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REVIEW ARTICLE

Toxins-antitoxins: diversity, evolution and function

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Abstract

Genes for toxin-antitoxin (TA) complexes are widespread in prokaryote genomes, and species frequently possess tens of plasmid and chromosomal TA loci. The complexes are categorized into three types based on genetic organization and mode of action. The toxins universally are proteins directed against specific intracellular targets, whereas the antitoxins are either proteins or small RNAs that neutralize the toxin or inhibit toxin synthesis. Within the three types of complex, there has been extensive evolutionary shuffling of toxin and antitoxin genes leading to considerable diversity in TA combinations. The intracellular targets of the protein toxins similarly are varied. Numerous toxins, many of which are sequence-specific endoribonucleases, dampen protein synthesis levels in response to a range of stress and nutritional stimuli. Key resources are conserved as a result ensuring the survival of individual cells and therefore the bacterial population. The toxin effects generally are transient and reversible permitting a set of dynamic, tunable responses that reflect environmental conditions. Moreover, by harboring multiple toxins that intercede in protein synthesis in response to different physiological cues, bacteria potentially sense an assortment of metabolic perturbations that are channeled through different TA complexes. Other toxins interfere with the action of topoisomersases, cell wall assembly, or cytoskeletal structures. TAs also play important roles in bacterial persistence, biofilm formation and multidrug tolerance, and have considerable potential both as new components of the genetic toolbox and as targets for novel antibacterial drugs.

Keywords: horizontal gene transfer; translation inhibitors; selfish genes; transcriptional regulator; stress response

From plasmids to chromosomes

Toxin-antitoxin (TA) systems were discovered originally on plasmids in the mid 1980s. The *ccd* operon identified on *Escherichia coli* F plasmid initially was thought to couple host cell division to F plasmid replication (*ccd* for *c*ontrol of *c*ell *d*ivision) (Ogura & Hiraga, 1983). However, it subsequently was shown that *ccd* does not prevent plasmid loss, but rather acted by killing progeny that did not inherit a plasmid copy after cell division (Jaffe *et al.*, 1985). Therefore, *ccd* was redefined as a plasmid maintenance system (Jaffe *et al.*, 1985). Concomitantly, the analogous *hok-sok* system of *E. coli* R1 plasmid was discovered (Gerdes *et al.*, 1986). Subsequent studies revealed that the molecular components and mechanisms of the two systems were different. The *ccd* operon is composed of the *ccdA* and *ccdB* genes encoding, respectively, the CcdB toxic protein whose activity is negatively regulated by the CcdA protein antitoxin (Bex *et al.*, 1983; Karoui *et al.*, 1983) (Figure 1A). In the *hok-sok* locus, the *hok* gene encodes a toxic protein, and its expression is regulated negatively at the post-transcriptional level by the antisense *sok* RNA (Gerdes *et al.*, 1988) (Figure 1B). Phenotypically, both systems lead to an apparent stabilization of otherwise unstable replicons coupled with the production of non-viable daughter cells devoid of plasmid copies. The term 'post-segregational killing' (PSK) was proposed to describe this phenomenon (Gerdes *et al.*, 1986), although it took a few more years to unravel the molecular mechanisms that underlie it. PSK relies on a differential stability of the toxin and antitoxin components, the antitoxin being less stable than the toxin. Therefore, in plasmid-free progeny, the antitoxin component is degraded releasing the toxin from inhibition leading to killing of plasmid-free progeny. Antitoxin degradation is achieved either by endoribonucleases if

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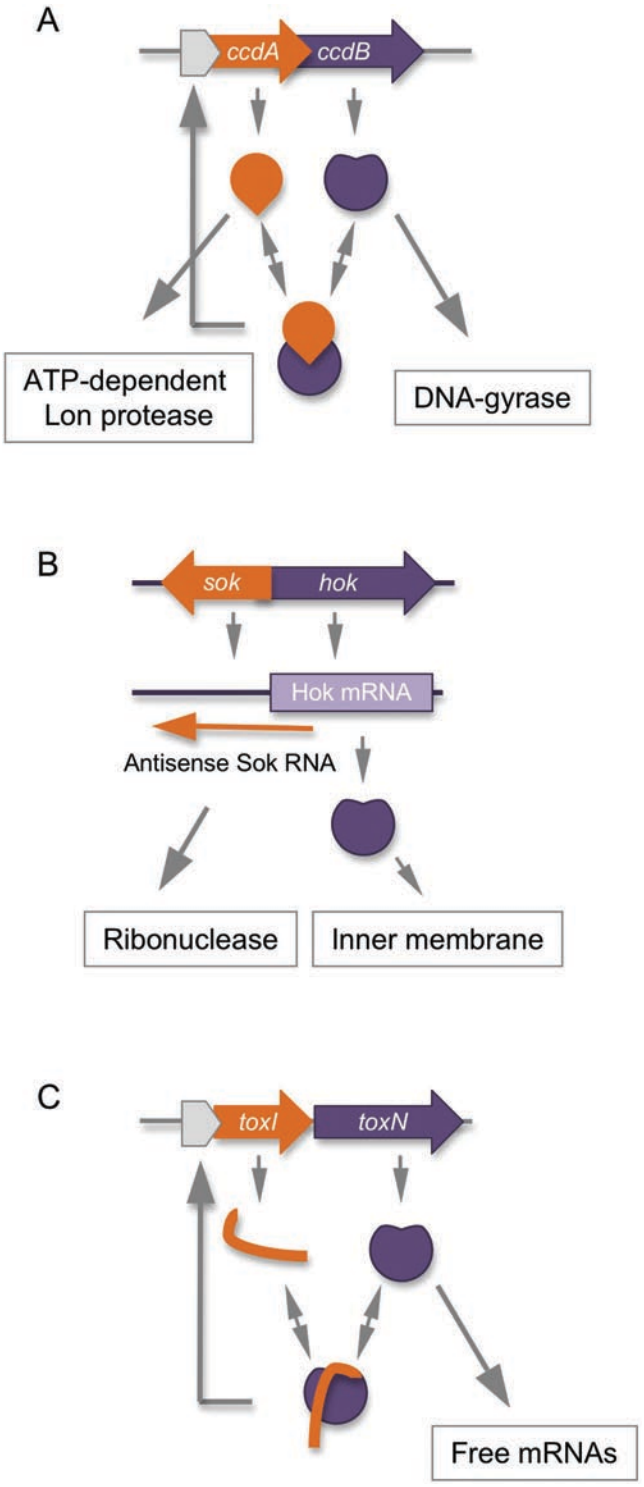

Figure 1. Three paradigms for plasmid-encoded TA complexes. (A) The *ccd* genes represent the paradigm for type II TAs. This locus is composed of the *ccdA* gene, encoding the CcdA antitoxin, and the *ccdB* gene encoding the CcdB toxin. CcdA interacts with CcdB to inhibit its toxic activity. This complex is also responsible for negative autoregulation of the operon. In the complex, CcdA is the DNA-binding element (Figure 5). The target of CcdB is DNA gyrase. CcdA is degraded by the Lon ATP-dependent protease. (B) The *hok-sok* locus represents the paradigm for type I TAs. The *hok* gene encodes the Hok toxic protein. The *sok* gene encodes the Sok antisense RNA. Sok inhibits Hok translation by interacting with the leader region of the *hok* mRNA and promoting degradation of the duplex by ribonucleases. Intricate regulations comprising a translational coupling of the *mok* small ORF (not shown in the scheme) located upstream the *hok* ORF as well as secondary structures within the Hok mRNA inhibiting Hok translation account for a tight control of *hok* expression. (C) The *toxIN* cassette represents the paradigm for type III TAs. The ToxN toxin is a protein whose toxic activity is inhibited by binding to the ToxI RNA antitoxin. This complex also negatively regulates the operon at the transcriptional level. The molecular mechanism underlying toxin activation is unknown.

the antitoxin is an RNA species (Gerdes *et al.*, 1992) or by ATP-dependent proteases if the antitoxin is a labile protein (Tsuchimoto *et al.*, 1992; Van Melderen *et al.*, 1994). The PSK phenomenon is also denoted as 'addiction' since a cell becomes addicted to the presence of the plasmid and *in extenso* to that of the TA system (Yarmolinsky, 1995). Subsequently, complexes in which the toxin is neutralized by an antitoxin RNA that inhibits toxin translation were categorized as type I systems, whereas those in which both the toxin and antitoxin are proteins were termed type II complexes (Hayes, 2003).

The *hok-sok* and *ccd* systems became the paradigms for type I and type II systems, respectively. Following their discovery, novel type II systems were rapidly identified on plasmids, e.g., the R1 *kis-kid* system (Bravo *et al.*, 1987) which is identical to the R100 *pem* system (Tsuchimoto *et al.*, 1988), the RK2 *parDE* system (Roberts *et al.*, 1990), the R485 *stbDE* system (Hayes, 1998) and the *phd-doc* system located on the P1 bacteriophage (Lehnherr *et al.*, 1993). For type I systems, homologs of *hok-sok* were identified on plasmids such as *srnB-srnC* on the F plasmid and *pndA-pndB* on the R483 plasmid (for a recent review, see Fozo *et al.*, 2008).

A third type of TA cassette (type III) recently was revealed on a plasmid of *Erwinia carotovora* (Fineran *et al.*, 2009) (Figure 1C). As in type I and II systems, the ToxN toxin is a protein. However, although the ToxI antitoxin is an RNA, its mode of action does not rely on expression inhibition but rather on inhibition of activity: it sequesters the toxin by complex formation (Blower *et al.*, 2009, 2011a). Interestingly, this TA system was discovered as an abortive infection that limits bacteriophage infection in a bacterial population by promoting cell death. This property of TAs might be generalized for the three types since *hok-sok* was found to exclude bacteriophage T4 (Pecota & Wood, 1996) and the type II *mazEF* locus provokes P1 exclusion (Hazan & Engelberg-Kulka, 2004).

A few years after the discovery of plasmid-encoded TAs, homologs of type I *hok-sok* and type II *kis-kid* modules were identified in the *E. coli* chromosome (Poulsen *et al.*, 1989; Masuda *et al.*, 1993). Following that, other type I and II modules were identified rapidly and extensively studied. The paradigm for type II chromosomally encoded systems are the *mazEF* and *relBE* cassettes (see below) (Aizenman *et al.*, 1996; Christensen *et al.*, 2001). Since then, chromosomally encoded TA systems have gained in attention their biological role(s) remains a debated issue (see below).

With the development of large-scale sequencing projects and the increasing number of bacterial genome sequences, the number of type II TA systems detected in bacterial genomes has been growing exponentially (Gerdes, 2000; Pandey & Gerdes, 2005; Guglielmini *et al.*, 2008; Ramage *et al.*, 2009; Leplae *et al.*, 2011). In a recent survey performed on 2181 prokaryotic genomes (including chromosomes, phages and plasmids), more than 7000 toxin and 10,000 antitoxin sequences were detected (Leplae *et al.*, 2011). Novel families of type II toxins and antitoxins were predicted. As only a small proportion of these predictions were tested experimentally and successfully validated, numerous TA families might still be discovered (Leplae *et al.*, 2011). The occurrence of toxin and antitoxin families varies (Pandey & Gerdes, 2005; Jorgensen *et al.*, 2009; Leplae *et al.*, 2011) and the ParE/RelE toxin family appears to be the most prevalent in our recent survey (Leplae *et al.*, 2011). Accordingly, metagenomic analysis of the human gut mobile microbiome showed that the *relBE* genes were overrepresented (Jones *et al.*, 2010).

