Mobile Genetic Elements 3:5, e26219; September/October 2013; © 2013 Landes Bioscience

Toxin–antitoxin systems
Biology, identification, and application

Simon J Unterholzner, Brigitte Poppenberger, and Wilfried Rozhon*

Biotechnology of Horticultural Crops; Technische Universität München; Freising, Germany

Keywords: toxin, antitoxin, RNA interferase, translation, cloning, protein expression

Toxin–antitoxin (TA) systems are small genetic elements composed of a toxin gene and its cognate antitoxin. The toxins of all known TA systems are proteins while the antitoxins are either proteins or non-coding RNAs. Based on the molecular nature of the antitoxin and its mode of interaction with the toxin the TA modules are currently grouped into five classes. In general, the toxin is more stable than the antitoxin but the latter is expressed to a higher level. If supply of the antitoxin stops, for instance under special growth conditions or by plasmid loss in case of plasmid encoded TA systems, the antitoxin is rapidly degraded and can no longer counteract the toxin. Consequently, the toxin becomes activated and can act on its cellular targets. Typically, TA toxins act on crucial cellular processes including translation, replication, cytoskeleton formation, membrane integrity, and cell wall biosynthesis. TA systems and their components are also versatile tools for a multitude of purposes in basic research and biotechnology. Currently, TA systems are frequently used for selection in cloning and for single protein expression in living bacterial cells. Since several TA toxins exhibit activity in yeast and mammalian cells they may be useful for applications in eukaryotic systems. TA modules are also considered as promising targets for the development of antibacterial drugs and their potential to combat viral infection may aid in controlling infectious diseases.

Introduction

The first TA system, *ccdA*/*ccdB*, was described 30 years ago as a module enhancing the stability of the F plasmid by post-segregational killing of plasmid-free daughter cells.[1] Subsequently, further plasmid encoded TA systems were identified by their ability to enhance plasmid stability but also a number of TA systems were detected by homology searches on bacterial chromosomes. Intriguingly, bacteria were identified that contain dozens of putative TA systems per genome, for instance *Mycobacterium tuberculosis*[2,3] and *Nitrosomonas europaea*.[2] While the function of TA systems on plasmids was obvious, their presence on chromosomes remained enigmatic for a long time and it is only now that some of the proposed functions have been confirmed by experimental evidence. Exhaustive homology searches in sequence databases and novel bioinformatic approaches allowed the identification of numerous TA systems and today more than 10000 (putative) TA modules are known.[2,4-6] This nearly ubiquitous presence has increased the interest in TA systems considerably. Moreover, they represent convenient systems to address basic scientific questions including gene regulation, stress response and persister cell formation and are versatile tools for biotechnological applications.

In this review we provide a brief description of how TA systems are composed at the molecular level, highlight novel findings about the action of TA toxins on their cellular targets and discuss functions proposed for TA systems. In addition, approaches for identification and characterization of putative TA systems are described. A special focus of this review is also the application of TA systems in biotechnology and basic research. Finally, the potential of TA systems for combating pathogens is discussed.

*Correspondence to: Wilfried Rozhon; Email: wilfried.rozhon@wzw.tum.de
Submitted: 06/27/13; Revised: 08/16/13; Accepted: 08/19/13
Citation: Unterholzner SJ, Poppenberger B, Rozhon W. Toxin-antitoxin systems: Biology, identification, and application. Mobile Genetic Elements 2013; 3:e26219; http://dx.doi.org/10.4161/mge.26219

Biology of TA Modules

The toxins of all characterized bacterial TA systems are proteins, while the antitoxins are either proteins or small RNAs (sRNAs). In general, the toxin is more stable than the antitoxin. Currently, TA systems are assigned to five classes (I–V) according to their genetic structure and regulation. In type I and III TA modules the antitoxins are small noncoding RNAs, while the antitoxins of the other TA classes are small proteins.

Type I

Type I antitoxins are unstable antisense sRNAs. In most cases expression of the toxin is downregulated by base-pairing of the antitoxin sRNA with the stable mRNA of the toxin.[7] This interaction was shown to prevent binding to the ribosome and thereby arrests translation of the toxin mRNA.[8] An example for a type I TA system regulated by this mode is the *symR*/*symE* module of *Escherichia coli*[9] (**Fig. 1A**). Additional levels of regulation involve the control of *symE* expression by LexA,[10] a SOS-response regulated transcriptional repressor, and the degradation of SymE by the protease Lon.[9] Further examples of TA systems regulated by inhibition of toxin mRNA translation are *tisB*/*istR-1*[11] and *ibs*/*sib*,[12] both encoded by the chromosome of *E. coli*, *fstI*/*rnaII* from plasmid pAD1[13] and *hok*/*sok* encoded by plasmid R1 (**Fig. 1B**). In case of the latter regulation is more complex. The RNA antitoxin *sok* is expressed from a strong promoter but the transcript has a very short half-life of

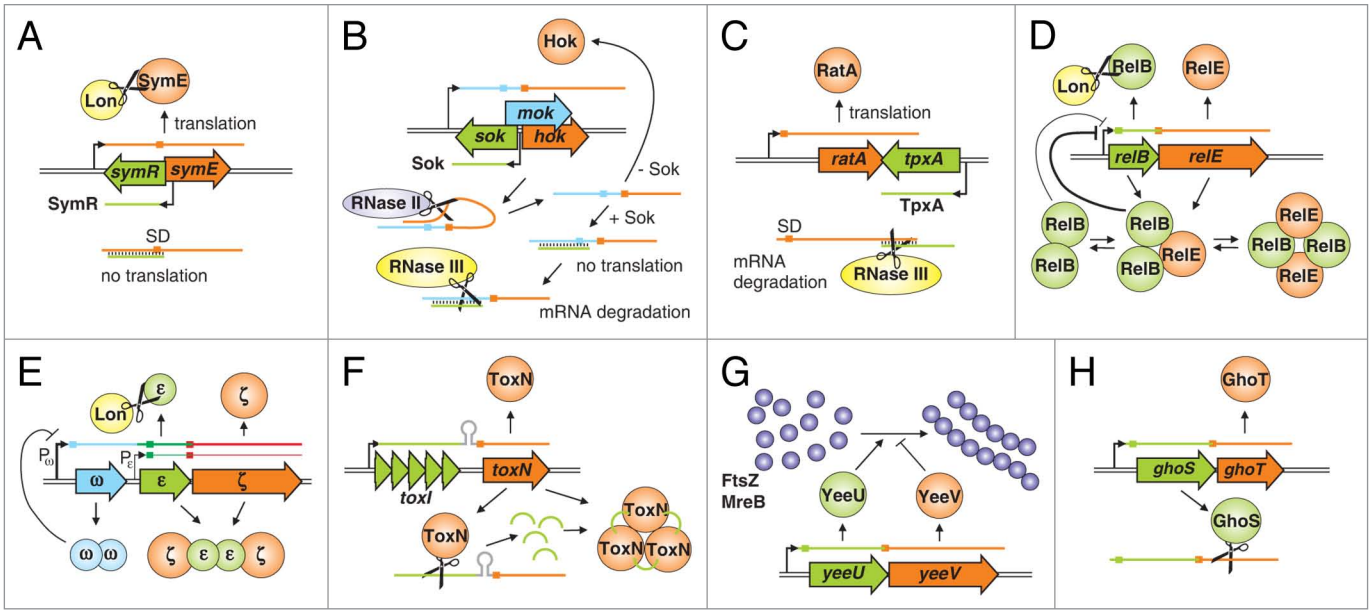

Figure 1. Types of TA systems. (**A**) The *symR/symE* module of *E. coli* as an example for a type I system regulated by interference of toxin mRNA translation. SD, Shine-Dalgarno sequence. (**B**) Regulation of the type I system *hok/sok* of plasmid R1. (**C**) The *ratA/tpxA* module from *Bacillus subtilis* represents a type I system where toxin mRNA degradation is promoted. (**D**) The *relB/relE* two module type II system from *E. coli*. (**E**) The ω-ε-ζ three module type II systems from *Streptococcus pyogenes* plasmid pSM19035. (**F**) The *toxI/N* type III system from the *Erwinia carotovora* plasmid pECA1039. (**G**) The *yeeU/yeeV* type IV system of *E. coli*. (**H**) The *ghoS/ghoT* type V system of *E. coli*. In this and all subsequent figures the toxin and its encoding gene are shown in orange while the antitoxin and its encoding gene are shown in green.

approximately 0.5 min. In contrast, the *hok* mRNA is expressed from a very weak promoter but has a half-life of approximately 20 min.[14] The *hok* transcript shows extensive secondary structure and the 3′ end folds back to the 5′ end. The folded full-length *hok* mRNA is neither accessible for the ribosome nor the Sok-RNA antitoxin. Processing by RNase II removes a part of the 3′ terminus causing a major structural rearrangement including the 5′ part of the *hok* mRNA. This allows translation but also binding of the Sok-RNA.[15] However, the Sok antisense-RNA does not show complementarity to the Shine-Dalgarno sequence of *hok*. Interestingly, the *hok/sok* locus contains in addition to the *hok* toxin and *sok* antitoxin a third gene called *mok* for modulator of killing, which overlaps with almost the entire *hok* gene. Analysis of point mutations revealed that prevention of *mok* translation abolished efficient expression of *hok*, indicating that the *hok* and *mok* open reading frames are translationally coupled and that the Sok-RNA regulates *hok* translation indirectly by preventing translation of *mok*.[16] Finally, the *hok* mRNA/Sok-RNA hybrids are cleaved by RNase III, which is the initial step for decay of the *hok* mRNA but not essential for regulation.[17] In contrast, for some type I modules including *txpA/ratA*[18] (**Fig. 1C**), *bsrG/sr4*[19], and *yonT/as-yonT*[18] of *Bacillus subtilis* cleavage of double-stranded RNA regions created by binding of the antitoxin RNA to the toxin mRNA by RNase III is crucial for regulation of toxin expression.

Type I TA modules were found in plasmids and on chromosomes. Interestingly, the majority of characterized plasmid encoded sRNA antitoxins have extensive complementarity with the toxin mRNA, while many sRNAs of chromosomally encoded type I TA systems act by base pairing with targets that have limited complementarity.[20]

Type II

The type II TA systems are the best studied class of TA modules. Within this class both the toxin and the antitoxin are small proteins. The antitoxin forms a protein-protein complex with the toxin resulting in its neutralization (**Fig. 1D**). While the toxin protein is stable the antitoxin is rapidly degraded by proteases[21] of the Clp family[22-25] or by Lon.[26-29] The operon of type II TA modules typically comprises two small open reading frames where the upstream gene encodes the antitoxin. However, exceptions of this conserved gene organization are known, for instance the *higB/higA* TA module, where the toxin gene *higB* is located upstream of the antitoxin gene *higA*.[30] Typically, transcription of the TA operon is autoregulated by binding of the antitoxin or by the toxin–antitoxin complex to the promoter.[31] Depending on the stoichiometric ratio of the antitoxin to the toxin several types of complexes may be formed with distinct affinities to the promoter. For instance, in presence of an excess of RelB over RelE dimers of RelB ($RelB_2$) and the 2:1 complex $RelB_2RelE$ are formed, which both inhibit the *relB/relE* promoter[32] (**Fig. 1D**). The $RelB_2RelE$ has a stronger inhibitory effect on the *relB/relE* promoter than $RelB_2$ and thus RelE acts as a transcriptional co-repressor.[33,34] In contrast, in the presence of an excess of RelE the 2:2 complex ($RelB_2RelE_2$) is formed,[35] which cannot bind the promoter and, consequently, transcription is activated.[32] This mode of regulation is frequently called conditional cooperativity and is believed to be important for stabilization of the antitoxin level in rapidly growing cells to minimise random induction of

relB/*relE*.[36] Also other TA systems including *phd-doc* from the *E.coli* bacteriophage P1[37] and *vapB*/*vapC* of *Salmonella enterica*[38] are regulated by conditional cooperativity.

