Toxin-antitoxin (TA) pairs were first identified on plasmids, where they serve as stabilization systems – maintaining plasmids in bacterial culture by killing plasmid free daughters of plasmid-bearing cells. However, it has recently become clear that TA pairs are ubiquitous components of bacterial genomes where they are frequently present in multiple copies.1

A typical TA operon encodes one mRNA and two cistrons (or protein-coding regions). The second cistron codes for a highly toxic protein. Whereas, the first codes for an antitoxin protein which neutralizes the toxin by binding and deactivating it. Another feature exhibited by TA pairs is autorepression mediated by the antitoxin protein. Repression keeps the cellular pool of toxin molecule relatively low.2

Because maintenance of the bacterial chromosome does not require added stabilization elements, the selective advantage of genomic TA pairs is not readily apparent. Many hypotheses involving plasmid segregation3, stress response4, and stabilization of specific genetic elements have been advanced5. However, these hypotheses are rarely reproducibly confirmed.6

One well-characterized TA system is the parDE operon from plasmid RK2. We have bioinformatically identified a network of eight parE toxin homologues from the gram-negative bacterium Caulobacter crescentus. Each is adjacent to a putative antitoxin genes. In order to elucidate their biological role, we are using a compound genetic and biochemical approach to characterize these operons systematically. Owing to the considerable homology observed between TA operons on different genomes, conclusions made from the C. crescentus model should be generalizable to other bacterial species.

References:

Please Read the abstract and first three sections of the following review for background.
Toxin–antitoxin modules as bacterial metabolic stress managers

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Bacterial genomes frequently contain operons that encode a toxin and its antidote. These ‘toxin–antitoxin (TA) modules’ have an important role in bacterial stress physiology and might form the basis of multidrug resistance. The toxins in TA modules act as gyrase poisons or stall the ribosome by mediating the cleavage of mRNA. The antidotes contain an N-terminal DNA-binding region of variable fold and a C-terminal toxin-inhibiting domain. When bound to toxin, the C-terminal domain adopts an extended conformation. In the absence of toxin, by contrast, this domain (and sometimes the whole antidote protein) remains unstructured, allowing its fast degradation by proteolysis. Under silent conditions the antidote inhibits the toxin and the toxin–antidote complex acts as a repressor for the TA operon, whereas under conditions of activation proteolytic degradation of the antidote outpaces its synthesis.

Introduction

Approximately 25 years ago, an operon that couples plasmid proliferation with cell division was identified on the F-plasmid of the bacterium Escherichia coli [1] and was subsequently called ccd. Soon thereafter, it was discovered that ccd acts by killing cells that become plasmid-free [2] (Figure 1). This finding led to the term ‘plasmid addiction’ [3] because the ccd operon effectively renders the cell ‘addicted’ to the plasmid.

Many other evolutionarily seemingly unrelated addiction systems have been subsequently identified on several plasmids [4]. All of them show similar phenotypic effects and have the same genetic organization. In each case, a gene encoding a small toxin of ~100 amino acids is preceded by a gene encoding a protein of ~80 amino acids (the antidote or antitoxin) that can antagonize this toxic activity. In all systems examined, the antidote has a much shorter in vivo lifetime than the toxin and is degraded by a specific protease such as Lon, ClpXP or ClpAP [5–8]. Toxin and antidote form a complex, resulting in inhibition of the toxin. The antidote–toxin complex also functions as a repressor–co-repressor pair for the operon [7,9–12].

For nearly 15 years after their discovery, plasmid addiction systems such as ccd were considered as quirky and relatively unimportant operons, and they attracted little attention outside the field. In 1993, however, two homologs of plasmid addiction operons were discovered in the E. coli chromosome [7,13]. Like bona fide plasmid addiction systems, these two operons, mazEF and the closely related chpBIK, were found to stabilize plasmids. Recently, large-scale sequencing projects have shown that the genomes of bacteria and archaea often contain several operons related to plasmid addiction systems [12,14–17]. These operons are now known as toxin–antitoxin (TA) modules and have become a main topic of interest and discussion among microbiologists [18].

