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Toxin–antitoxin systems: why so many, what for?

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Toxin–antitoxin (TA) systems are small genetic modules that are abundant in bacterial genomes. Three types have been described so far, depending on the nature and mode of action of the antitoxin component. While type II systems are surprisingly highly represented because of their capacity to move by horizontal gene transfer, type I systems appear to have evolved by gene duplication and are more constrained. Type III is represented by a unique example located on a plasmid. Type II systems promote stability of mobile genetic elements and might act at the selfish level. Conflicting hypotheses about chromosomally encoded systems, from programmed cell death and starvation-induced stasis to protection against invading DNA and stabilization of large genomic fragments have been proposed.

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Introduction

Toxin–antitoxin (TA) systems are small genetic modules consisting in general of 2 components, a stable toxin and its labile antitoxin. TA systems are of 3 types, depending on the antitoxin nature and mode of action. While toxins are always proteins, antitoxins are either RNAs (type I and III) or proteins (type II) (see Box 1). Type I and II systems were discovered on plasmids in the 80s [1,2], while type III was discovered in 2009 and is represented so far by a unique example [3••]. Plasmid-encoded TA systems participate in plasmid stabilization by a mechanism denoted as post-segregational killing [4] or addiction [5]. The molecular mechanism underlying this phenomenon relies on differential stability of the 2 components. When a plasmid copy is not transmitted to daughter bacteria, the antitoxin and toxin pool is not replenished. Since the antitoxin is labile and rapidly degraded, the toxin is released from inhibition, leading to the killing of plasmid-free cells. As a consequence at the population level, the plasmid prevalence is increased (number of plasmid containing cells/total number of cells).

Homologues of TA systems were subsequently found in chromosomes of eu- and archaea bacteria. Type II systems appear to be widespread in chromosomes and often found in multiple copies within genomes (see Table 1 for the currently known type II toxins and their characteristics) [6•,7–9]. Type I systems appear to be less represented [10••]. The surprising abundance of these genetic entities, at least type II systems, in bacterial genomes raises interesting questions regarding their possible biological roles, their evolution and their mobility.

Biological roles of TA systems

Coping with stress

Current hypotheses propose that TA systems are involved in stress management either by promoting altruistic sacrifice of a large fraction of the population (programmed cell death hypothesis, PCD) or by inducing a dormant stage that allows cells to cope with stress (stasis). These hypotheses have emerged mainly from the study of 2 *E. coli* K-12 type II systems (*mazEF* and *relBE*) in which toxins are mRNAs interferases and inhibit translation. The general principle for TA systems activation relies on the differential stability of antitoxin and toxin proteins, as described for plasmid-encoded TA systems. As MazE and RelB antitoxins are unstable, their degradation in conditions in which neo-synthesis is impaired (such as transcription and/or translation inhibition) will liberate the toxin activity. Free toxins will then cause translation inhibition.

The *mazEF* system was shown by Engelberg-Kulka's group to be responsible for PCD upon numerous unrelated stresses (such as amino acid starvation, high temperature, oxidative stress, thymine less death and antibiotic treatments). PCD appears to require ppGpp that shuts-down *mazEF* transcription in addition to a quorum sensing-like small peptide (EDF for extracellular death factor) [11,12], although it is unclear whether both are required under all the stress conditions tested. However, *mazEF*-dependent PCD does not seem to be a 'universal' phenomenon, as it has not been reproduced by several groups [13–16]. Moreover, addition of synthetic EDF to cells subjected to stress treatments did not promote PCD either, leaving the PCD issue controversial (Gerdes and Van Melderen, unpublished).

