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Review Article

REVIEW ARTICLE

One cannot rule them all: Are bacterial toxins-antitoxins druggable?

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One sentence summary: We consider various approaches to develop the toxins of the type II family as possible candidates to drug discovery; druggability of toxins-antitoxins could be possible as antivirals. As antibacterials, they might be considered as druggable but delivery and formulation may not be simple so far.

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ABSTRACT

Type II (proteic) toxin–antitoxin (TA) operons are widely spread in bacteria and archaea. They are organized as operons in which, usually, the antitoxin gene precedes the cognate toxin gene. The antitoxin generally acts as a transcriptional self-repressor, whereas the toxin acts as a co-repressor, both proteins constituting a harmless complex. When bacteria encounter a stressful environment, TAs are triggered. The antitoxin protein is unstable and will be degraded by host proteases, releasing the free toxin to halt essential processes. The result is a cessation of cell growth or even death. Because of their ubiquity and the essential processes targeted, TAs have been proposed as good candidates for development of novel antimicrobials. We discuss here the possible druggability of TAs as antivirals and antibacterials, with focus on the potentials and the challenges that their use may find in the 'real' world. We present strategies to develop TAs as antibacterials in view of novel technologies, such as the use of very small molecules (fragments) as inhibitors of protein–protein interactions. Appropriate fragments could disrupt the T:A interfaces leading to the release of the targeted TA pair. Possible ways of delivery and formulation of Tas are also discussed.

Key words: toxin–antitoxin operons; drug discovery; drug delivery; antibacterials; antivirals; inhibitors of protein–protein interactions; persistence

INTRODUCTION

Humans have long searched for all kind of medicines that could cure their bodies, restore their health and prolong their short lifespan. Thereby, search for a *panákeia* (*pan* = *all; akés* = *a cure*) as a universal drug that could overcome all kind of diseases started as early as Medicine itself. Such an ecumenical view also appears within the tolkienian fantasy, where there was the One Ring of Power, the One that would rule all the lesser magical rings (http://www.tolkien.co.uk). Unfortunately, there was no One Ring in real life; sadly enough to say, a panacea also does not exist, at least for now. Back to the reality, in this review, we will focus on the present problems of the emerging antimicrobial resistance of many pathogenic bacteria as well as spread of viral infections. We will discuss that there is an impending need to research on the discovery of novel antimicrobials, to find alternatives to classical treatments as well as to use novel approaches that will provide not only one, but multiple solutions to tackle human infections. We shall also discuss the possible

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Table 1. Modes of action of some known antibiotics.[a]

Type	Example/representative	Function inhibited
β-Lactams	Penicillins, cephalosporins	Biosynthesis of bacterial cell wall
Aminoglycosides	Streptomycin[b], Gentamicin	Ribosomal-mediated protein translation
Glycopeptides	Vancomycin[c]	Cell-wall biosynthesis
Tetracyclines	Tetracycline[b]	Ribosomal-mediated protein translation
Macrolides	Erythromycin	Ribosomal-mediated protein translation
Lincosamides	Clindamycin	Ribosomal-mediated protein translation
Phenicols	Chloramphenicol[b]	Ribosomal-mediated protein translation
Quinolones	Ciprofloxacin	DNA gyrase
Pyrimidines	Trimethoprim	C_1 metabolism
Sulfonamides	Sulfamethoxazole[d]	C_1 metabolism
Rifamycins	Rifampin	RNA polymerase
Cationic peptides	Colistin	Cell membrane biosynthesis

View it in a separate window

[a] Adapted from (Davies, 2010 #4750)

[b] Not widely used due to high-resistant bacteria.

[c] Vancomycin was considered as the 'last' antibiotic. Mechanisms of resistance to this antibiotic were reported by (Walsh, 1996 #4751).

[d] Hardly used due to low effectiveness.

druggability of the bacterial toxins–antitoxins (TAs) family of proteins as well as the pros and cons of these approaches.

THE GLOOM SCENARIO

Infectious diseases constitute one of the highest causes of deaths of human beings throughout the world, by the millions every year. The World Health Organization (WHO: www.who.int/research/) stated that 'deaths from acute respiratory infections, diarrhoeal diseases, measles, AIDS and malaria account for more than 85% of the mortality from infection worldwide' (WHO Reports 2010 and 2013). The total amount of children died under 5 years of age in 2012 summed up to 6.6 million worldwide, and nearly 60% of these deaths were due to infectious diseases. Pneumonia is the largest single cause of death in under-fives and about 80% of them could be cured by prevention and proper antibiotic treatment. These figures apply mostly for the under-developed countries. In developed countries, albeit the rates of mortality are much lower, there is high-risk populations formed by children, elderly and immuno-compromised people, which are generally subjected to antibiotic treatments, either ambulant or in hospital. The problem can be much bigger than envisaged with the appearance of bacteria exhibiting multi- or even pan-drug resistance (Falagas and Karageorgopoulos 2008), or the recent epidemics outbreak of viral infections like Ebola, avian influenza and Middle East respiratory syndrome (http://www.cdc.gov/outbreaks/). Thus, the global figure of people suffering from microbial infections may reach the hundreds of millions. This poses huge ethical, medical and economical concerns that must be tackled in the most urgent way. One of the main problems found in the everyday practice of treating these patients is the steady emergence of multidrug-resistant bacteria. This problem dictates the need for major efforts to discover new classes of antimicrobial drugs. The current portfolio of compounds in clinical trials includes mostly derivatives of existing antibiotics for which underlying resistance mechanisms already exist (WHO, Global report on antimicrobial resistance 2014; http://www.who.int/drugresistance/documents/surveillancereport/en/). This scenario is particularly worrisome in the case of hospital infections caused by highly resistant pathogens, although some advances have been achieved (Bassetti *et al.*, 2013). Antibiotic resistance (AbR) is not a new conundrum and it seems that there is no ultimate denouement but just a temporary regime to alleviate the problems. The emergence of resistance to the few antibiotics that are still useful for the treatment of nosocomial infections, as well as the knowledge accumulated on the mechanisms of action of antibiotics (Blair *et al.*, 2015), urges the need for novel infection prevention and control strategies (Table 1).

In spite of the fact that AbR compromises our ability to deal with infectious diseases, a recent report performed in the UK showed that the funds devoted to this area are small and clearly insufficient (Head *et al.*, 2014). Furthermore, most large pharmaceutical companies have stopped investing in new antibiotic research and development: the number of industries working on active discovery and development programs for antibacterial agents have dropped in the last 20 years from more than 20 companies to only 4 in 2013 (Shlaes *et al.*, 2013). In-depth analyses of the Food and Drug Administration (FDA)-approved antimicrobials have shown that the number of antibacterials attained a peak by the end of the 1990s and decreased very fast afterwards, concomitantly with the decline of pharmaceutical companies introducing new molecules with antibacterial activity into the market (Kinch *et al.*, 2014). Reasons for this withdrawal include that drug development is a very time consuming and costly process: from target discovery to lead compound optimization, followed by preclinical studies and final clinical development and marketing authorization can take typically more than 10 years. Thus, the pricing pressure (mostly due to the marketing of antibiotic generic compounds) and severe regulatory hurdles have accelerated the withdrawal of companies from the antibacterial drug market which, in turn, has led to the reduced number of new drugs approved by the US-FDA. The '10 $\times$ 20' initiative, launched in 2010 to promote the development of 10 new antibiotics by 2020 has resulted, so far, in only two new molecules, and the number of new drugs in the market is steadily decreasing, generating a situation that enhances the risks of returning to the pre-antibiotic era (Boucher *et al.*, 2013). This gloom scenario led to the proposal of rebooting the FDA's entire approach to antibiotic development (Shlaes *et al.*, 2013). In fact, actions in this direction have been taken by the designation of the Generating Antibiotic Incentives Now program, included in the FDA Safety and Innovation Act. This gives the FDA, through the Department of Health and Human Services, the

Table 2. Alternative strategies to tackle AbR.

Classical strategies	Novel strategies
Chemical derivatives of the known antibiotics	Entirely novel antibiotics
Antibiotic cycling or rotation	Collateral sensitivity
Biologic approaches	Novel small molecules
- Phage therapy	- Antivirulence
- Vaccines	- Antipersisters
- Peptides	
Search for essential genes as targets	Search for novel targets
	- Virulence factors
	- TAs
Change antibiotic treatment until new resistance appears	Target the spread of AbR
	- Mobilome: conjugation inhibitors (COINS)
–	Translational research and interactions among healthcare cadres

urgency to come up with a list of pathogens that qualifying drugs must target and to develop regulations for the antibiotic incentives (www://bioworld.com).

In the case of antivirals, the discoveries paralleled those of antibiotics and, again, novel compounds have not been so frequently introduced in the market. Since antiviral drugs are highly specific for a single infectious agent, accurate diagnosis is needed before any antiviral can be prescribed, therefore limiting the number of diseases of commercial interest. The existence of effective vaccines and vaccination programs is also important to determine whether the market needs new antivirals. For instance, in the case of hepatitis B, even though an effective vaccine already exists, the number of patients already infected is still very high and they do constitute a significant population that is in need of antiviral therapy. Due to the social and economical impact, the main efforts are focused on the development of drugs against human immunodeficiency virus (HIV) and hepatitis C virus (HCV) infections (Littler and Oberg 2005). The guidelines on the use of vaccines and antivirals especially during pandemic are very crucial. The current market-based system does not have the capacity to supply a sudden demand of vaccine when pandemic is declared. Stockpiling of vaccines for pandemic is not practical as vaccine composition relies on the responsible virus, which can only be identified after it appears at the beginning of the pandemic situation. The antiviral, like influenza, is likely still effective in the prophylaxis and treatment of disease caused by a new pandemic virus. As antiviral drugs are relatively stable, thus stockpiling is feasible. Vaccines still remains the primary mode of prevention but antivirals will still have its role to complement the situation (http://www.who.int/csr/resources/publications/influenza/11_29_01_A.pdf). The emergence and re-emergence of viral infections pose serious problems to the public health due to virus dormancy, improper diagnosis and development of resistance to antivirals. Furthermore, treatment is also hampered by the lack of specificity and the likelihood of antivirals being toxic to the patient (Saxena, Mishra and Saxena 2009).