Type I loci appear to be less prevalent although identifying these systems is quite challenging because of the hydrophobic nature and small size of the toxin and the difficulty in predicting small RNA (Wozniak & Waldor, 2009). Nevertheless, novel families were recently mined and successfully validated (Fozo *et al.*, 2010). A small number of *toxIN* type III homologs were detected in both Gram-positive and Gram-negative bacteria (Fineran *et al.*, 2009), although other type III systems presenting the same organization but devoid of sequence similarity might still be discovered.

Thus, both type I and II TA genes are abundant in bacterial chromosomes, although their copy numbers greatly vary between genomes (Pandey & Gerdes, 2005; Jorgensen *et al.*, 2009; Fozo *et al.*, 2010). Some genomes contain almost 100 predicted type II TA sequences representing 1.5% of the total number of open reading frames, whereas other chromosomes are entirely lacking in TA loci (Leplae *et al.*, 2011). Interestingly, no correlation can be drawn between the total number of coding sequences and the number of type II TA systems (Leplae *et al.*, 2011). Even small genomes that have been subjected to reductive evolution, such as those of obligate intracellular bacteria, contain predicted TA systems. In the *Rickettsia* genus, whereas strains such as *R. prowazekii* and *R. typhi* are devoid of any predicted TA systems, others such as *R. bellii* and *R. felis* contain as many as 32 and 36 predicted TA systems representing 2.2 and 2.6%, respectively, of the total number of coding sequences (Leplae *et al.*, 2011). The TA content of the *E. coli* K-12 MG1655 laboratory strain is illustrated in Table 1. Sixteen type I and twelve type II systems have been identified thus far.

In view of the frequency and distribution of chromosomal TA genes, the question of their origin arises. Although it has not been studied in a systematic way, it is evident that type II TAs have invaded chromosomes through horizontal gene transfer. The chromosomally encoded systems typically are parts of genomic islands or constitute genomic islets by themselves. They are detected within cryptic prophages such as *rnlAB* and *relBE* located on CP4-57 and Qin, respectively, in *E. coli* K-12 (Table 1) as well as on conjugative transposons (Wozniak & Waldor, 2009). In species such as *Vibrio cholerae*, type II TAs are mobile gene cassettes that are part of the superintegron (for a recent review, see Cambray *et al.*, 2010). In other cases, they are inserted

Table 1. Toxin-antitoxin systems in the *E. coli* K-12 MG1655 strain.

Systems	Position in *E. coli* K-12 genome (strand)	Activity	Genomic location
Type I			
hokC-sokC	16,751 (lagging)	–	Transposase DDE *insL* gene (15,445)
hokE-sokE	607,059 (leading)	–	Transposase DDE *insL* gene (607,588)
ldrA-rdlA	1,268,391 (lagging)	ND	3 systems linked
ldrB-rdlB	1,268,926 (lagging)	ND	
ldrC-rdlC	1,269,461 (lagging)	ND	
hokB-sokB	1,489,986 (lagging)	–	REP116 (1,487,728)
hokD-sokD	1,643,143 (lagging)	–	Qin cryptic prophage-upstream *relBE*
ibsA-sibA	2,151,373 (lagging)	+	REP148 (2,149,698) 2 systems are linked
ibsB-sibB	2,151,705 (lagging)	+	
shoB-ohsC	2,698,139 (lagging)	+	/
ibsC-sibC	3,059,412 (lagging)	+	/
ibsD-sibD	3,192,788 (leading)	+	2 systems are linked
ibsE-sibE	3,193,163 (leading)	+	
ldrD-rdlD	3,698,003 (lagging)	+	/
istR-tisB	3,851,576 (leading)	+	REP279 (3,851,735)
symER	4,577,522 (lagging)	+	Immigration control region element (ICR)
Type II			
dinJ-yafQ	245,961 (lagging)	+	REP17 (244,129)/REP18 (247,458)
yafNO	252,005 (leading)	+	REP19 (248,147)/REP20 (254,217)
hicAB	1,507,310 (leading)	+	REP117 (1,505,815)
hipBA	1,590,200 (lagging)	+	REP123 (1,590,655)
relBE	1,643,657 (lagging)	+	Qin cryptic prophage
yefM-yoeB	2,087,486 (lagging)	+	REP143 (2,095,261) at 7.7 kb (*his* operon)
rnlAB	2,763,940 (leading)	+	CP4-57 cryptic prophage
mazEF	2,909,113 (lagging)	+	REP202 (2,915,910)
mqsRA	3,166,270 (lagging)	+	REP223 (3,161,666)
ygjMN	3,232,163 (lagging)	+	REP233 (3,232,660)
prlF-yhaV	3,275,024 (leading)	+	REP238 (3,275,841)
chpB	4,446,470 (leading)	+	REP336 (4,448,967)
Other			
yafW-ykfI	262,914 (leading)	+	CP4-6 cryptic prophage
yeeUV	2,075,136 (lagging)	+	CP4-44 cryptic prophage

For type I, the orientation of the toxin gene is considered. REP sequences or transposase genes flanking TA systems within 5000 bp are indicated except for the *yefM-yoeB* which is separated from a REP sequence by the *his* operon.
/ = no REP detected within 5000 bp ND, not determined.

in intergenic regions such as ccd_{O157} in *E. coli* O157:H7 (Wilbaux *et al.*, 2007; Mine *et al.*, 2009) or between genes organized in operons such as *yafNO* and *mazEF* in *E. coli* K-12 (Singletary *et al.*, 2009) (Figure 2). In K-12, type II TA modules are located in close proximity to REP (repetitive extragenic palindromic) sequences which might be an indicator that these TA genes are mobile (Table 1). Indeed, REP sequences are conserved inverted repeats representing ~1% of the *E. coli* genome (Higgins *et al.*, 1988) and are often associated with mobile genetic elements (Tobes & Pareja, 2006).

Comparison of their genomic distribution in different isolates of the same species further supports the idea that type II TA loci are part of the bacterial accessory genome (Figure 2). The occurrence of fourteen type II TA cassettes in three *E. coli* isolates is illustrated in Table 2. Whereas the laboratory strain MG1655 and the enterohemorragic strain O157:H7 contain 12 and 10 TAs, respectively, the uropathogenic strain CFT1073 possesses 3 of the 14 systems. Interestingly, the 'common' genome shared by these three isolates is represented by 40% of the total number of coding sequences (Welch *et al.*, 2002). Type II TAs clearly contribute to genetic diversity in bacterial genomes. Whether type II TA systems possessed the capacity to move independently during their evolution is unknown. Nevertheless, associating with mobile genetic elements provides an efficient mechanism to colonize bacterial genomes.

Evolution of type II systems

Macroevolution

Toxins of type II systems are sub-divided into 12 superfamilies based on sequence similarity (Leplae *et al.*, 2011) (Table 3). Three dimensional structures have been solved for members of 6 of these 12 superfamilies (for a recent review, see Blower *et al.*, 2011b). A perfect correlation between both sequence- and structure-based classifications is observed indicating that, although toxins within one structural group show only limited

Table 2. Occurrence of type II TA systems in three *E. coli* isolates.

TA genes	MG1655	O157:H7	CFT073
dinJ-yafQ	+	+	–
yafNO	+	+	–
hicAB	+	+	–
hipBA	+	+	+
relBE	+	–	-
yefM-yoeB	+	–	+
rnlAB	+	–	–
mazEF	+	+	–
ygiUT (mqsRA)	+	–	–
ygjNM	+	+	–
prlF-yhaV	+	+	+
chpB	+	+	–
ccd_{O157}	–	+	–
paaR2-paaA2-parE2	–	+	–

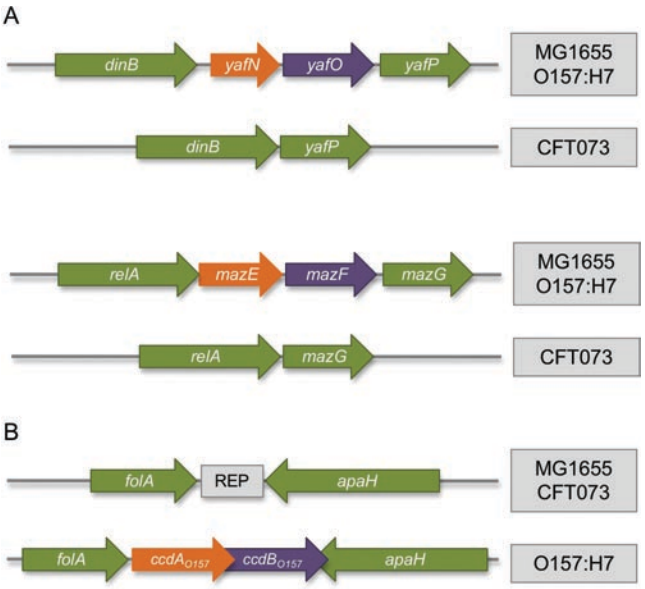

Figure 2. Type II TA genes constitute genomic islands. (A) The *yafNO* and the *mazEF* modules are inserted within operons. They are detected in the *E. coli* laboratory strain MG1655 and in *E. coli* enterohemorragic O157:H7 strain, but are absent from uropathogenic CFT073 strain. (B) The ccd_{O157} module is located between two convergently transcribed genes in *E. coli* O157:H7. The module is absent from MG1655 and CFT073 and a REP sequence separates the flanking genes in MG1655.

primary sequence similarities, the sequence-based classification is accurate. However, only the resolution of the three dimensional structures of members of all the groups will unambiguously solve how well the two classification schemes match. Thus, these data suggest that the 12 toxin superfamilies do not share any evolutionary relationship and that they originate from distinct ancestors. Interestingly, most of the type II toxins are translation inhibitors, except for the ζ toxins, which inhibit peptidoglycan synthesis, and the CcdB/Kid and ParE/RelE superfamilies both containing members that are DNA gyrase or translation inhibitors. The reason why translation inhibition is more prevalent than other types of toxicity is unknown. In this large population of translation inhibitors, various mechanisms are described and some of them will be detailed below (Figure 3).

Retracing evolution of the antitoxins might be complicated as they are composed in general of two distinct domains, a DNA-binding region and a toxin-binding domain. DNA-binding motifs among antitoxins can be of various folds such as helix-turn-helix (HTH), ribbon-helix-helix (RHH) and AbrB (for a review, see Gerdes *et al.*, 2005, and see below). The three dimensional structures of free antitoxins have been obtained in some cases (Figure 5), and a general property seems to be a lack of structure of the toxin-binding domain (Van Melderen *et al.*, 1994, 1996; Christensen *et al.*, 2001; Takagi *et al.*, 2005; Cherny *et al.*, 2007; Overgaard *et al.*, 2009; Shinohara *et al.*, 2010). This probably explains the susceptibility of antitoxins to ATP-dependent proteolysis. This domain in general adopts a structure upon binding to the toxin, which renders the antitoxin less unstable in the presence of the toxin (Van Melderen *et al.*, 1994, 1996; Christensen *et al.*, 2001; Takagi *et al.*, 2005; Cherny *et al.*, 2007; Overgaard *et al.*, 2009; Shinohara *et al.*, 2010). Even in absence of the toxin, antitoxin degradation is not a fast process. Half-lives measured *in vivo* are estimated between 20 and 40 minutes. Evolution might have selected poor protease substrates to secure TA systems. Based on sequence similarity, antitoxins are classified in 20 superfamilies (Leplae *et al.*, 2011). Not included in these superfamilies is the MqsA antitoxin belonging to the *mqsRA* complex (Brown *et al.*, 2009). This antitoxin appears to be folded throughout its entire sequence in contrast with canonical antitoxins. Another distinctive feature is that MqsA regulates expression of other *E. coli* genes, unrelated to TA loci, and the *mqsRA* system is involved in persistence as well as in quorum-sensing and biofilm formation regulation (see below) (Gonzalez Barrios *et al.*, 2006). Whether other types of antitoxins are able to regulate expression of non-TA genes remains to be shown.

