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REVIEW

Bacterial toxin-antitoxin systems targeting translation

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Summary
Toxin-antitoxin systems (TAS) emerged more than 25 years ago and have since developed as an important field in molecular microbiology. TAS are autoregulated operons coding a stable toxin and an unstable antitoxin found in the plasmids and chromosomes of Bacteria and Archaea. The conditional activation of their toxins interferes with cell growth/viability and, depending on the context, can influence plasmid maintenance, stress management, bacterial persistence, cell differentiation and, it is likely also bacterial virulence. This review summarizes recent results on the *parD* system of plasmid R1 and on the chromosomal *relBE* systems found in *Escherichia coli* and in *Streptococcus pneumoniae* with a focus on the RNase activity of their toxins, their regulation and their biomedical applications and implications.

Key words: toxin-antitoxin systems; plasmid maintenance; cell growth regulation; toxin RNases; protein synthesis inhibition

INTRODUCTION

Toxin-antitoxin systems (TAS) were reported more than 25 years ago as plasmid auxiliary maintenance cassettes. Three plasmid-borne TAS contributed initially to establishing this field in microbiology: the *parB (hok, sok)* and *parD (kis, kid)* systems of plasmid R1 and the *ccd (ccdA, ccdB)* system of plasmid F (Gerdes et al. 1986, Hiraga et al. 1986, Bravo et al. 1987). TAS are based on the activity of two intracellular components, a stable toxin and a constitutively unstable antitoxin; the differential decay of the antitoxin, occurring also in cells losing the plasmid at cell division, allows activation of the toxin in these cells and its subsequent interference with cell growth/viability. This favours the preferential propagation of plasmid containing TAS and contributes to their maintenance in bacterial populations. The three systems described, initially define two main TAS types: *hok/sok* is the prototype of Type I TAS in which the toxin is a protein and the antitoxin is an RNA, while *ccd* and *parD* define type II TAS in which both the antitoxin and the toxin are proteins. Most of the focus has been so far concentrated on type II systems (Hayes 2003, Buts et al. 2005, Gerdes et al. 2005, Condon 2006, Kamphuis et al. 2007a, Van Melderen and Saavedra De Bast 2009).

Following the initial reports, many type II systems were found in plasmids and also in the chromosomes of many Eubacteria and Archaea (Pandey and Gerdes

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2005). Up to 9 different type II TA families have been defined. TA systems can contribute to stress management in bacterial populations, to genome stability or to complex phenotypes such as bacterial persistence and cell differentiation and eventually as anti-addiction modules (Van Melderen and Saavedra De Bast 2009).

This review summarizes recent contributions made in our two groups (RDO and MEP) on the TAS systems *parD (kis, kid)* of plasmid R1 and *relBE* of *Streptococcus pneumoniae*, whose toxins cleave mRNAs. We focus on the mechanisms of action of the toxins of these TAS which inhibit translation, and on the way they are regulated. The biomedical implications/applications of these studies is briefly addressed.

THE *parD* SYSTEM OF PLASMID R1: INHIBITION OF PROTEIN SYNTHESIS BY mRNA CLEAVAGE IN THE ABSENCE OF RIBOSOMES

Identification of the parD *system and its specific role in the maintenance of plasmid R1*

parD (kis, kid) system was reported by our laboratory in 1987 (Bravo et al. 1987). This TAS is a bicistronic operon of plasmid R1 which is close to the basic replicon of the plasmid. It codes two short proteins, an antitoxin, Kis (killer suppressor), and a toxin, Kid (killing determinant). An identical system to *parD*, *pem*, was also found in plasmid R100 (Tsuchimoto et al. 1988) and two chromosomal TA systems homologous to *pem (chpA/mazEF* and *chpB)* were later identified by the same authors (Masuda et al. 1993). A marginal cross-talk between these systems and *parD* that could be enhanced by mutations affecting the *chpAI/mazE* and *chpBI* antitoxins could be detected (Santos-Sierra et al. 1997, 1998). Under standard growth conditions *parD* is inefficient as a plasmid maintenance system and for this reason it was initially undetected. It was identified by an unexpected mutation in the *kis* antitoxin gene that greatly increased the stability of the plasmid and in combination with other mutations, revealed the toxin and antitoxin activities of Kis and Kid (Bravo et al. 1987, 1988). The first *kis* mutant also indicated a key role for the antitoxin in the transcriptional regulation of the *parD* operon. Unexpectedly, the *parD* system was activated when replication of a low copy mini-R1 plasmid was compromised and, surprisingly, this activation contributed to recover the plasmid replication (Ruiz-Echevarría et al. 1995b). This result suggested the specialized role of the system in recovering inefficient plasmid replication, and this recovery was clarified following the identification of the RNase activity of Kid (see below): it seems that the activated Kid toxin modulates the efficiency of replication by down-regulating the mRNA levels of CopB, the auxiliary R1 copy number controller, via specific RNA cleavage within the polycistronic *copB-repA* mRNA (Pimentel et al. 2005). These results suggested a functional coupling between the replication and *parD* modules in plasmid R1.