Within this group of TA modules also three-component TA systems were reported. One example for this extraordinary genetic organization is the ω-ε-ζ TA module encoded by the *Streptococcus pyogenes* plasmid pSM19035 (**Fig. 1E**). The cytotoxic effect of the ζ protein is counteracted by the dimeric ε_2 antitoxin, which forms a stable $\zeta_2\varepsilon_2$ heterotetramer complex.[39-41] In contrast to other type II TA systems neither the ζ toxin nor the ε antitoxin or the $\zeta_2\varepsilon_2$ complex regulate their own expression. Instead, the activity of promoter P_ω is regulated by dimeric ω_2, a global regulator of transcription.[39,42] In addition, basal levels of ε and ζ are expressed from the constitutive but very weak promoter Pε.[43,44] Also the *paaR-paaA-parE* TA module encoded by *E. coli* O157:H7 represents a three-component system where the upstream located *parR* is necessary for transcriptional control of the TA module. However, in contrast to ω-ε-ζ, the ParE-PaaA complex is involved in the regulation of its own transcription.[45] The prototype of the third group of the three-component type II TA systems is the *pasA*/*pasB*/*pasC* module of plasmid pTF-FC2 from *Thiobacillus ferrooxidans*, where autoregulation is achieved by the antitoxin PasA and its complex with the toxin PasB.[46] The third component, PasC, is not involved in regulation of expression but enhances toxin–antitoxin complex formation.[47]

Originally type II TA systems were grouped into 8–14 families based on sequence similarity and gene structure[2,20] and it was assumed that each toxin family is associated with a specific antitoxin family. However, it has now become evident that many hybrid systems exist where a TA locus contains a toxin of one class and an antitoxin of another class. The functionality of a number of such hybrid systems has already been confirmed.[48-50] Thus, it was recently suggested to classify toxin and antitoxin families independently and 13 type II toxin superfamilies and 20 antitoxin superfamilies have been proposed.[4,51] In addition, 4 superfamilies of 'solitary' toxins were identified, which showed inhibition of growth if overexpressed in *E. coli* but experimental proof of antitoxin activity of open reading frames flanking these toxins failed.[4] However, it cannot be excluded that the antitoxin activity might be provided by a small RNA similar to type I and III systems.[51] With respect to that it is interesting to note that toxin/antitoxin shuffling may also occur between the different types of TA systems since the toxin ToxN of the type III TA module *toxI*/*toxN* shows 3D structure similarity to the type II toxin MazF.[52]

Type III

Similar to type I systems the antitoxin of type III modules is a sRNA. However, the mode of interaction is different. So far the only characterized member of this class is the *toxI*/*toxN* TA module of pasmid pECA1039 from *Pectobacterium carotovoum*,[53] which was originally described as a protection system of bacteria against bacteriophage infection.[54] The *toxN* gene is preceded by a short inverted repeat and by a tandem array of direct repeats (**Fig. 1F**). The inverted repeat serves as a terminator for regulating the amount of antitoxic sRNA and toxin mRNA. The ToxN protein has RNase activity and cleaves the *toxI*/*toxN* transcript at the direct repeats to release the active 36 nt RNA antitoxin.[53] In contrast to type I TA modules the RNA antitoxin neutralises the toxin protein directly by binding. Studies showed that the protein-RNA interaction results in the formation of a heterohexameric triangular assembly of three ToxN proteins, which are interspersed by three 36 nt ToxI sRNA pseudoknots[52] and that this binding reaction has an exquisite molecular specificity.[55] In a phylogenetic study a total of 125 putative type III systems were identified and assigned to 3 families, *toxIN*, *cptIN*, and *tenpIN*.[53] The majority of the type III systems is encoded by bacterial chromosomes but approximately 15% of the *toxIN* and the *tenpIN* modules are encoded by plasmids and one *toxIN* module is encoded by a prophage. The functionality of selected *cptIN* and *tenpIN* systems was confirmed in *E. coli* by assessing the toxicity of the proposed toxin gene and the ability of the cognate antitoxic repeats to inhibit the lethal effects.[53]

Type IV

A type IV TA system designation was proposed for the *yeeU*/*yeeV* (also named *ctbA*/*ctbB*) TA module of *E. coli*.[56] The functional analysis of this TA module revealed that the toxin YeeV interacts with MreB and FtsZ and thereby interferes with their polymerization and therefore with cytoskeleton assembly (**Fig. 1G**). The YeeU antitoxin protein counteracts YeeV by stabilizing MreB and FtsZ polymers.[56] A similar mode of action was also reported for *cptA*/*cptB* (*ygfX*/*ygfY*), another TA module of *E. coli*.[57] While the toxin and antitoxin of all other TA classes interact either at the RNA or the protein level, the toxin and antitoxin of this TA class do not directly interact.

Type V

Recently, the *ghoS*/*ghoT* TA module of *E. coli* was designated as a type V TA system (**Fig. 1H**). The antitoxin protein GhoS has a sequence specific endoribonuclease activity for the cleavage of the GhoT toxin mRNA and thereby prevents the translation of the toxin.[58]

Cellular targets of the toxin compound

Toxin proteins of TA modules target a wide range of cellular processes and structures (**Table 1**). Most characterized toxins act as translational inhibitors and a multitude of mechanisms has been identified how they impact on protein biosynthesis. Many toxins function as mRNA interferases, either in a ribosome-dependent or independent manner. Typical examples for toxins cleaving free mRNAs are MazF,[63] Kid,[66] ChpBK,[74] MqsR,[75] and HicA.[67] While HicA has no specific consensus recognition motif,[67] Kid shows a preference for UA(A/C)[66] and ChpBK, MqsR, and MazF cleave specifically at UAC,[74] GCU,[76] and ACA[63] sites, respectively. The recognition motif among MazF homologs varies and consist of 3, 5, or even 7 nucleotides.[77] MazF can also target the 3′ terminus of the 16S rRNA within the 30S ribosomal subunit to remove 43 nucleotides containing the anti-Shine-Dalgarno sequence required for initiation of translation. Interestingly, these modified ribosomes do not recognize canonical mRNAs containing Shine-Dalgarno sequences but were shown to translate so called leaderless mRNAs generated by MazF by cleaving ACA sites at or closely upstream of the AUG start codon of some specific mRNAs.[64] Recently, it was shown that mycobacterial MazF-mt6 cleaves mRNA at the sequence UUCCU and that it targets an

Table 1. Cellular targets of selected well characterized TA-toxins

Toxin	Antitoxin/ molecular species	Type	Toxin activity	Cellular process	Ref.
Hok	Sok / RNA	I	Integrates into the inner cell membrane	ATP synthesis	59
TisB	IstR-1 / RNA	I	Integrates into the inner cell membrane	ATP synthesis	60
SymE	SymR / RNA	I	mRNA cleavage	Translation	9
CcdB	CcdA / Protein	II	Inhibition of DNA gyrase	Replication	61
ParE	ParD / Protein	II	Inhibition of DNA gyrase	Replication	62
MazF	MazE / Protein	II	Ribosome-independent mRNA cleavage and cleavage of 16S rRNA	Translation	63,64
MazF-mt6	MazE-mt6 / Protein	II	Ribosome-independent mRNA cleavage and cleavage of 23S rRNA	Translation	65
Kid	Kis / Protein	II	Ribosome-independent mRNA cleavage	Translation	66
HicA	HicB / Protein	II	Ribosome-independent mRNA cleavage	Translation	67
RelE	RelB / Protein	II	Cleavage of ribosome-bound mRNA	Translation	68
VapC	VapB / Protein	II	Cleavage of $tRNA^{fMet}$	Translation	69
Doc	Phd / Protein	II	Binds to the 30S ribosomal subunit	Translation	70
RatA	RatB / Protein	II	Binds to the 50S ribosomal subunit	Translation	71
HipA	HipB / Protein	II	Phosphorylation of EF-Tu	Translation	72
ζ	ε / Protein	II	Phosphorylation of UDP-N-acetylglucosamine	Peptidoglycan synthesis	73
ToxN	ToxI / RNA	III	RNA cleavage?	Translation?	54
YeeV	YeeU / Protein	IV	Inhibition of FtsZ and MreB polymerization	Cytoskeleton	56
CptA	CptB / Protein	IV	Inhibition of FtsZ and MreB polymerization	Cytoskeleton	57
GhoT	GhoS / Protein	V	Integrates into the inner cell membrane	ATP synthesis	58

evolutionarily conserved region of the 23S rRNA in the ribosomal A site, which inhibits translation and destabilizes the association of the 30S and 50S ribosomal subunits.[65] Structural similarities with MazF indicate an action as endoribonuclease also for ToxI, the toxin of the type III TA system *toxI*/*toxN*,[54] however, the targeted RNA species remains elusive. RelE is the best characterized example of a RNA interferase cleaving mRNAs in a ribosome-dependent manner. RelE cleaves the mRNAs in the A site of the ribosome and was reported to show some sequence preferences.[68] Also the type II TA toxin VapC exhibits RNase activity but, in contrast to the toxins mentioned above, shows specificity for the $tRNA^{fMet}$ in vivo.[69] Another way in which TA modules can interfere with mRNA synthesis is by modifying the ribosomal activity via direct interaction with different subunits of the ribosome. Direct association of the toxin Doc with the 30S ribosomal subunit stopped elongation of translation resulting in a translational arrest.[70] Similarly, the RatA toxin of the *ratA*/*ratB* type II system was shown to bind to the 50S ribosomal subunit, which prevents its association with the 30S ribosomal subunit and consequently the formation of 70S ribosomes.[71] In contrast, the toxin HipA, a protein kinase, mediates inhibition of translation by phosphorylation of the elongation factor EF-Tu, preventing its interaction with tRNA.[72]

Also the toxin ζ of the ω-ε-ζ type II module shows kinase activity but its cellular target is distinct from HipA: ζ phosphorylates the peptidoglycan precursor UDP-N-acetylglucosamine (UNAG), which inhibits MurA, the enzyme catalyzing the initial step in peptidoglycan synthesis, and consequently impairs formation of the bacterial cell wall.[73]

In addition to the cell wall the inner membrane may also be targeted by TA toxins. Most type I systems encode small, hydrophobic proteins that seem to function like phage holins introducing pores into the inner cell membrane,[7] which leads to loss of the membrane potential[59] and consequently impairs ATP synthesis.[60] A similar mechanism is also anticipated for GhoT, the toxin of the type V system *ghoS*/*ghoT*. GhoT is a small highly hydrophobic protein with two predicted transmembrane domains and its induction causes formation of lysed cells with damaged membranes, which are also referred to as ghost cells.[58]

The cytoskeleton is targeted by the type IV toxin YeeV, which interacts with FtsZ and inhibits its polymerization and GTPase activity. In addition, YeeV was also shown to disrupt MreB polymers, which are important for cell division and maintenance of the cell shape.[78] Similar results were reported for the CptA toxin.[57]

TA toxins can also inhibit DNA replication. The type II toxins ParE and CcdB were shown to inhibit GyrA,[61,62] a subunit of an essential type II topoisomerase, which relaxes positive supercoils that arise by unwinding the DNA double strand by helicase during replication. Gyrase poisoning induces DNA double strand

breaks, activates the SOS response and efficiently triggers programmed cell death. However, it must be emphasized that most TA toxins including HipA,[72] StbE,[50] and RelE[79] do not cause cell death but induce a static condition in which the cells are still viable but unable to proliferate.