Microevolution

As discussed above, TAs appear to be evolutionary successful. They are present in nearly all bacterial species and multiple copies of different and homologous systems often are found. One of the forces that drive TA evolution certainly is the coexistence of homologs in a single genome, whether they are encoded within the chromosome or on mobile genetic elements.

Homologs belonging to the *ccd* family located in the chromosome and on plasmid coexist in *E. coli* O157:H7 strains (Wilbaux *et al.*, 2007). Interestingly, the plasmid-encoded locus is functional for PSK in these strains as the chromosomally encoded antitoxin is unable to counteract toxicity of the plasmid-encoded toxin. Phylogenetic analysis revealed that chromosomally encoded *ccd* toxins and antitoxins form monophyletic groups that are distantly related to plasmid-encoded versions, indicating that co-evolution might have lead to enough

divergence to ensure the absence of cross-talk (Saavedra De Bast *et al.*, 2008). *A contrario*, homologs that are sufficiently related are able to cross-talk, providing a clue to a potential role for chromosomally encoded systems in the protection against PSK. This phenomenon was called anti-addiction (Saavedra De Bast *et al.*, 2008). Molecular evolution analysis also provided information regarding the type of evolutionary forces that were brought to bear on chromosomally- and plasmid-encoded TA loci. dN/dS (non-synonymous/synonymous mutations) analysis for plasmid-encoded toxins showed that they are under negative selection (values <<1), indicating that they are very constrained and therefore functional. On the contrary, the dN/dS ratio for chromosomally encoded toxins shows that they are under neutral selection (values close to 1), which reflects a high rate of mutation that inactivates a significant proportion of these toxins (Mine *et al.*, 2009). Thus, chromosomally encoded ccd_{O157} might be devoid of any biological role (Mine *et al.*, 2009). Based on these observations, a model retracing the microevolution of homologous TAs was proposed (Saavedra De Bast *et al.*, 2008). Upon integration in the chromosome, newly-acquired TA loci might serve as anti-addiction modules and provide a fitness gain to the host. This

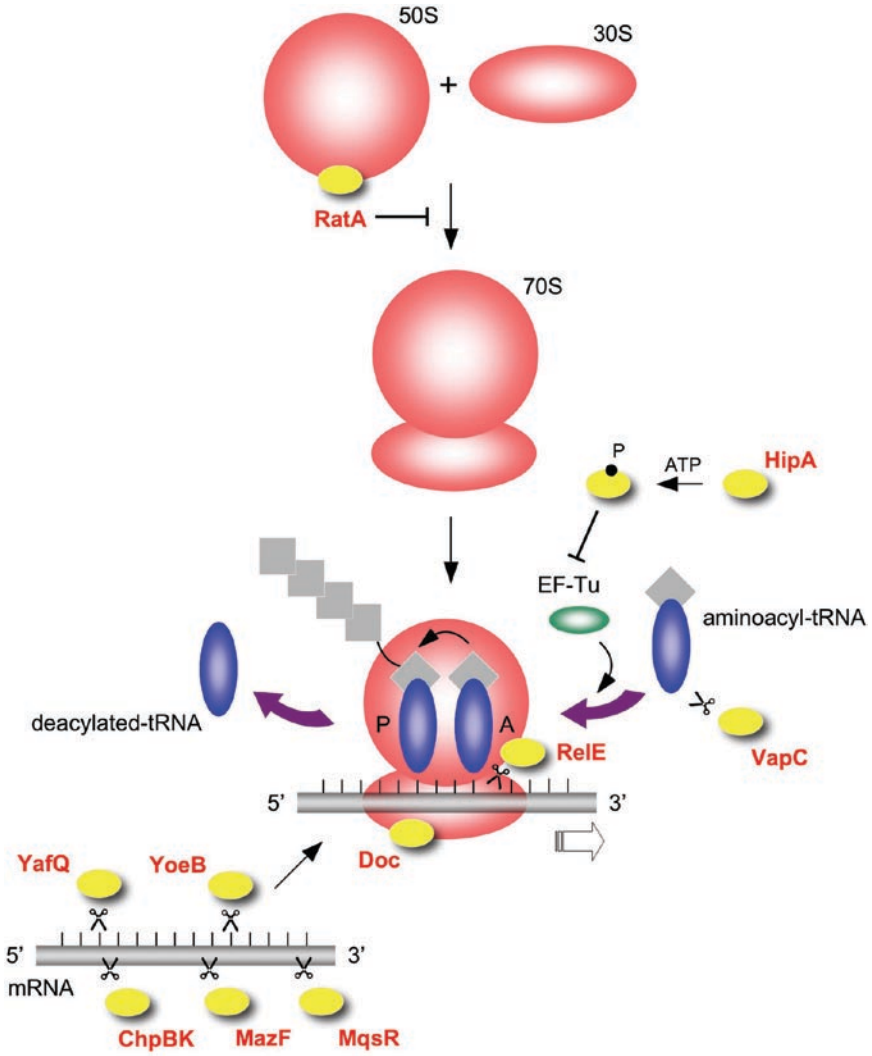

Figure 3. Simplified representation of the protein biosynthesis machinery illustrating known targets of selected toxin factors of TA complexes. The 70S ribosome comprises the 30S and 50S subunits (red ovals). The mRNA (grey bar) binds to the former. Aminoacyl-tRNA (purple ellipse) bearing an amino acid (grey diamond) enters the ribosomal A-site. The incoming amino acid is added to the growing polypeptide chain with formation of a new peptide bond. Deacylated tRNA exits from the P-site. Movement of the ribosome to the next codon (open arrow) is accompanied by transfer of the peptidyl-tRNA to the P-site so that the A-site is available for the following aminoacyl-tRNA. The RatA toxin associates with the 50S subunit and blocks assembly of the intact ribosome. Doc of bacteriophage P1 binds to the 30S subunit thereby stabilizing ribosome-bound mRNA and blocking further translation. The RelE endoribonuclease associates with the 50S subunit and cleaves mRNA located in the A-site. The HigB and YafO toxins (not shown) also are ribosome-dependent endoribonucleases. By contrast, ChpBK, MazF and MqsR are sequence-specific ribonucleases of free mRNA. YafQ and YoeB also can cleave free mRNA, but their activities are modified by association with the ribosome. VapC cleaves the initiator $tRNA^{fMet}$ in the anticodon stem-loop, but not elongator tRNAs. EF-Tu is an essential elongation factor involved in delivery of aminoacyl-tRNA molecules to the A-site. Phosphorylation of EF-Tu by HipA is predicted to inactivate translation. Autophosphorylation (filled circle) of HipA also is required for toxicity.

Table 3. The twelve superfamilies of type II toxins.

Sequence based superfamilies	Tertiary structures	Activities	Overexpression phenotype	References[a]
ParE/RelE	RNase T1 fold	Cleave mRNAs in the ribosome at the A site Cleave free mRNAs	Inhibition of translation	Takagi *et al.*, 2005; Francuski & Saenger, 2009
		Target DNA-gyrase	Inhibition of replication/SOS induction	Dalton & Crosson, 2010
CcdB/Kid	CcdB/Kid fold	Cleave free RNAs	Inhibition of translation	Hargreaves *et al.*, 2002; Kamada *et al.*, 2003
		Target DNA-gyrase	Inhibition of replication/SOS induction	Loris *et al.*, 1999
Doc	Fic fold, AvrB fold (FIDO superfamily)	Association with 30S ribosomal subunit	Inhibition of translation	Garcia-Pino *et al.*, 2008; Arbing *et al.*, 2010
ζ	Phosphotransferase fold	Phosphorylates UDP-Glc-NAc	Inhibition of peptidoglycan synthesis	Meinhart *et al.*, 2003
VapC	Pin-domain fold	Cleavage of $tRNA^{fMet}$	Inhibition of translation	Miallau *et al.*, 2009
HipA	Eukaryotic Ser/Thr kinase fold	Phosphorylation of the EF-Tu elongation factor	Inhibition of translation	Schumacher *et al.*, 2009
VapD	ND	ND	ND	
YafO	ND	Association with 30S ribosomal subunit	Inhibition of translation	
GinA	ND	ND	Inhibition of translation	
GinB	ND	ND	Inhibition of translation	
GinC	ND	ND	Inhibition of translation	
GinD	ND	ND	Inhibition of translation	

[a]Only the references related to the tri-dimensional structure are indicated.

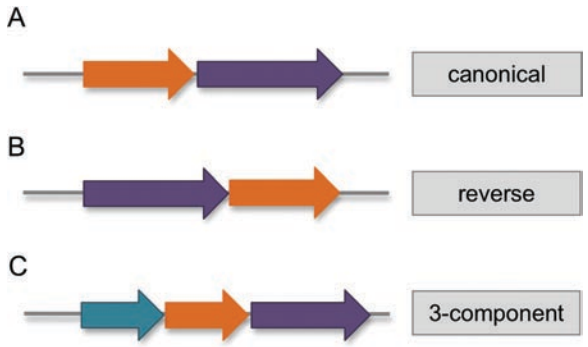

Figure 4. Different flavors of type II TA gene organization. (A) Canonical organization. The antitoxin gene (orange) is located upstream of the toxin gene (purple). (B) Reverse organization. The toxin gene is located upstream of the antitoxin gene. (C) Three-component organization. The transcriptional regulation activity (blue) and the antitoxin activity are encoded by different genes.