The RNase activity of Kid toxin

The discovery that RelE toxin belonging to the *relBE* system inhibits translation by cleaving the mRNA on the ribosome (Pedersen et al. 2003), triggered a search for similar activities in the toxins of other TAS. It was found that Kid/PemK, the identical toxin of the *parD/pem* systems of plasmid R1 and R100 (Tsuchimoto et al. 1988), are specific endoribonucleases that, in contrast to RelE, can cleave RNA in the absence of ribosomes (Zhang et al. 2003, Zhang et al. 2004, Muñoz-Gómez et al. 2004, Muñoz-Gómez et al. 2005). This cleavage inhibits the potential of the cells to synthesize proteins. Kid toxin cleaves RNA as RNase A or RNase T1 do (Kamphuis et al. 2006). This cleavage is initiated by a nucleophilic attack of the scissile phosphate by the oxygen of an adjacent 2'-OH residue and requires a catalytic acid, a catalytic base and additional stabilizing interactions. The structure of the functional dimeric Kid toxin is available (Hargreaves et al. 2002) and the model of a structure of the complex of Kid with a mimetic RNA substrate based in NMR data has been proposed (Kamphuis et al. 2006) (see Fig. 1). Residues proposed by this model to play key roles in RNA cleavage and in specific interactions with the substrate have been evaluated using a collection of specific mutants in the toxin (Diago-Navarro et al. 2009a). The results of these studies have supported the predictions of the model. Kis/PemI and MazE antitoxins are able to neutralize the RNase activity of their respective toxins in addition to their potential in inhibiting protein synthesis (Muñoz-Gómez et al. 2004, 2005, Zhang et al. 2003, 2004). The available structures of the $MazF_2$-$MazE_2$-$MazF_2$ heterohexamer (Kamada et al. 2003) and of the Kid_2-Kis_2-Kid_2 heterohexamer, modelled on the MazE-MazF heterohexamer (Kamphuis et al. 2007b), explain this neutralization as the result of an interference of the C-terminal region of the antitoxin with toxin residues involved in RNA binding or cleavage. Neutralization of Kid RNase activity by the Kis antitoxin occurs either in prokaryotic or eukaryotic cells (de la Cueva-Méndez et al. 2003, Muñoz-Gómez et al. 2005).