LOOKING FOR ALTERNATIVES

Many alternatives to the classical treatments have been proposed (Kaplan and Mason 1998, Mandell *et al.*, 2007, Imamovic and Sommer 2013). There is not a single drug that can cure all infections, and combination of different therapies seem to be the more contingency, if not the only, approach especially when development of new antimicrobials seems to be still far ahead. We can envisage some variants of the classical alternatives (Table 2), such as (i) development of chemical derivatives of the known antibiotics by chemical modification of the active molecule; (ii) alternate use of available antibiotics, the so-called antibiotic cycling, in which one category of antibiotics is rotated with one or more different antibiotic classes that exhibit comparable activity, although cautions have been raised against this approach (Brown and Nathwani 2005), and (iii) implementation of more biologic approaches like phage therapy (reviewed in Deresinski 2009), new vaccines and antimicrobial peptides. More novel approaches include (i) development of entirely novel classes of antibiotics; (ii) the use of new small molecules as antivirulence or antipersisters drugs (Conlon *et al.*, 2013); (iii) employment of virulence factors as antibacterials (Marra 2004) and (iv) search for novel targets (Alonso *et al.*, 2007). The finding that bacteria that have developed resistance to one antibiotic may display a greater sensitivity to a second one from a distinct structural class (a phenomenon known as collateral sensitivity) has opened new and exciting approaches to design antibiotic treatments (Imamovic and Sommer 2013). Furthermore, the identification of virulence genes which are associated with the bacterial mobilome (plasmids, bacteriophages and integrative and conjugative elements, ICEs) has thrown light on mechanism of spread of AbR among pathogenic bacteria and on how these bacteria are able to evolve very rapidly as to adapt to new niches and environments (Wellington *et al.*, 2013). It is clear that the mobilome as a joint bacterial response to the selective pressure posed by humans plays a central role in the 'arms race' between pathogens and humans. Thus, an interesting strategy that would be worth exploring further was initiated with the identification of inhibitors that target the transfer of DNA among bacteria, which were termed conjugation inhibitors (Fernandez-Lopez *et al.*, 2005).

Despite all these possible approaches to be developed at a medium term, the everyday practice in hospitals need protocols to deal with infectious outbreaks that are easy and reliable to implement. Therefore, in addition to new antibacterials there is a need to develop translational research and interactions among healthcare cadres leading to direct and fluent communications between researchers, hospitals and the companies involved in drug development, e.g. knowledge transfer partnership. This, in due time, should lead to concepts such as drug-likeness approaches, so that the finding of novel bacterial targets and the drug development process could be expedited. It is also important to keep in mind the need to understand the biology of the bacterial species we are aiming, since biological and, especially,

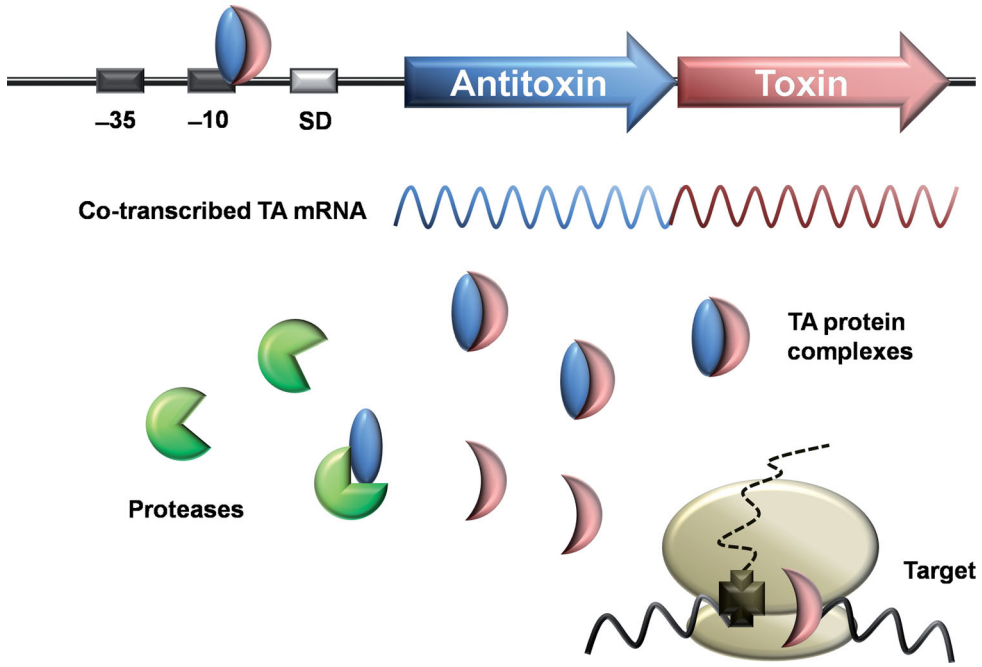

Figure 1. Features of type II TAs. Typical type II TAs consists of two genes organized as an operon. The antitoxin gene precedes the toxin one. Both genes usually overlap and are co-transcribed from one or two promoters. TA genes encode both antitoxin (oval) and toxin (crescent) proteins that bind to each other and generate a harmless complex under normal conditions. The antitoxin protein also binds to its own promoter to negatively autoregulate the TA operon. The toxin protein is not able to bind to the promoter by itself, but serves as a co-repressor upon binding of the antitoxin to the promoter, to further repress transcription of the operon. Under stressful circumstances, the antitoxin protein which is more labile, is degraded more rapidly by the host proteases and thus liberate the toxin protein to act on the cell target.

genetic approaches will be a significant aid in the drug discovery process (Trauner, Sassetti and Rubin 2014).

Strategies that have been pointed out as potential for drug discovery like exploiting the bacterial TAs, although constitute a dispute on its druggability, yet still comprise compelling rationales that worth to be explored (Alonso *et al.*, 2007; Mutschler and Meinhart 2011; Gerdes 2013). In this review, we will discuss the possible druggability of TAs, which is not as simple as previously envisaged, but also in terms of choosing the proper toxin (or combination of two), their delivery and their targets, because many biologically active molecules can show a high activity *in vitro*, but would not reach the clinical phases due to lack of intestinal absorption and/or poor *in vivo* metabolic stability and tissue distribution, the so-called 'ADME' (absorption, distribution, metabolism and excretion) properties. In summary, development of novel antimicrobial drugs is a combination of chemical, biological and industrial approaches in order to design the active compound, validate its usefulness and developing it for clinical use (De Clercq 2010).

BACTERIAL TAs: WHAT ARE THEY?

TAs are usually composed of two genes: the toxin gene encodes a toxin protein that has toxic effect to the cells, whereas the antitoxin can be either RNA or protein, which counteracts the toxicity of its cognate toxin. Depending on the mode of the antitoxin to neutralize the toxin, TAs have been grouped into five types so far: (i) type I, the antitoxin antisense RNA prevents translation of the toxin protein by binding to its complementary toxin mRNA; (ii) type II, the antitoxin protein binds to the toxin protein through protein–protein interactions (PPI); (iii) type III, the antitoxin mRNA interacts directly to the toxin protein; (iv) type IV, the antitoxin protein interferes the binding of the toxin protein to the cell target, and (v) type V, the antitoxin protein cleaves specifically its cognate toxin mRNA (Hayes and Van Melderen 2011; Masuda *et al.*, 2012; Wang *et al.*, 2012).

We will analyse the potential use of type II TAs as antimicrobial targets since they are the most abundant and better studied among TAs, and their mechanisms of action have been generally unveiled. Type II TAs exhibit typical features (Fig. 1) such as (i) the antitoxin gene is usually preceding the toxin gene; (ii) both genes are co-transcribed from a promoter located upstream of the antitoxin gene and (iii) they are translationally coupled. The operon is negatively autoregulated by the antitoxin upon binding to an operator region that overlaps the single promoter of the operon, whereas the toxin acts as an efficient co-repressor. The antitoxin protein is labile and more susceptible to degradation by the host proteases, while the toxin protein is much more stable. The ratio of the toxin to antitoxin is important to determine the stoichiometry of the TA protein complex and also to regulate the transcription of the operon, so that the antitoxin is replenished to shield the toxicity in normal condition (Monti *et al.*, 2007; Cataudella *et al.*, 2012). However, under certain stressful circumstances such as nutritional stress, colonization of new hosts, DNA-damaging agents, etc., transcription of the TA will be triggered, and the labile antitoxin will be degraded more rapidly, unleashing the toxin protein to act on their targets.

The classification of type II TA pairs is not an easy task as the toxin homologues that share sequence similarities can pair with different antitoxins with little sequence similarities (e.g. the *yefM–yoeB* TA genes encode antitoxin from the Phd family and toxin from the RelE family) (Anantharaman and Aravind 2003; Grady and Hayes 2003; Hayes and Van Melderen 2011). Toxins that share similar structures, despite no notable sequence

Table 3. The 12 superfamilies of type II toxins.

Superfamilies according sequence similarity	Modes of action
RelE/ParE	- RelE inhibits translation by cleaving translating mRNAs (Christensen 2001 #29) - ParE inhibits replication by hindering DNA-gyrase activity (Jiang 2002 #5)
CcdB/MazF/Kid	- CcdB inhibits replication by hindering DNA-gyrase activity (van Melderen 2002 #4317) - MazF/Kid inhibits translation by cleaving free mRNAs (Zhang 2003 #53)
Doc	Inhibition of translation by phosphorylating the translation elongation factor EF-Tu (Castro-Roa 2013 #4557)
Zeta	Inhibition of peptidoglycan synthesis by phosphorylating the peptidoglycan precursor uridine diphosphate-N-acetylglucosamin (Mutschler 2011 #3597)
VapC	Inhibition of translation by cleaving $tRNA^{fMet}$ in the anticodon stem-loop (Winther 2011 #3641)
HipA	Inhibition of translation by hindering aminoacyl-tRNA synthetase (Germain 2013 #4561)
YafO	Inhibition of translation by association with 50S ribosomal subunit (Zhang, Yamaguchi and Inouye 2009 #3720)
VapD	Inhibition of translation by cleaving free mRNAs (Kwon 2012 #4334)
GinA	Inhibition of translation[a] (Leplae 2011 #2223)
GinB	Inhibition of translation[a] (Leplae 2011 #2223)
GinC	Inhibition of translation[a] (Leplae 2011 #2223)
GinD	Inhibition of translation[a] (Leplae 2011 #2223)

[a]Target not determined

similarities, bind to different targets (e.g. CcdB binds to DNA gyrase but Kid, which has similar structure as CcdB, cleaves free mRNAs) (Hargreaves *et al.*, 2002). Further, toxins that were thought to belong to different families have similar mode of actions (e.g. ParE and CcdB act on DNA gyrase) (Jiang *et al.*, 2002; van Melderen 2002). To complicate the picture more, a toxin might have more than one target: for instance, toxin MazF-mt6 from *Mycobacterium tuberculosis* cleaves free mRNA at the sequence 5′-UU↓CCU-3′ (being ↓ the cleaving site), but also cleaves 23S rRNA at a single 5′-UUCCU-3′ sequence in the ribosomal A site that contacts tRNA and ribosome recycling factor (Zhu *et al.*, 2009; Schifano *et al.*, 2013). Thus, instead of classifying the TA gene pair, 12 toxin superfamilies (Table 3) and 20 antitoxin superfamilies have been identified (Hayes and Van Melderen 2011; Leplae *et al.*, 2011) based on sequence similarities. However, the number of the TA genes that we know up to date seems to be still below the actual number (Sberro *et al.*, 2013).