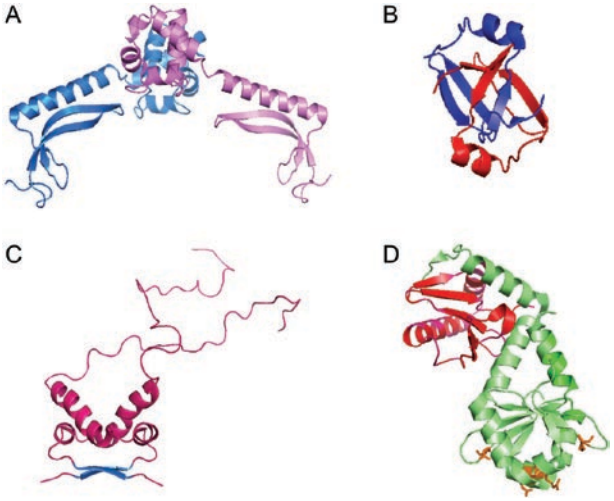

Figure 5. Tertiary structures of representative DNA binding antitoxins. (A) Structure of the apo-MqsA dimer that possesses a HTH motif (Brown *et al.*, 2011). The monomers are shown in blue and magenta. The N-terminal domains undergo extensive rearrangement when bound to DNA around which they form a clamp-like structure. (B) AbrB-like fold shown by dimeric MazE (Bobay *et al.*, 2005; Loris *et al.*, 2003). Monomers are colored red and blue. (C) RHH fold adopted by the N-terminal domain of CcdA antitoxin dimer (Madl *et al.*, 2006). The antiparallel β-strands that are the principal DNA binding determinants are highlighted in blue. The flexible C-terminal regions become structured when bound by CcdB toxin. (D) Tertiary structure of the $YefM_2$-YoeB heterotrimer (Kamada & Hanaoka, 2005). YoeB is colored red and the two YefM monomers are depicted in green. The positions of the R10 and R31 residues in each YefM monomer that are implicated in DNA binding are highlighted in orange (Bailey & Hayes, 2009).

could favor their fixation within populations subjected to the lethal activity of the plasmid-encoded homologs. In turn, this could drive the selection of plasmid-encoded TAs in which the toxin is no longer recognized by the anti-addiction module. Thus, with time, chromosomally encoded systems would lose their anti-addiction properties and degenerate.

In general, contemporary chromosomally encoded systems do not cross-talk (Ramage *et al.*, 2009; Hallez *et al.*, 2010; Fiebig *et al.*, 2010; Goeders & Van Melderen, unpublished data). Cross-talk might lead to the loss of activity as suggested above and divergence might be therefore essential for maintenance of these systems. Nevertheless, some exceptions to this in the case of *relBE* complexes are discussed below.

TA module organization: shuffling genes within a common framework

Genetic organization

As briefly explained above, a general framework for type II TAs can be drawn. In general, these modules are composed of two genes organized in an operon, the downstream gene encoding a toxic protein and the upstream gene specifying the antitoxin protein (Figure 4A). A first variation on the theme is a 'reverse' organization with the gene encoding the toxin preceding the antitoxin gene (Figure 4B). This organization was first observed for the *higBA* cassette located on the Rts1 plasmid (Tian *et al.*, 1996) and its homologs subsequently discovered on the *V. cholerae* superintegron (Christensen-Dalsgaard & Gerdes, 2006; Budde *et al.*, 2007). *E. coli* K-12 MG1655 contains several systems with reverse organization: *hicAB*, *ygjNM*, *mqsRA* (*ygiUT*) and *rnlAB* (Table 1). Although the YgjM and HigA antitoxins belong to the HigA superfamily and the MqsR and HigB toxins belong to the ParE/RelE superfamily, these systems do not share significant similarities with each other (Leplae *et al.*, 2011, and data not shown), suggesting that TA cassettes with reverse order do not descend from a common ancestor. Among the novel families of toxin and antitoxin recently discovered, a significant fraction of TAs showed this reverse organization, suggesting that they might be more widespread than previously anticipated (Leplae *et al.*, 2011).

At the protein level, domain shuffling is observed for the MqsA antitoxin (Brown *et al.*, 2009). In general, antitoxin proteins are composed of a pair of domains, a DNA-binding region located at the N-terminus of the protein and a toxin-binding domain located at the C-terminus. Within the antitoxin-toxin complex, the antitoxin is responsible for DNA binding (see below). In the MqsA antitoxin, the HTH DNA-binding domain is located at the C-terminus of the protein (Figure 5) (Brown *et al.*, 2009). This domain organization does not appear to be widespread in antitoxins; however, more detailed analysis might be required to define precisely antitoxin functional domains.

In a third flavor of organization, DNA-binding and antitoxin functions are split and encoded by different genes (Figure 4C). Two examples have been described: the ωζε locus on the Gram-positive *Streptococcus pyogenes*

pSM19035 plasmid (de la Hoz *et al.*, 2000) and the *paaR2-paaA2-parE2* system located on the CP933P cryptic prophage in *E. coli* O157:H7 (Hallez *et al.*, 2010). In both cases, the gene encoding the transcriptional regulator is upstream of the antitoxin-toxin pair. These systems may be less abundant as they might represent an ancestral form of TA gene organization. Considering that evolution tends to fuse domains into larger proteins with multiple activities (Trifonov & Frenkel, 2009), it is tempting to propose that the two functions encoded by separate polypeptides are ancestral and have evolved to multi-domain proteins. As antitoxins are unstable, a single polypeptide carrying both activities is likely to more stably maintain a proper antitoxin:toxin ratio. Nevertheless, molecular mechanisms underlying regulation of three component TA complexes remain to be determined.

Genetic organization also varies among the type I TAs (for a recent review, see Fozo *et al.*, 2008). Briefly, the RNA antitoxin gene and the toxin gene are encoded on opposite strands and overlap either at the 5'-end or 3'-end of the mRNAs. In a few cases, both genes are encoded divergently (*trans*-acting RNA) within the same intergenic region.

Mix and match between toxin and antitoxin superfamilies

For some time, it was thought that one toxin was always associated with a specific antitoxin, and TAs were classified based on the association of the two components. As an example, the gene for the ParE toxin was associated with the ParD antitoxin gene to form the canonical *parDE* module. A first bioinformatics analysis revealed that a toxin from one family could be associated with antitoxins from multiple families (Anantharaman & Aravind, 2003) and form hybrid systems. This hypothesis was validated experimentally by the discovery and characterization of the *yefM-yoeB* locus that encodes an antitoxin from the Phd superfamily and a toxin from the ParE/RelE superfamily—the canonical association would be *phD-doc* and *relB-relE* (Grady & Hayes, 2003), as well as of the *prlF-yhaV* cassette which associates a ParE/RelE toxin and an antitoxin belonging to the VapB superfamily—the canonical association would be *vapB-vapC* (Schmidt *et al.*, 2007). These observations have implications not only for classification of type II TAs, but also on their evolution. Regarding their classification, a simple categorization would be to refer to antitoxin and toxin families instead of families of systems (Leplae *et al.*, 2011). Regarding their evolution, these findings support the idea that type II antitoxins and toxins were assembled multiple times (Anantharaman & Aravind, 2003). *In situ* displacement of genes encoding functionally related but evolutionary unrelated proteins could account for this 'mix and match' phenomenon with a strong constraint on the operon structure as well as on regulation of expression (Anantharaman & Aravind, 2003).

One step further in the 'mix and match' phenomenon is illustrated by the type III *toxIN* genes. The ToxN toxin is an endoribonuclease presenting a fold similar to that of toxins belonging to the CcdB/Kid superfamily (Blower *et al.*, 2011a). This might suggest shuffling between type II and type III mechanisms of toxicity. However, it is not excluded that ToxN might be evolutionary unrelated to type II toxins and that the CcdB/Kid-fold arose by convergent evolution (Blower *et al.*, 2011a).

Toxins adopting the fold of an antitoxin and vice versa have also been described. Functional shift from a transcriptional factor or antitoxin to a toxin has been suggested to occur in the case of the type I SymE toxin (Kawano *et al.*, 2007). This toxin shows an AbrB-fold, as does the type II MazE antitoxin (Figure 5). Functional shift of toxin to an antitoxin appears to have occurred in the case of the YeeU antitoxin which shows a ParE/RelE-fold (Arbing *et al.*, 2010). Note that the *yeeU-yeeV* is not a canonical type II locus and that the YeeU mode of antitoxicity is still unknown (Brown & Shaw, 2003).

Transcriptional regulation: keeping toxins under control

In view of the numerous type II TA modules that populate most bacterial genomes (Table 1), unregulated expression of toxin genes could exert disastrous effects on cell growth and survival. It is imperative that cognate toxins and antitoxins are produced in a balanced ratio under steady state conditions. Control is achieved by transcriptional autoregulation of the TA operons (Gerdes *et al.*, 2005). Most typically, the antitoxin is a DNA-binding factor that recognizes an operator site that overlaps the operon promoter and thereby inhibits function of the transcription machinery. However, the antitoxin alone usually is insufficient for full repression of the module. Instead, the toxin, which itself lacks DNA-binding activity, is a repression enhancer. The toxin corepressor activity often mediates stabilization of the antitoxin within the complex. This stabilization serves a dual purpose, as it also protects the labile antitoxin from protease degradation. As described above, the N-terminal region of the antitoxin directs DNA-binding and the C-terminal region is involved in neutralization of the toxin. For example, *E. coli* and many other species encode the YefM-YoeB TA complex (Grady & Hayes, 2003) (Table 1). Although the YefM antitoxin originally was described as intrinsically disordered (Cherny & Gazit, 2004), it is well structured and at least partially folded (Kędzierska *et al.*, 2007; Nieto *et al.*, 2007; Kumar *et al.*, 2008). The flexible C-terminal region of YefM becomes stabilized when it interacts with the structured YoeB toxin concealing the latter's endoribonuclease fold (Kamada *et al.*, 2003; Kumar *et al.*, 2008). Free YefM binds its cognate operator site via the N-terminal region of the protein, sequentially recognizing adjacent long and short DNA palindromes that share a common core motif. The YefM-YoeB complex binds the

Table 4. Cleavage specificity of endoribonuclease toxins.

Toxin	Antitoxin	Organism	Ribosome Dependency of Cleavage	RNA (5'-3') Cleavage Specificity *in vivo*	RNA (5'-3') Cleavage Specificity *in vitro*	Reference
MazE-like toxins						
ChpBK	ChpBI	*Escherichia coli*	Independent	A↓C↓Y	A↓C↓(A/G/U)	Zhang *et al.* (2005b)
EndoA (YdcE)	YdcD	*Bacillus subtilis*	Independent	NT	↓U↓AC	Pellegrini *et al.* (2005)
Kid/PemK	Kis/PemI	Plasmid R1/R100 (*Escherichia coli*)	Independent	U↓A↓C	U↓A↓(A/C/U)	Zhang *et al.* (2004)
				UU↓ACU	U↓A(A/C)	Muñoz-Gómez *et al.* (2005); Pimentel *et al.* (2005)
MazF	MazE	*Escherichia coli*	Independent	A↓CA	A↓CA	Zhang *et al.* (2003)
				NT	(U/A)↓AC	Muñoz-Gómez *et al.* (2004)
				↓A↓CA	↓ACA	Zhang *et al.* (2005a)
MazF-mt1	MazE-mt1	*Mycobacterium tuberculosis*	Independent	CU↓ACC/UU↓ACA	U↓AC	Zhu *et al.* (2006)
MazF-mt3	MazE-mt3	*Mycobacterium tuberculosis*	Independent	NT	(C/U)U↓CCU	Zhu *et al.* (2008)
MazF-mt6	MazE-mt6	*Mycobacterium tuberculosis*	Independent	(U/C)U↓(A/U)C(U/C)	(U/C)U↓(A/U)C(U/C)	Zhu *et al.* (2006)
MazF-mt7	MazE-mt7	*Mycobacterium tuberculosis*	Independent	NT	U↓CGCU	Zhu *et al.* (2008)
MazF-mx	MrpC	*Myxococcus xanthus*	Independent	NT	(G/A)U↓UGC	Nariya & Inouye (2008)
MazFSa	MazESa	*Staphylococcus aureus*	Independent	(A/C/G)UU(A/C/G)	(A/C/G/)UU(A/C/G)	Fu *et al.* (2007)
				NT	U↓ACAU	Zhu *et al.* (2009)
RelE-like toxins						
HigB	HigA	*Escherichia coli*	Dependent	NN↓N	NT	Christensen-Dalsgaard *et al.* (2010)
HigB	HigA	Plasmid Rts1 (*Proteus vulgaris*)	Dependent	↓A↓A↓A↓	NC	Hurley & Woychik (2009)
HP0894 (RelE)	HP0895	*Helicobacter pylori*	Independent	NT	(U/C)↓A	Han *et al.* (2011)
MqsR (YgiU)	MqsA (YgiT)	*Escherichia coli*	Independent	GC↓(A/U)	NT	Christensen-Dalsgaard *et al.* (2010)
RelE	RelB	*Escherichia coli*	Dependent	A↓A↓A, NN↓G, UU↓A, UA↓G	UA↓G, UCG, CAG[a]	Christensen & Gerdes (2003); Pedersen *et al.* (2003)
				5' region of transcripts	NT	Hurley *et al.* (2011)
YafO	YafN	*Escherichia coli*	Dependent	11–13 bases downstream of initiation codons	11–13 bases downstream of initiation codons	Zhang *et al.* (2009)
				NN↓N	NT	Christensen-Dalsgaard *et al.* (2010)
YafQ	DinJ	*Escherichia coli*	Ribosome Modified	AAA↓(G/A)	Flanking GG	Prysak *et al.* (2009)
YoeB	YefM	*Escherichia coli*	Ribosome Modified	NT	Purine-specific	Kamada & Hanaoka (2005)
				Adenine tracts	NT	Christensen *et al.* (2004)