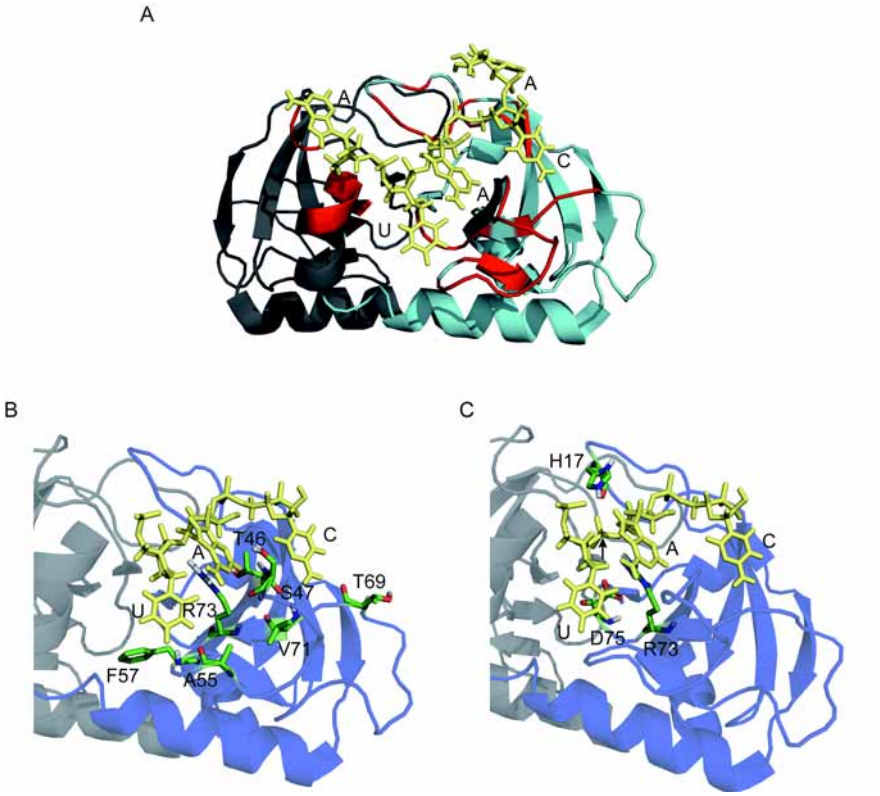

Fig. 1. **Modelled mechanism of binding and cleavage of RNA by Kid toxin**. A) RNA binding pocket of Kid. Kid monomers are coloured in grey and cyan. Residues involved in one of the symmetric RNA binding surfaces are shown in red. B) and C) show respectively Kid residues (sticks) involved in specific interactions with the RNA core sequence and these forming the active site of the protein. The arrow points to the scissile phosphate. RNA bases (AUACA) are coloured in pale yellow.

The RNase activity of Kid toxin explains several other results obtained before this primary activity was discovered: the recovery of compromised plasmid R1 replication was due to the RNase activity of the Kid toxin (Pimentel et al. 2005), and the inhibition of ColE1 replication by the Kid toxin observed *in vitro* and *in vivo* (Potrykus et al. 2002, Muñoz-Gómez et al. 2005) could be explained by the cleavage of the RNA transcript required to make the RNA that primes replication of this plasmid. This cleavage was shown using purified RNA ColE1 pre-primer as a substrate of Kid; however a detailed analysis of this cleavage occurring during transcription remains to be carried out. The ability of Kid to inhibit cell growth/viability both in prokaryotes and eukaryotes is related to the inhibition of protein synthesis due to its RNase activity (Muñoz-Gómez et al. 2005).

Interestingly the Kid toxin shows structural homology to the CcdB toxin (Hargreaves et al. 2002) even if CcdB rather than cleaving RNA targets DNA gyrase (Bernard and Couturier 1992).

Regulation of the parD *system*

TAS are subjected to strict control to prevent the deleterious effects of their toxins. The activity of the toxins is neutralized by direct interactions with their antitoxins (see above). Interestingly these interactions lead to complexes that regulate the operon and maintain, the toxin-antitoxin transcripts at low levels. Recent studies of the transcriptional regulation of *parD* showed that dimers of the antitoxin pilot the interactions of the repressor complex with specific sequences of the promoter-operator region and that the relative levels of Kis and Kid proteins influence this regulation: a hetero-octameric complex formed in excess of the antitoxin binds in the promoter-operator region efficiently, suggesting that it is involved in repression, and a heterohexameric Kis-Kid complex formed in excess of the toxin is the main species involved in toxin neutralization (Monti et al. 2007, Kamphuis et al. 2007b). This complex binds with low affinity to the promoter-operator region thus favouring deregulation of the operon and reposition of the antitoxin levels. The interplay of the two proteins

could restore the regulated situation when the antitoxin decays. This hypothesis explains the requirement of two different proteins to regulate the system.

Post-transcriptional regulatory mechanisms (Ruiz-Echevarría et al. 1995a) contribute to maintain, under standard growth conditions, an A/T ratio close to 2 that results in a repressed situation. The selective activity of the Lon protease on the Kis antitoxin (Tsuchimoto et al. 1992) can unbalance this situation.