ROLE OF TYPE II TAs

Several mechanisms of action of the different toxins have been identified (Table 3) via inhibition of replication by targeting the DNA gyrase (e.g. CcdB or ParE) (Jiang *et al.*, 2002; van Melderen 2002); inhibition of peptidoglycan synthesis by phosphorylating the peptidoglycan precursor uridine diphosphate-N-acetylglucosamine (e.g. Zeta/PezT) (Mutschler *et al.*, 2011); and inhibition of translation by several alternative mechanisms such as (i) cleaving free RNA (e.g. MazF, Kid/PemK, HicA or MqsR) (Pimentel, Madine and de la Cueva-Mendez 2005; Nariya and Inouye 2008; Jørgensen *et al.*, 2009; Yamaguchi, Park and Inouye 2009; Agarwal *et al.*, 2010); (ii) cleaving translating RNA (e.g. RelE, YoeB, HigB or YafO) (Christensen *et al.*, 2001; Hurley and Woychik 2009; Zhang and Inouye 2009; Zhang, Yamaguchi and Inouye 2009); (iii) cleaving $tRNA^{fMet}$ in the anticodon stem-loop (e.g. VapC) (Winther and Gerdes 2011); (iv) inhibiting aminoacyl-tRNA synthetase (e.g. HipA) (Germain *et al.*, 2013), or (v) phosphorylating the conserved threonine of the translation elongation factor EF-Tu (e.g. Doc) (Castro-Roa *et al.*, 2013). The toxic effect could be bacteriostatic or bactericidal, depending upon the type of toxin and time of exposure (Nieto *et al.*, 2010).

TAs operons can reside on chromosomes, on plasmids or on both, in single or multiple copies. In several instances, they are also found within horizontal mobile elements like pathogenic islands, ICEs, integrative mobilizable elements and on large superintegrons (Rowe-Magnus *et al.*, 2003; Szekeres *et al.*, 2007; Wozniak and Waldor 2009; Gerdes 2013). From the beginning of the discovery of TAs, plasmid-encoded TAs were believed to only function in exerting post-segregational killing (PSK) of daughter cells that do not inherit the parental TA-encoded plasmid (Bravo, de Torrontegui and Díaz 1987; Bravo *et al.*, 1988; López-Villarejo *et al.*, 2012). However, an additional role of plasmid-encoded TA ccd_F as a transmissible persistence factor was reported (Tripathi *et al.*, 2012), indicating that the role of plasmid-encoded TAs might be far more complex than envisaged. On the other hand, the functions of the chromosomally encoded TAs are even more debatable (van Melderen and Saavedra De Bast 2009; van Melderen 2010; Makarova *et al.*, 2011). They have been suggested to be involved in at least the following processes: (i) global stress response leading to inhibition of cell growth under unfavourable condition, but the toxicity is reversible when a normal environment is restored (Christensen *et al.*, 2001); (ii) bacterial programmed cell death, i.e. an altruistic behaviour in which some of the cells sacrifice themselves to release nutrients for the benefit of the siblings or community as a whole when nutrients are scarce (Engelberg-Kulka and Glaser 1999); (iii) anti-addiction module: the chromosomally encoded TAs protect the cells against PSK mediated by the plasmid-encoded TA homologues (de Bast, Mine and van Melderen 2008); (iv) persistence leading to inhibition of cells growth mediated by TAs to reduce the susceptibility of the cells to antibiotic (Gerdes and Maisonneuve 2012); (v) biofilm formation (Ren *et al.*, 2004; Kim *et al.*, 2009; Kolodkin-Gal *et al.*, 2009); (vi) maintenance of the bacterial mobilome by stabilization of the dispensable chromosomal regions to prevent extensive genetic loss (Rowe-Magnus *et al.*, 2003; Szekeres *et al.*, 2007); (vii) increase survival rate and virulence (Ren, Walker and Daines 2012), and (viii) colonization of niches (Norton and Mulvey 2012).

Employment of the toxins from TAs to use in a number of biotechnological applications have been proposed and, in several cases, successfully developed for commercial purposes such as positive selection plasmid vectors (Stieber, Gabant and Szpirer 2008). In this instance, *Escherichia coli* strains harbour plasmid vectors expressing the lethal toxin gene product, which is counteracted by expression of the cognate antitoxin in trans. Cloning of genes is achieved by transformation of a strain devoid of the antitoxin, thus allowing the selection of recombinants in which only the inactivated toxin genes are recovered (Bernard *et al.*, 1994). Other applications of the TAs include the controlled release of genetically modified organisms into the biosphere. Balance of the benefits versus possible risks has been tackled by creating genetic traps, termed active containment systems that avoid bacterial escape. They are based on the controlled expression of lethal genes that would be turned 'on' in the case of escape into the environment. Toxins with a broad spectrum, such as MazF or Kid, have been chosen as good candidates as genetic traps (Ramos *et al.*, 1994; Guan *et al.*, 2013) and, in some cases, combination of two toxins acting on different targets and controlled by different regulatory signals has been considered as the best alternative because the risk of escape in these containment conditions was estimated to be lower than 10^{-8} (Torres *et al.*, 2003). Further applications in biosensors and bioremediations, the GeneGuard system, have been developed within a synthetic biology framework, to be used in a variety of bacteria (Wright *et al.*, 2014). In the food industry, employment of TAs to construct plasmid vectors in which the number of plasmid-free cells is decreased as well as vectors to overproduce plasmid-encoded proteins in the absence of antibiotic selection have proved to be a useful approach (Pecota *et al.*, 1997). Excellent reviews on the biotechnological applications of TAs have been published (Suzuki *et al.*, 2005; Inouye 2006; Stieber, Gabant and Szpirer 2008; Unterholzner, Poppenberger and Rozhon 2013).

TAs AS ANTIVIRALS

Among the anti-infective drugs recently approved for clinical use, the antiviral agents have been the more numerous, following a parallel path of that set in the past by antibiotics. Above 40 antivirals have been licensed, more than half of them being used in the treatment of HIV infections. Other antivirals have been licensed for the treatment of infections by herpes virus, hepatitis (B and C) and influenza (De Clercq 2010). Even for those viral infections that have already been controlled by therapeutic modalities, search for additional molecular targets and new treatment strategies still have to be followed. It is within this context that TAs has offered, probably, the most promising solutions.

MazEF is a well-studied TA operon that constitutes an attractive candidate for antivirals. As mentioned above, MazF toxin is an endoribonuclease, which preferentially cleaves single-stranded RNA between A and C residues at the ACA recognition sequence in a manner independent of ribosome and thus inhibiting protein synthesis (Zhang *et al.*, 2003), whereas MazE is its cognate antitoxin which nullifies MazF toxicity. There have been, as far as we are aware, a few reports on possible use of MazEF as antivirals (summarized in Fig. 2). The first report contemplates the HIV-encoded transactivator of transcription (Tat) protein, an early viral regulator, as a possible target (Chono *et al.*, 2011a,b). Tat is produced early after HIV-1 infection and binds to the transactivation response (TAR) sequence, inducing the subsequent expression of other HIV-1 proteins. Thus, a Tat-dependent MazF expression system of a retroviral vector was constructed in which the *mazF* gene was inserted downstream the TAR sequence (Fig. 2a). Consequently, *mazF* was to be expressed when the HIV-1 viral Tat protein was produced and so to cleave the mRNA specifically at ACA codon in the infected cells. The *mazF* gene contains nine ACA sequences, which were engineered to avoid self-cleavage without altering the amino acid sequence and maintained its toxicity. On the other hand, the HIV RNA contains more than 240 ACA sequences, and thus the viral RNAs can hardly escape from the MazF attack. The experiment was conducted by transducing the recombinant plasmid into human T lymphoid line CEM-SS cells, which are highly susceptible to HIV infection. Strikingly, when the transduced cells were infected with HIV-1 IIIB, the replication of the infected virus was inhibited and the CD4 level was also not affected (as depicted in Fig. 2a). In addition, the level of MazF induced was not enough to cause serious cell damage and thus maintaining the normal cell growth. Similar results were observed when the Tat-dependent MazF system were investigated on rhesus macaque primary CD4+ T cells from monkeys that were infected with the chimerical virus SHIV 89.6P (Chono *et al.*, 2011a,b).

Another line of work made use of a typifying characteristic of viral proteases, which have very specific cleavage site, to target explicitly the infected cells instead of the innocent ones (Park, Yamaguchi and Inouye 2012). Non-structural serine protease, NS3–4A, is an HCV protein that is essential for the HCV replication. An NS3-activated MazF system was constructed in which the NS3 protease cleavage site linker was fused in between MazF and truncated C-terminal of MazE (as we interpret in Fig. 2b). These fusion proteins are inert, and when incubated with NS3 protease the MazF toxin could be activated via cleavage at the linker in between MazE and MazF complex (Park, Yamaguchi and Inouye 2012). Other viral protease cleavage sites worked the same with their corresponding proteases (e.g. HIV-1 protease and factor Xa) (Park, Yamaguchi and Inouye 2012). A similar construct was done by another group which they termed it as 'zymoxin' (Shapira *et al.*, 2012). While this construct was activated by NS3 protease (in HEK293 T-REx cells that harbour tetracycline-inducible NS3–4A constructs), NS3-mediated activation of MazF that inhibited cellular protein synthesis was observed; however, cytotoxic effect was also evident even when NS3 was mildly induced (Shapira *et al.*, 2012). The possible undesirable effects of MazF cleaving the eukaryotic mRNAs could be avoided by careful control of the dosage of the toxin, so that MazF was able to cleave the viral, but not damaging the cells (Chono *et al.*, 2011a,b).