NC, no cleavage; NT, not tested.
[a]Other codons cleaved at lower rates.

palindromes more avidly than free antitoxin via cooperative interactions (Kędzierska *et al.*, 2007; Bailey & Hayes, 2009). Other TA factors, including CcdA-CcdB, Kis-Kid, MazE-MazF, and RelB-RelE, similarly show complex interactions with their regulatory DNA sites (Afif *et al.*, 2001; Marianovsky *et al.*, 2001; Dao-Thi *et al.*, 2002; Zhao & Magnuson, 2005; Monti *et al.*, 2007; Overgaard *et al.*, 2008, 2009; Brown *et al.*, 2011; Garcia-Pino *et al.*, 2010).

Binding of antitoxins to regulatory sites can be achieved either by HTH motifs (Khoo *et al.*, 2007; Brown *et al.*, 2009; Schumacher *et al.*, 2009; Arbing *et al.*, 2010) or by RHH folds (Takagi *et al.*, 2005; Madl *et al.*, 2006; Mattison *et al.*, 2006; Oberer *et al.*, 2007; Li *et al.*, 2008; Dalton & Crosson, 2010). In addition, the YefM/Phd group of antitoxins comprises a distinctive family with unique DNA-binding motifs, and MazE and related antitoxins possess an AbrB-like fold (Anantharaman & Aravind, 2003; Kamada *et al.*, 2003; Loris *et al.*, 2003; Bobay *et al.*, 2005; Kumar *et al.*, 2008; Bailey & Hayes, 2009) (Figure 5). The diversity in DNA-binding folds among antitoxin transcriptional regulators is another illustration of the 'mix and match' arrangements adopted by TA complexes referred to previously.

Not all TA cassettes conform to the canonical pattern of transcriptional regulation in which the antitoxin is the principal repressor with the toxin as corepressor. For example, transcription of genes for the ζ toxin-ε antitoxin complex of plasmid pSM19035 is repressed by ω, a global regulator of genes on this plasmid. The toxin and antitoxin have no role in transcriptional control (de la Hoz *et al.*, 2000). Analogously, the genomic *mazEF* operon of *Staphylococcus aureus* is not autoregulated. Instead, the global transcriptional regulator SarA activates the cassette, whereas the alternative sigma factor σ^B represses transcription, most probably indirectly (Donegan & Cheung, 2009). As referred to above, the tripartite *paaR-paaA-parE* locus in *E. coli* O157:H7 (Table 2) presents another variation on regulation of TA gene expression. In this case, the PaaA antitoxin-ParE toxin complex partially represses transcription from a promoter upstream of the cassette which the PaaR protein independently represses more effectively. All three proteins together fully downregulate the promoter (Hallez *et al.*, 2010). Finally, the MqsR-MqsA complex autoregulates its own expression, but also negatively controls and binds the promoter of the gene encoding the DNA replication inhibitor, CspD (Kim *et al.*, 2010). These examples hint that understanding transcriptional control of TA gene expression and its interplay with other genetic circuits will be a key to deciphering the signals that drive TA activity and evolution.

TA function: inhibiting protein synthesis for cell death or stasis

The RelE-RelB archetype: unleashing an endoribonuclease toxin when times are bad

The effects of the RelE and MazF toxins on macromolecular synthesis and physiology in *E. coli* and the antagonistic roles of the respective antitoxins were established soon after their discovery (Aizenman *et al.*, 1996; Gotfredsen & Gerdes, 1998). However, the precise intracellular targets of RelE and MazF temporarily remained a conundrum (Engelberg-Kulka & Glaser, 1999; Gerdes, 2000; Galvani *et al.*, 2001; Hayes, 2003). In light of evidence that TA genes were pervasive on bacterial genomes and potentially were of greater biological significance than previously anticipated, the observation that RelE inhibited translation and associated with ribosomes provided vital clues to the mechanism by which numerous chromosomally encoded toxins exert their effects (Christensen *et al.*, 2001; Galavani *et al.*, 2001). Subsequently, RelE was shown to be a sequence-specific endoribonuclease that cleaves single-stranded mRNA situated in the ribosomal A-site with high codon specificity (Table 4). RelE initially was shown to recognize both sense and stop codons differentially: UCG and CAG sense codons were cleaved most efficiently, whereas UAG, UAA and UGA stop codons were cleaved at fast, intermediate and slow rates, respectively (Christensen & Gerdes, 2003; Pedersen *et al.*, 2003). In contradiction with these findings, a more recent study has reported that mRNA cleavage by RelE is entirely codon-independent *in vivo*, but that the protein instead preferentially cuts within the 5' ends of transcripts (Hurley *et al.*, 2011). Interestingly, the YafO toxin in *E. coli* is a RelE homolog that also cleaves mRNA without strong sequence specificity downstream of initiation codons. Unlike RelE, cleavage by YafO apparently occurs outside of the ribosomal A-site (Zhang *et al.*, 2009).

Although conflicting data have been presented concerning its cleavage specificity, it is clear that RelE only digests translated mRNA, failing to target free mRNA (Pedersen *et al.*, 2003), which reflects a mandatory requirement for interaction of the protein with the 50S subunit of the 70S ribosome (Hurley & Woychik, 2009). When RelE enters the ribosomal A-site, it blocks access by tRNA and translation factors, including polypeptide release factor RF1, and preferentially cuts between the second and third nucleotides of mRNA codons (Pedersen *et al.*, 2003; Diago-Navarro *et al.*, 2009; Neubauer *et al.*, 2009). RelE-mediated cleavage of mRNA involves a rearrangement of these two bases which are stabilized by interaction with conserved residues in the toxin and 16S rRNA (Neubauer *et al.*, 2009). In addition, the action of the toxin in the ribosomal A-site necessitates the interaction of several basic residues in RelE with different regions of 16S rRNA (Neubauer *et al.*, 2009) which partly explains the promiscuity of RelE proteins across the three domains of life in which the corresponding rRNA domains are conserved (Kristoffersen *et al.*, 2000; Yamamoto *et al.*, 2002; Christensen & Gerdes, 2003; Andreev *et al.*, 2008). Because RelE possesses an incomplete RNase fold, its interactions within the ribosome are crucial to support the protein's endoribonuclease activity. By contrast, the YoeB toxin is a structural homolog of RelE, but exhibits an intact RNase fold which permits ribosome-independent cleavage of RNA *in vitro* (Kamada & Hanaoka, 2005). Nevertheless, cleavage by YoeB is modified *in vivo* and

in vitro by association with the translation machinery, similar to the activity of another RelE homolog, YafQ (Kamada & Hanaoka, 2005; Christensen-Dalsgaard & Gerdes, 2008; Prysak *et al.*, 2009; Zhang & Inouye, 2009). As YoeB preferentially cleaves immediately downstream of initiation codons, it has been proposed that this toxin specifically inhibits translation initiation (Yoshizumi *et al.*, 2009; Zhang & Inouye, 2009) (Figure 3).

The intracellular RelB:RelE ratio normally is balanced so that RelE-mediated cleavage of mRNA is inhibited. RelB antitoxin blocks the action of RelE by sequestering the protein into a catalytically inert complex: this involves displacement of a critical α-helix from its active configuration in free RelE (Li *et al.*, 2009). The antitoxin within this high affinity complex is protected from protease degradation to which it is otherwise highly susceptible (Christensen *et al.*, 2001; Overgaard *et al.*, 2009; Shinohara *et al.*, 2010). The differential protease sensitivity of free RelB and within the RelB:RelE complex reflects that the unbound antitoxin is partially disordered and hence more prone to degradation, but acquires more tertiary structure when bound to RelE (Takagi *et al.*, 2005; Cherny *et al.*, 2007). This is a recurring theme among TA complexes. Certain environmental stresses, including amino acid starvation and global inhibition of translation, induce the *relBE* cassette and related loci that encode RelE homologs in *E. coli* (Christensen-Dalsgaard *et al.*, 2010). The RelB-RelE complex also is implicated in recovery from stalled chromosomal replication forks that might otherwise cause cellular lethality (Godoy *et al.*, 2006). Induction of RelB-RelE activity during stress requires Lon protease which preferentially degrades RelB thereby unleashing the toxic effects of RelE (Christensen *et al.*, 2001; Christensen-Dalsgaard *et al.*, 2010). However, the toxicity of RelE is transient and bacteriostatic, rather than permanent and bactericidal. Resuscitation of cells from RelE-mediated mRNA cleavage requires a low level of continued protein synthesis. Recovery also is dependent on, first, relief from the prevailing stress conditions and, second, tmRNA. The latter, also denoted SsrA or 10Sa RNA, enters and clears stalled ribosomes bearing aberrant mRNA (Moore & Sauer, 2007), including RelE-cleaved transcripts (Christensen *et al.*, 2003). Interestingly, tmRNA sequences of diverse bacterial species have evolved a significant bias against cleavage sites for the MazF endoribonuclease supporting the view that intact tmRNA is required to recycle ribosomes that are blocked by toxin-cleaved transcripts (Baik *et al.*, 2009). Thus, the RelB-RelE complex is a conduit through which a variety of nutritional and other stresses are relayed so that cellular metabolism, specifically translation, is ablated temporarily until physiological conditions have improved.