RelBE TYPE SYSTEMS: PROTEIN INHIBITION BY CLEAVAGE OF mRNA ON THE RIBOSOMES

One of the most studied TAS members is the *relBE* gene, present in the chromosome of many eu- and archae- bacteria and firstly reported in *E. coli* (Christensen et al. 2001). Homologues were found in Bacteria, Archaea (Gerdes et al. 2005, Pandey and Gerdes 2005), and in plasmids (Gronlund and Gerdes, 1999, Hayes, 2003). In addition to the *E. coli* operon (Gotfredsen and Gerdes 1998), the nature as *bona fide* TAS *relBE* loci has been confirmed for *S. pneumoniae* (Nieto et al. 2006), *Mycobacterium tuberculosis* (Korch et al. 2009), *Pyrococcus horikoshii* (Takagi et al. 2005), and *Methanococcus jannaschii* (Francuski and Saenger 2009). In *E. coli* cultures subjected to nutritional stress, especially in conditions that impair protein synthesis, transcription of *relBE* increased and toxin RelE was activated due to degradation of its cognate RelB antitoxin by the Lon protease. This resulted in cell growth arrest, concomitantly with inhibition of translation (Christensen et al. 2001, Gotfredsen and Gerdes, 2002). Over-expression of *Ec*RelE induced stasis from which cells can recover by antitoxin production (Pedersen et al. 2002). A similar behaviour was found in *E. coli* by activation of the pneumococcal *Sp*RelE toxin but, in this case, prolonged exposure of the cells to the toxin led to cell death rather than to cell stasis (Nieto et al. 2006). Therefore, chromosomal *relBE* could act as a stress response locus more than a cell killing system, adjusting the rates of protein synthesis under unfavourable growth conditions.

RelE mRNA cleavage mechanism

RelE was shown to inhibit translation by cleaving mRNAs, both *in vivo* and *in vitro* by RelE positioning at the ribosomal A site (Christensen and Gerdes, 2003, Pedersen et al. 2003). Determination of the RelE structure showed that the protein has a shape similar to the C-terminal region of the translation elongation factor EF-G (Takagi et al. 2005), thus allowing its access into the A site of the ribosome. RelE-mediated cleavage occurred preferentially between the second and the third bases of stop and sense codons with a G at third position (Pedersen et al. 2003). In agreement with the entrance of RelE in the ribosomal A-site was the finding that the release factor I (RFI), which binds to the ribosomal A site in the translation termination stage prevented *in vitro* cleavage of mRNA mediated by RelE (Pedersen et al. 2003). RFI-mutants showed increased sensitivity to the RelE and Kid toxins (Diago-Navarro et al. 2009b). In the case of RelE, overproduction of tmRNA was able to rescue stalled ribosomes, thus counteracting its toxic effect (Christensen and Gerdes 2003). *Ec*RelE only cleaved ribosome-bound mRNAs (Christensen and Gerdes, 2003, Pedersen et al. 2003). However, *E. coli* strains with deletions in genes encoding toxins (RelE, MazF, ChpBK, YoeB, YafQ and YhaV) were shown not to be involved in cleavage of a particular mRNA composed of rare Arg codons that caused ribosome pausing (Garza-Sánchez et al. 2008); in addition, arginine starvation was shown to induce mRNA cleavage at specific codons. However, cleavage occurred with the same specificity in the strain lacking the toxins, indicating that mRNA cleavage occurring during arginine starvation was independent of these known TA systems. Similar results were found by Aiba's group (Li et al. 2008b). Interestingly, comparison of the fitness of two isogenic *E. coli* strains, one wild type (wt) and the other having deletions in five TAS *(mazEF, relBE, chpBK, yefM-YoeB, dinJ-yafQ)* subjected to short-term stress conditions (amino acid starvation, acidic stress, antibiotic treatment, and long term stationary phase) showed no significant differences among them (Tsilibaris et al. 2007), suggesting that TAS could be involved only in long-term evolution (Van Melderen and Saavedra De Bast 2009).