TYPE II TAs AS TARGETS FOR ANTIBACTERIAL DRUG DEVELOPMENT

One of the most challenging aspects in drug discovery is to find novel chemical or biological molecules that may act as antibacterials. The discovery pipeline includes new natural products or products that result from new biosynthetic pathways (e.g. molecules that control cell communication), although they also may include novel chemical molecules or antibacterial protein/peptides. Potential targets for the development of novel antibacterials could be making use of the toxins of the bacterial TA genes, partly because they are widely distributed in bacteria and archaea but not in eukaryotic cells (Inouye 2006; Alonso *et al.*, 2007; Hayes 2011; Makarova *et al.*, 2011; Mutschler and Meinhart 2011; Chan *et al.*, 2012). Some toxin homologues were also identified *in silico* in fungi, but their functionality has not been experimentally validated, and it is also unknown

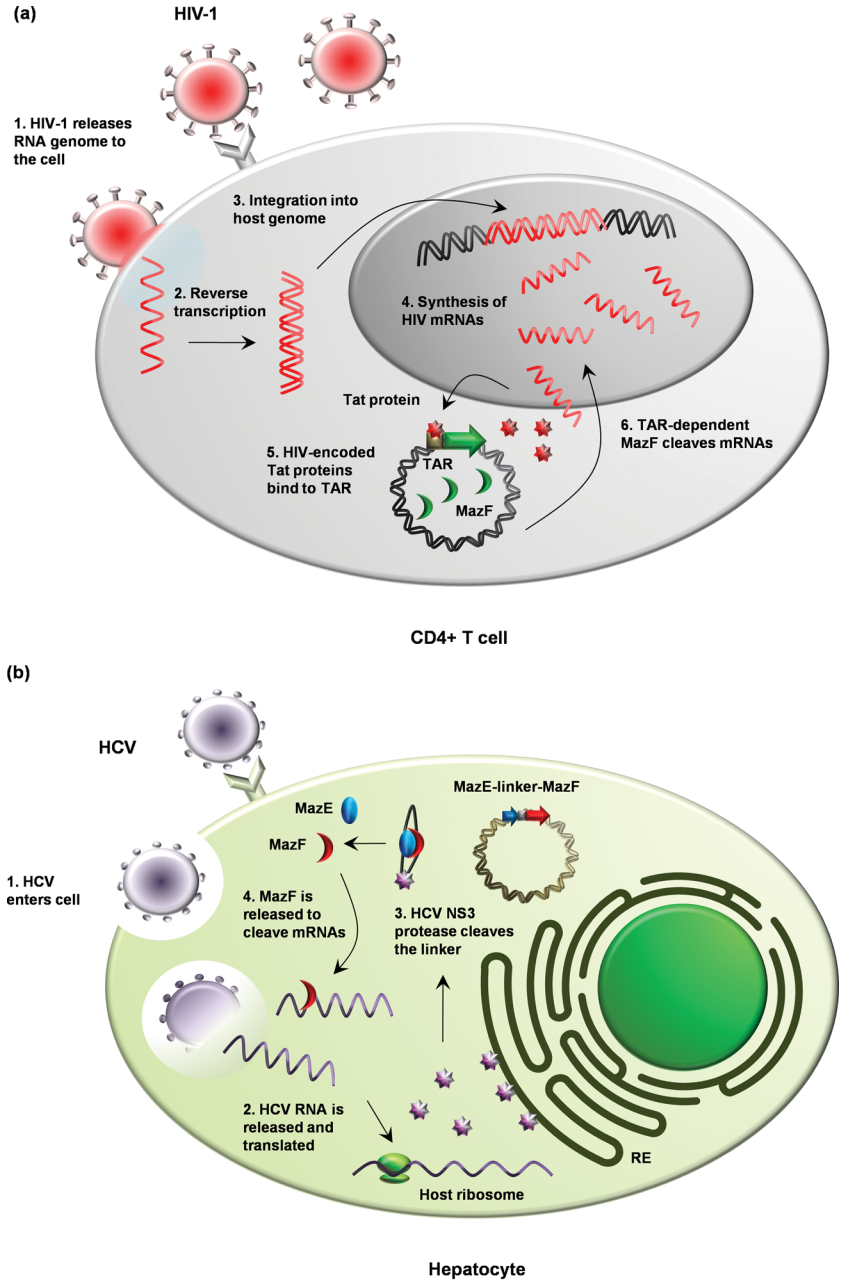

Figure 2. Conceptual models of potential approaches using TA as antivirals. (**a**) Activation of engineered viral promoter-MazF by early viral regulator protein. HIV-encoded Tat protein is an early viral regulator that binds to TAR sequence. A Tat-dependent MazF toxin (crescent) expression system of a retroviral vector was designed in which the *mazF* gene was inserted downstream the TAR sequence. MazF is an endoribonulease that cleaves free mRNA at the ACA codons. The *mazF* gene was engineered to avoid self-cleavage by changing the base sequences but conserving its amino acid sequence to preserve its toxicity to cleave the viral mRNAs. The vector was then transduced into human T lymphoid line CEM-SS cells. When HIV-1 attempts to enter the cell, interactions between cell surface molecules and viral envelope proteins allow the envelope to fuse with the cell membrane and subsequently viral RNA genome is released to the cell (1). The viral single-strand RNA genome is transcribed into double-strand DNA (2), and then integrated into a host chromosome (3). The proviral genome can consequently be transcribed into viral mRNA (4) for translation into HIV proteins. The early viral protein Tat will bind to the TAR sequence to induce MazF production (5) to cleave viral mRNAs (6). It is worth to mention that this system will only be triggered in the HIV-1-infected cells but not the innocent ones. (**b**) Cleavage of specific linker by viral protease to trigger MazF. NS3–4A is an HCV protein that has a very specific cleavage site. A recombinant vector was constructed that produced a complex in which the NS3 protease cleavage site linker was fused in between MazF (crescent) and truncated C-terminal of MazE (oval). Once the HCV enters the hepatocyte (1), HCV will take over parts of the intracellular machinery to replicate (2). NS3–4A will be produced and cleave specifically to the MazEF-linker (3) and thus liberating MazF to cleave viral mRNAs (4).

whether the cognate antitoxins are present. Obligate intracellular organisms were proposed to be devoid of, or have less, TA cassettes but, in contrast, free-living slow growing prokaryotes have many TA cassettes (Pandey and Gerdes 2005); more recent analyses, however, have not confirmed this observation (Leplae *et al.*, 2011). Interestingly, comparison of 12 genomes of dangerously epidemic bacteria with those of closely related non-epidemic species showed that TAs could participate in the virulence repertoire (Georgiades and Raoult 2011; Georgiades 2012).

Comparative genomic studies showed that the presence of TA modules is significantly associated with the pathogenicity of bacteria (De la Cruz *et al.*, 2013). For example, *M. tuberculosis* contains at least 30 functional TA genes (88 putative TA gene pairs were tested) (Ramage, Connolly and Cox 2009), whereas its non-pathogenic counterpart, *M. smegmatis* has only three functional TA genes (Robson *et al.*, 2009; McKenzie *et al.*, 2012). Furthermore, the number of TA genes reported so far can be a serious underestimation of the real figure in the microbial world. A more thorough way to identify functional TA genes, instead of *in silico* screening, was done via shotgun cloning which had revealed that many toxin genes could only be cloned when the neighbouring antitoxin genes were present (Sberro *et al.*, 2013). The situation could be more complex, as exemplified by the possible type II TAs present in the chromosome of the pathogenic bacterium *Streptococcus pneumoniae*: bioinformatics approaches predicted the existence of up to 10 different putative TAs (Chan *et al.*, 2012, 2013). However, genetic and physiological validation of the candidates showed that only four out of the 10 predicted operons were bona fide functional TAs. Even though some of the putative toxin genes exhibited toxicity when overexpressed, their cognate gene pairs did not counteract the toxicity respectively and thus were ruled out as 'classical' TAs (Chan *et al.*, 2014). The presence of more than one copy of the same TA may be a source on uncertainty because closely related antitoxins may functionally interact with each other, so that the paradigm of one antitoxin–one toxin may not be correct (Zhu *et al.*, 2010). It could be thought that cross-talks between chromosomally encoded toxins and antitoxins do exist perhaps as means to adapt the bacteria to environmental changes that the populations encounter along their colonization/invasion of new niches (Zhu *et al.*, 2010). If this indeed were the case, the scenario would be much more complex than envisaged.

Taken together, all the above features make the exploitation of TAs as antibacterials a major challenge: due to their redundancy, target validation as well as the specificity and selectivity (off-target activity) of any drug candidate targeting a specific TA would be very difficult to assess. Despite of that, the toxins of the TA pairs could be an attractive antibacterial targets to be used as stand-alone drugs or in combination with classical antibiotics (Mutschler and Meinhart 2011; Park, Mann and Li 2013; Unterholzner, Poppenberger and Rozhon 2013). However, it seems that the combination with current approved antibiotics would have, at least at present, another major challenge in terms of matching the formulation to generate a combination product. The route to approach these problems can vary depending upon the way the toxin effect is envisaged. In the first place, if the toxin is going to be the candidate itself, then the fact that TAs are absent in human is irrelevant. The toxins of the TA pairs as all novel targets to be considered for drug discovery must comply with a set of requirements, such as lack of toxicity to eukaryotic cells and target only the infecting bacteria. However, the toxins of TA genes are also harmful to the human cells unless there is a careful monitoring of the toxin dosage (as mentioned above in the antiviral tests) and safe delivery systems are applied, in addition to provision for the replenishment of commensal flora. If, however, the TA is going to be used as a target for drug ligands, then all of the above arguments are relevant, keeping in mind the challenges related to redundancy. Several strategies to disrupt the interface between antitoxins and their cognate toxins by inhibitors of PPI have been envisaged as well as the possible use of the toxins in combined strategies (Alonso *et al.*, 2007; Mutschler and Meinhart 2011). We will summarize below some of the reported studies as well as our views on whether employment of TAs could be considered as promising candidates for future use in the biopharmaceutical industry. We will also discuss the pros and cons of the use of these toxins as druggable antimicrobial targets.