The *relBE* system is considered the paradigm for the stasis hypothesis and similar data have been obtained

Box 1 Characteristics of the 3 types of TA systems

Three types of TA systems have been described so far. In most of the type I systems described so far (reviewed in [50,51]), the genes encoding the toxin protein and the RNA antitoxin are located on opposite strands and overlap either at the 5′ or at the 3′ of the toxin genes. In a few cases, TA genes are adjacent, located on the same strand, transcribed divergently and sharing complementarity. A simple view for toxin expression inhibition by type I antitoxins relies on base-pairing across the ribosome binding site, blocking of translation initiation and mRNA degradation, although regulation is more intricate in several cases. The unique example of type III RNA antitoxin is composed of 5.5 of nearly identical 36 nucleotide repeats and precedes the toxin gene. The 2 genes are co-transcribed. The RNA antitoxin interferes with the toxin activity instead of preventing its expression, although the precise molecular mechanism is still unknown [3••]. Type II antitoxins are proteins that interact tightly with their cognate toxins and inhibit thereby their activity (reviewed in [52]). In general, the antitoxin gene precedes that of the toxin. They are co-transcribed and their expression is autoregulated at the transcriptional level by the antitoxin–toxin complex. Antitoxin proteins in general contain an amino-terminal DNA-binding domain and a carboxy-terminal toxin-binding domain. In some cases, the regulatory and the anti-toxicity domains are encoded on 2 different polypeptides [42,53].

In all 3 types, the antitoxin molecule decays more rapidly than the toxin which allows, in conditions that impair neo-synthesis such as gene loss or transcription and/or translation inhibition, the toxin to be expressed (type I) or active (type II and III).

with other systems, notably *mazEF* [17], *relBE* homologous systems in *E. coli* [18], *relBE* and *parDE* in *Caulobacter crescentus* [19] and VapC- and RelE-homologous systems in *Mycobacterium tuberculosis* [20,21]. Interestingly, these systems appear to be differentially induced by stresses. As an example, in *M. tuberculosis*, among the 30 functional TA systems, 2 are induced specifically during hypoxia while 2 others are specifically induced during macrophage infection [20]. This indicates that TA systems might respond to different environmental cues to promote specific adaptation. However, single deletion of other *relBE* systems of *M. tuberculosis* did not trigger sensitivity to stress (hypoxia, nitrosative or oxidative conditions) nor defects in chronic persistence in mouse tissues although they were activated in such conditions [21]. Finding phenotypes for type II systems deletion mutants has been unsuccessful in *E. coli* K-12 too. A mutant deleted of 5 type II systems (including *relBE* and *mazEF*) did not show any disadvantage in competition experiments with the wild-type strain in various stress conditions [13]. Similarly, a deletion mutant of *yafNO* (not included in the 5 systems deleted and under the SOS system control) is not impaired for stress-induced mutagenesis, does not present increased sensitivity to SOS-inducing treatments [22] nor it is affected in recovery from SOS induction (Hallez, Geeraerts and Van Melderen, unpublished).

Several questions remain to be answered such as how is the specificity of activation dictated, is antitoxin degradation increased in stress conditions and what is the phenotype of multiple mutants deleted of the TA systems in adverse conditions?

A related phenomenon to stress resistance is persistence, a stochastic phenomenon that confers a dormant multidrug resistance state to a very small fraction of the population (reviewed in [23]). Involvement of type II systems in persistence of *E. coli* K-12 has been indicated by transcriptomics and toxin ectopic overexpression although single deletion phenotype analyses failed to support this hypothesis [24]. Note that the *hipBA* mutant which showed a defect in persister cells formation turned out to be deleted of the *dif* site [25], ruling out *hipBA* as a major contributor to persistence. Moreover, ectopic mild-overexpression of unrelated proteins also induces persistence [26]. This indicates that persistence might be the result of stochastic expression of a broad variety of genes, some of them encoding products that may become toxic [26]. More recently, the type I *tisAB/itsR1* system was shown to play a role in persister formation in *E. coli* K-12 [27]. Like several type I and II TA systems in *E. coli* K-12, *tisAB/itsR1* is under the control of the SOS system. Lewis

Table 1

The current 10 toxin families.