Even though RelE has a microbial RNase fold it lacked both basic and acidic catalytic residues suggesting that they should be provided by the ribosome (Buts et al. 2005, Condon 2006). The solution of the three-dimensional structures of *Ec*RelE, alone and bound to programmed *Thermus thermophilus* 70S ribosomes in both pre- and post-cleavage states solved this puzzle (Neubauer, 2009). In the crystal, RelE occupies the A site, establishing direct contacts with the 16S rRNA, and thus preventing access of translation factors and tRNA to the ribosome. The overall structure of RelE hardly changed upon binding to the ribosome, but its interaction with the A site on the 30S subunit seemed to reorganize the mRNA, promoting a 2'-OH hydrolysis between codon position two and three

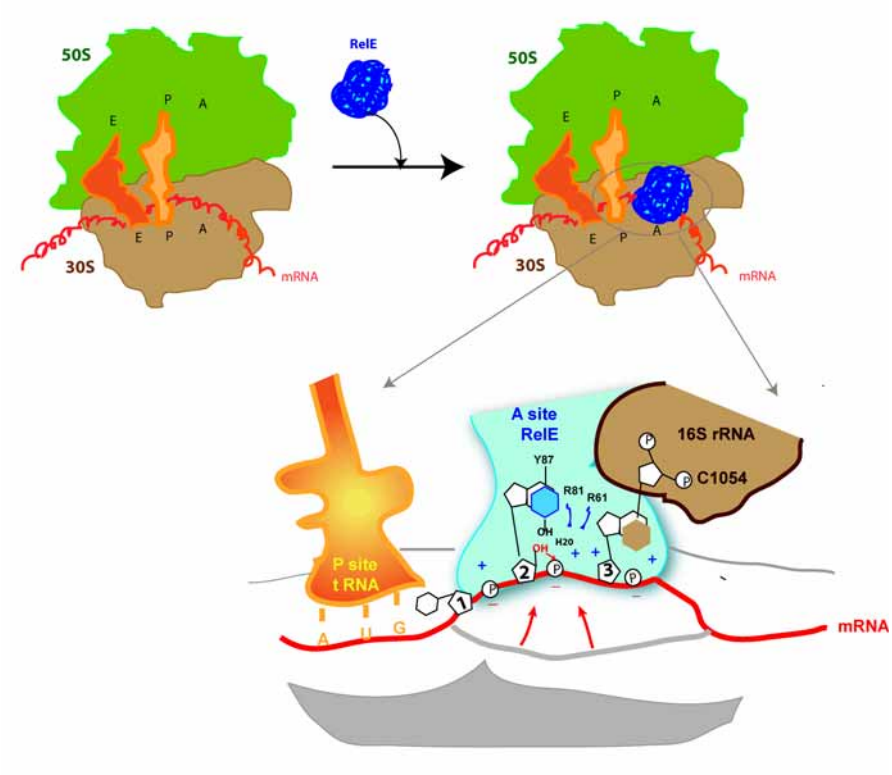

Fig. 2. **Mechanism of cleavage of RNA on the ribosome.** A) Schematic representation of RelE binding on the ribosome. E, P, and A denotes specific ribosomal sites involved in translation. B) Model of endonucleolytic reaction of RelE on translated mRNA. RelE approaches the mRNA to its active site through interactions between RelE basic side chains (+) (R45, R81, R61, K52, K54, and R56) and the phosphate group in the mRNA (–). The stacking of the second nucleotide with tyrosine 87(Y87) and of the third nucleotide with the residue C1054 of 16S rRNA are indicated. The red arrow indicates the nucleophilic attack of 2'O$^-$ on the phosphate group. The water molecule and residues R61 and R81 present in the active sites of RelE are also shown.

(Fig. 2). Considering the structural and functional data, a model for RelE-mediated cleavage of mRNA was proposed (Neubauer et al. 2009). Even though cleavage of the mRNA on the ribosome is carried out by RelE and not by the ribosome itself, this cell particle is essential for the RelE catalysis.