TOXICITY TO EUKARYOTIC CELLS

Most of the known toxins from TAs act as endoribonucleases that cleave mRNAs associated or not to translating ribosomes, independently of the mRNA origin, prokaryotic or eukaryotic. Thus, the toxin could act as an antitumoural agent and, in fact, it was proposed that the Kid toxin from plasmid R1 could be used as an efficient anti-cancer compound (de la Cueva Méndez *et al.*, 2003). One of the approach could be using tumour-specific promoter to direct therapeutic expression of toxin in cancer cells (Yang *et al.*, 2004). More recently, reversion of induced solid tumours in mice has been reported by employment of an engineered version of the MazF toxin; nevertheless, the rate of success in reversion was around 50%, probably due to loss of functional toxin (Shimazu *et al.*, 2014). Such a loss of activity was not observed for toxins VapC from *M. tuberculosis* or PasB from plasmid pTF-FC2 of *Thiobacillus ferrooxidans* when expressed into human cancer cells (Wieteska *et al.*, 2014). These findings leave a door open to testing more thoroughly the possibilities of employment of toxins into tumoural cells. However, under the light of possible use of toxins as antibacterials, it is important to consider that if the toxins have to be delivered directly to the patients to kill the pathogens, the adverse effect of the toxins on the normal commensal flora and the human cells could also be difficult to avoid. Perhaps, as shown the antiviral activity of the MazF interferase, the dosage of the toxin is a critical factor when testing its activity on eukaryotic cells (Chono *et al.*, 2011a,b). The more efficient approaches would include the more specific targeting of the pathogens, or the bacteria that harbour TAs by triggering the release of the toxins, which are not found in human cells.

EFFECT ON THE INFECTING BACTERIA

Some toxins are bactericidal, as it should be for an 'ideal' antibacterial, so that the infecting bacteria would be killed by the toxin (Zielenkiewicz and Ceglowski 2005; Mutschler and Meinhart 2011). However, stasis and dormancy due to temporal reduction of the cell growth is a more common effect of the toxin, and this bacteriostatic effect can be reversed by induction of the antitoxin (Pedersen, Christensen and Gerdes 2002; Maisonneuve *et al.*, 2011; Germain *et al.*, 2013). Recovery of the dormancy due to the toxin may be only effective during a given time period, and prolonged exposure of the bacteria to a toxin like RelE can lead to cell lethality (Nieto *et al.*, 2006). In addition, there are also some other factors to be considered if toxin alone is the one to be administered, since there are instances in which the toxin may have a different panorama of toxicity in different hosts, e.g. expression of toxin Zeta of plasmid pSM19035, which was discovered in *S. pyogenes*, is bactericidal for the Gram-positive *Bacillus subtilis* and bacteriostatic for the Gram-negative *E. coli* (Zielenkiewicz and Ceglowski 2005).

PERSISTENCE AND BIOFILM

The first gene that was discovered to be involved in persistence was *hipA* from *E. coli* K-12, which is also the toxin gene of *hipBA* TA pair (Moyed and Bertrand 1983). Toxin HipA

inhibits aminoacylation and it is counteracted by its cognate antitoxin HipB which, in turn, is degraded by Lon protease (Gerdes, Christensen and Lobner-Olensen 2005; Schumacher *et al.*, 2009; Hansen *et al.*, 2012; Germain *et al.*, 2013). Upon exposure to ampicillin, the wild-type strain showed a persister cell ratio of 10^{-5} to 10^{-6}; however, a double mutant of *hipA*, the *hipA7* allele that abolished the toxicity of HipA, showed an increased frequency of persisters ratio up to 10^{-2} under the same experimental conditions (Moyed and Bertrand 1983). Overexpression of wild-type HipA also increased the frequency of persisters to the levels conferred by the HipA7 allele (Korch and Hill 2006). HipA toxin was shown to phophorylate Ser^{239}, which is near the active centre of $tRNA^{Glu}$-bound glutamyl-tRNA synthetase, thus accumulating uncharged $tRNA^{Glu}$ (Germain *et al.*, 2013). This finding was taken as an indication that the entering of uncharged $tRNA^{Glu}$ into the A site of the ribosome would trigger activation and release of RelA, which is an alarmone pentaphosphate (pppGpp) synthetase. This in turn would lead to dramatic increases of pppGpp, whose levels are positively correlated to the persistence level (Germain *et al.*, 2013; Maisonneuve, Castro-Camargo and Gerdes 2013). In another study by the same group, formation of persisters was shown to be modulated by the signalling nucleotide pppGpp through a cascade involving inorganic polyphosphate (PolyP; a long linear polymer of orthophosphate residues), Lon protease and TAs. The concentration of PolyP is controlled by pppGpp, which competitively inhibits exopolyphosphatase from degrading PolyP. PolyP was demonstrated to stimulate Lon-mediated degradation of the antitoxins of TA pairs, stochastically, in small subpopulation of cells. This would lead to the release of toxins that eventually would promote inhibition of cellular biosynthesis, cessation of cell growth and persistence (Maisonneuve, Castro-Camargo and Gerdes 2013). Previous studies showed that prolonged exposure to antibiotics activated transcription of TAs encoding mRNases in *E. coli* K-12; this activation led to inhibition of global cellular translation, rendering the cells to a state of dormancy and persistence. Single deletion of a toxin mRNase did not affect persister formation, and it required deletion of 10 TAs encoding mRNAses to cumulatively reduce the levels of persisters (Maisonneuve *et al.*, 2011). Redundancy of TAs can increase the frequency of persister cells and stochastic fluctuations can spontaneously switch on the TAs giving rise to the bistable state of normal cells and persisters (Fasani and Savageau 2013).

TAs has also been shown to influence biofilm formation. In certain instances, deletion of TA loci decreased biofilm formation (Kim *et al.*, 2009; Kolodkin-Gal *et al.*, 2009; Wen, Behiels and Devreese 2014) that coincided with other instance in which several TAs were upregulated in biofilm-forming cells (Mitchell *et al.*, 2009). The mechanisms involved are still not well understood. Perhaps the most direct evidence of involvement of TA in biofilm formation, albeit through a different pathway, was the case of the antitoxin MqsA of the MqsA–MqsR TA loci. MqsA serves as a global regulator that can repress the expression of CsgD, which is the master regulator of biofilm formation. Repression of CsgD by MsqA leads to reduction of the signalling nucleotide c-di-GMP, curli production and thereby reduction in biofilm formation (Harrison *et al.*, 2009; Soo and Wood 2013).

Persistence poses significant challenges for the treatment of bacterial infections such as latent infections and post-treatment relapse; and biofilm also raises problem by forming a host for the survival of persisters (Lewis 2007, 2008, 2010; Zhang *et al.*, 2014). Knowing that activation of TAs increases persisters and that most of the common antibiotics kill only growing bacteria, we could conjecture to turn the risk into opportunity: reduce bacterial cell growth and increase persister cells formation by activation of TAs, and then treat the persister cells with persister drug-like PZA. PZA is an unconventional persister drug for tuberculosis that only act on non-growing persister cells at acidic condition. It has shortened the tuberculosis treatment period from 9–12 months to 6 months by killing the subpopulation of persisters that are not killed by the tuberculosis drug (Zhang *et al.*, 2014). Consequently, in line with the development of TAs as antibacterials, new drugs that target persisters should also be developed. Other approaches that deal with persisters could be 'waking up' the persisters, enhance activities of antibiotics and harness the host immune system (reviewed by Zhang *et al.*, 2014).

DISRUPTION OF TA COMPLEX BY INHIBITORS OF PPIS (I-PPIS)

One of the proposed approaches to the druggability of bacterial toxins is to identify peptides or small molecules that are able to disrupt the protein (antitoxin)–protein (toxin) interactions based on their binding interface (PPI-approaches; Labbé *et al.*, 2013). The binding affinities of the TA pair varies, making it difficult to estimate the dosage of the inhibitors: for instance the PezAT pair exhibited very strong binding affinities (binding constant in the femtomolar range), which is three to eight orders of magnitude higher than other TAs such as Phd-Doc, RelBE, CcdAB and even Epsilon–Zeta, which is a homologue of PezAT (Gazit and Sauer 1999; Camacho *et al.*, 2002; Dao-Thi *et al.*, 2005; Overgaard, Borch and Gerdes 2009; Mutschler, Reinstein and Meinhart 2010). Even though PPI interfaces are very interesting targets to develop novel molecules, there are still not promising examples of drugs designed against TA interfaces. The use of high-throughput screening techniques to identify hits that act as i-PPIs in conjunction with the development of techniques to optimize the possible hits to discover proper leads (hit-to-lead techniques) is a matter not fully solved yet.

A specific bioluminiscence resonance energy transfer (BRET) assay (Xu, Piston and Johnson 1999) to test libraries of peptides that could disrupt the T:A interfaces was developed (Nieto *et al.*, 2006). This tool is an *in vivo* assay, amenable for high-throughput scaling up, and with clear advantages over fluorescence resonance energy transfer (FRET)-based assays because BRET is not susceptible to photo-bleaching, and thus yields more reliable results. BRET has been proven to be a constructive tool to identify potential hits for T:A interruption (Fig. 3) (Lioy *et al.*, 2010). In this study, several libraries encompassing various millions of peptides were used in attempt to disrupt the Epsilon–Zeta TA protein complex. Two positive 17 amino-acid-long hits were found in two sublibraries; however, no new records on further progresses have been reported (Lioy *et al.*, 2010). It also remains unknown whether the peptides were binding to the antitoxin or to the toxin. Besides disruption of the interfaces of both proteins, it is important to ensure that the binding of the peptides, if to the toxin, does not hinder the catalytic activity of the toxin, so that the released toxin can still act on its target to kill the pathogens. As shown in another study, peptides designed based on antitoxin sequences were able to disrupt the T:A interface, but the toxicity of the toxin was also reduced (Agarwal *et al.*, 2010). We suggest that peptides designed based on toxin sequence to bind to antitoxin would be a better approach. Employments of peptides of sizes ranging from 6 to 60 amino acids to tackle disruption of PPIs have important advantages such as