Functions of TA systems

While the role of TA modules located on plasmids is quite clear, the widespread appearance of TA modules in chromosomes remained enigmatic for a long time.[7] Plasmid encoded TA modules are important for plasmid stabilization. If the plasmid bearing a TA module is not transmitted to a daughter cell the unstable antitoxin is degraded while the stable toxin remains and acts on its cellular targets to kill or to inhibit growth of the plasmid-free cells (**Fig. 2A**). This effect is called post-segregational killing[80] or addiction.[81] In addition, plasmid encoded TA systems are also important for mediation of exclusion of co-existent compatible plasmids.[82] Conjugation can create cells containing two plasmids of the same incompatibility group, which cannot be stably maintained in the same host. Loss of the plasmid possessing the TA module will kill the daughter cell while loss of the other plasmid leaves the cell unaffected (**Fig. 2B**). Over several rounds of conjugation and subsequent exclusion the plasmid containing a TA module can outcompete the second plasmid from the bacterial population.[83]

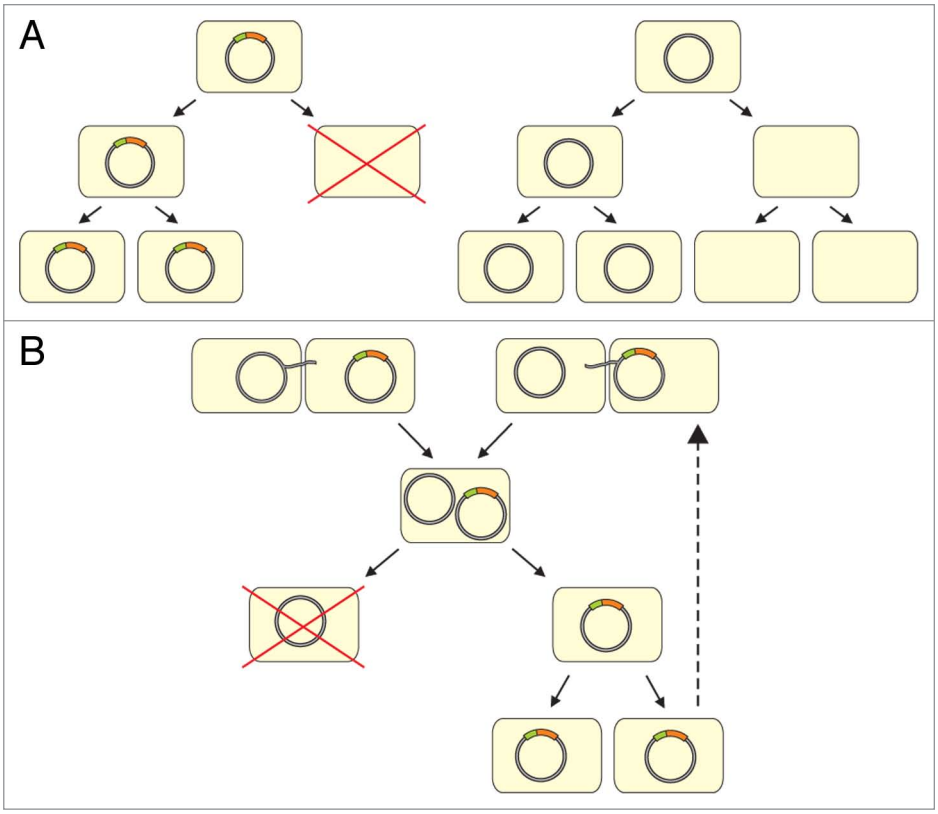

Figure 2. Functions of plasmid encoded TA systems. (**A**) Stabilization of plasmids by post segregational killing. (**B**) Exclusion of co-existent compatible plasmids.

Some TA systems present on chromosomes may fulfil a similar function and mediate stabilization of superintegrons. This type of genetic element encodes for proteins with adaptive functions like resistance, virulence and metabolic activities and has been found to frequently contain TA modules.[84] In the genome of *Vibrio fischeri* a stabilizing role of a superintegron was suggested for the type II TA module *ccdA/ccdB* and in a superintegron of *V. cholera* putative TA modules with similarities to *parA/parD*, *higA/higB*, and *phd/doc* systems were found.[85] In addition, the introduction of *relB/relE* and *parD/parE* into the chromosome of *E. coli* resulted in the stabilization of the flanking DNA regions.[84]

Chromosomal TA modules may also play a role in protection against invading DNAs such as plasmids and phages. Bacteria have evolved multiple phage resistance mechanisms, including abortive infection, during which the bacteriophage-infected cells commit suicide to prevent spreading of phages in the bacterial population. The type I TA module *hok/sok* of plasmid R1 was shown to exclude T4 pages in *E. coli*[86] and the chromosomal *mazE/mazF* type II TA module induced abortive infection upon P1 bacteriophage attack.[87] A further example is the *toxI/toxN* type III TA module isolated from *Erwinia carotovora*. Upon infection of bacterial cells with phages the toxin ToxN is activated and kills the cells.[54] Recent findings showed that some phages evolved sequences encoding mimics of the bacterial antitoxin *toxI* that can neutralise bacterial ToxN and thus evade host cell defense systems.[88,89]

Several studies indicate a role of TA systems in the formation of bacterial persisters upon stress conditions. When bacterial populations are exposed to bactericidal factors some cells enter a dormant state in which they are recalcitrant to the unfavorable conditions. Persister cell formation in *E. coli* after ampicillin treatment was suggested to be regulated by chromosomal TA modules. The toxin HipA of the *hipA/hipB* TA module was identified as an important determinant for persister cell formation.[90-92] In addition, analysis of cellular fractions enriched for persisters revealed that these cells show increased levels of TA mRNAs.[90,93] A recent study illustrates the role of several mRNase toxins in the formation of persister cells and suggests that the different TA loci encoding mRNases cumulatively contribute to this phenomenon. Since all these mRNase toxins are degraded by Lon, whose activity is regulated by environmental factors, it was suggested that the level of persister cells can be modulated by environmental conditions in a Lon-dependent way.[94] With respect to that it is interesting to note that cross-activation between different TA systems has been described recently.[95,96] The observation that TA toxin activation induces perciceter cell formation demonstrated that TA systems can enhance survival

of bacteria under unfavorable conditions. However, other studies indicate that at least some TA systems mediate programmed cell death. For instance, a number of publications suggest that MazF promotes cell death in response to nutritional stress,[97] phage infection,[87] treatment with antibiotics[98,99] or extracellular signal peptides[100-102] while other studies report that MazF induces a reversible bacteriostatic condition[79,103] and contributes to persister cell formation.[94] Thus it remains controversially discussed whether MazF is bactericidal or induces a bacteriostatic stage.[104,105]

Other possible functions of chromosomally encoded TA systems include regulation of biofilm formation and action as global regulators and were recently reviewed by Wang and Wood.[106] Although a number of questions regarding the function of chromosomally encoded TA systems remain, their role is no longer as enigmatic as it was a few years ago.

Methods for Identification of TA Systems and Confirmation of Their Activity

While the first described TA systems were discovered by their plasmid stabilizing effect,[1] novel TA systems are now usually identified by BLAST, TBLASTN, and PSI-BLAST database searches using sequences of characterized TA modules.[2,5,53] To remove false positives additional filters were developed that make use of certain typical properties of TA modules, mainly the close physical association of the toxin and the antitoxin gene and the sizes of the predicted genes.[4,107] Also a number of sequence independent search algorithms were established. For instance, in a search for pairs of small open reading frames with sizes of 65 to 135 amino acids each and a spacing of less than 150 bp, 18 pairs of putative TA systems were identified in the genome of *E. coli* MG1655. Subsequent assays for toxicity revealed that 6 genes inhibited cellular growth upon overexpression.[108] One of them, *yeeV*, was recently shown to inhibit cytoskeleton polymerization and represents the toxic compound of the first type IV TA system described.[56] For identification of type I TA modules Fozo et al.[5] used an algorithm based on searches for small ORFs, transmembrane domain predictions and enrichment of polar or aromatic amino acids at the C-terminus. In addition, also the tendency of some type I TA modules to be tandemly duplicated in bacterial genomes was applied for identification of novel TA modules. These approaches allowed the identification of functional type I modules that could not be detected by extensive PSI-BLAST and TBLASTN searches performed in the same study.

An experimental approach for identification of type I TA modules involves the cloning or microarray-based hunt for small untranslated RNAs and the subsequent search of small ORFs in close proximity. The *shoB*/*ohsC*, *symE*/*symR*, and *tpxA*/*ratA* modules could be identified by this strategy.[8] In a recent study novel functional type II TA systems were discovered by shotgun cloning.[6] This approach is based on the fact that a functional toxin can only be cloned together with its cognate antitoxin (**Fig. 3A**). During whole-genome shotgun Sanger sequencing the microbial genome is randomly fragmented, ligated into a vector and transformed in *E. coli*. If a toxin is detached from its cognate antitoxin growth of the *E. coli* clone is arrested and, consequently, such clones are not obtained. Searches in more than 300 bacterial and archaeal genomes for genes, which are absent from clones (the putative toxin) unless the adjacent gene (the putative antitoxin) is present, were performed using publicly available data from genome sequencing projects. After application of statistical tests and additional selection criteria 8 putative, previously unidentified families of TA systems were identified and 6 of them could be experimentally evaluated. Subsequent experiments revealed that *sanaTA* from *Shewanella sp* and *rlegTA* from *Rhizobium leguminosarum*, two representatives of the newly identified TA systems, could mediate partial resistance of *E. coli* against infection with T7 phages. Approaches similar to "shotgun cloning" may also be suitable for detection of other types of TA systems. Important drawbacks of this method are that TA modules with very small antitoxin genes are difficult to detect (the probability of fragmentation of such a gene is too low) and that only TA systems active in *E. coli* can be obtained. In addition, microbial genome data are currently mainly obtained by cloning-independent next generation sequencing techniques and such data are unsuitable for analysis with the "shotgun cloning" approach.

A versatile although rarely used method for isolation of TA systems uses their ability to stabilize plasmids (**Fig. 3B**). The genome or plasmid to be investigated is randomly fragmented and ligated into pALA136, a vector containing the ColE1 origin and the P1 replicon. Since this plasmid replicates in wild-type *E. coli* by the ColE1 origin to a moderate copy number such a gene library can be prepared with ease. The library is then transformed into a *polA* deficient host where the ColE1 origin is non-functional and replication switches to a low copy number under control of the P1 replicon.[109] The transformants are subsequently replica-plated several times under non-selective conditions and finally maintenance of the plasmid is investigated by plating on an antibiotic-containing selective medium. This approach allowed the isolation of the first representatives of the *stbD*/*stbE* TA systems,[109] which belong to the *relE*/*parE* superfamily and were later shown to inhibit translation.[50]

To confirm the functionality of a putative TA system a number of criteria have been established. The toxin should arrest growth of its host if overexpressed in the absence of the antitoxin. Co-expression of the antitoxin should revert this effect. Such studies are often performed with a two plasmid system where one contains an IPTG-inducible *lac* promoter and the second an inducible *ara* promoter.[4,45,50,56] Such experiments are also frequently used to determine the so called "point of no return", a delay time after which cells cannot be rescued anymore even if the antitoxin is expressed.[45] However, it must be mentioned that the two plasmid system is artificial, especially with respect to the expression level and thus the results of such "point of no return" experiments should be interpreted with care. Another commonly used assay for functionality of TA systems is the stabilization of a plasmid showing a high loss rate.[50,109,110]

Morphological changes of the bacterial cell after toxin induction can provide first evidence for the targeted process.