Toxin family	Target	Activity	Cellular process
CcdB	DNA gyrase	Generates DS breaks	Replication
RelE	Translating ribosome	Induces mRNAs cleavage	Translation
MazF	RNAs	Endoribonuclease	Translation
ParE	DNA gyrase	Generates DS breaks	Replication
Doc	Translating ribosome	Induces mRNAs cleavage	Translation
VapC[1]	RNAs	Endoribonuclease	Translation
ζ	ND	Phosphotransferase	ND
HipA	EF-Tu	Protein kinase	Translation
HigB	Translating ribosome	Inducdes mRNAs cleavage	Translation
HicA[2]	RNAs	Induces mRNAs cleavage	Translation

The activity and cellular process targeted by the 10 toxin families currently described are indicated. Adapted from [51] except for [1]see [54] and [2] not determined whether the HicA activity is direct or not [40].

and collaborators showed that ciprofloxacin causes persistence by inducing TisB expression. The frequency of persister cells was drastically reduced (10- to 100-fold) in a strain that did not produce the TisB toxin (Δ*tisAB* strain). TisB is a small hydrophobic protein spanning the cytoplasmic membrane impairing ATP production under overproduction conditions [28]. The direct role of TisB in persistence remains to be elucidated.

Guarding against DNA loss

In a way reminiscent to their role in plasmid stabilization, TA systems have been found to participate in the maintenance of other types of MGEs (*m*obile *g*enetic *e*lement*s*). In a recent elegant study, Waldor's group identified the *mosAT* genes as a type II TA system essential for SXT high stability. SXT is an integrative and conjugative element (ICE) found in many clinical isolates of *Vibrio cholerae* and carrying antibiotic resistance genes [29••]. When integrated in the chromosome, *mosAT* expression is shut down by MosA. To undergo conjugation, SXT has to excise from the chromosome and circularize. In this condition, *mosAT* is expressed and enables stabilization, most likely through post-segregational killing.

Another type of protection against DNA loss is exemplified by the multiple TA systems found in gene cassettes of superintegrons (SIs) (reviewed in [30]). SIs are large stable chromosomal genetic elements consisting of dozens of gene cassettes that are integrated by site-specific recombination. Two TA systems (*relBE* and *parDE*) from the *Vibrio vulnificus* SI were introduced into the *E. coli* chromosome and were shown to prevent deletion of flanking DNA [31•]. As SIs contain numerous repeats that might recombine and lead to excision, TA systems are likely to counter-select bacteria in which such deletions occur [31•]. Interestingly, some chromosomally encoded systems (namely *dinJ-yafQ* of *E. coli* K-12 homologue and ccd_{O157} of *E. coli* O157:H7) were unable to do so [31•,32], indicating that different systems might have different functions depending on their location. This is further suggested by the work of Gerdes and collaborators showing that 2 *higBA* systems from the *V. cholerae* SI are very efficient at stabilization of an unstable replicon [33•].

Protection against invading DNA

Several lines of research indicate that chromosomal TA systems might serve as a protection against mobile genetic elements such as plasmids and phages. As a recent example, a novel type of system was discovered on a cryptic plasmid of *Erwinia carotovora*. The toxin protein (ToxN) of this type III *toxIN* system is identical to Abi proteins (phage abortive infection) [3••]. *toxIN* confers resistance to different phages most likely by preventing mature particle formation, although the precise molecular mechanisms remain to be shown [34]. A similar function was proposed for the type I *hok-sok* system and for the type II *mazEF* system [35,36]. TA systems might also function as anti-addictive modules [37]. If the antitoxin protein of a chromosomally encoded TA system is able to counteract the toxin activity of a plasmid-encoded TA system, the plasmid can be lost without any harmful consequences for the host bacteria. This phenomenon might be essential for both plasmid and chromosomal TA systems evolution (see below).

Diversity, abundance, origin and evolution

Type II TA systems are thought to be part of the mobilome and to move from one genome to another through horizontal gene transfer [38,39]. This certainly accounts for the surprisingly high number of type II TA systems present in most eu- and archaea-bacterial chromosomes [6•,7–9]. In addition to the 10 current families of toxins (see Table 1), predictions revealed the existence of a dozen novel toxin and antitoxin families ([6•], Geeraerts, Leplae, Hallez and Van Melderen, in preparation).