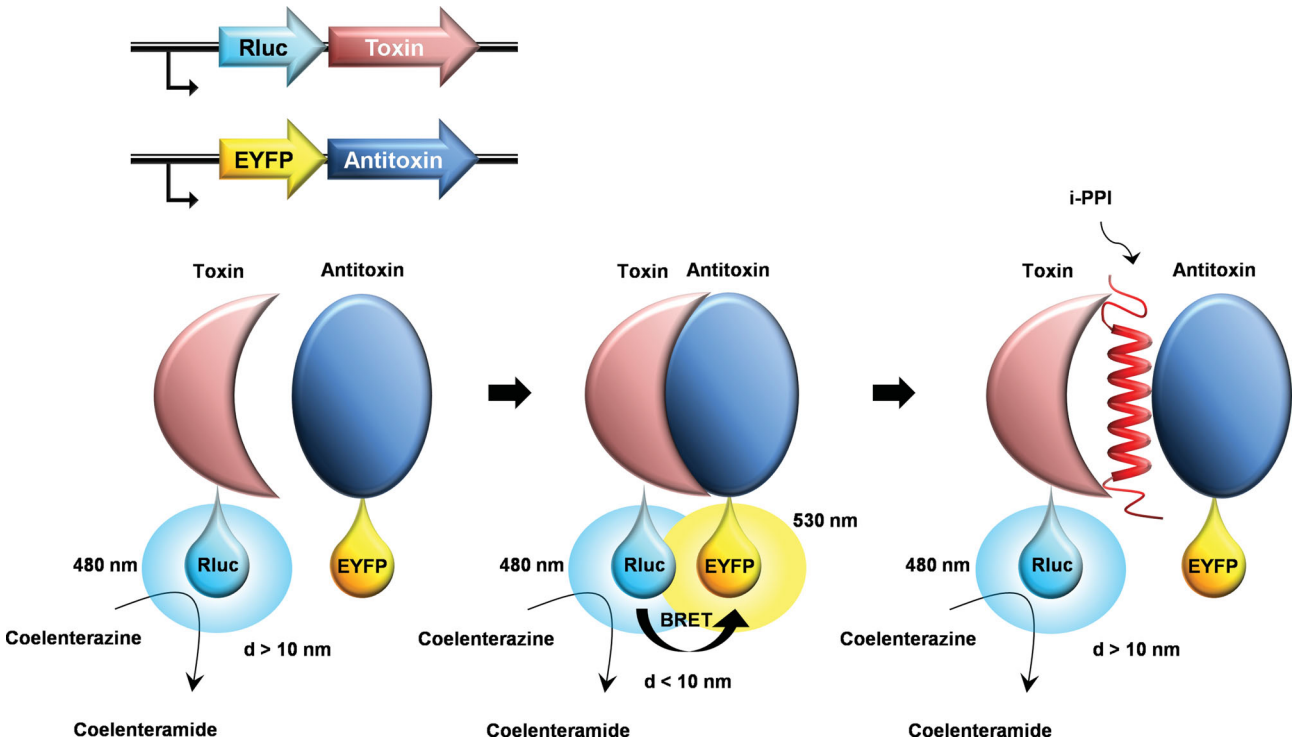

Figure 3. Detection of disruption of TA protein complex by i-PPI via BRET assays. The BRET assay technology is based on the efficient resonance energy transfer between a bioluminescent donor moiety and a fluorescent acceptor moiety. The bioluminescent *Renilla* luciferase (RLUC) that was fused with toxin protein catalyses the coelenterazine substrate to coelenteramide with concomitant light emission at 480 nm. When the acceptor enhanced yellow fluorescent protein (EYFP)-antitoxin is in close proximity to RLUC, EYFP will absorb the energy emitted by the RLUC/coelenterazine reaction and emit yellow light that can be measured at 530 nm. If the interactions between the toxin and antitoxin proteins are disrupted by i-PPI, RLUC and EYFP will be too far apart for resonance energy transfer and only the blue-emitting spectrum of RLUC will be detected.

high specificity and potentially lower failure rates after its inclusion into the drug pipeline of clinical trials. However, biological molecules have shown several shortcomings, such as poor oral availability and high costs of bioprocessing.

Alternatively to traditional high-throughput screening of libraries, fragment-based methods for drug discovery (Erlanson, McDowell and O'Brien 2004) has emerged as a stunning approach and mainstream for the discovery of new drugs within most pharmaceutical companies and many academic groups (Fig. 4). Comparison of putative candidates for novel drugs indicated that drugs derived from fragment-based screens could uncover novel compounds with more drug-like properties than those derived from more conventional drug discovery techniques. The search of these fragments is driven by the Rule of Three: molecular mass <300 Da, the number of hydrogen bond donors and acceptors each ≤3 and the clogP ≤3 (Congreve *et al.*, 2003). The fragment-based lead discovery deals with low molecular mass and low affinity molecules, so that later on they can be optimized into drug leads (Park, Mann and Li 2013). This fragment-based approach has been very successful since it has allowed the marketing of a drug, vemurafenib (a drug for metastatic melanoma), in only six years (Baker 2013). In addition, several groups in the UK have joined to develop the 3D Fragment Consortium (http://www.3DFrag.org) devoted to the building of a shared library (500–3000 fragments) with enhanced three dimensionality of compounds that seem to be more advantageous than the traditional approach.

New technologies aimed at characterization of the contact surface between two interacting proteins, in conjunction to methodologies based on structure-based design, have facilitated the detection of small molecules that act as i-PPIs. The identification of compounds that may act as i-PPIs seems to be attainable by computational drug design (Bienstock 2012). There are a number of strategies that have been employed to check for hotspot regions in the protein–protein interfaces, such as protein crystallography to elucidate the 3D protein structure, molecular docking, virtual screening and approaches to design novel drug libraries (reviewed in depth by Bienstock 2012 and Huang 2014), although there is a strong need to have curated databases to help in the discovery of PPI-modulators (see, for instance: http://www.vls3d.com/). Development of novel databases to assist the finding and design of novel i-PPIs should prove to be useful for these purposes (Labbé *et al.*, 2013). A number of TA structures have been solved, which would lead to a more thorough approach to find small molecules that are able to disrupt the T:A interactions, for which the design should be customized since hydrophobic residues seem to dominate the interfaces between the toxin and its cognate antitoxin (Loris *et al.*, 1999; Hargreaves *et al.*, 2002; Kamada, Hanaoka and Burley 2003; Takagi *et al.*, 2005; Kamphuis *et al.*, 2007; Oberer *et al.*, 2007; Kumar *et al.*, 2008; Brown *et al.*, 2009; Francuski and Saenger 2009; Miallau *et al.*, 2009; Arbing *et al.*, 2010; Dienemann *et al.*, 2011; Bøggild *et al.*, 2012; Heaton *et al.*, 2012; Kwon *et al.*, 2012; Feng *et al.*, 2013; Samson *et al.*, 2013; Schureck *et al.*, 2014). Structural

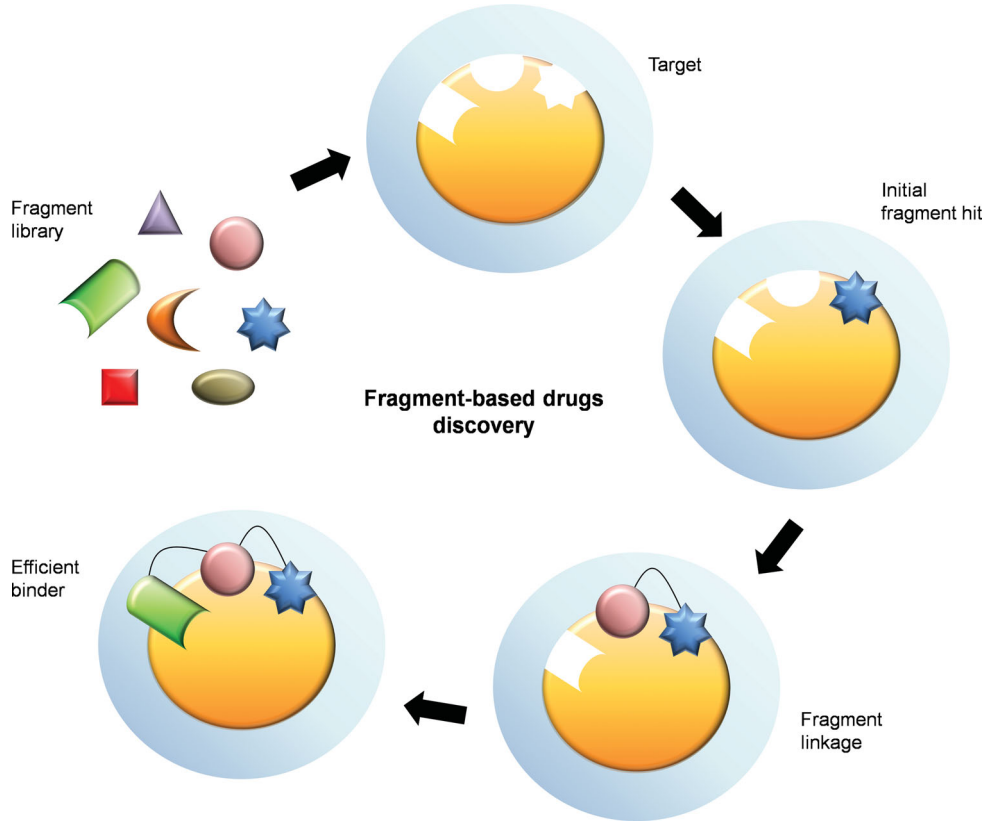

Figure 4. Combination of combinatorial chemistry with high throughput has contributed to the development of large screening libraries of compounds. However, the largest imaginable collection of compounds falls short of potential chemical diversity space. As molecular size decreases, the number of possible molecules decreases exponentially. Thus, at least from a theoretical point of view, it would result easier to screen large collections of very small molecules ('fragments') and, later on proceed to expand, merge or link them. Fragment screening is an excellent method for the identification and validation of lead compounds that can later on be tested for development of therapeutic agents. Fragments are small (MW <300 Da) and can provide the sampling of chemical space more effectively than other screening methods. Highly ligand efficient hits have been identified for several soluble proteins and for i-PPIs purposes. Determination of the 3D structure of the target proteins in conjunction to compounds with a greater degree of 3D shape is a good method to increase the diversity of libraries. Finally, through different rounds of chemical modifications and/or combination with other molecules, fragments with increased affinity for the target protein can be developed.

knowledge on TAs should enable us to get a better understanding on the surface contacts between the two protein pairs so that development of inhibitors is facilitated (Park, Mann and Li 2013). These approaches could be refined by employment of selected databases dedicated to PPIs that have already known inhibitors, so that small molecules can be taken as examples to construct guidelines to characterize i-PPIs (http://2p2idb.cnrs-mrs.fr).