Several common families of TA modules have been identified on the chromosomes of bacteria and archaea: relBE, higBA, mazEF, ccdAB, vapBC, parDE, phd–doc and yoeB–yefM. Genetic and structural analyses have shown evolutionary relationships between several TA families [14,19–21]. The relBE, parDE and higBA families share weak but significant sequence similarities and could define a class of related TA systems. The YoeB family of toxins also shows clear sequence homology with the RelE family, but the corresponding antidotes RelB and YefM are unrelated. The toxins from the mazEF and ccdAB families are closely related structurally and distinct from the toxins of the relBE family, but share no detectable sequence identity. Thus, mazEF and ccdAB define a second class. VapBC defines a third subclass, in which the toxins are predicted to have a PIN domain fold [22]. It is not known whether phd–doc belongs to any of these three classes. In this review we will focus on the biochemical functions of TA systems. These have recently been resolved for several TA families and provide insight in the mechanisms of stress management by bacteria.

Stable toxins and labile antidotes

It is well established that TA systems rely on a difference in lifespan between toxin and antidote [5]. Whereas the toxin is highly resistant to proteases, the lifespan of the antidote is shortened through degradation by a specific protease. Susceptibility to proteases seems to result from a combination of low thermodynamic stability and intrinsically unfolded domains [23–32]. The crystal structures of the antidote MazE in its free state and bound to its toxin MazF provide a clear picture of this mechanism (Figure 2a).

In the MazE–MazF complex, MazE consists of a globular N-terminal dimerization domain followed by an extended C-terminal half that interacts with MazF [24].

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In the absence of MazF, the C-terminal half of MazE becomes unstructured [23], which potentially explains its high vulnerability to proteases. The N-terminal dimerization domain of MazE also functions as a DNA-binding domain. Thus, the two binding activities (for DNA and toxin) are separated in the molecule. Despite its defined three-dimensional structure, the N-terminal domain of MazE is only marginally stable [26,27]. It unfolds in a two-state transition from folded dimer into unfolded monomer. Its low thermodynamic stability means that a significant fraction of molecules are in the unfolded state and thus vulnerable to proteolytic degradation. Because MazE is a dimer, the fraction of folded protein is dependent on concentration. It has been inferred that in the concentration range that is relevant in the cell, most MazE molecules that do not interact with either DNA or toxin would be unfolded [26,27]. The system thus allows for continuous synthesis and breakdown, which in turn keeps the number of molecules in the cell to a minimum and permits fast switching on or off.

A similar modular structure has been shown for YefM [33] (Figure 2b) and is also inferred for several other antidotes, such as ParD, Phd and CcdA [29,34,35]. Similarly, the N-terminal domains of CcdA, ParD and Phd are only marginally stable [26–30]. By contrast, YefM is completely unstructured in its isolated state [32,36], despite the presence of a globular dimerization domain in the crystal structure of the YoeB–YefM complex [33].

The antidote RelB from the archaeon Pyrococcus horikoshii is also completely unstructured in its native state. When bound to RelE, RelB adopts an extended, largely helical structure that wraps around RelE [25] and sterically shields the toxin from the target (Figure 2c).

Targeting gyrase and mRNA
Two targets of TA toxins, gyrase and mRNA, have been identified. Members of the CcdB [37] and ParE [38] families target gyrase, an enzyme that can induce and relax supercoils in DNA, as well as resolve interlinked plasmids. It is essential to disentangle DNA during replication and chromosome separation. CcdB has been
found to function as a gyrase poison, turning this crucial enzyme into a harmful agent [37]. The crystal structure of CcdB bound to a fragment of the A subunit of gyrase shows that the toxin acts as a wedge that stabilizes a dead-end covalent gyrase–DNA adduct [39]. This complex forms an obstruction for DNA and RNA polymerases [40] (Figure 3).

RelE mediates cleavage of mRNA in a ribosome-dependent manner [41], thereby affecting the level of protein synthesis. Thus, RelE might not be a ribonuclease per se. Rather, it activates an RNase activity that is present in the ribosome itself [42]. This function implies that RelE acts as a ribosome poison: it modulates the normal catalytic activity of the ribosome and converts it into a harmful agent, paralleling the action of CcdB on gyrase.

Toxins from the MazF and YoeB families have been found to show in vitro RNase activity [33,43,44]. In contrast to most other ribonucleases, they are highly specific and cut at only one or a few sites. MazF specifically cleaves at ACA sequences [43,45,46], whereas YoeB is a purine-specific ribonuclease that cleaves at adenine tracts [8]. The catalytic residues of YoeB have been determined from a combination of crystallography and site-specific mutagenesis [33]. The catalytic residues of MazF, by contrast, are unknown. The MazF family of proteins does not contain the conserved histidine residue that is usually present in RNases; thus, its reaction mechanism remains a puzzle.