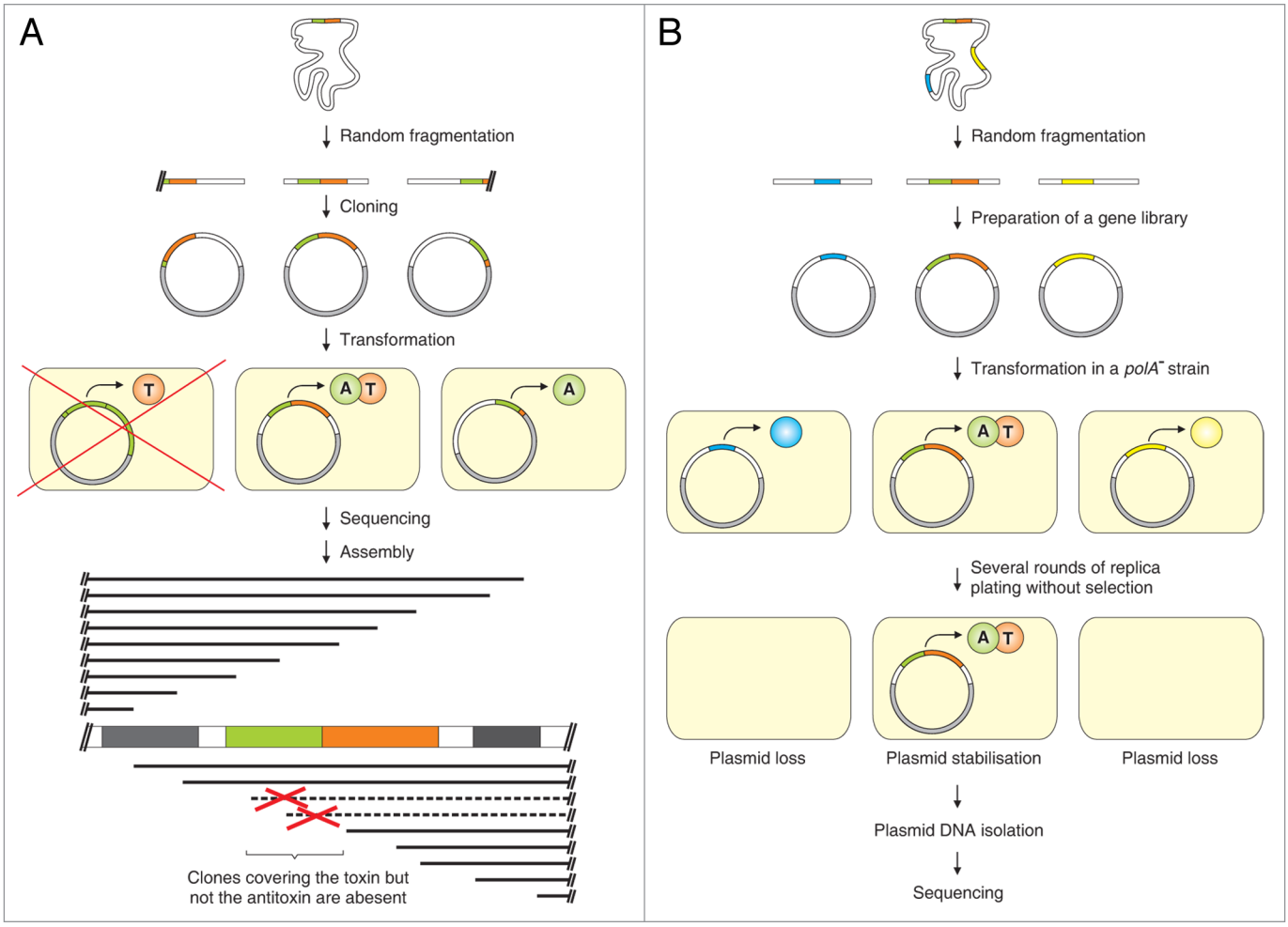

Figure 3. Experimental approaches for identification of TA systems. (**A**) Shotgun cloning: the genome to be investigated is randomly fragmented, cloned and transformed into *E. coli*. Clones comprising the toxin but not the antitoxin do not proliferate and are absent in the assembly. (**B**) Plasmid stabilization: the fragmented DNA is cloned in a vector that can normally replicate in wild type hosts but that is highly unstable in *polA*⁻ strains. After several rounds of replica plating under non selective conditions only colonies with an insert mediating plasmid stability still contain plasmids at a high frequency.

Cell filamentation may be indicative for inhibition of DNA replication,[62] bulge formation was observed in cells with impaired cell wall biosynthesis[73] and a lemon to spherical cell shape was reported for toxins targeting the cytoskeleton.[56] However, such morphological changes are only indicative and require confirmation by additional methods. Pulse-chase experiments using radio-labeled precursors like ^{3}H-thymidine, ^{3}H-uracile, and ^{35}S-methionine (or ^{3}H-leucine) whose incorporation is specific for DNA, RNA, and protein biosynthesis, respectively, are frequently used to investigate whether the toxin effects one of these processes.[4,50,68,70,95,111] Targeting of the cell membrane can be investigated with several staining techniques and evaluation by fluorescence microscopy.[50,60] Membrane staining techniques[73] but also the quantification of the ATP level and the activity of NAD(P)H-dependent oxidoreductases[50] are useful for distinguishing living and dead cells. It is likely that the application of such methods will increase in the investigation of TA systems since there is a general debate whether TA systems are bacteriostatic or bactericidal.[6]

Application of TA Systems

TA systems as tools for biotechnology and molecular biology

A major challenge in DNA cloning is the low frequency of insertion of fragments into linearized vectors. A number of vectors for positive selection of inserts contain a toxin gene, typically *ccdB* from the F-plasmid, that is inactivated upon insertion of foreign DNA,[31,112] allowing only insert-containing clones to grow (**Fig. 4A**). The *ccdA*/*ccdB* module was also used in the StabyCloning™ system, where the vector contains a truncated version of the *ccdA* antitoxin. Attachment of a 14 bp sequence to the 5′ end of the DNA fragment to be cloned, for instance by including it in the PCR primer, restores the active antitoxin, which is capable to counteract the toxin that has been introduced into the genome of the host cell.[112] Thus, only cells containing a vector with an insert in the desired orientation can form colonies (**Fig. 4B**). The use of antibodies is not necessary for this system. The commonly used Gateway™ system is based on the recombination system of phage λ. The *attB1* and *attB2* sites are

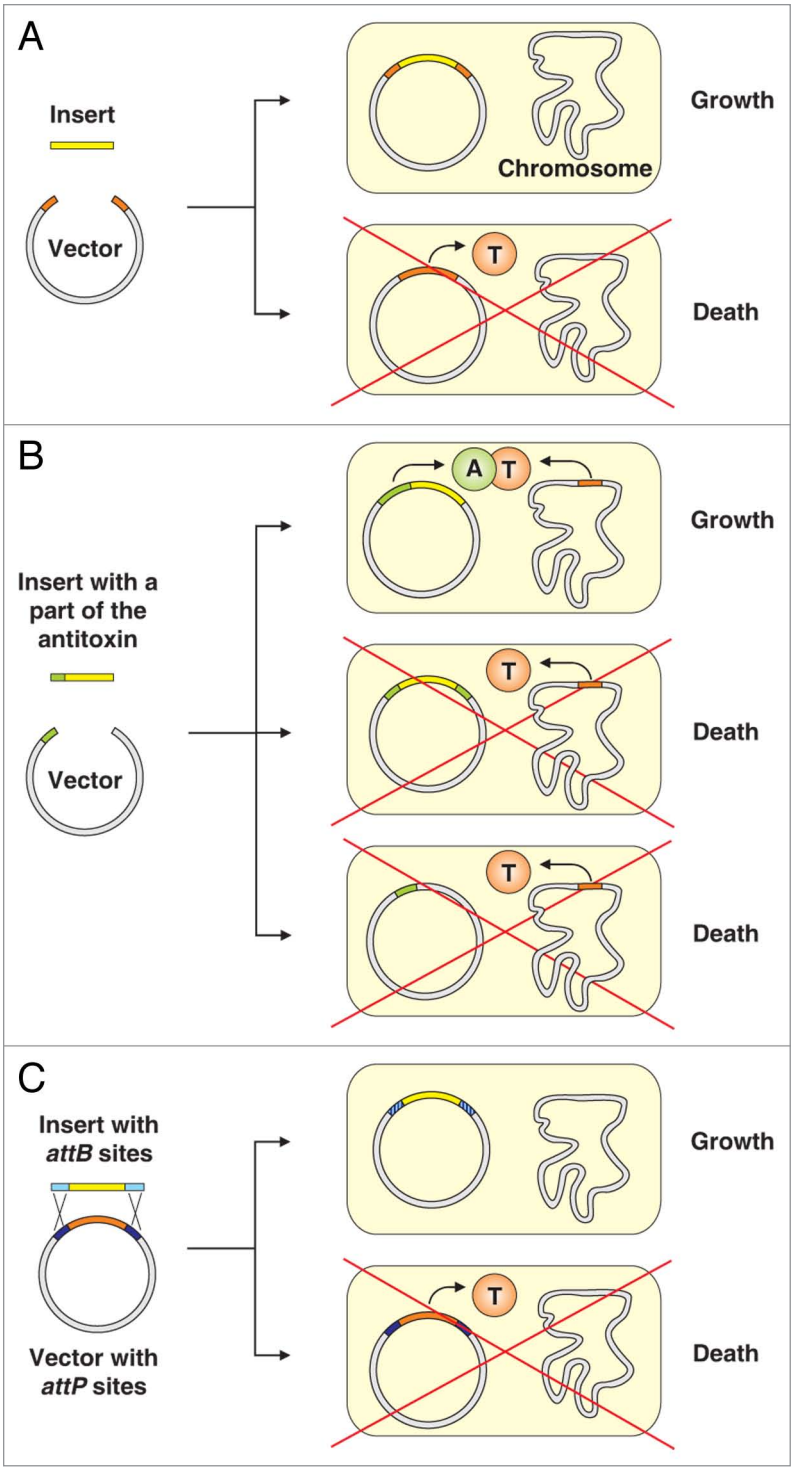

Figure 4. Application of TA systems for DNA cloning. (**A**) Insertion of the gene of interest destroys the toxin gene and allows the bacteria to growth. (**B**) Principle of the StabyCloning™ system. (**C**) Principle of the selection used in the Gateway cloning™ system.

attached by PCR to the 5′ and 3′ end of the DNA to be cloned, respectively. Subsequently, an in vitro recombination reaction is performed using the PCR fragment, the vector containing the *attP1* and *attP2* sites, the λ integrase INT and the integration host factor (IHF) from *E. coli*. To distinguish between vectors containing the desired fragment and empty vectors the *ccdB* toxin gene is placed between the *attP1* and *attP2* sites providing powerful selection against empty vectors (**Fig. 4C**). In the subsequent cloning steps the same strategy is used to distinguish between empty and insert-containing vectors.[112]

Plasmid instability can be a severe problem for the production of proteins in microorganisms. Cells that had lost the expression plasmid have usually a significantly higher growth rate than construct-containing cells and can rapidly overgrow the latter. Thus antibiotic selection pressure must be maintained during the whole fermentation process in order to obtain high yields. This practice is costly, poses a risk of contaminating the product with antibiotics and requires special waste treatment to avoid the release of antibiotics into the environment. Moreover, in dense cultures it might be difficult to maintain selective pressure because the antibiotics are rapidly inactivated by resistant cells. As an alternative strategy, TA systems can be used that kill their hosts upon plasmid loss. Supplementing an existing expression vector with one or several TA systems can significantly enhance its stability.[113] Importantly, the obtained constructs have the same requirements as the original expression vectors and can be used with standard host strains allowing for maximal flexibility. However, this approach can only delay but not completely prevent the appearance of plasmid-free cells.[113] This disadvantage can be circumvented by integrating the toxin gene into the genome while placing the antitoxin gene on the expression vector. This separated-component-stabilization allows stabilization without the use of antibiotics, increases the yield of recombinant protein and does not require modification of the culture conditions.[112] However, special host strains are necessary. Recently, a system was developed that makes use of the ACA-specificity of MazF, the toxic component of the *E. coli mazE/mazF* module, for expression of a single protein in living *E. coli* cells.[114] The gene of interest is engineered to encode an ACA-less mRNA and expressed in *E. coli* together with MazF. The latter induces bacteriostasis by cleaving mRNAs at ACA sites. Upon toxin induction protein synthesis is shut down except for the engineered gene. Despite growth arrest, the cells retain essential metabolic and biosynthetic activities for energy metabolism, transcription, translation and biosynthesis of nucleotides and amino acids for several days allowing production of the target protein in high yields. In addition, bacteriostasis offers the opportunity to work with much denser cultures than traditional methods. This system is ideal for production of stable isotope-labeled proteins as required for NMR studies, since the amount of costly stable isotope-labeled precursors can be kept at a minimum.[115]

In eukaryotic cells gene silencing due to epigenetic effects is a major drawback in the generation of stably expressing cell lines. Moreover, transfection of animal cell lines leads to a heterogeneous population of clones expressing the transgene at different levels. Since high expression often reduces the growth rate, cells with low or no expression are selected over time. Recently, a novel technique was described circumventing these problems: the transgene is tightly co-expressed with the Kis antitoxin in cells that expresses the Kid toxin, a ribonuclease digesting free mRNA. This system provides a positive selection for cells expressing the transgene and the antitoxin at high levels since they can overcome Kid toxicity. Indeed, a significant enrichment of cells with strong transgene/antitoxin expression was observed over time.[116] The Kid toxin was also suggested as a tool for selectable elimination of cells in tissue cultures or even whole organisms.[117] Other TA toxins with proven activity in mammalian cell lines include RelE[118] and MazF[119] and might be useful for similar techniques.