RelE homologs are encoded widely by both bacteria and archaea (Pandey & Gerdes, 2005; Guglielmini *et al.*, 2008; Makarova *et al.*, 2009; Leplae *et al.*, 2011) (Table 3). Although some homologs possess low amino acid sequence identity, they share common folds in which residues crucial for RNA recognition and cleavage are conserved (Kamada & Hanaoka, 2005; Takagi *et al.*, 2005; Motiejunaite *et al.*, 2007; Francuski & Saenger, 2009; Dalton & Crosson, 2010; Barbosa *et al.*, 2010). The ribonuclease activities of several RelE homologs have been characterized. Like the prototypical RelE specified by *E. coli*, the homologs are sequence-specific RNAses, although diverse homologs exhibit different cleavage characteristics. Moreover, some RelE relatives either act independently of the ribosome or cleavage activity differs dependent on whether the enzyme is free or ribosome-associated (Table 4). Each RelE homolog is counteracted by its cognate antitoxin (Grady & Hayes, 2003; Takagi *et al.*, 2005; Motiejunaite *et al.*, 2007; Nieto *et al.*, 2007; Hurley & Woychik, 2009; Christensen-Dalsgaard *et al.*, 2010; Han *et al.*, 2011). In hosts bearing multiple *relBE*-like cassettes, the paralogs may function independently and respond dissimilarly to stress conditions, or may exhibit extensive cross-talk with other *relBE* modules (Christensen-Dalsgaard *et al.*, 2010; Fiebig *et al.*, 2010; Yang *et al.*, 2010). Interactions between RelB-RelE homologs and heterologous TA systems also have been described (Garcia-Pino *et al.*, 2008; Winther & Gerdes, 2009). Thus, dependent on the host background, the numbers of complexes and their interactions, the RNA cleavage patterns of the toxins, and the triggers by which they are activated, a suite of RelB-RelE complexes may mediate an intricate repertoire of physiological responses that are induced by an array of environmental signals (Magnuson, 2007; Van Melderen & Saavedra De Bast, 2009).

The MazE-MazF complex: a different flavor of endoribonuclease

The MazF family, which is unrelated evolutionarily to the RelE group, comprises a second major class of endoribonuclease toxins (Table 3). The activity of MazF is blocked by the labile MazE antitoxin which is susceptible to degradation by the ClpAP chaperone-protease complex (Aizenman *et al.*, 1996). The toxin is activated in *E. coli* by a range of signals including amino acid starvation, elevated temperatures, exposure to selected antibiotics that block transcription or translation, oxidative stress, DNA damage, thymine starvation, and bacteriophage infection (Sat *et al.*, 2001, 2003; Hazan & Engelberg-Kulka, 2004; Hazan *et al.*, 2004; Godoy *et al.*, 2006; Kolodkin-Gal & Engelberg-Kulka, 2006; Kolodkin-Gal *et al.*, 2007, 2009). MazF cleaves 5'-ACA-3' sequences in *E. coli* transcripts. Cleavage occurs independently of the ribosome and of translation, and thus the sequence specificity displayed *in vivo* is maintained *in vitro* (Zhang *et al.*, 2003, 2005a; Munoz-Gomez *et al.*, 2004; Christensen-Dalsgaard & Gerdes, 2008). Analogous observations have been made with numerous MazF homologs from a diverse range of bacterial species, although the mRNA sequences recognized by these homologs often differ from the *E. coli* archetype, as well as from each other (Yamaguchi & Inouye, 2009) (Table 4). The molecular basis for these differences is

unknown, but species such as *Mycobacterium tuberculosis* that possess multiple MazF homologs with varying sequence specificities may employ these and a battery of other TA complexes to respond to diverse environmental signals (Zhu *et al.*, 2010). Thus, mRNAs with particular codon usage patterns may be vulnerable when toxins with corresponding cleavage specificities are activated. Other transcripts may be resistant to these endoribonucleases, but may be targeted when toxins that recognize alternative sequences are triggered.

The *mazEF* cassette is located downstream of the *relA* gene in *E. coli*. When the ribosomal A-site is occupied by deacylated tRNA species during stressful conditions such as amino acid limitation, the RelA protein is activated and catalyzes production of the alarmone guanosine 5'-diphosphate, 3'-diphosphate (ppGpp). Overproduction of ppGpp via *relA* overexpression represses the *mazEF* operon. This induces decreased synthesis of MazE antitoxin thereby liberating the more stable MazF toxin to act intracellularly (Aizenman *et al.*, 1996). The *mazG* gene that is cotranscribed with *mazEF* provides another regulatory layer to the action of MazF. MazG is a nucleoside triphosphate pyrophosphohydrolase that hydrolyzes dNTPs, and depletes ppGpp levels *in vivo*. The activity of MazG is ablated by the MazE-MazF complex. However, when starvation conditions elicit elevated ppGpp concentrations with a resultant reduced level of the MazE-MazF complex, the activity of MazG is no longer suppressed by the complex and ppGpp levels decrease. Thus, the action of the MazE-MazF complex is balanced by the antagonistic activities of RelA which is involved in ppGpp formation and MazG that depletes alarmone levels (Gross *et al.*, 2006).

The impact of the MazE-MazF complex on cell survival is contentious. One viewpoint asserts that the complex is part of a programmed cell death mechanism that is activated by selected metabolic conditions within a defined time window following exposure to the stress (Amitai *et al.*, 2004; Kolodkin-Gal & Engelberg-Kulka, 2006, 2009; Engelberg-Kulka *et al.*, 2009; Kolodkin-Gal *et al.*, 2009). It may be altruistic for a subset of cells within a population to commit suicide under certain circumstances, e.g., to prevent spread of bacteriophage within the culture or by lysing to provide a source of nutrients for sibling cells (Engelberg-Kulka *et al.*, 2006). Although the activity of MazF reduces global translation levels, the continued expression of protein subsets that mediate cell death or survival may be important in determining which subpopulations perish or remain viable (Amitai *et al.*, 2009). In further support of MazE-MazF as a programmed cell death mechanism, induction of the toxin recently was shown to be a population-dependent phenomenon that involves a quorum-sensing pentapeptide, extracellular death factor (EDF) in *E. coli* (Kolodkin-Gal *et al.*, 2007, 2008). EDF is generated from a protein precursor, glucose-6-phosphate dehydrogenase, and is extruded into the extracellular medium (Kolodkin-Gal & Engelberg-Kulka, 2008). The peptide activates the endoribonuclease activity of MazF under dense growth conditions when an appropriate concentration of EDF accumulates. MazF possesses dual mRNA processing sites; occupation of one of these sites by MazE antitoxin prevents substrate binding to the second site (Li *et al.*, 2006). By competing with MazE for the same binding sites on MazF, EDF simultaneously blocks access by the antitoxin and enhances mRNA cleavage by the toxin by an unknown mechanism (Belitsky *et al.*, 2011). Although EDF is the first example of a quorum-sensing peptide in *E. coli*, these peptides are commonplace among Gram-positive bacteria. However, none is known to be an enhancer of ribonuclease activity in these species suggesting that quorum-sensing peptides may be a latent pool of TA complex activators in Gram-positives (Hayes, 2011).

In contradiction with earlier findings (Aizenman *et al.*, 1996), amino acid starvation has been reported to stimulate, instead of repress, *mazEF* transcription and ppGpp was not required for MazF induction in *E. coli*. Moreover, conflicting evidence has emerged that induction of the MazF toxin is not lethal but bacteriostatic, is counteracted by tmRNA, and that cells can recover by subsequent production of MazE antitoxin (Pedersen *et al.*, 2002; Christensen *et al.*, 2003; Tsilibaris *et al.*, 2007). Doubt also has been cast on the role of EDF in eliciting MazF-mediated programmed cell death (Van Melderen, 2010). Thus, an alternative viewpoint of MazE-MazF function in *E. coli* asserts that, like RelB-RelE, the toxin is activated under certain stress conditions and transiently reduces global translation levels, but without affecting cell viability. The dormant population can revive later when conditions have ameliorated (Gerdes *et al.*, 2005). Similar observations were made in the opportunistic pathogen, *S. aureus*, and in the oral bacterium, *Streptococcus mutans*, in which ectopic overproduction of the native MazF homologs instigated bacteriostasis, with only small fractions of the populations undergoing death (Fu *et al.*, 2007, 2009; Syed *et al.*, 2011). Certain cellular mRNAs that are important for viability of *S. aureus* apparently are protected from MazF cleavage by a cryptic RNA-binding protein which would only be necessary if the affected cells need to revive at a later stage (Fu *et al.*, 2009). MazF may also preferentially target gene transcripts that play key roles in pathogenesis by this bacterium (Zhu *et al.*, 2009).

Toxins that inhibit protein synthesis without mRNA degradation

Although genes for RelE- and MazF-like proteins are widespread and the properties of numerous homologs have been investigated, other toxins recently have emerged that inhibit protein synthesis without mRNA cleavage. Thus, the widespread PIN domain toxins, the Doc toxin of bacteriophage plasmid P1, and the Hha and RatA toxins encoded by *E. coli* interfere with different facets of the protein synthesis machinery, revealing that translation has developed as a prime target for diverse toxins (Figure 3).

PIN (PilT N-terminus) domains (~140 amino acids) are disseminated widely in proteins of both procaryotes and eucaryotes (Table 3). These domains share structural similarity with the RNaseH superfamily and a quartet of acidic residues that potentially coordinate Mg^{2+} ions are conserved within the active sites of both families suggesting that PIN domain proteins are involved in nucleic acid metabolism (Arcus *et al.*, 2011). Genes for PIN domain toxins accompanied by one of a range of genes encoding putative DNA-binding antitoxins are widespread in both archaea and bacteria (Anantharaman & Aravind, 2003). These toxins and antitoxins often are denoted VapC and VapB, respectively (for virulence associated protein). As examples, *M. tuberculosis* encodes >40 *vapBC* cassettes and the thermophilic crenarchaeota *Pyrobaculum aerophilum* and *Sulfolobus solfataricus* possess at least 12 and 26 *vapBC* operons, respectively (Pandey & Gerdes, 2005; Cooper *et al.*, 2009; Arcus *et al.*, 2011). VapC-mediated inhibition of translation induces bacteriostasis that can be overcome by expression of the cognate VapB verifying that the protein pairs comprise a TA complex in which the toxin targets protein synthesis (Daines *et al.*, 2007; Robson *et al.*, 2009; Winther & Gerdes, 2009). Structural studies confirmed that VapC toxins possess PIN domains and are bound tightly by the cognate antitoxin. The insertion of an arginine residue from the C-terminus of the antitoxin within the toxin's acidic cleft may block the latter's activity (Mattison *et al.*, 2006; Miallau *et al.*, 2008). There had been conflicting evidence on whether translation inhibition by VapC homologs reflected direct RNA cleavage or whether inhibition occurred by another mode. Among five VapC proteins analyzed from different species, two were found to degrade RNA and one was observed to be a DNA exonuclease *in vitro* (Arcus *et al.*, 2004; Daines *et al.*, 2007; Miallau *et al.*, 2009). By contrast, two enteric VapC homologs degraded neither DNA nor RNA, but instead activated the YoeB toxin *in vivo* by an unknown mechanism (Winther & Gerdes, 2009). These discrepancies were resolved recently when it was shown that VapC is a ribonuclease that cleaves specifically in the stem-loop region of the initiator $tRNA^{fMet}$, but does not recognize elongator tRNAs, mRNA, rRNA or tmRNA, and does not associate with the ribosome. The effects of VapC can be negated by the VapB antitoxin or by excess $tRNA^{fMet}$ (Winther & Gerdes, 2011). Considering the prevalence of *vapB-vapC* loci and indications that pathogenic bacterial species may be enriched in these and other TA genes (Goulard *et al.*, 2010; Georgiades & Raoult, 2011), more precise elucidation of the molecular mechanisms that underpin VapB-VapC activation, action and control will provide invaluable insights into an important class of putative stress response factors.