Few experimental data are available for the VapC toxins, but their predicted homology with PIN domains suggests that these toxins also have RNase activity [22,47]. In eukaryotes, PIN domain proteins are ribonucleases involved in nonsense-mediated RNA degradation [48], a quality control process that removes aberrant mRNAs containing nonsense mutations in their coding regions. The crystal structure of an archaeal PIN domain protein has shown that this protein has a fold closely related to those of RNase H from phage T4 and the exonuclease domain of Thermophilus aquaticus polymerase [49].

Evolution of toxin folds

The crystal structures of six TA toxins, either in their free state or in complex with their antidote or target, have been solved. Four of them, belonging to the same superfamily, have surprisingly similar 3D structures and probably evolved from a common ancestor (Figure 4a). These toxins are CcdB from plasmid F [21], MazF from the E. coli chromosome [24] and two homologs of MazF, Kid from plasmid R1 [19] and YdcE from the chromosome of Bacillus subtilis [20]. This structural similarity is striking because CcdB is a gyrase poison, whereas MazF, Kid and YdcE are ribonucleases. It is possible that the common ancestor acted against both targets and that in some (or most) toxins one or both activities is lost or has not been recognized as yet. The crystal structure of CcdB in complex with a gyrase fragment and that of the MazE–MazF complex, together with mutagenesis data on Kid, show that the RNase and gyrase-binding activities are separated spatially [19] and thus could potentially coexist on the same protein (Figure 4b).

So far, however, such ‘moonlighting’ has not been reported for any TA toxin. Depending on different functional contexts, one of the two activities (RNase or gyrase poisoning) might be sufficient. Given the proposed roles of TA systems (see later), however, we can easily see the advantage of combining both activities. To shut down a cell efficiently, either for the purpose of killing the cell or for the purpose of reversibly halting metabolism, it is better to act simultaneously at the level of both DNA replication and protein synthesis. Gene duplication followed by a division of tasks early in evolution might have further fine-tuned the activities of TA modules, enabling them to react in a more directed way on specific types of stress.

The toxins RelE [25] and YoeB [33] are also structurally related and adopt a fold reminiscent of that of the microbial RNases barnase and RNase T1 (Figure 5). Superposition of barnase and RNase T1 with RelE shows the absence of catalytic residues in this
toxin, in keeping with the proposition that RelE triggers an RNase activity in the ribosome. YoeB, by contrast, has intrinsic ribonuclease activity [33], and good candidates for the general acid (His83), general base (Glu46) and electrostatic catalyst (Arg65) can be proposed on the basis of comparison with RNase T1 and barnase.

Lastly, VapC toxins are predicted to adopt a PIN domain fold [49]. This fold is completely z-helical and unrelated to CcdB, MazF or RelE, but is closely related to RNase H from phage T4.

Antidote action
The antidote CcdA in the ccd system competes with gyrase for binding the toxin CcdB. Thus, if gyrase and CcdA are present in the cell in roughly equal numbers, then CcdB must have a higher affinity for CcdA than for gyrase. The structure of the complex between CcdA and CcdB is not known, but it is likely to resemble the complex between MazE and MazF [24] because the toxins have the same fold and weak sequence similarity between the antidotes CcdA and MazE has been detected. CcdB stalls the activity of gyrase by stabilizing a covalent gyrase–DNA

![Figure 4. Toxins with CcdB-like folds. (a) Crystal structures of the dimeric toxins CcdB (i), MazF (ii) and Kid (iii). CcdB and MazF have strikingly similar folds and form the same type of dimer, despite different activities and a lack of detectable sequence identity. Kid is a homolog of MazF found on E. coli plasmid R1. (b) Biochemical activities of the structurally related toxins CcdB (i), MazF (ii) and Kid (iii) mapped onto the crystal structures. The molecular surface of each toxin is shown. The contact surface of CcdB with gyrase is in blue, that of MazF with MazE in green. The residues that have been shown by mutagenesis to have a role in the toxicity of Kid are in red. The orientations of the toxins in the top row correspond to those of CcdB, MazF and Kid in (a). The orientations in the bottom row correspond to a 180° rotation.](image-url)
adduct that forms a ‘roadblock’ for DNA replication and transcription. Retraction of CcdB from gyrase by CcdA, a process called ‘rejuvenation’ [50,51], is sufficient to resolve these covalent adducts and to restore the normal activity of gyrase.