In addition to the *E. coli* RelE, the structure of the complex RelE-RelB, has been determined for *P. horikoshii* (*Ph*RelBE) (Takagi et al. 2005) and *M. jannaschii* (*Mj*RelBE) (Francuski and Saenger 2009). *Ph*RelBE forms a tetrameric complex, in which a molecule of RelB wraps around a compact RelE dimer generating a heterodimer. Two heterodimers generate the heterotetramer through interactions between *Ph*RelB from one heterodimer and *Ph*RelE from another. The extensive wrapping of RelB around RelE makes the dimer bigger, thus hampering the entrance of RelE into the ribosome A-site. *Mj*RelBE forms also a heterotetrameric complex with a different pattern. Data on the RelBE complex from *E. coli* indicate that the C-terminal region of RelB could be responsible for RelB-RelE interaction. This region showed high sensitivity to proteases, so that its interaction with RelE could confer resistance to degradation; thus the RelB C-terminal region could be involved in both self-association and RelE binding (Cherny et al. 2007). NMR studies showed that RelB-RelE complex provokes a helix displacement near the RelE mRNA interferase active site resulting in the neutralization of the positively charge catalytic sites by acidic residues from RelB antitoxin (Li et al. 2009). The *E. coli* RelB N-terminal domain may form a dimeric ribbon-helix-helix structure (Cherny et al. 2007, Li et al. 2008a), making it likely that the antitoxin would use this motif for DNA binding (Li et al. 2008a, Overgaard et al. 2008). However, the three-dimensional structure of *Ph*RelB showed the antitoxin would recognize its DNA target as a dimer via a leucine zipper motif (Takagi et al. 2005). In conclusion, the structural data collected regarding RelB indicate that despite the sequence and structure

homology between the toxins from *E. coli*, *M. jannaschii* and *P. horikoshii*, the antitoxins may differ in the mechanism they use to optimize their DNA binding and their stability properties, adapting them to their own host.

Auto-regulation

Two models have been proposed to explain the mechanism of autorepression of the *E. coli relBE* operon. The first considers the possibility that RelBE complex could bind as a tetramer to the operator sequence and that RelB has two levels of regulation (Li et al. 2008a). The second model (Overgaard et al. 2008) proposes that RelBE complex could control *relBE* transcription by binding to the DNA operator as a trimer, $RelB_2$-RelE. Thus, RelE would have two RelB binding sites, one of low and another of high affinity each playing different roles depending on the toxin:antitoxin ratios (Overgaard et al. 2008).

YoeB mRNA interferase

Another mRNA interferase homologous to RelE, YoeB, has been studied, albeit in less detail than RelE. It has been grouped as belonging to the RelE superfamily (Pandey and Gerdes 2005, Makarova et al. 2009), and studied in *E. coli* and in other microorganisms. Gene *yoeB* encodes toxin YoeB belonging to the TA pair YefMYoeB, which was identified as a homolog of the Txe toxin (Grady and Hayes 2003) in *E. coli*. The Axe-Txe TA pair was identified in a plasmid from *Enterococcus faecium* (Grady and Hayes 2003), and homologs have been studied in *S. pneumoniae* (Nieto et al. 2007), *Staphylococcus aureus* (Yoshizumi et al. 2009), and *M. tuberculosis* (Kumar et al. 2008). The structure of the *E. coli* YoeB and the complex with its cognate antitoxin, YefM, has been elucidated and the residues involved in YoeB catalysis have been identified. YoeB showed a structure similar to RNase Sa and Barnase, two proteins with a characteristic microbial RNase fold (Kamada and Hanaoka 2005); it also exhibited similarities with RelE from *E. coli* and with *Ph*RelE monomer (Takagi et al. 2005, Neubauer et al. 2009). YoeB acts as a ribosome-dependent mRNA interferase (Christensen-Dalsgaard and Gerdes 2008). All experimental data suggest that YoeB inhibits protein synthesis, but its primary function as a blockade for this process is unclear. Whereas YoeB might act as a sequence-specific endoribonuclease or an mRNA interferase (Kamada and Hanaoka 2005, Christensen-Dalsgaard and Gerdes 2008), it could be that the endoribonuclease activity of YoeB is not primarily required for its inhibitory function for protein synthesis but to prevent the formation of the initiation complex (Zhang and Inouye 2009). In this latter case, YoeB would bind to the 50S ribosomal subunit in 70S ribosomes, and would interact with the A site, impairing formation of the initiation complex and, as a consequence, inhibiting protein synthesis. In turn, this inhibition would activate the latent endoribonuclease activity of either ribosomes or YoeB, resulting in cleavage of mRNA at the A site. Interaction of YoeB with its cognate antitoxin YefM could induce a conformational rearrangement of the RNase catalytic site of YoeB leading to the movement of some of the residues involved in catalysis, away from the active site. This conformational change in the catalytic site of YoeB could explain the mechanism employed by YefM to neutralize YoeB toxicity. Regulation of the expression of this TAS is similar to the other examples mentioned: YefM is the primary repressor and YoeB acts as a repressor enhancer (Kedzierska et al. 2007). However, this picture might be an oversimplification of the regulatory circuit (W. T. Chang and C. C. Yeo, personal communication).