In summary, TA systems and their components are currently successfully applied in DNA cloning and protein expression in microorganisms but may also be useful tools for manipulation of eukaryotic cells.

Strategies to use TA systems for pathogen control

Since TA systems are ubiquitously present in bacterial genomes and have a considerable potency to repress growth or even kill cells they have been proposed as potential targets for development of antibacterial drugs.[20,120-122] Because of their proteinaceous nature type II systems seem to be the most feasible targets for artificial activation of the toxin compound. The most straight forward approach for activation of the toxin would be a drug that disrupts TA complexes (**Fig. 5A**) or prevents their formation (**Fig. 5B**), which would directly activate the toxin. Alternatively, activation of the cellular proteases would lead to enhanced degradation of the proteolytically highly sensitive antitoxin and thereby activate the toxin (**Fig. 5C**). Similarly, repression of transcription of the TA operon would prevent de novo synthesis of the labile antitoxin (**Fig. 5D**). However, transcription of TA systems is usually autorepressed by binding of the antitoxin or the TA complex to its own promoter, which might counteract the latter strategy. In contrast, a drug interfering with autoregulation would increase the TA complex pool. After removal of the drug the excess of TA complexes would tightly repress de novo synthesis of the labile antitoxin and thus a critical level of free active toxin might be generated by proteolytic cleavage (**Fig. 5E**). Finally, plasmid-located TA systems might be artificially activated by inducing plasmid loss, for instance by interfering with the replication system of the plasmid (**Fig. 5F**). However, there are considerable difficulties in the application of these strategies. The targeted TA system must be widespread in strains of the pathogenic bacterial species to ensure reliable efficacy. In addition, the activation of TA systems bears the danger of persister cell induction, clearly an undesirable effect.[121] Thus the TA systems to be targeted must be carefully selected. So far several peptides were reported to induce TA systems in vitro.[120,123,124] One example called EDF (extracellular death factor) was reported to induce death of *E. coli* also upon

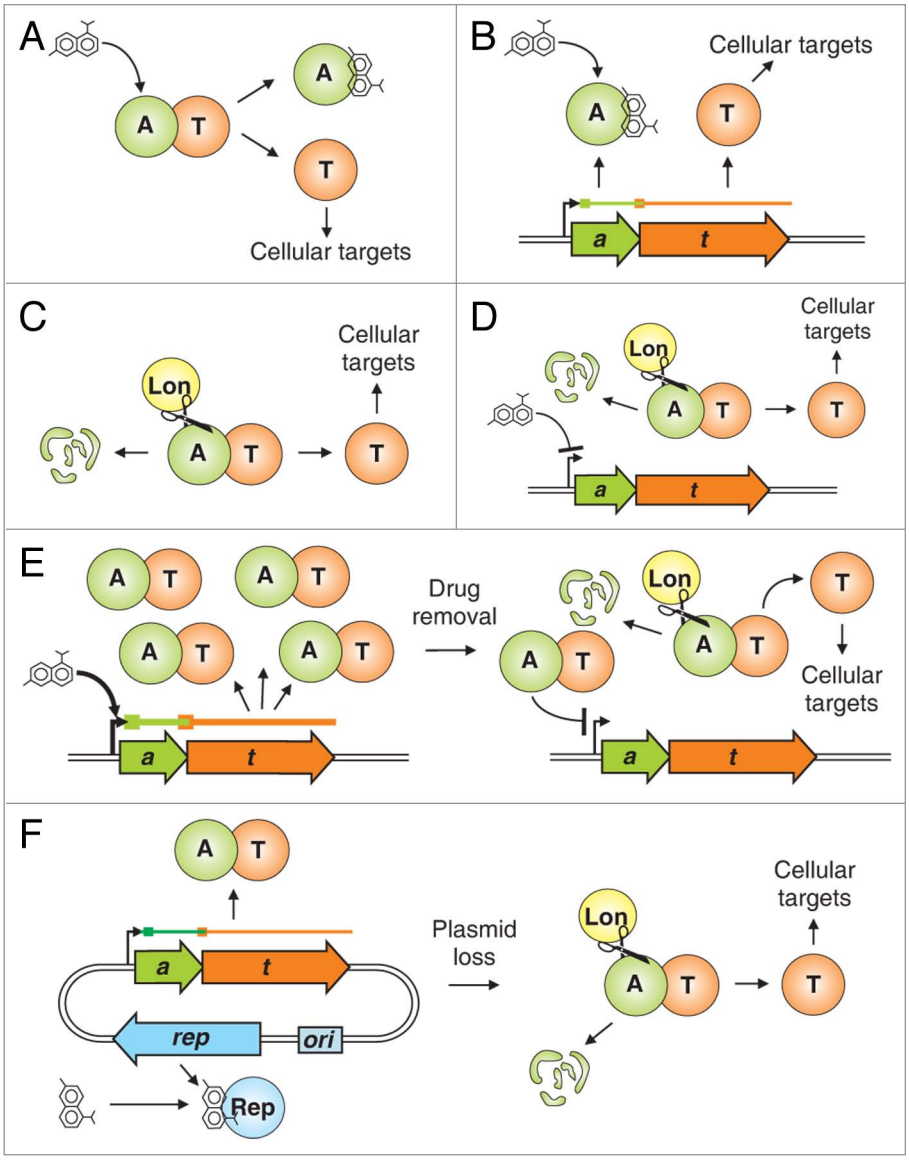

Figure 5. Strategies for artificial activation of TA systems. (**A**) Disruption of TA complexes. (**B**) Prevention of complex formation. (**C**) Activation of cellular proteases, for instance Lon or Clp. (**D**) Inhibition of TA transcription. (**E**) Overexpression of the TA system and subsequent removal of the activating drug. (**F**) Induction of plasmid loss (for plasmid encoded TA systems).

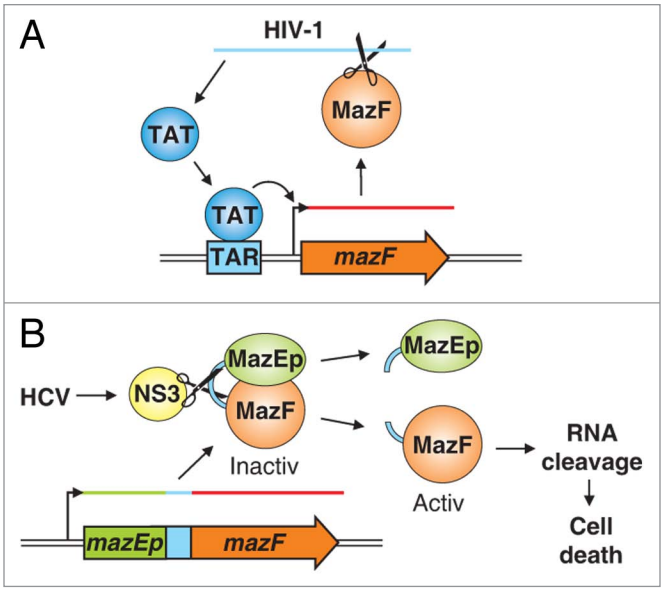

Figure 6. TA systems as antiviral tools. (**A**) CD4+ cells were transfected with a construct containing *mazF* under control of TAR. After infection of CD4+ cells with HIV-1 the viral TAT protein is produced, which binds to the TAR sequence and triggers expression of MazF. The active MazF protein cleaves RNA including HIV-1 and prevents thereby its replication. (**B**) Cells were transformed with a construct containing a part of *mazE* (*mazEp*), a linker and *mazF* as a polyprotein. The polyprotein remains inactive until the hepatitis C virus (HCV) encoded protease NS3 cleaves the linker. The released active MazF protein cleaves RNA and triggers cell death.

extracellular treatment by activating MazF.[100-102,125] EDF is a NNWNN pentapeptide, which is likely derived proteolytically from glucose-6-phosphate dehydrogenase in a ClpXP-dependent manner[101] and subsequently secreted into the medium. Treatment of exponentially growing *E. coli* cells with culture supernatant of EDF producing bacteria or with synthetic EDF activates MazF[100] and its homolog ChpBK[102] by overcoming the inhibitory effects of the antitoxins MazE and ChpBI, respectively, and induces thereby cell death. Recently, it was shown that also the culture supernatants of other bacterial species contain peptides that can trigger MazF activation.[126] Although these data are promising, further studies are required to evaluate the potential of EDF or homologous peptides as a novel class of antibiotics.

Another potential use of TA systems may be in gene therapy against viral infections. Recently, a retroviral vector was developed containing the *E. coli mazF* gene under control of the TAR promoter from HIV-1 (**Fig. 6A**). The HIV infection cycle starts with expression of the viral Tat protein, which binds to the TAR sequence to induce transcription of the entire HIV-1 genome. Infection of CD4+ cells containing the TAR-*mazF* construct with HIV-1 induced the expression of MazF, which in turn efficiently cleaved the viral mRNA and thus prevented replication of HIV-1 in such cells in vitro.[127] An alternative approach was recently investigated for removal of Hepatitis C virus (HCV) infected cells. An expression cassette was constructed encoding a polypeptide incorporating MazF and a fragment of MazE (MazEp) linked via a stretch containing a NS3 cleavage site (**Fig. 6B**). NS3 is a viral serine protease essential for processing of polyproteins encoded by HCV. Cells containing the *mazF*-linker-*mazEp* construct can grow since MazF toxicity is neutralised by the covalently attached MazEp. In contrast, in cells infected with HCV the viral NS3 protease activates MazF proteolytically, which efficiently shuts down protein biosynthesis and eradicates infected cells.[128] This strategy may also be useful for other viruses, since many of them, for instance HIV, depend on viral proteases.

Conclusion

Our knowledge about TA systems and their functions has greatly increased since their discovery 30 y ago. However, it must be emphasized that still many scientific questions remain. For instance, in 2012 two novel types of TA systems were reported. There is also a general debate whether TA systems are bactericidal or bacteriostatic and it is becoming evident that particularly chromosome encoded TA systems may have a multitude of physiological functions including protection against phage infections, induction of persister cell formation, general stress response, regulation of biofilm formation and action as global regulators. This raises also the question for host specificity of TA systems, a topic that has been little addressed as yet. Currently, TA systems and their components are used in DNA cloning and protein expression in bacterial cells while their application in eukaryotes is just emerging. Since several biotechnological applications require TA systems with specific properties, for instance certain RNA cleaving sites, there is clearly a demand for the development of novel high-throughput methods for identification and particularly for biochemical characterization of TA systems. TA systems may also offer an exciting opportunity for development of novel strategies to control pathogens. However, additional research including in vivo studies are required to fully assess their potential.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The work in our laboratory is supported by the Technische Universität München and by the Austrian Science Fund FWF (project P22734). We thank Mamoona Khan for critical reading of the manuscript.