In the relBE system, the toxin RelE interacts with the ribosome and the antidote RelB sterically inhibits this interaction [25]. After inactivation of RelE by RelB, tmRNA (a special type of RNA with properties of both tRNA and mRNA) is recruited to remove the defective mRNA from the ribosome and to restore normal ribosome activity [52]. tmRNA is present in all eubacteria and reactivates stalled ribosomes by a process called trans-translation [53,54].

The antidote in the mazEF and yoeB–yefM systems directly inhibits the ribonuclease activity of the toxins. In agreement with mutagenesis data on Kid [19], the presumed mRNA-binding site of MazF overlaps with the MazE-binding site [24] (Figure 4b). The latter is a long groove in which the C-terminal half of MazE binds in an extended fashion. This groove could also fit an extended piece of single-stranded RNA. Similarly, the C-terminal half of the antidote YefM shields the catalytic site of YoeB, in which it also induces a conformational change [33].

DNA binding
In general, the repressor of the TA operon is the complex formed between the toxin and the antidote [9–12]. Only the antidote interacts directly with DNA, but the toxin can assist by introducing cooperativity into the system.

In CcdA and MazE, the folded N-terminal domain is responsible for both dimerization and DNA binding, whereas the C-terminal domain interacts with the dimeric toxin. Thus, a situation is created in which toxin dimers can bridge antidote dimers bound at two or more distinct sites on the DNA, such that the interaction becomes cooperative [55,56]. This type of cooperative interaction is indeed what happens in the CcdA and MazE systems. The mazEF promoter on the E. coli chromosome contains three adjacent binding sites for MazE dimers [57], which are presumably bridged by MazF dimers (Figure 6). ccdAB on the F plasmid has an unusually long operator sequence (~120 bp) to which several CcdA dimers can bind with low affinity and high on and off rates [55]. CcdB enhances the affinity by bridging the CcdA dimers. The interaction between toxin and antidote has been further studied in this system by a biophysical approach that has confirmed that chains of alternating CcdA and CcdB dimers are formed, in agreement with the DNA-binding model [55].

Phd, the antidote from the phd–doc TA system, exists as a monomer in the absence of Doc, but it also has a modular structure in which the DNA-binding and toxin-inhibiting activities are separated [34]. The operator region of phd–doc from phage P1 consists of two adjacent palindromes that each bind two Phd monomers cooperatively [30]. Doc enhances binding of Phd only if both palindromes are present, which again suggests that two antidote dimers are bridged by a toxin dimer.

In contrast to ccdAB, mazEF and phd–doc, only a single palindrome (and no palindrome repeats) is found...
upstream of the E. coli relB coding region [58]. RelB occurs as a monomeric unfolded peptide in the absence of RelE [25], but it can still act as a repressor in this toxin-free state [59]. Binding of RelB to RelE enhances repressor activity. Dimerization of RelB has been proposed to occur through the formation of a leucine zipper [25], but only when the antidote is bound to DNA. Thus, as earlier, a situation can be envisaged in which antidote dimers are bridged by toxin dimers.

No cooperativity has been observed for DNA binding by the proteins from parDE on plasmid RK2. Although the ParD dimer has a modular structure similar to that of MazE and CcdA, the toxin ParE is not required for either DNA binding or repression of transcription [60].

Physiological roles of TA systems
The function of chromosome-borne TA systems has been highly debated but a central role in bacterial stress physiology is emerging [19]. Originally, TA systems were identified as stabilizing factors on plasmids of low copy number. In this context, they can be considered to act as selfish operons that ensure the inheritance of themselves and neighboring genes [14,61]. Compatible with this hypothesis is the observation in Vibrio cholerae that all chromosomal TA systems cluster in its mega-integron, a mobile DNA fragment that can move from one chromosomal site to another [14,62]. Several TA systems are active on plasmids even in organisms other than the one in which they were originally derived, suggesting that these ‘parasites’ have a relatively broad host range. If TA operons are indeed essentially selfish DNA fragments that parasitize their hosts, one might expect that they would be difficult to delete from genomes. Surprisingly, however, they can be deleted easily and several groups have constructed knockout mutants of chromosomal TA systems in E. coli [7,63,64].