OTHER STRATEGIES LEADING TO TOXIN DRUGGABILITY

A number of different strategies have been suggested as possible means to the druggability of toxins (Mutschler and Meinhart 2011; Chan *et al.*, 2013; Gerdes 2013; Unterholzner, Poppenberger and Rozhon 2013). These strategies are based on the finding that the antitoxin is more susceptible to degradation by the host proteases than its cognate toxin. Therefore, by inhibiting the replenishment of the antitoxin, the toxin will be released and can exhibit its toxicity to the cells while its cognate antitoxin is degraded. We can conceive a number of strategies, all having their pros and cons, as schematized in Fig. 5.

Firstly, one approach could be the inhibition of transcription of the TA pair so that autoregulation would not take place and synthesis of the TA would be hindered. By use of the different half-lives of the two proteins, the likelihood of degradation of the antitoxin and release of the toxin could be increased. How to achieve this goal? One possibility could be the use of a mutant of the antitoxin with enhanced DNA binding affinity ('super-repressor') but devoid of the ability to complex to its cognate toxin. This could be achieved as usually the binding site of the antitoxin to the DNA is at the N-terminal moiety whereas to its cognate toxin is at the C-terminus. Perhaps the challenge would be the binding affinity, as the antitoxins are, in general, weak repressors; exceptions have been reported as in the case of the TA pairs *mqsA–mqsR* from *E. coli* (Brown *et al.*, 2013) and *parDE* from plasmid RK2 (Davis, Helinski and Roberts 1992; Oberer *et al.*, 2002, 2007). Characterization of these features could be easily followed by a novel technique based on differential repression of the green fluorescent protein expression (Chan *et al.*, 2014).

Secondly, we could envisage the inhibition of translation of the antitoxin by antisense molecules. Even though both TA genes are co-transcribed as a single mRNA transcript, the toxin gene usually carries its own Shine–Dalgarno sequence that is embedded within its upstream antitoxin gene (Chan *et al.*, 2012). Thus, antisense RNA silencing of the antitoxin could be achieved by the binding of short RNA sequences (or specific oligonucleotides) to the antitoxin coding region or to the initiation of translation signals of the antitoxin, without

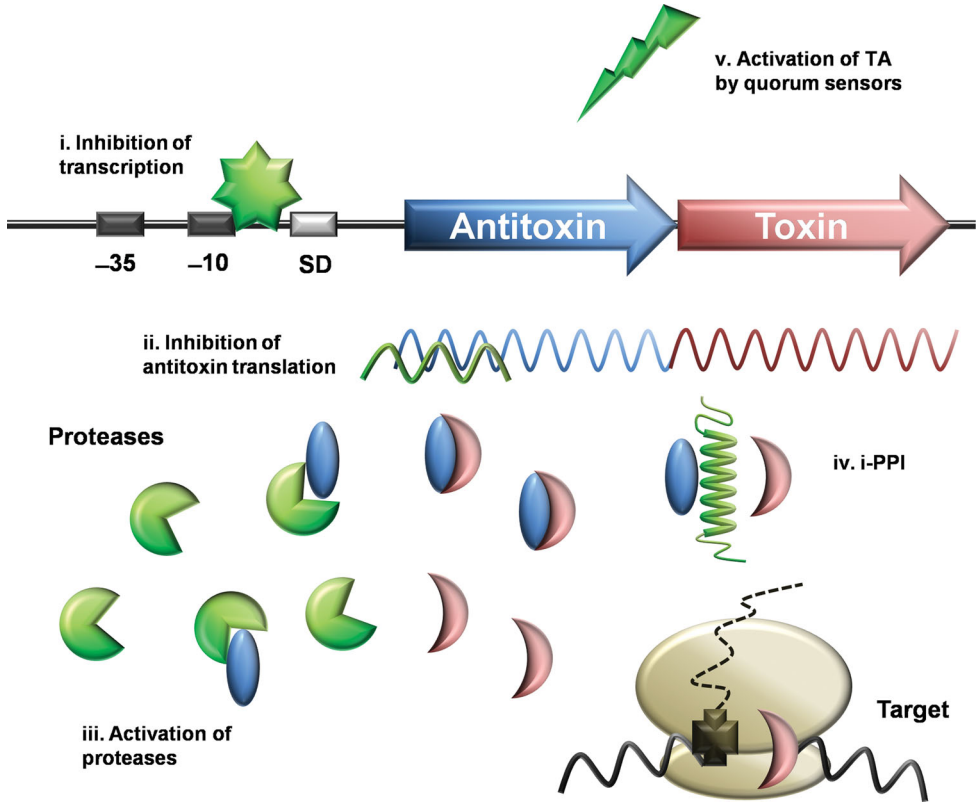

Figure 5. Proposed strategies to use TA as antimicrobials. A few approaches have been suggested to make use of the toxin (crescent) of the pathogen itself for self-killing: Inhibition of TA transcription (I) or inhibition of antitoxin (oval) translation (II), thus antitoxin cannot be replenished and once the remaining antitoxin is degraded, the toxin will be free to act on the bacterial cell; Activation of host proteases (III) to rapidly degrade the labile antitoxin proteins and disruption of TA protein complex by i-PPI (IV) to liberate the toxin, as well as triggering activation of TA by quorum sensors (V).

affecting translation of the toxin. However, a few factors have to be considered, such as the efficiency of the antisense to bind to the mRNA, the length of the antisense molecule, possible interference of the translation of the toxin protein, the stability of the antisense molecule (i.e. degradation by bacterial RNases) and also the double-stranded nature of the generated RNA after binding to the mRNA target.

Antisense antibacterial strategies had been developed since two decades ago by continuous refining of antisense oligodeoxynucleotides (AS-ODNs) to silence essential genes at mRNA/DNA levels. Unmodified RNA/DNA is labile and prone to degradation by nucleases, in addition to exhibiting poor pharmacokinetics properties. A whole plethora of AS-ODNs have been developed to improve the stability and intrinsic affinity to their targets, mainly via modification of the backbone, phosphodiester bond and sugar ring. Examples of the AS-ODNs derivatives include phosphorothioate oligodeoxyribonucleotides (PS-ODNs) (first generation); 2′-O-methyloligoribonucleotides (2′-OMes) and 2′-Omethoxyethyl oligonucleotides (2′-MOE) (second generation); locked nucleic acids, phosphorodiamidate morpholino oligonucleotides (PMOs), thiophosphoroamidate oligonucleotides and peptide nucleic acids (PNAs) (third generation) (Bai and Luo 2012). Among these molecules, PNAs and PMOs had gone through preclinical and clinical evaluation and proved to be promising antisense drugs (Bai and Luo 2012) (Nielsen and Egholm 1999). PNAs molecules are synthetic analogues of nucleic acids in which the phosphate-sugar backbone is replaced by a flexible pseudo-peptide polymer to which the bases are attached. Thus, PNAs can mimic and pair to DNA or RNA generating duplexes that are resistant to enzymatic degradation. Although PNAs have been proven as good silencer, delivery would be one of the major challenges that are not easy to tackle (Ray and Nordén 2000). The coupling of antisense molecules with modified peptides called membrane-penetrating peptides has been explored as useful antibacterials. One of the fascinating examples is peptide-conjugated PMOs. These compounds are synthetic oligomers mimicking the nucleic acid structure, with one end attached to a short peptide. The peptide is designed with a repeating sequence of cationic and non-polar amino acid residues that facilitate the oligomer to penetrate the Gram-negative outer membrane. These conjugated compounds can be targeted to silencing specific genes and that have been shown to be an active antibacterial (Geller *et al.*, 2013).

A third approach could be to target the proteases that cleave the antitoxin, which appears to be either Lon or ClpP (Van Melderen *et al.*, 1996; Christensen *et al.*, 2004), although there may be other not yet identified proteases. Induction of the proteases would lead to antitoxin degradation and release of the cognate toxin. Activation of proteases that degrade the antitoxins can lead to killing of bacterial persisters by the freed toxin (Williams and Hergenrother 2012); this approach, in combination to antibiotics, resulted in biofilm eradication *in vitro*. We consider that this could be a novel avenue to treatment chronic infectious diseases (Conlon *et al.*, 2013), like endocarditis, bronchitis, otitis, etc., or to tackle diseases that derive from the infection of internal prosthesis or catheter-related interventions (http://www.cdc.gov/hicpac/bsi/bsi-guidelines-2011.html).

Another interesting strategy, developed for the *mazEF* TA (a widespread TA among many bacteria, many of them pathogenic) has been proposed (Kumar and Engelberg-Kulka 2014). This involves triggering of the TA from outside by extracellular death factor (EDF), a short peptide that acts as a quorum sensor. Although EDF is species-specific, it seems that they can provoke triggering of *mazEF* in heterologous hosts: the *Pseudomonas aeruginosa* or the *B. subtilis* EDF can activate the *E. coli mazEF*, leading to death of the latter bacterial cells (Kumar, Kolodkin-Gal and Engelberg-Kulka 2013). This clever strategy might be enough as a starting point to develop a novel antibacterial that, however, would activate only this particular TA (Kumar and Engelberg-Kulka 2014).

Last but not least, since different TAs have different targets, selection of more than a kind of TAs could be an alternative to explore: the combined use of TAs that target cell walls (e.g. PezAT) with those that target mRNAs (MazEF) could lead to an antibacterial with more broad spectrum. However, even if any of the above approaches could be followed (and some of them merit a thorough experimental exploration), we believe that they would confront some major problems, such as the delivery strategies.

DRUG DELIVERY

One of the hurdles to overcome in designing new drugs is how to deliver them to the appropriate tissue target. Research in drug delivery is multidisciplinary, requiring knowledge of (i) how drugs work, (ii) their chemical and physical properties, (iii) how these properties affect the *in vivo* behaviour of the drugs, (iv) how these drugs reach their targets and (vi) what could be done to formulate these drugs to potentially solve any delivery problems associated with them. Therefore, drug delivery research necessitates interdisciplinary collaborations both at a national and international level. Although a range of delivery systems is available, the delivery of sensitive drugs such as peptides, nucleic acid-based therapeutics (including antisense RNA and small interfering RNAs), simple and complex carbohydrates, and synthetic vaccines presents a major challenge to the pharmaceutical industry. Formulation for novel molecules and drug delivery are very costly, since nearly 10% of the costs of drug development are devoted to these area (Allen and Cullis 2012). Some of the above-mentioned biologics could be formulated in ways that can be expensive or inconvenient: for instance, replacement of powders with ready-to-use liquids may be an important improvement, but storage of the material (cold versus shelf storage) may constitute an important bias against employment of proteins, so that improving heat stability will lead to simpler transportation and distribution processes.