References

1. Ogura T, Hiraga S. Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc Natl Acad Sci U S A 1983; 80:4784-8; PMID:6308648; http://dx.doi.org/10.1073/pnas.80.15.4784
2. Pandey DP, Gerdes K. Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. Nucleic Acids Res 2005; 33:966-76; PMID:15718296; http://dx.doi.org/10.1093/nar/gki201
3. Gupta A. Killing activity and rescue function of genome-wide toxin-antitoxin loci of *Mycobacterium tuberculosis.* FEMS Microbiol Lett 2009; 290:45-53; PMID:19016878; http://dx.doi.org/10.1111/j.1574-6968.2008.01400.x
4. Leplae R, Geeraerts D, Hallez R, Guglielmini J, Drèze P, Van Melderen L. Diversity of bacterial type II toxin-antitoxin systems: a comprehensive search and functional analysis of novel families. Nucleic Acids Res 2011; 39:5513-25; PMID:21422074; http://dx.doi.org/10.1093/nar/gkr131
5. Fozo EM, Makarova KS, Shabalina SA, Yutin N, Koonin EV, Storz G. Abundance of type I toxin-antitoxin systems in bacteria: searches for new candidates and discovery of novel families. Nucleic Acids Res 2010; 38:3743-59; PMID:20156992; http://dx.doi.org/10.1093/nar/gkq054
6. Sberro H, Leavitt A, Kiro R, Koh E, Peleg Y, Qimron U, Sorek R. Discovery of functional toxin/antitoxin systems in bacteria by shotgun cloning. Mol Cell 2013; 50:136-48; PMID:23478446; http://dx.doi.org/10.1016/j.molcel.2013.02.002
7. Brantl S. Bacterial type I toxin-antitoxin systems. RNA Biol 2012; 9:1488-90; PMID:23324552; http://dx.doi.org/10.4161/rna.23045
8. Fozo EM, Hemm MR, Storz G. Small toxic proteins and the antisense RNAs that repress them. Microbiol Mol Biol Rev 2008; 72:579-89; PMID:19052321; http://dx.doi.org/10.1128/MMBR.00025-08
9. Kawano M, Aravind L, Storz G. An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. Mol Microbiol 2007; 64:738-54; PMID:17462020; http://dx.doi.org/10.1111/j.1365-2958.2007.05688.x
10. Fernández De Henestrosa AR, Ogi T, Aoyagi S, Chafin D, Hayes JJ, Ohmori H, Woodgate R. Identification of additional genes belonging to the LexA regulon in *Escherichia coli*. Mol Microbiol 2000; 35:1560-72; PMID:10760155; http://dx.doi.org/10.1046/j.1365-2958.2000.01826.x
11. Wagner EG, Unoson C. The toxin-antitoxin system *tisB-istR1*: Expression, regulation, and biological role in persister phenotypes. RNA Biol 2012; 9:1513-9; PMID:23093802; http://dx.doi.org/10.4161/rna.22578
12. Fozo EM. New type I toxin-antitoxin families from "wild" and laboratory strains of *E. coli*: Ibs-Sib, ShoB-OhsC and Zor-Orz. RNA Biol 2012; 9:1504-12; PMID:23182878; http://dx.doi.org/10.4161/rna.22568
13. Greenfield TJ, Ehli E, Kirshenmann T, Franch T, Gerdes K, Weaver KE. The antisense RNA of the *par* locus of pAD1 regulates the expression of a 33-amino-acid toxic peptide by an unusual mechanism. Mol Microbiol 2000; 37:652-60; PMID:10931358; http://dx.doi.org/10.1046/j.1365-2958.2000.02035.x
14. Gerdes K, Helin K, Christensen OW, Løbner-Olesen A. Translational control and differential RNA decay are key elements regulating postsegregational expression of the killer protein encoded by the *parB* locus of plasmid R1. J Mol Biol 1988; 203:119-29; PMID:2460630; http://dx.doi.org/10.1016/0022-2836(88)90096-4
15. Franch T, Gultyaev AP, Gerdes K. Programmed cell death by *hok*/*sok* of plasmid R1: processing at the *hok* mRNA 3′-end triggers structural rearrangements that allow translation and antisense RNA binding. J Mol Biol 1997; 273:38-51; PMID:9367744; http://dx.doi.org/10.1006/jmbi.1997.1294
16. Thisted T, Gerdes K. Mechanism of post-segregational killing by the *hok*/*sok* system of plasmid R1. Sok antisense RNA regulates hok gene expression indirectly through the overlapping mok gene. J Mol Biol 1992; 223:41-54; PMID:1370544; http://dx.doi.org/10.1016/0022-2836(92)90714-U
17. Gerdes K, Nielsen A, Thorsted P, Wagner EG. Mechanism of killer gene activation. Antisense RNA-dependent RNase III cleavage ensures rapid turn-over of the stable *hok, srnB* and *pndA* effector messenger RNAs. J Mol Biol 1992; 226:637-49; PMID:1380562; http://dx.doi.org/10.1016/0022-2836(92)90621-P
18. Durand S, Gilet L, Condon C. The essential function of *B. subtilis* RNase III is to silence foreign toxin genes. PLoS Genet 2012; 8:e1003181; PMID:23300471; http://dx.doi.org/10.1371/journal.pgen.1003181
19. Jahn N, Preis H, Wiedemann C, Brantl S. BsrG/SR4 from *Bacillus subtilis*--the first temperature-dependent type I toxin-antitoxin system. Mol Microbiol 2012; 83:579-98; PMID:22229825; http://dx.doi.org/10.1111/j.1365-2958.2011.07952.x
20. Park SJ, Son WS, Lee BJ. Structural overview of toxin-antitoxin systems in infectious bacteria: a target for developing antimicrobial agents. Biochim Biophys Acta 2013; 1834:1155-67; PMID:23459128; http://dx.doi.org/10.1016/j.bbapap.2013.02.027
21. Brzozowska I, Zielenkiewicz U. Regulation of toxin-antitoxin systems by proteolysis. Plasmid 2013; 70:33-41; PMID:23396045; http://dx.doi.org/10.1016/j.plasmid.2013.01.007
22. Lehnherr H, Yarmolinsky MB. Addiction protein Phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of *Escherichia coli*. Proc Natl Acad Sci U S A 1995; 92:3274-7; PMID:7724551; http://dx.doi.org/10.1073/pnas.92.8.3274
23. Cherny I, Gazit E. The YefM antitoxin defines a family of natively unfolded proteins: implications as a novel antibacterial target. J Biol Chem 2004; 279:8252-61; PMID:14672926; http://dx.doi.org/10.1074/jbc.M308263200
24. Aizenman E, Engelberg-Kulka H, Glaser G. An Escherichia coli chromosomal "addiction module" regulated by guanosine [corrected] 3′,5′-bispyrophosphate: a model for programmed bacterial cell death. Proc Natl Acad Sci U S A 1996; 93:6059-63; PMID:8650219; http://dx.doi.org/10.1073/pnas.93.12.6059
25. Diago-Navarro E, Hernández-Arriaga AM, Kubik S, Konieczny I, Díaz-Orejas R. Cleavage of the antitoxin of the *parD* toxin-antitoxin system is determined by the ClpAP protease and is modulated by the relative ratio of the toxin and the antitoxin. Plasmid 2013; 70:78-85; PMID:23419648; http://dx.doi.org/10.1016/j.plasmid.2013.01.010
26. Smith AS, Rawlings DE. Efficiency of the pTF-FC2 *pas* poison-antidote stability system in *Escherichia coli* is affected by the host strain, and antidote degradation requires the lon protease. J Bacteriol 1998; 180:5458-62; PMID:9765581
27. Christensen SK, Mikkelsen M, Pedersen K, Gerdes K. RelE, a global inhibitor of translation, is activated during nutritional stress. Proc Natl Acad Sci USA 2001; 98:14328-33; PMID:11717402; http://dx.doi.org/10.1073/pnas.251327898
28. Roberts RC, Ström AR, Helinski DR. The *parDE* operon of the broad-host-range plasmid RK2 specifies growth inhibition associated with plasmid loss. J Mol Biol 1994; 237:35-51; PMID:8133518; http://dx.doi.org/10.1006/jmbi.1994.1207
29. Van Melderen L, Thi MH, Lecchi P, Gottesman S, Couturier M, Maurizi MR. ATP-dependent degradation of CcdA by Lon protease. Effects of secondary structure and heterologous subunit interactions. J Biol Chem 1996; 271:27730-8; PMID:8910366; http://dx.doi.org/10.1074/jbc.271.44.27730
30. Tian QB, Ohnishi M, Murata T, Nakayama K, Terawaki Y, Hayashi T. Specific protein-DNA and protein-protein interaction in the *hig* gene system, a plasmid-borne proteic killer gene system of plasmid Rts1. Plasmid 2001; 45:63-74; PMID:11322821; http://dx.doi.org/10.1006/plas.2000.1506
31. Bukowski M, Rojowska A, Wladyka B. Prokaryotic toxin-antitoxin systems--the role in bacterial physiology and application in molecular biology. Acta Biochim Pol 2011; 58:1-9; PMID:21394325
32. Overgaard M, Borch J, Gerdes K. RelB and RelE of *Escherichia coli* form a tight complex that represses transcription via the ribbon-helix-helix motif in RelB. J Mol Biol 2009; 394:183-96; PMID:19747491; http://dx.doi.org/10.1016/j.jmb.2009.09.006
33. Overgaard M, Borch J, Jørgensen MG, Gerdes K. Messenger RNA interferase RelE controls *relBE* transcription by conditional cooperativity. Mol Microbiol 2008; 69:841-57; PMID:18532983; http://dx.doi.org/10.1111/j.1365-2958.2008.06313.x
34. Li GY, Zhang Y, Inouye M, Ikura M. Structural mechanism of transcriptional autorepression of the *Escherichia coli* RelB/RelE antitoxin/toxin module. J Mol Biol 2008; 380:107-19; PMID:18501926; http://dx.doi.org/10.1016/j.jmb.2008.04.039
35. Bøggild A, Sofos N, Andersen KR, Feddersen A, Easter AD, Passmore LA, Brodersen DE. The crystal structure of the intact *E. coli* RelBE toxin-antitoxin complex provides the structural basis for conditional cooperativity. Structure 2012; 20:1641-8; PMID:22981948; http://dx.doi.org/10.1016/j.str.2012.08.017
36. Cataudella I, Trusina A, Sneppen K, Gerdes K, Mitarai N. Conditional cooperativity in toxin-antitoxin regulation prevents random toxin activation and promotes fast translational recovery. Nucleic Acids Res 2012; 40:6424-34; PMID:22495927; http://dx.doi.org/10.1093/nar/gks297
37. Garcia-Pino A, Balasubramanian S, Wyns L, Gazit E, De Greve H, Magnuson RD, Charlier D, van Nuland NA, Loris R. Allostery and intrinsic disorder mediate transcription regulation by conditional cooperativity. Cell 2010; 142:101-11; PMID:20603017; http://dx.doi.org/10.1016/j.cell.2010.05.039
38. Winther KS, Gerdes K. Regulation of enteric *vapBC* transcription: induction by VapC toxin dimer-breaking. Nucleic Acids Res 2012; 40:4347-57; PMID:22287572; http://dx.doi.org/10.1093/nar/gks029
39. Camacho AG, Misselwitz R, Behlke J, Ayora S, Welfle K, Meinhart A, et al. *In vitro* and *in vivo* stability of the $\varepsilon_2\zeta_2$ protein complex of the broad host-range *Streptococcus pyogenes* pSM19035 addiction system. Biol Chem 2002; 383:1709-13; http://dx.doi.org/10.1515/BC.2002.191
40. Meinhart A, Alonso JC, Sträter N, Saenger W. Crystal structure of the plasmid maintenance system ε/ζ: functional mechanism of toxin ζ and inactivation by $\varepsilon_2\zeta_2$ complex formation. Proc Natl Acad Sci U S A 2003; 100:1661-6; PMID:12571357; http://dx.doi.org/10.1073/pnas.0434325100
41. Zielenkiewicz U, Kowalewska M, Kaczor C, Ceglowski P. *In vivo* interactions between toxin-antitoxin proteins epsilon and zeta of streptococcal plasmid pSM19035 in *Saccharomyces cerevisiae.* J Bacteriol 2009; 191:3677-84; PMID:19346303; http://dx.doi.org/10.1128/JB.01763-08
42. Zielenkiewicz U, Ceglowski P. The toxin-antitoxin system of the streptococcal plasmid pSM19035. J Bacteriol 2005; 187:6094-105; PMID:16109951; http://dx.doi.org/10.1128/JB.187.17.6094-6105.2005