Bacteriophage P1 lysogenizes *E. coli* as a low copy number plasmid. The maintenance of lysogenic P1 is mediated in part by a TA cassette that specifies a toxin, Doc, and antitoxin, Phd that form a stable complex in which the toxin is sequestered. As noted with other antitoxins, Phd is intrinsically unstable and protease-susceptible, but the acquisition of more extensive secondary structure when associated with the toxin enhances Phd stability (Garcia-Pino *et al.*, 2008, 2010; Arbing *et al.*, 2010). Plasmid-free cells in which Phd cannot be replenished are acted on post-segregationally by Doc (Lehnherr *et al.*, 1993). The mechanism of Doc toxicity was revealed by observations that ectopic induction of *doc* stabilized mRNA *in vivo* which is in contrast to RelB and MazF toxins which, as alluded to above, dramatically reduced global mRNA levels under comparable conditions (Liu *et al.*, 2008). Doc stabilizes transcripts by associating with the 30S ribosomal subunit in a manner analogous to that of the aminoglycoside antibiotic, hygromycin B. Thus, the antibiotic and protein compete for binding to the 30S subunit, and a ribosomal mutation that blocks hygromycin B sensitivity also imparts resistance to Doc. In view of these observations, Doc is thought to occlude the ribosomal A, P and E sites thereby hindering the coupled translocation of mRNA and tRNA through the ribosome (Liu *et al.*, 2008) (Figure 3). As a consequence of the inhibition of protein synthesis by Doc, the RelE endoribonuclease is activated via Lon-dependent decay of the RelB antitoxin. This activation may be a strategy developed by the P1 plasmid to amplify the toxicity of Doc, or it may be the host's attempt to clear ribosomes that are stalled by the toxin (Garcia-Pino *et al.*, 2008). MazF also has been reported to be activated by Doc, but this result is disputed (Hazan *et al.*, 2001; Garcia-Pino *et al.*, 2008). Although Doc elicits its effect postsegregationally on cells that have failed to acquire a copy of the P1 plasmid at cytokinesis, ribosomal poisoning is reversible if production of the toxin ceases (Garcia-Pino *et al.*, 2008). This raises the intriguing question whether Phd-Doc is indeed a PSK complex, or instead whether Doc instead only maims plasmid-free cells sufficiently that they are outcompeted by plasmid-bearing rivals.

The RatA protein is toxic in *E. coli* (Brown & Shaw, 2003). It is unclear whether RatA comprises part of a TA complex as an antitoxin has not been identified, nor is it apparent under which conditions it is induced or how it is regulated. Nevertheless, like Doc, ectopic expression of *ratA* induction provokes inhibition of protein synthesis without diminishing mRNA levels (Zhang & Inouye, 2011). However, unlike Doc that targets the 30S ribosomal subunit, RatA associates with the 50S subunit to prevent assembly of intact ribosomes. The toxin has low sequence similarity with initiation factor 3 (IF3) which also has ribosome anti-association properties, and both RatA and IF3 are blocked by the aminoglycoside antibiotic paromomycin. Paradoxically, IF3 binds to the 30S subunit indicating that the mechanisms of action of RatA and IF3 are not identical (Zhang & Inouye, 2011).

Whereas Doc and RatA interfere with ribosome function and assembly, respectively, the Hha toxin inhibits

protein translation by repressing transcription of rare codon tRNAs, including the *argU*, *ileX*, *ileY*, and *proL* genes, in *E. coli*. Toxicity is ablated by the cognate antitoxin, YbaJ (Garcia-Contreras *et al.*, 2008). By decreasing the availability of rare tRNAs, cell growth is reduced and the production of fimbriae is inhibited as a result of which biofilm formation is impaired. Moreover, the ClpXP and Lon proteases are induced causing perturbations in expression of the *dinJ-yafQ*, *relB-relE* and *yefM-yoeB* TA modules. Activation of these proteases also switches on cell lysis genes encoded by prophages resident in the chromosome. Thus, the Hha-YbaJ TA complex intersects with multifarious cellular processes crucial for cell growth, survival and dispersal (Garcia-Contreras *et al.*, 2008).

Playing havoc with chromosome transactions: toxins that poison DNA gyrase

As outlined above, the stability of the F plasmid stems in part from the action of a plasmid-encoded toxin, CcdB, which is liberated in plasmid-free segregants (Ogura & Hiraga, 1983). The CcdA antitoxin counteracts the toxin and comprises an N-terminal DNA-binding domain and an intrinsically flexible C-terminal region that becomes structured when bound to CcdB (Madl *et al.*, 2006) (Figure 5). However, the labile CcdA antitoxin is degraded by Lon protease in plasmid-free cells and cannot be replenished due to the plasmid's absence, whereas the CcdB toxin is more long-lived (Van Melderen *et al.*, 1994, 1996) (Figure 1). Free CcdB perturbs the action of DNA gyrase (Bernard & Couturier, 1992; Maki *et al.*, 1992; Miki *et al.*, 1992). Gyrase is an essential topoisomerase that uniquely induces negative supercoiling in DNA, thereby balancing the positive supercoiling that accumulates ahead of replication forks as they circumnavigate the chromosome (Nöllmann *et al.*, 2007). Numerous DNA transactions, including transcription, recombination, segregation and replication, are highly sensitive to the supercoiling status of the substrate DNA. Gyrase comprises two subunits, GyrA and GyrB, which assemble as a heterotetramer. GyrA is involved in DNA binding and strand cleavage, whereas the GyrB subunits possess ATP binding and hydrolysis activities. The interaction of CcdB with GyrA traps a cleaved DNA intermediate complex. This stalled nucleoprotein species is a barrier for passage of RNA polymerase thereby preventing transcription (Critchlow *et al.*, 1997; Dao-Thi *et al.*, 2005; Smith & Maxwell, 2006; Simic *et al.*, 2009). Aberrant double strand DNA breaks and induction of the SOS response ensue followed by cell death (Van Melderen, 2002).

In addition to sequestering the toxin in plasmid-bearing cells, the CcdA antitoxin also can rescue CcdB-poisoned gyrase complexes (Bahassi *et al.*, 1999). Although gyrase shields the bulk of the CcdA binding sites on CcdB, the flexible C-terminal region of the antitoxin retains a low affinity for CcdB within the complex. This initial binding provokes a conformational change in the toxin that elicits release of gyrase with the concomitant interaction of a second, high affinity binding site of CcdA with CcdB (De Jonge *et al.*, 2009). As the process cannot take place in plasmid-free cells that lack a renewable source of the antitoxin, the circumstances under which *CcdA*-mediated rejuvenation of CcdB-poisoned gyrase complexes occurs are unresolved. However, rescue of these complexes may be important when perturbations in expression of the *ccdA-ccdB* operon or excess degradation of the labile antitoxin have temporarily caused an increase in the CcdB concentration. It has been proposed that plasmids which encode TA complexes hold a competitive advantage over plasmids that lack these elements (Cooper & Heinemann, 2000; Van Melderen & Saavedra De Bast, 2009). Rescue of CcdB-trapped gyrase complexes by CcdA may also be important in this advantage.

The *parDE* TA operon was identified and characterized originally on the RK2 plasmid of *E. coli* in which it behaves as an archetypal PSK system (Johnson *et al.*, 1996; Roberts *et al.*, 1994). More recently, functional homologs have been identified on other plasmids as well as on chromosomes (Fiebig *et al.*, 2010; Hallez *et al.*, 2010; Yuan *et al.*, 2010, 2011). Although it lacks extensive sequence homology, the ParE toxin is related to the widespread RelE family of endoribonucleases (Anantharaman & Aravind, 2003; Dalton & Crosson, 2010) (Table 3). However, ParE possesses no apparent RNA cleavage activity, but instead poisons DNA gyrase. The ParD antitoxin neutralizes ParE and blocks its interaction with the target (Jiang *et al.*, 2002; Oberer *et al.*, 2007). Like CcdB, ParE traps a gyrase-DNA complex by binding to the GyrA subunit. However, the mechanisms by which ParE and CcdB impede gyrase may differ (Yuan *et al.*, 2010). Comparison of ParE-gyrase tertiary structures with those of CcdB-gyrase (Dao-Thi *et al.*, 2005) will reveal how these different toxins both poison a common essential target.

In contrast with gyrase, type I topoisomerases relax negatively supercoiled DNA thereby counterbalancing the torsional strain generated during replication and other DNA transactions, as well as by the opposing activity of gyrase. Like other MazF homologs, MazF-mt7 (also known as Rv1495) from *M. tuberculosis* is a sequence-specific endoribonuclease (Zhu *et al.*, 2008) (Table 4). Unexpectedly, MazF-mt7 interacts with and inhibits topoisomerase I from this host, ablating both its DNA cleavage and relaxation activities. Conversely, mycobacterial topoisomerase I partially blocks RNA digestion by MazF-mt7 *in vitro*. The cognate antitoxin was unable to prevent the anti-topoisomerase activity of MazF-mt7 suggesting that the interfaces used by the toxin to bind the interacting proteins do not overlap (Huang & He, 2010). It will be intriguing to determine whether these interactions are universal features shared by MazF homologs and topoisomerase I enzymes in other hosts. Furthermore, whereas the signals that provoke MazF endoribonuclease activity are comparatively well described, it is unknown whether MazF-mt7 inhibits topoisomerase I in response to the same or different signals.

Nucleic acids metabolism is not the only toxin target

Nucleic acid metabolism and related processes have evolved as prime targets for the toxin components of TA complexes: as outlined above, numerous toxins are endoribonucleases or block other aspects of protein synthesis, whereas others interfere with the activity of topoisomerases. However, novel intracellular targets—cell wall assembly and the cell division apparatus—for two toxins recently have been identified. Uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) is a precursor in the biosynthesis of peptidoglycan, lipid A, lipopolysaccharide, and teichoic acids. Lipopolysaccharide is the principal component of the Gram-negative outer membrane to which it is tethered by lipid A, whereas peptidoglycan is the major constituent of the bacterial cell wall. Teichoic acids confer rigidity on cell walls of Gram-positive species (Kotnik *et al.*, 2007). The first committed step of peptidoglycan formation is catalyzed by the MurA enzyme which transfers an enolpyruvyl moiety from phosphoenolpyruvate to UDP-GlcNAc producing enolpyruvyl-UDP-GlcNAc and inorganic phosphate. As alluded to above, plasmid pSM19035 specifies the ζ toxin and ε antitoxin (Ceglowski *et al.*, 1993; Zielenkiewicz & Ceglowski, 2005). Homologs of ε and ζ that are sometimes denoted PezA and PezT, respectively, are encoded by certain Gram-positive species (Khoo *et al.*, 2007). The ε/PezA and ζ/PezT proteins form a tight complex in which an N-terminal helix of the antitoxin occludes a nucleotide binding site on the partner protein ensuring that toxicity is negated (Meinhart *et al.*, 2003; Mutschler *et al.*, 2011). Free ζ/PezT toxin is a phosphoryltransferase that directs a kinase reaction from ATP and/or GTP to its substrate. The substrate was shown recently to be UDP-GlcNAc to which ζ attaches a phosphoryl group at the 3'-hydroxyl site of the *N*-acetylglucosamine moiety. The phosphorylated form of UDP-GlcNAc that accumulates following ζ toxin action is a competitive inhibitor of the MurA enzyme resulting in impaired peptidoglycan assembly. This leads to the disruption of cell wall integrity, gross changes in cell morphology, membrane permeabilization, and extensive lethality (Lioy *et al.*, 2006; Mutschler *et al.*, 2011). Toxicity of ζ/PezT is ablated by mutagenesis of key residues predicted to be involved in nucleotide binding and hydrolysis, or phosphoryltransferase activity (Meinhart *et al.*, 2003; Lioy *et al.*, 2006). The plasmid-encoded ζ toxin is induced by artificial inhibition of transcription or translation which leads to more rapid decay of the ε antitoxin by Lon protease (Camacho *et al.*, 2002). These conditions mimic loss of the pSM19035 plasmid when ε can no longer be replenished. However, the signals that fire the PezT chromosomal homolog have yet to be revealed (Mutschler *et al.*, 2010).