SOME POSSIBLE BIOMEDICAL APPLICATIONS/IMPLICATIONS OF TAS

The structural and functional information available on TA interactions in several systems make it possible to search for or to design molecules able to interfere with these interactions and trigger the activity of the toxin. BRET (Bioluminiscence Resonance Energy Transfer) technology has been used to monitor toxin-antitoxin interactions (Nieto et al. 2006, Lioy et al. 2010). These assays could act as a powerful tool in the search for possible new antibiotics against cells containing endogenous or acquired toxin-antitoxin systems. The role of some TAS, particularly, the HipBA system of *E. coli* in bacterial persistence has been pointed out (Moyed and Bertrand 1983). A 10,000 increase in the numbers of persistent cells was associated with a mutation, *hipA7*, in the toxin gene. However, in spite of the well characterized mechanism of action of the toxin and of its neutralization by the antitoxin (Schumacher et al. 2009), the pathway involved in the activation of this epigenetic phenomenon, affecting a small fraction of the population, is not well understood. Persistent cells have important implications in combating antibiotic resistances in the clinical context as these cells are tolerant to antibiotics (Bigger 1944).

TAS were first characterized as virulence-associated determinants on the basis of their prevalence in the chromosomes of virulent versus avirulent strains of specific human pathogens (Hopper

et al. 2000, Daines et al. 2007). Moreover, TAS are widely conserved in plasmids from common hospital pathogens, including vancomycin-resistant enterococci (VRE) and aminoglycoside-resistant *E. coli* (Moritz and Hergenrother 2007, Perichon et al. 2008). The sequenced strains of *Mycobacterium tuberculosis* have 60 TAS including 7 homologues of *mazEF* (Pandey and Gerdes 2005). The contribution of these systems to the pathogenic character of this micro-organism remains to be fully explored. It is noteworthy that in bacterial species such as *Vibrio cholerae*, up to 13 TAS cluster in a mega-integron structure (Pandey and Gerdes 2005). Thus TAS could contribute to the genetic stability of mobile genetic elements and could play a role in stabilizing virulent traits during the evolution of pathogenic bacteria. Measurable attenuation of the virulence of pathogenic bacteria through the genetic inactivation of TAS using suitable animal infection models remains to be provided to properly address the possible role of TAS during infection.

Even before RNA was identified as the direct target of Kid, the bacterial Kid toxin was known to prevent proliferation in eukaryotic cells including yeast, oocites of *Xenopus laevis* and HeLa cells (de la Cueva-Méndez et al. 2003). Kid induced apoptosis in the later cells and the antitoxin Kis was protecting from these effects. This indicates that regulated expression of Kid and Kis might be used to kill particular cells, including tumour cell lines, in a selective way. This may be achieved by expressing the *kid* and *kis* genes under the control of promoters that are, respectively, induced and repressed in these cells, and that have the inverse behaviour in normal cells. Since Kid also inhibits the growth of embryonic cells, a similar strategy might be used to prevent the growth of particular cell lineages during development. This approach could have value in studies of differentiation, organogenesis or degenerative disorders (de la Cueva-Méndez et al. 2003). Indeed differential expression of the toxin and antitoxin genes of the *parD* system in fertilized embryos of zebrafish has been used to eliminate selectively the germ line of this fish and to study its role in sex differentiation (Slanchev et al. 2005). Similarly the RelE protein from *E. coli* acts as a global inhibitor of translation not only in bacteria or archaea but also in eukaryotic cells. In *Saccharomyces cerevisiae* the expression of the bacterial *relE* gene is toxic to yeast cells (Kristoffersen et al. 2000), and in a human osteosarcoma cell line the toxin RelE retards growth and leads to cell death by apoptosis (Yamamoto et al. 2002).

The growing structural and functional information on toxin-antitoxin systems open important avenues to the exploration of their biomedical and biotechnological implications and applications both in prokaryotic and eukaryotic cells.

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