New developments in drug delivery research are likely to have enormous economic impacts upon the pharmaceutical and biotechnology industries. In fact, drug delivery research represents a US$70 billion a year industry. Centres for Drug Delivery and Formulation have been established all over the world to promote research, development and training in drug delivery science. There has also been a focus on realizing the commercial potential of innovative molecules (e.g. peptides, nucleic acid based therapies and vaccines), or delivery technologies that are developed from the basic research centres. Thus, it is no wonder that a number of novel clever approaches have been proposed to deliver new drugs, as it has to be the case if bacterial toxins are going to be developed as antimicrobials. One interesting system, albeit still hypothetical, could be the employment of bacterial minicells for delivery (Fig. 6; de la Cueva-Méndez 2013). Minicells are produced by bacteria defective in cell division; they lack chromosomal DNA, but plasmids do segregate into them and continue to be metabolically active. Thus, as early shown in many laboratories, ours among them (Espinosa, Lopez and Lacks 1984; Lacks *et al.*, 1986), plasmids in minicells are able to synthesize *de novo* the products they encode, including TA proteins, as shown for the *kis–kid* pair (Bravo, de Torrontegui and Díaz 1987; Bravo *et al.*, 1988). Plasmids encoding the desired TA construction will segregate into the minicells, making them good delivery candidates to the targeted cells (MacDiarmid *et al.*, 2007). We must remark that minicells do not seem to be appropriate enough for the treatment of bacterial infections as minicells will not be engulfed by prokaryotic cells; they could be envisaged, however, as useful delivery solutions for the treatment of cancer cells (MacDiarmid *et al.*, 2007; de la Cueva-Méndez 2013).

Another interesting delivery approach is to make use of cell-penetrating peptides (CPPs) as carrier peptides (Sebbage 2009). CPPs are peptides (up to 30 amino acids) that can be internalized by energy-free mechanisms not well understood yet. CPPs have been shown to be readily internalized by mammalian cells and be effective carriers for drug development (Zorko and Langel 2005). However, CPPs approaches would seem more appropriate to use the toxins as antivirals (Chono *et al.*, 2011a,b) than antibacterials because of these peptides would not overcome the barrier offered by the bacterial cell wall. We could imagine that coupling the toxins to microcin peptides (e.g. lasso peptides; Rebuffat 2012) that penetrate the bacterial cell wall might offer an alternative to employment of CPPs. Novel approaches, yet to be fully developed are those carriers constituted by carbon nanotubes (CNTs). They are nanomolecules made by layers of graphene that can be disposed in layers that can be built as needle-shaped cylinders (Bai and Luo 2012). CNTs have been used to inject plasmid DNA into *E. coli* cells (Rojas-Chapana *et al.*, 2005). CNTs have been approached as useful carriers for delivery of some antimicrobials, although their use as anticancer drugs is more advanced in drug therapy (reviewed in Wong *et al.*, 2013). However, so far it seems that cytotoxicity of these molecules to patients has not been overcome.

Perhaps a more elegant strategy, but limited because it is species-specific, would be the use of recombinant bacteriophages, defective for lysis and harbouring the desired toxin gene cloned under the control of an early (usually very strong) promoter. Upon injection of the phage DNA, the toxin gene will be transcribed and translated, releasing the toxin which will kill the pathogens (Huys *et al.*, 2013; Samson *et al.*, 2013).

CONCLUSIONS AND FINAL THOUGHTS

We have presented here a number of plausible, and sometimes, speculative, approaches to tackle the druggability of TAs. We have also tried to discuss different angles on the subject as well as to present it in a thought-provoking manner. Fortunately, an increasing number of protein TA structures are being solved, which will facilitate the study of the TA interfaces. This, in turn, will permit to perform *in silico* docking experiments to design molecules that may disrupt the T: A interactions. In our opinion, a fragment-based drug design to deal with the disruption of the TAs interactions seems to be one of the most likely approaches to develop TAs as good candidates to enter into the antibacterials pipeline. Perhaps the most useful approach would be (i) rationale design of the fragments library based on the known structures of the TAs solved so far (Park SJ *et al.*, 2013), and

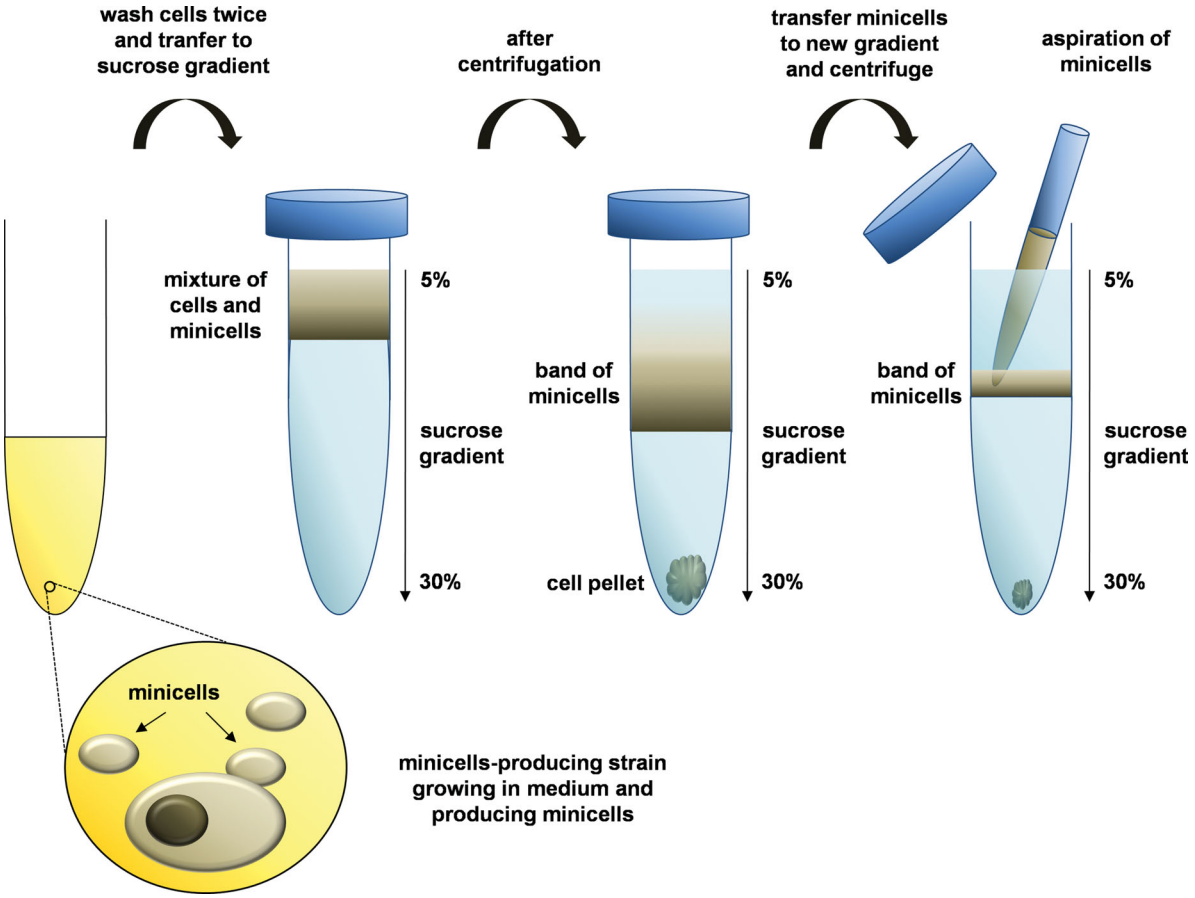

Figure 6. What are minicells? Bacterial cells that harbour mutations in some of their genes involved in cell division (like *min* mutants in *E. coli* or *divIV* mutants in *B. subtilis*) were shown to have a septum abnormally positioned, resulting in a cell of normal size and a minicell. Because of their small size (around 400 nm), minicells can be separated and purified from normal-sized cells by employment of two successive buoyant density sucrose gradients. Purified minicells can be stored with 10% glycerol at –70 °C without loss of their biological activity. Due to the abnormal chromosome segregation, minicells lack chromosomal DNA; however, they are metabolically active and have all the biochemical machinery to synthesize proteins. When a minicell-producing strain harbours a plasmid, these plasmids would segregate into the normal cells and into the minicells. Thus, determination of *de novo* protein synthesis by minicells has been successfully employed to characterize plasmid-encoded proteins, including TAs (Lacks *et al.*, 1986, Bravo *et al.*, 1987, 1988).

(ii) employment of fragments designed to bind to the C-terminal moiety of the antitoxin, which seems to be less structured than the N-terminal domain (the appropriateness of the chemical space between the two proteins). An exciting alternative to explore is the employment of antisense small RNAs as the molecules to inhibit the translation of the operon (Ji *et al.*, 1999; Faridani *et al.*, 2006; Makarova, Grishin and Koonin 2006; Kawano, Aravind and Storz 2007; Mruk and Kobayashi 2014) in combination to an appropriate delivery system, perhaps engineered bacteriophages or peptides.

However, and before there is a good proof of principle on the druggability of the TAs proteins as effective antibacterials, other approaches could be envisaged. One of them would be to diagnose first several of the TAs encoded by the infecting bacterium through PCR amplification using sets of specific oligonucleotides per each TA pair. Identification of a set (or of all) TAs could be easily achieved by entering into an appropriate database like, for instance, TADB (http://bioinfo-mml.sjtu.edu.cn/TADB/). This could be followed by administration of the most indicated drug for the identified infecting agent rather than a random drug use (and perhaps abuse).

All in all, we believe that research on new antibacterials should meet the needs of the day-to-day clinicians working in hospitals, where fast responses should be granted within easy technological access to user-friendly databases. If we could conceive such a scenario, then novel avenues will open to explore real novel drugs.

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