43. de la Hoz AB, Ayora S, Sitkiewicz I, Fernández S, Pankiewicz R, Alonso JC, Ceglowski P. Plasmid copy-number control and better-than-random segregation genes of pSM19035 share a common regulator. Proc Natl Acad Sci U S A 2000; 97:728-33; PMID:10639147; http://dx.doi.org/10.1073/pnas.97.2.728
44. de la Hoz AB, Pratto F, Misselwitz R, Speck C, Weihofen W, Welfle K, Saenger W, Welfle H, Alonso JC. Recognition of DNA by omega protein from the broad-host range *Streptococcus pyogenes plasmid* pSM19035: analysis of binding to operator DNA with one to four heptad repeats. Nucleic Acids Res 2004; 32:3136-47; PMID:15190131; http://dx.doi.org/10.1093/nar/gkh633
45. Hallez R, Geeraerts D, Sterckx Y, Mine N, Loris R, Van Melderen L. New toxins homologous to ParE belonging to three-component toxin-antitoxin systems in *Escherichia coli* O157:H7. Mol Microbiol 2010; 76:719-32; PMID:20345661; http://dx.doi.org/10.1111/j.1365-2958.2010.07129.x
46. Smith AS, Rawlings DE. Autoregulation of the pTF-FC2 proteic poison-antidote plasmid addiction system (*pas*) is essential for plasmid stabilization. J Bacteriol 1998; 180:5463-5; PMID:9765582
47. Smith AS, Rawlings DE. The poison-antidote stability system of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2. Mol Microbiol 1997; 26:961-70; PMID:9426133; http://dx.doi.org/10.1046/j.1365-2958.1997.6332000.x
48. Grady R, Hayes F. Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant, clinical isolate of *Enterococcus faecium*. Mol Microbiol 2003; 47:1419-32; PMID:12603745; http://dx.doi.org/10.1046/j.1365-2958.2003.03387.x
49. Schmidt O, Schuenemann VJ, Hand NJ, Silhavy TJ, Martin J, Lupas AN, Djuranovic S. *prlF* and *yhaV* encode a new toxin-antitoxin system in *Escherichia coli*. J Mol Biol 2007; 372:894-905; PMID:17706670; http://dx.doi.org/10.1016/j.jmb.2007.07.016
50. Unterholzner SJ, Hailer B, Poppenberger B, Rozhon W. Characterisation of the *stbD/E* toxin-antitoxin system of pEP36, a plasmid of the plant pathogen *Erwinia pyrifoliae*. Plasmid 2013; 70:216-25; dx.doi.org/10.1016/j.plasmid.2013.1004.1002; PMID:23632277; http://dx.doi.org/10.1016/j.plasmid.2013.04.002.
51. Guglielmini J, Van Melderen L. Bacterial toxin-antitoxin systems: Translation inhibitors everywhere. Mob Genet Elements 2011; 1:283-90; PMID:22545240; http://dx.doi.org/10.4161/mge.18477
52. Blower TR, Pei XY, Short FL, Fineran PC, Humphreys DP, Luisi BF, Salmond GP. A processed noncoding RNA regulates an altruistic bacterial antiviral system. Nat Struct Mol Biol 2011; 18:185-90; PMID:21240270; http://dx.doi.org/10.1038/nsmb.1981
53. Blower TR, Short FL, Rao F, Mizuguchi K, Pei XY, Fineran PC, Luisi BF, Salmond GP. Identification and classification of bacterial Type III toxin-antitoxin systems encoded in chromosomal and plasmid genomes. Nucleic Acids Res 2012; 40:6158-73; PMID:22434880; http://dx.doi.org/10.1093/nar/gks231
54. Fineran PC, Blower TR, Foulds IJ, Humphreys DP, Lilley KS, Salmond GP. The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. Proc Natl Acad Sci U S A 2009; 106:894-9; PMID:19124776; http://dx.doi.org/10.1073/pnas.0808832106
55. Short FL, Pei XY, Blower TR, Ong SL, Fineran PC, Luisi BF, Salmond GP. Selectivity and self-assembly in the control of a bacterial toxin by an antitoxic noncoding RNA pseudoknot. Proc Natl Acad Sci U S A 2013; 110:E241-9; PMID:23267117; http://dx.doi.org/10.1073/pnas.1216039110
56. Masuda H, Tan Q, Awano N, Wu KP, Inouye M. YeeU enhances the bundling of cytoskeletal polymers of MreB and FtsZ, antagonizing the CbtA (YeeV) toxicity in *Escherichia coli*. Mol Microbiol 2012; 84:979-89; PMID:22515815; http://dx.doi.org/10.1111/j.1365-2958.2012.08068.x
57. Masuda H, Tan Q, Awano N, Yamaguchi Y, Inouye M. A novel membrane-bound toxin for cell division, CptA (YgfX), inhibits polymerization of cytoskeleton proteins, FtsZ and MreB, in *Escherichia coli*. FEMS Microbiol Lett 2012; 328:174-81; PMID:22239607; http://dx.doi.org/10.1111/j.1574-6968.2012.02496.x
58. Wang X, Lord DM, Cheng HY, Osbourne DO, Hong SH, Sanchez-Torres V, Quiroga C, Zheng K, Herrmann T, Peti W, et al. A new type V toxin-antitoxin system where mRNA for toxin GhoT is cleaved by antitoxin GhoS. Nat Chem Biol 2012; 8:855-61; PMID:22941047; http://dx.doi.org/10.1038/nchembio.1062
59. Gerdes K, Bech FW, Jørgensen ST, Løbner-Olesen A, Rasmussen PB, Atlung T, Boe L, Karlstrom O, Molin S, von Meyenburg K. Mechanism of postsegregational killing by the hok gene product of the *parB* system of plasmid R1 and its homology with the *relF* gene product of the *E. coli relB* operon. EMBO J 1986; 5:2023-9; PMID:3019679
60. Unoson C, Wagner EG. A small SOS-induced toxin is targeted against the inner membrane in *Escherichia coli*. Mol Microbiol 2008; 70:258-70; PMID:18761622; http://dx.doi.org/10.1111/j.1365-2958.2008.06416.x
61. Miki T, Park JA, Nagao K, Murayama N, Horiuchi T. Control of segregation of chromosomal DNA by sex factor F in *Escherichia coli*. Mutants of DNA gyrase subunit A suppress *letD* (*ccdB*) product growth inhibition. J Mol Biol 1992; 225:39-52; PMID:1316444; http://dx.doi.org/10.1016/0022-2836(92)91024-J
62. Jiang Y, Pogliano J, Helinski DR, Konieczny I. ParE toxin encoded by the broad-host-range plasmid RK2 is an inhibitor of *Escherichia coli* gyrase. Mol Microbiol 2002; 44:971-9; PMID:12010492; http://dx.doi.org/10.1046/j.1365-2958.2002.02921.x
63. Zhang Y, Zhang J, Hoeflich KP, Ikura M, Qing G, Inouye M. MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *Escherichia coli*. Mol Cell 2003; 12:913-23; PMID:14580342; http://dx.doi.org/10.1016/S1097-2765(03)00402-7
64. Vesper O, Amitai S, Belitsky M, Byrgazov K, Kaberdina AC, Engelberg-Kulka H, Moll I. Selective translation of leaderless mRNAs by specialized ribosomes generated by MazF in *Escherichia coli*. Cell 2011; 147:147-57; PMID:21944167; http://dx.doi.org/10.1016/j.cell.2011.07.047
65. Schifano JM, Edifor R, Sharp JD, Ouyang M, Konkimalla A, Husson RN, Woychik NA. Mycobacterial toxin MazF-mt6 inhibits translation through cleavage of 23S rRNA at the ribosomal A site. Proc Natl Acad Sci U S A 2013; 110:8501-6; PMID:23650345; http://dx.doi.org/10.1073/pnas.1222031110
66. Muñoz-Gómez AJ, Lemonnier M, Santos-Sierra S, Berzal-Herranz A, Díaz-Orejas R. RNase/anti-RNase activities of the bacterial *parD* toxin-antitoxin system. J Bacteriol 2005; 187:3151-7; PMID:15838042; http://dx.doi.org/10.1128/JB.187.9.3151-3157.2005
67. Jørgensen MG, Pandey DP, Jaskolska M, Gerdes K. HicA of *Escherichia coli* defines a novel family of translation-independent mRNA interferases in bacteria and archaea. J Bacteriol 2009; 191:1191-9; PMID:19060138; http://dx.doi.org/10.1128/JB.01013-08
68. Christensen SK, Gerdes K. RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. Mol Microbiol 2003; 48:1389-400; PMID:12787364; http://dx.doi.org/10.1046/j.1365-2958.2003.03512.x
69. Winther KS, Gerdes K. Enteric virulence associated protein VapC inhibits translation by cleavage of initiator tRNA. Proc Natl Acad Sci U S A 2011; 108:7403-7; PMID:21502523; http://dx.doi.org/10.1073/pnas.1019587108
70. Liu M, Zhang Y, Inouye M, Woychik NA. Bacterial addiction module toxin Doc inhibits translation elongation through its association with the 30S ribosomal subunit. Proc Natl Acad Sci U S A 2008; 105:5885-90; PMID:18398006; http://dx.doi.org/10.1073/pnas.0711949105
71. Zhang Y, Inouye M. RatA (YfjG), an *Escherichia coli* toxin, inhibits 70S ribosome association to block translation initiation. Mol Microbiol 2011; 79:1418-29; PMID:21323758; http://dx.doi.org/10.1111/j.1365-2958.2010.07506.x
72. Schumacher MA, Piro KM, Xu W, Hansen S, Lewis K, Brennan RG. Molecular mechanisms of HipA-mediated multidrug tolerance and its neutralization by HipB. Science 2009; 323:396-401; PMID:19150849; http://dx.doi.org/10.1126/science.1163806
73. Mutschler H, Gebhardt M, Shoeman RL, Meinhart A. A novel mechanism of programmed cell death in bacteria by toxin-antitoxin systems corrupts peptidoglycan synthesis. PLoS Biol 2011; 9:e1001033; PMID:21445328; http://dx.doi.org/10.1371/journal.pbio.1001033
74. Zhang Y, Zhu L, Zhang J, Inouye M. Characterization of ChpBK, an mRNA interferase from *Escherichia coli*. J Biol Chem 2005; 280:26080-8; PMID:15901733; http://dx.doi.org/10.1074/jbc.M502050200
75. Yamaguchi Y, Park JH, Inouye M. MqsR, a crucial regulator for quorum sensing and biofilm formation, is a GCU-specific mRNA interferase in *Escherichia coli*. J Biol Chem 2009; 284:28746-53; PMID:19690171; http://dx.doi.org/10.1074/jbc.M109.032904
76. Kasari V, Kurg K, Margus T, Tenson T, Kaldalu N. The *Escherichia coli mqsR* and *ygiT* genes encode a new toxin-antitoxin pair. J Bacteriol 2010; 192:2908-19; PMID:20233923; http://dx.doi.org/10.1128/JB.01266-09
77. Yamaguchi Y, Nariya H, Park JH, Inouye M. Inhibition of specific gene expressions by protein-mediated mRNA interference. Nat Commun 2012; 3:607; PMID:22215082; http://dx.doi.org/10.1038/ncomms1621
78. Tan Q, Awano N, Inouye M. YeeV is an *Escherichia coli* toxin that inhibits cell division by targeting the cytoskeleton proteins, FtsZ and MreB. Mol Microbiol 2011; 79:109-18; PMID:21166897; http://dx.doi.org/10.1111/j.1365-2958.2010.07433.x
79. Pedersen K, Christensen SK, Gerdes K. Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. Mol Microbiol 2002; 45:501-10; PMID:12123459; http://dx.doi.org/10.1046/j.1365-2958.2002.03027.x
80. Gerdes K, Rasmussen PB, Molin S. Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. Proc Natl Acad Sci U S A 1986; 83:3116-20; PMID:3517851; http://dx.doi.org/10.1073/pnas.83.10.3116
81. Yarmolinsky MB. Programmed cell death in bacterial populations. Science 1995; 267:836-7; PMID:7846528; http://dx.doi.org/10.1126/science.7846528
82. Cooper TF, Heinemann JA. Postsegregational killing does not increase plasmid stability but acts to mediate the exclusion of competing plasmids. Proc Natl Acad Sci U S A 2000; 97:12643-8; PMID:11058151; http://dx.doi.org/10.1073/pnas.220077897
83. Van Melderen L, Saavedra De Bast M. Bacterial toxin-antitoxin systems: more than selfish entities? PLoS Genet 2009; 5:e1000437; PMID:19325885; http://dx.doi.org/10.1371/journal.pgen.1000437

