific family of toxins as the specificity of the interaction between toxin and antitoxin is dictated by the toxin-neutralizing domain[16,193]. Type II systems are therefore modular. Bioinformatics and experimental studies showed that 'mixing and matching' occurs, which indicates that type II systems have been assembled from these toxin and antitoxin superfamilies on different occasions most likely by in situ displacement[16,193,195,196].

Reservoir for polymorphic toxic systems

Homologues of most of the toxins encoded by toxin–antitoxin systems can be found in other contexts. Polymorphic toxin systems are secreted effectors that function in intragenomic conflicts[37]. They typically consist of an amino-terminal domain that is recognized by a secretion machinery, on which a high diversity of modular toxic domains can be ligated to the carboxy terminus. Some of these toxic domains show structural

similarities with toxin–antitoxin toxins; for example, some polymorphic toxins encode RNase domains with a ParE–RelE-like fold[37,197]. Furthermore, recently described RES fold-containing NAD-degrading toxins and small alarmone synthetase (ToxSAS) toxins were also identified as type III and type VI secretion system-delivered toxins[198,199]. Antitoxins (usually called 'immunity proteins' in the polymorphic toxin system context) are necessary for toxin-secreting bacteria to avoid self-intoxication and the killing of siblings. As in the case of toxin–antitoxin modules, the toxin–immunity protein pairs are most often encoded adjacently. Several studies have traced back evolutionary links between toxin–antitoxin systems and secreted effectors, suggesting that toxin–antitoxin systems could serve as a reservoir for novel secreted effectors or vice versa. For example, a family of type IV secretion system effectors from *Bartonella* comprises type II FicT toxic domains fused to a type IV secretion signal[38,200]. Another study found that the *Xanthomonas* type III secretion system effector AvrRxo1 is related to type II toxins from the ζ family[201,202]. A recent study identified a class of type VI secretion system effectors that are neutralized by immunity proteins comprising a DNA-binding domain. Moreover, these toxin–immunity protein pairs can be found as stand-alone operons reminiscent of toxin–antitoxin architecture[203]. Some phage defence modules also share structural similarities with toxin–antitoxin systems[56], and Cas2 proteins are structurally similar to VapD toxins[204]. For example, some type I and type III CRISPR-associated nucleases encode a HEPN-like domain. VapC-like and HEPN-like domains can also be found in the restriction component of restriction-modification systems[56,205]. Although still speculative, this phenomenon could represent a form of exaptation in which toxin–antitoxin modules would acquire particular properties, such as being able to branch on secretion or defence machineries.

Conclusions and future perspectives

Despite more than 30 years of research on toxin–antitoxin systems, many questions remain unanswered. Probably the most puzzling one concerns their formidable expansion in bacterial genomes. These modules (at least type II) are abundant and generally not conserved even in closely related genomes. So far, bioinformatics analysis indicates that they tend to be associated with genomic islands, such as transposons, cryptic and active prophages or defence islands, or constitute themselves small genomic islets[20,67,184,206]. Extensive bioinformatics analysis including all toxin–antitoxin types might provide better insights into whether the systems that are located in chromosomes are always part of the accessory genome, whether some are conserved across broader bacterial clades and whether general rules can be established for the different types of toxin–antitoxin system. In addition, these analyses might provide a broader view of the potential decay of toxins, as already observed in specific cases[17,20,189,190]. Complementary to that, with the exponential increase of genomic and metagenomic data, it might be timely to re-evaluate the diversity and distribution of toxin–antitoxin systems, and to assess whether they are over-represented in specific species with particular lifestyles or in particular MGEs.

The other critical question concerns the conditions in which toxin–antitoxin systems are activated. Because of the difference in half-life between the toxin and antitoxin components, the classic view proposes that any condition impairing antitoxin synthesis would, in principle, favour an excess of toxins and activate the system. In the case of some chromosomal *E. coli* type II systems, recent data indicate that it is not the case as toxin-bound antitoxins do not seem to be prone to degradation, even in conditions in which antitoxin expression is inhibited[85]. However, in the case of the type III *toxIN* system, transcription shut-off mediated by the T4 phage leads to antitoxin degradation and activation of the ToxN toxin[158]. Whether the *E. coli* type II systems that have been studied represent particular cases in which the toxin cannot be liberated from the antitoxin complex in the conditions tested remains to be investigated[85]. A related but unexplored question is whether chromosomal toxin–antitoxin systems retain the capacity to mediate PSK. If this property is conserved for some chromosomal systems, does it correlate with a potential role, for instance the ability to stabilize their genetic neighbourhood? As toxin–antitoxin systems are so diverse in terms of toxin activities, antitoxicity modes, regulation and genetic neighbourhood, they should be studied on a case-by-case basis. Moreover, the properties of the various toxin–antitoxin systems might be different depending on the experimental conditions (in vitro versus in vivo in a host) and their bacterial host species, further complicating the establishment of general rules.

Recent advances point towards the idea that toxin–antitoxin systems are small selfish modules involved in genomic conflicts, favouring the maintenance of the replicon that carries them. One could wonder whether expansion of toxin–antitoxin systems in bacterial genomes is a consequence of the constant arms races among these different replicons and whether this is a universal scenario for all toxin–antitoxin systems, replicons and hosts. As the toxin–antitoxin system field stands, efforts should be made to unveil how these systems move between replicons and bacterial species, and what the driving forces for their evolutionary success are.

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