Interestingly, type II systems possess the characteristics of selfish genes. Antitoxin and toxin genes are closely linked and show a strong dependency: a functional antitoxin gene is essential for survival and a functional toxin gene might be essential to maintain a functional antitoxin. These properties might explain their evolutionary success in bacterial genomes. Unrelated bacteria such as *M. tuberculosis* and *Nitrosomonas europaea* contain more than 50 copies of type II systems [6•,20,40]. In *M. tuberculosis*, 37% of these systems are located on genomic islands [20].

A trend is observed between the number of systems and the genome size [6•]. Small genomes of obligate host-associated such as *Buchnera*, *Mycoplasma* and *Ricketsia belii* tend to contain no or very few TA systems [6•,7]. This might be the consequence of genome size reduction as well as a low frequency of horizontal gene transfer for such species living in closed environments. A contrario, *Ricketsia felis* (obligate host-associated) contains plasmids that are predicted to be conjugative and its chromosome carries at least 13 predicted type II systems [41], making it difficult to draw a strict correlation between genome size, lifestyle and type II TA content. Type I systems do not move via horizontal gene transfer but rather have evolved by lineage-specific duplication [10••] and therefore appear less widespread and abundant compared to type II systems. However, detection of such systems might be more arduous since both components are rather difficult to detect *in silico* (sRNAs and peptides smaller than 60 amino acids). Nevertheless, novel families of type I systems were identified and experimentally validated recently, indicating that these modules might be common in bacterial genomes [10••].

Co-existence of multiple homologous systems within a single genome is thus often observed. In general, these systems do not cross-talk (see as examples [33•,20,42,19]), which might reflect independent evolution, and therefore contribute to their persistence. Similar observations were made for the plasmid-encoded ccd_F system and its chromosomally encoded ccd_{O157} homologue, that is, the antitoxin encoded by the chromosomal system is unable to counteract the plasmid toxin, enabling the ccd_F system to be functional for addiction [32]. Heinemann and Cooper proposed that type II systems selection is the result of plasmid–plasmid competition and that plasmid-encoded TA have an advantage over chromosomal ones under post-segregational killing conditions [43,44]. The presence of TA systems in chromosomes might drive evolution of their plasmid-encoded counterparts, and plasmidic systems that 'escape' chromosomal ones might be selected [37]. Thus, anti-addiction mediated by chromosomally encoded systems might be effective only during a narrow time frame. These systems might in turn decay as observed for the $ccdB_{O157}$ toxin gene within the *E. coli* species [45]. It remains to be tested whether other chromosomally encoded systems undergo degeneration and at what frequency.

Concluding remarks

Although type II TA systems might in specific cases be hijacked by host regulatory networks such as the solitary MazF toxin from *M. xanthus* [46], it is tempting to speculate that they might operate at the selfish level to promote their own 'survival' at the expense of the host as proposed by Kobayashi for restriction-modification systems [47]. When located on mobile genetic elements, these systems appear to promote their stability as well as exclusion of competitors DNA which might be a consequence of their addictive property. For chromosomal type I systems, the function is still unknown although systems that are located on plasmids such as *hok-sok* are also involved in stabilization. For several others such as *tisAB/itsR1*, no plasmidic counterparts are reported [10••]. Multiple copies of type I systems are also found to co-exist within a single chromosome. Five copies of *hok-sok* were identified in *E. coli* K-12 and they all are inactivated by either IS insertion, point mutation or genetic rearrangement [48]. Similarly, 4 copies of *ldr* are found in *E. coli* K-12 [49]. Deletion of the 4 systems did not affect cell growth nor morphology and these systems were unable to stabilize an unstable replicon. These data, as well as those on type II systems mentioned above, suggest that chromosomally encoded systems might lose their addictive properties which might be the first sign of degeneration.

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