84. Szekeres S, Dauti M, Wilde C, Mazel D, Rowe-Magnus DA. Chromosomal toxin-antitoxin loci can diminish large-scale genome reductions in the absence of selection. Mol Microbiol 2007; 63:1588-605; PMID:17367382; http://dx.doi.org/10.1111/j.1365-2958.2007.05613.x
85. Rowe-Magnus DA, Guerout AM, Biskri L, Bouige P, Mazel D. Comparative analysis of superintegrons: engineering extensive genetic diversity in the Vibrionaceae. Genome Res 2003; 13:428-42; PMID:12618374; http://dx.doi.org/10.1101/gr.617103
86. Pecota DC, Wood TK. Exclusion of T4 phage by the *hok*/*sok* killer locus from plasmid R1. J Bacteriol 1996; 178:2044-50; PMID:8606182
87. Hazan R, Engelberg-Kulka H. *Escherichia coli mazEF*-mediated cell death as a defense mechanism that inhibits the spread of phage P1. Mol Genet Genomics 2004; 272:227-34; PMID:15316771; http://dx.doi.org/10.1007/s00438-004-1048-y
88. Blower TR, Evans TJ, Przybilski R, Fineran PC, Salmond GP. Viral evasion of a bacterial suicide system by RNA-based molecular mimicry enables infectious altruism. PLoS Genet 2012; 8:e1003023; PMID:23109916; http://dx.doi.org/10.1371/journal.pgen.1003023
89. Blower TR, Short FL, Fineran PC, Salmond GP. Viral molecular mimicry circumvents abortive infection and suppresses bacterial suicide to make hosts permissive for replication. Bacteriophage 2012; 2:234-8; PMID:23739522; http://dx.doi.org/10.4161/bact.23830
90. Keren I, Shah D, Spoering A, Kaldalu N, Lewis K. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J Bacteriol 2004; 186:8172-80; PMID:15576765; http://dx.doi.org/10.1128/JB.186.24.8172-8180.2004
91. Falla TJ, Chopra I. Joint tolerance to beta-lactam and fluoroquinolone antibiotics in *Escherichia coli* results from overexpression of *hipA*. Antimicrob Agents Chemother 1998; 42:3282-4; PMID:9835528
92. Moyed HS, Bertrand KP. *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol 1983; 155:768-75; PMID:6348026
93. Shah D, Zhang Z, Khodursky A, Kaldalu N, Kurg K, Lewis K. Persisters: a distinct physiological state of *E. coli*. BMC Microbiol 2006; 6:53; PMID:16768798; http://dx.doi.org/10.1186/1471-2180-6-53
94. Maisonneuve E, Shakespeare LJ, Jørgensen MG, Gerdes K. Bacterial persistence by RNA endonucleases. Proc Natl Acad Sci U S A 2011; 108:13206-11; PMID:21788497; http://dx.doi.org/10.1073/pnas.1100186108
95. Winther KS, Gerdes K. Ectopic production of VapCs from Enterobacteria inhibits translation and trans-activates YoeB mRNA interferase. Mol Microbiol 2009; 72:918-30; PMID:19400780; http://dx.doi.org/10.1111/j.1365-2958.2009.06694.x
96. Kasari V, Mets T, Tenson T, Kaldalu N. Transcriptional cross-activation between toxin-antitoxin systems of *Escherichia coli*. BMC Microbiol 2013; 13:45; PMID:23432955; http://dx.doi.org/10.1186/1471-2180-13-45
97. Engelberg-Kulka H, Hazan R, Amitai S. *mazEF*: a chromosomal toxin-antitoxin module that triggers programmed cell death in bacteria. J Cell Sci 2005; 118:4327-32; PMID:16179604; http://dx.doi.org/10.1242/jcs.02619
98. Sat B, Hazan R, Fisher T, Khaner H, Glaser G, Engelberg-Kulka H. Programmed cell death in *Escherichia coli*: some antibiotics can trigger *mazEF* lethality. J Bacteriol 2001; 183:2041-5; PMID:11222603; http://dx.doi.org/10.1128/JB.183.6.2041-2045.2001
99. Dorsey-Oresto A, Lu T, Mosel M, Wang X, Salz T, Drlica K, Zhao X. YihE kinase is a central regulator of programmed cell death in bacteria. Cell Rep 2013; 3:528-37; PMID:23416055; http://dx.doi.org/10.1016/j.celrep.2013.01.026
100. Kolodkin-Gal I, Hazan R, Gaathon A, Carmeli S, Engelberg-Kulka H. A linear pentapeptide is a quorum-sensing factor required for *mazEF*-mediated cell death in *Escherichia coli*. Science 2007; 318:652-5; PMID:17962566; http://dx.doi.org/10.1126/science.1147248
101. Kolodkin-Gal I, Engelberg-Kulka H. The extracellular death factor: physiological and genetic factors influencing its production and response in *Escherichia coli*. J Bacteriol 2008; 190:3169-75; PMID:18310334; http://dx.doi.org/10.1128/JB.01918-07
102. Belitsky M, Avshalom H, Erental A, Yelin I, Kumar S, London N, Sperber M, Schueler-Furman O, Engelberg-Kulka H. The *Escherichia coli* extracellular death factor EDF induces the endoribonucleolytic activities of the toxins MazF and ChpBK. Mol Cell 2011; 41:625-35; PMID:21419338; http://dx.doi.org/10.1016/j.molcel.2011.02.023
103. Christensen SK, Pedersen K, Hansen FG, Gerdes K. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. J Mol Biol 2003; 332:809-19; PMID:12972253; http://dx.doi.org/10.1016/S0022-2836(03)00922-7
104. Tsilibaris V, Maenhaut-Michel G, Mine N, Van Melderen L. What is the benefit to *Escherichia coli* of having multiple toxin-antitoxin systems in its genome? J Bacteriol 2007; 189:6101-8; PMID:17513477; http://dx.doi.org/10.1128/JB.00527-07
105. Gerdes K, Maisonneuve E. Bacterial persistence and toxin-antitoxin loci. Annu Rev Microbiol 2012; 66:103-23; PMID:22994490; http://dx.doi.org/10.1146/annurev-micro-092611-150159
106. Wang X, Wood TK. Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. Appl Environ Microbiol 2011; 77:5577-83; PMID:21685157; http://dx.doi.org/10.1128/AEM.05068-11
107. Sevin EW, Barloy-Hubler F. RASTA-Bacteria: a web-based tool for identifying toxin-antitoxin loci in prokaryotes. Genome Biol 2007; 8:R155; PMID:17678530; http://dx.doi.org/10.1186/gb-2007-8-8-r155
108. Brown JM, Shaw KJ. A novel family of *Escherichia coli* toxin-antitoxin gene pairs. J Bacteriol 2003; 185:6600-8; PMID:14594833; http://dx.doi.org/10.1128/JB.185.22.6600-6608.2003
109. Hayes F. A family of stability determinants in pathogenic bacteria. J Bacteriol 1998; 180:6415-8; PMID:9829958
110. Gotfredsen M, Gerdes K. The *Escherichia coli relBE* genes belong to a new toxin-antitoxin gene family. Mol Microbiol 1998; 29:1065-76; PMID:9767574; http://dx.doi.org/10.1046/j.1365-2958.1998.00993.x
111. Robson J, McKenzie JL, Cursons R, Cook GM, Arcus VL. The *vapBC* operon from *Mycobacterium smegmatis* is an autoregulated toxin-antitoxin module that controls growth via inhibition of translation. J Mol Biol 2009; 390:353-67; PMID:19445953; http://dx.doi.org/10.1016/j.jmb.2009.05.006
112. Stieber D, Gabant P, Szpirer C. The art of selective killing: plasmid toxin/antitoxin systems and their technological applications. Biotechniques 2008; 45:344-6; PMID:18778262; http://dx.doi.org/10.2144/000112955
113. Pecota DC, Kim CS, Wu K, Gerdes K, Wood TK. Combining the *hok/sok, parDE*, and *pnd* postsegregational killer loci to enhance plasmid stability. Appl Environ Microbiol 1997; 63:1917-24; PMID:9143123
114. Suzuki M, Zhang J, Liu M, Woychik NA, Inouye M. Single protein production in living cells facilitated by an mRNA interferase. Mol Cell 2005; 18:253-61; PMID:15837428; http://dx.doi.org/10.1016/j.molcel.2005.03.011
115. Suzuki M, Mao L, Inouye M. Single protein production (SPP) system in *Escherichia coli*. Nat Protoc 2007; 2:1802-10; PMID:17641648; http://dx.doi.org/10.1038/nprot.2007.252
116. Nehlsen K, Herrmann S, Zauers J, Hauser H, Wirth D. Toxin-antitoxin based transgene expression in mammalian cells. Nucleic Acids Res 2010; 38:e32; PMID:20007149; http://dx.doi.org/10.1093/nar/gkp1140
117. de la Cueva-Méndez G, Mills AD, Clay-Farrace L, Díaz-Orejas R, Laskey RA. Regulatable killing of eukaryotic cells by the prokaryotic proteins Kid and Kis. EMBO J 2003; 22:246-51; PMID:12514130; http://dx.doi.org/10.1093/emboj/cdg026
118. Andreev D, Hauryliuk V, Terenin I, Dmitriev S, Ehrenberg M, Shatsky I. The bacterial toxin RelE induces specific mRNA cleavage in the A site of the eukaryote ribosome. RNA 2008; 14:233-9; PMID:18083838; http://dx.doi.org/10.1261/rna.693208
119. Shimazu T, Degenhardt K, Nur-E-Kamal A, Zhang J, Yoshida T, Zhang Y, Mathew R, White E, Inouye M. NBK/BIK antagonizes MCL-1 and BCL-XL and activates BAK-mediated apoptosis in response to protein synthesis inhibition. Genes Dev 2007; 21:929-41; PMID:17403773; http://dx.doi.org/10.1101/gad.1522007
120. Lioy VS, Rey O, Balsa D, Pellicer T, Alonso JC. A toxin-antitoxin module as a target for antimicrobial development. Plasmid 2010; 63:31-9; PMID:19800365; http://dx.doi.org/10.1016/j.plasmid.2009.09.005
121. Williams JJ, Hergenrother PJ. Artificial activation of toxin-antitoxin systems as an antibacterial strategy. Trends Microbiol 2012; 20:291-8; PMID:22445361; http://dx.doi.org/10.1016/j.tim.2012.02.005
122. Williams JJ, Hergenrother PJ. Exposing plasmids as the Achilles' heel of drug-resistant bacteria. Curr Opin Chem Biol 2008; 12:389-99; PMID:18625335; http://dx.doi.org/10.1016/j.cbpa.2008.06.015
123. Agarwal S, Mishra NK, Bhatnagar S, Bhatnagar R. PemK toxin of *Bacillus anthracis* is a ribonuclease: an insight into its active site, structure, and function. J Biol Chem 2010; 285:7254-70; PMID:20022964; http://dx.doi.org/10.1074/jbc.M109.073387
124. Chopra N, Agarwal S, Verma S, Bhatnagar S, Bhatnagar R. Modeling of the structure and interactions of the *B. anthracis antitoxin*, MoxX: deletion mutant studies highlight its modular structure and repressor function. J Comput Aided Mol Des 2011; 25:275-91; PMID:21336656; http://dx.doi.org/10.1007/s10822-011-9419-z
125. Erental A, Sharon I, Engelberg-Kulka H. Two programmed cell death systems in *Escherichia coli*: an apoptotic-like death is inhibited by the *mazEF*-mediated death pathway. PLoS Biol 2012; 10:e1001281; PMID:22412352; http://dx.doi.org/10.1371/journal.pbio.1001281
126. Kumar S, Kolodkin-Gal I, Engelberg-Kulka H. Novel quorum-sensing peptides mediating interspecies bacterial cell death. MBio 2013; 4:e00314-13; PMID:23736285; http://dx.doi.org/10.1128/mBio.00314-13
127. Chono H, Matsumoto K, Tsuda H, Saito N, Lee K, Kim S, Shibata H, Ageyama N, Terao K, Yasutomi Y, et al. Acquisition of HIV-1 resistance in T lymphocytes using an ACA-specific *E. coli* mRNA interferase. Hum Gene Ther 2011; 22:35-43; PMID:20649483; http://dx.doi.org/10.1089/hum.2010.001
128. Shapira A, Shapira S, Gal-Tanamy M, Zemel R, Tur-Kaspa R, Benhar I. Removal of hepatitis C virus-infected cells by a zymogenized bacterial toxin. PLoS One 2012; 7:e32320; PMID:22359682; http://dx.doi.org/10.1371/journal.pone.0032320