An intriguing but rather controversial hypothesis attributes a role of altruistic killing to TA modules [7,65,66]. These systems might be devices that are activated by various stressful conditions such as a subpopulation of cells in a bacterial culture dies to permit the survival of the bacterial population as a whole [7,15]. TA systems are activated under conditions of extreme stress, such as amino acid starvation [67,68], thymine starvation [69], other DNA damage [69], the presence of antibiotics [70] or infecting phages [71]. Thereafter the cells die or, in other words, they reach an irreversible state with a ‘point of no return’ [68]. Several roles have been suggested for TA-mediated cell death [15], including prevention of the spread of phage infection [71], protection of the bacterial chromosome [15] and a response to severe nutritional stress [7,67,68]. In the last possibility, the death of a subpopulation would provide food for the surviving cells. Such cannibalism is reported during sporulation of Bacillus subtilis, although this organism has a death system that differs from the toxin–antitoxin systems [66].

The altruistic killing hypothesis can seem at odds with Darwinian evolution. A mutation that inactivates TA systems will generate defector cells that always choose cannibalism over altruistic cell death. After a single episode of stress, those cells are expected to survive preferentially, eliminating altruism from the population. In this view, therefore, bacterial populations are not simple collections of individual cells: possibly as a consequence of intercell communication, they acquire differentiation properties that are normally associated with multicellular organisms. No such elaborate interactions between bacterial cells have been described so far, but lower-level intercellular communication mechanisms such as quorum sensing are well known [72].

More widespread is the idea that TA toxins do not kill cells but induce a reversible stasis to enable the cells to survive episodes of extreme nutritional stress [64]. When conditions improve, at least part of the population is capable of recovery and resumes normal cell physiology. Indeed, cells expressing MazF have been recently shown to retain metabolic activity despite a lack of growth [73]. Consistent with this hypothesis is the fact that TA modules are extremely common in the genomes of bacteria that are confronted with periodic changes in environment. Moreover, they are absent in organisms that live in a constant environment such as obligate intracellular parasites [17].

In agreement with the reversible stasis model is the putative role of TA toxins in the generation of persister cells that lead to multidrug tolerance [74]. It is well established that a small fraction of the cells in biofilms are persister cells that are resistant to high doses of many different antibiotics. This resistance does not seem to originate simply from difficulties in getting antibiotics to diffuse into biofilms or from the generally slower metabolism of biofilm cells [75]. Recent data suggest that the few persister cells that seem to be responsible for multidrug tolerance in E. coli upregulate TA modules such as RelBE and MazEF [75]. The activity of TA modules in persister cells has been proposed to halt their metabolism to such an extent that antibiotics (which rely on metabolic activity or growth) can no longer harm the cells. In this context, it is worth noting that biofilm formation is under the control of quorum sensing [76], thereby providing a potential link between TA systems and multicellular-like behavior of bacterial populations.

Although the importance of TA systems is unequivocally recognized, their physiological interpretation remains enigmatic despite 25 years of active research. Individually the different hypotheses do not provide an unambiguous answer. We believe, however, that the various propositions are not fully incompatible. Stress conditions might activate chromosomal TA systems that bring the bacterial population into stasis. Escape from this situation might occur randomly in individual cells. For example, it has been shown that activation of MazF does not lead to a complete metabolic shutdown; the cells do not divide but remain active and can be used to overexpress proteins, provided that their mRNA does not contain a MazF cleavage site [73]. Thus, the accidental escape of a few antidote-encoded mRNAs at the right time – namely, when the stress factor (such as a lack of nutrients or the presence of an antibiotic) is relieved – can restore growth. These ‘happy few’ might then feed opportunistically on their less fortunate relatives.
References

38 Jiang, Y. et al. (2002) ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of Escherichia coli gyrine. Mol. Microbiol. 44, 971–979
Christensen, S.K. et al. (2003) Toxin–antitoxin loci as stress-response elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. *J. Mol. Biol.* 332, 809–819


Zhang, Y. et al. (2005) Insights into the mRNA cleavage mechanism by MazF, an mRNA interferase. *J. Biol. Chem.* 280, 3143–3150


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