The YeeV-YeeU system is a member of a family of TAs identified in *E. coli* by screening gene pairs with lengths and organization characteristic of TA operons, but that were of unknown function. Ectopic expression of *yeeV* perturbs cell growth and viability which is counteracted by YeeU (Brown & Shaw, 2003). FtsZ, a homolog of eucaryotic tubulin, assembles into a midcell contractile ring and simultaneously recruits numerous other division factors to the septum during bacterial cytokinesis. Disruption of FtsZ ring formation blocks cell division progression (Erickson *et al.*, 2010). YeeV inhibits both GTPase activity and GTP-dependent polymerization of FtsZ *in vitro*. This probably is achieved by direct protein-protein interactions that sterically block FtsZ polymer assembly (Tan *et al.*, 2011). Interestingly, YeeV targets a second function of *E. coli*. The actin-like protein MreB organizes into helical structures that lie under the cytoplasmic membrane and help maintain cell shape in *E. coli* (Margolin, 2009). YeeV and MreB interact physically. Although ATP hydrolysis by MreB is unaffected, nucleotide-dependent polymerization of the protein is inhibited partially by the toxin. Thus, YeeV apparently can interact with two disparate factors—tubulin and actin homologs—to exert a highly toxic effect in *E. coli* (Tan *et al.*, 2011). The signals that trigger YeeV activity and whether activation of the toxin always involves concomitant poisoning of both FtsZ and MreB are unknown. Curiously, antitoxicity by YeeU may not involve a direct interaction with YeeV as is the case with canonical TA complexes. Instead, the antitoxin neutralizes YeeV by modulating its intracellular concentration although the mechanism by which this is achieved is unclear (Brown & Shaw, 2003).

Toxins-antitoxins, bacterial persistence and biofilms

Persistence is the capacity of bacterial sub-populations (as few as 10^{-6} cells) to tolerate and revive from exposure to antibiotics, albeit without developing resistance. Persister cells emerge stochastically and enter a transient state of dormancy during which antibiotic targets, e.g., the ribosome and cell wall biosynthesis, are refractive to drug action (Lewis, 2010). The *hipBA* genes that mediate persistence in *E. coli* were identified almost 30 years ago (Moyed & Bertrand, 1983), but only recently were recognized as encoding a TA complex. Thus, overproduction of the HipA toxin suppresses global cellular metabolism and increases the frequency with which persister cells emerge. These effects are abrogated by the HipB antitoxin (Correia *et al.*, 2006; Korch & Hill, 2006; Rotem *et al.*, 2010). Solution of the HipA structure demonstrated that it harbors a eucaryotic serine/threonine kinase-like fold that binds ATP with high selectivity. Moreover, HipA interacts with and phosphorylates the EF-Tu protein (Schumacher *et al.*, 2009). EF-Tu is an essential GTP-binding elongation factor that mediates the delivery and entry of charged tRNA molecules to the ribosomal A site (Krab & Parmeggiani, 2002). Phosphorylation by HipA is predicted to inactivate EF-Tu for translation and inhibit protein synthesis, thereby explaining HipA toxicity. HipA toxicity also requires autophosphorylation (Schumacher *et al.*, 2009) (Figure 3). In view of its broad effects on cellular metabolism, phosphorylation by HipA also may modulate the activity of other, as yet unknown, targets (Schumacher *et al.*, 2009).

Apart from HipA-HipB, other TA complexes also are implicated in establishing the persister state (Lewis, 2010). For example, the *relE* homolog, *mqsR* (Tables 1 and 4), is the locus that is most upregulated during persistence in *E. coli* and overproduction of RelE itself dramatically increases persister cell numbers (Keren *et al.*, 2004; Brown *et al.*, 2009). Similarly, genes of the type I TA complex, *istR-tisB* (Table 1), are implicated in persistence following exposure to the DNA-damaging antibiotic, ciprofloxacin (Dörr *et al.*, 2010). The link between TAs and persistence is not confined to *E. coli*: ectopic expression of the gene for the MazF-mt6 endoribonuclease toxin of *M. tuberculosis* (Table 4) induced higher levels of persistence in *Mycobacterium smegmatis* (Han *et al.*, 2010). This is of particular significance considering latent tuberculosis infections in which dormant bacilli that are recalcitrant to antibiotic treatment can endure for decades.

Persistence is of major clinical significance as it can diminish the efficacy of antibiotic therapy and prolong chronic bacterial infections. Persistence is intertwined with the formation of biofilms in which bacteria grow as monocultures or as consortia of different species. Cells occupying diverse zones of a biofilm are in different metabolic states and are subject to variable temporal and spatial gradients. Thus, cells on the biofilm surface may be growing actively, whereas cells deeper within the structure where nutrients are more limiting may be dormant or in a persistent state (Fey, 2010). It has been established recently that the expression of certain TA genes fluctuates dependent on whether cells are growing planktonically or within a biofilm, and that TA complexes can profoundly influence biofilm formation and dispersal (Kolodkin-Gal *et al.*, 2009; Wood, 2009; Mitchell *et al.*, 2010). For example, the *yefM* antitoxin gene is upregulated when *E. coli* is grown as a biofilm (Ren *et al.*, 2004). Additionally, although simultaneous deletion of *mazE-mazF, relB-relE, chpBI-chpBK,* yefM-yoeB, and dinJ-yafQ affected neither survival nor fitness (Tsilibaris *et al.*, 2007), an *E. coli* strain lacking the five TA modules displayed complex alterations in biofilm production and stability, as did strains in which each toxin alone was deleted or in which antitoxins were overproduced (Kim *et al.*, 2009). Our understanding of the intricate role of TA complexes in persistence, multidrug tolerance and biofilm maintenance is only developing: future studies will decipher the interplay between TA systems and their activation by physiological stimuli, the intracellular relays that transmit these signals, and the role of intercellular communication in persistence and biofilm development.

Putting toxins-antitoxins to work in biotechnology and medicine

The deleterious effects on cell growth and survival exerted by the intracellular release of the toxin components has sparked considerable interest in employing TA complexes as genetic tools, both in procaryotes and eucaryotes. The gene for the CcdB toxin has long been used in positive selection plasmid vectors (Bernard *et al.*, 1994). Vectors bearing *ccdB* can be propagated in *E. coli* strains with gyrase mutations that confer resistance to the toxin, but cannot be introduced into strains producing wild-type gyrase. Thus, insertion of cloned DNA fragments that disrupt *ccdB* allows positive selection of recombinant plasmids in wild-type strains. A variety of cloning strategies, including commercially available vectors, have been developed based on this technology (Stieber *et al.*, 2008).

The realization that numerous toxins were sequence-specific endoribonucleases (Table 4) has prompted the design of novel expression systems aimed at massive overproduction of single proteins. The mRNA of the gene of interest either naturally lacks recognition sites for the endoribonuclease or can be engineered to eliminate the target codons. Induction of the endoribonuclease abolishes synthesis of the bulk of cellular proteins in *E. coli*, but translation of the desired mRNA continues potentially converting the cell into a single protein production factory (Suzuki *et al.*, 2005, 2006; Inouye, 2006; Schneider *et al.*, 2009; Vaiphei *et al.*, 2010). As understanding of the sequence-specificity of endoribonuclease toxins evolves further, other expression technologies likely will emerge.

Observations that endoribonuclease toxins were active not only in bacteria, but also in single-celled and multicellular eucaryotes, were intriguing from a two-fold perspective (Kristoffersen *et al.*, 2000; Yamamoto *et al.*, 2002; de la Cueva-Mendez *et al.*, 2003). First, they revealed that the targets of these toxins—frequently free mRNA—were ubiquitous. Second, they have allowed the development of eucaryotic expression systems that shut down protein synthesis. For example, MazF-mediated inhibition of protein synthesis provided new insights into apoptotic pathways in mammalian cells (Shimazu *et al.*, 2007). MazF also has been used as an experimental tool to block HIV-1 replication (Chono *et al.*, 2011). Nehlsen *et al.* (2010) coupled expression of the gene for the Kis antitoxin (Table 4) with a transgene of interest in a mammalian cell line. Expression of the transgene was enhanced when the gene for the Kid toxin was induced due to strong selection for cells that maintained Kis production. These imaginative examples illustrate the potential that TA complexes have as elements of the future genetic toolbox.

The toxin components of TA systems are latent molecular timebombs whose intracellular explosion can cripple or kill bacterial cells that harbor them. The possibility of devising strategies to activate these natural toxins artificially as novel agents in the battle against bacterial pathogens is of prime interest (Engelberg-Kulka *et al.*, 2004; Williams & Hergenrother, 2008; Lioy *et al.*, 2010). Small molecules that interfere selectively with TA gene expression may imbalance the toxin:antitoxin ratio so that the toxin is liberated. Similarly, ligands that compete effectively for the interface between toxin and antitoxin, or which destabilize the complex in other ways, also may promote toxin activation. Interestingly, the EDF quorum-sensing peptide is an example of a naturally occurring ligand that competes for the TA interface (Belitsky *et al.*, 2011). Synthetic peptides that inhibit toxin-anitoxin interactions also have been designed

(Agarwal *et al.*, 2010; Chopra *et al.*, 2011). As described above, toxins engage with disparate cellular targets, including facets of the translation machinery (Figure 3), topoisomerases, cell wall synthesis, and cytoskeletal elements. Might these targets be exploited as vulnerabilities for new antibacterial drug development (Sengupta & Austin, 2011)?

TA systems in the selfish theory framework

As discussed above, type II TA systems are widespread in bacterial genomes. Their distribution does not follow the bacterial phylogeny, indicating that they integrated into chromosomes quite recently, raising the possibility that most of these systems have not been recruited yet in host regulatory circuits. This would imply that they do not have any role in bacterial physiology and are 'just' selfish entities. Toxin and antitoxin genes show a strong interdependency, are closely linked and move through horizontal gene transfer. TAs also maintain themselves very efficiently, even at the expense of their host survival when they are located on plasmids. Their addictive properties might be a secondary effect of their selfish behavior (Christensen-Dalsgaard & Gerdes, 2006; Budde *et al.*, 2007; Szekeres *et al.*, 2007; Wozniak & Waldor, 2009).

Declarations of interest:

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