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Review Article

REVIEW ARTICLE

Toxin-antitoxins and bacterial virulence

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One sentence summary: In this review, the authors discuss toxin–antitoxin systems due to their capacity to modulate bacterial physiology in response to stress, which, as a consequence, can influence host–pathogen interactions.
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ABSTRACT

Bacterial virulence relies on a delicate balance of signals interchanged between the invading microbe and the host. This communication has been extensively perceived as a battle involving harmful molecules produced by the pathogen and host defenses. In this review, we focus on a largely unexplored element of this dialogue, as are toxin–antitoxin (TA) systems of the pathogen. TA systems are reported to respond to stresses that are also found in the host and, as a consequence, could modulate the physiology of the intruder microbe. This view is consistent with recent studies that demonstrate a contribution of distinct TA systems to virulence since their absence alters the course of the infection. TA loci are stress response modules that, therefore, could readjust pathogen metabolism to favor the generation of slow-growing or quiescent cells 'before' host defenses irreversibly block essential pathogen activities. Some toxins of these TA modules have been proposed as potential weapons used by the pathogen to act on host targets. We discuss all these aspects based on studies that support some TA modules as important regulators in the pathogen–host interface.

Keywords: bacterial pathogen; toxin–antitoxin system; stress; survival; intracellular; lifestyle

INTRODUCTION

Toxin–antitoxin (TA) systems were discovered as plasmid maintenance modules. The first three systems described were *ccdAB* of plasmid F (Ogura and Hiraga 1983; Jaffe, Ogura and Hiraga 1985), and *hok-sok* (*parB*) and *kis-kid* (*parD*) encoded by the plasmid R1 (Gerdes, Rasmussen and Molin 1986; Bravo, de Torrontegui and Diaz 1987). These operons contain a stable toxin that is always a protein and an unstable antitoxin that can be either a protein, as CcdA or Kis, or a small RNA, as Sok. To neutralize the toxin, antitoxins must be continuously synthesized. In plasmid-free segregants, the unstable antitoxins cannot be replenished leaving the toxin free to eliminate or arrest growth of these cells. Elimination of plasmid-free segregants was termed 'post-segregational killing' or PSK (Gerdes, Rasmussen and Molin 1986). The net result in both cases is plasmid maintenance in the bacterial population. Although TA systems were originally discovered on plasmids, the advent of genome sequencing era further led the unexpected discovery of multiple TA systems encoded in the chromosome (Gerdes, Christensen and Lobner-Olesen 2005; Pandey and Gerdes 2005; Van Melderen and Saavedra De Bast 2009). Chromosomal TA modules are logically unrelated to plasmid stability and their functions have been linked to distinct metabolic processes and growth control in response to adverse environments (Wang and Wood 2011; Yamaguchi and Inouye 2011). It has been proposed

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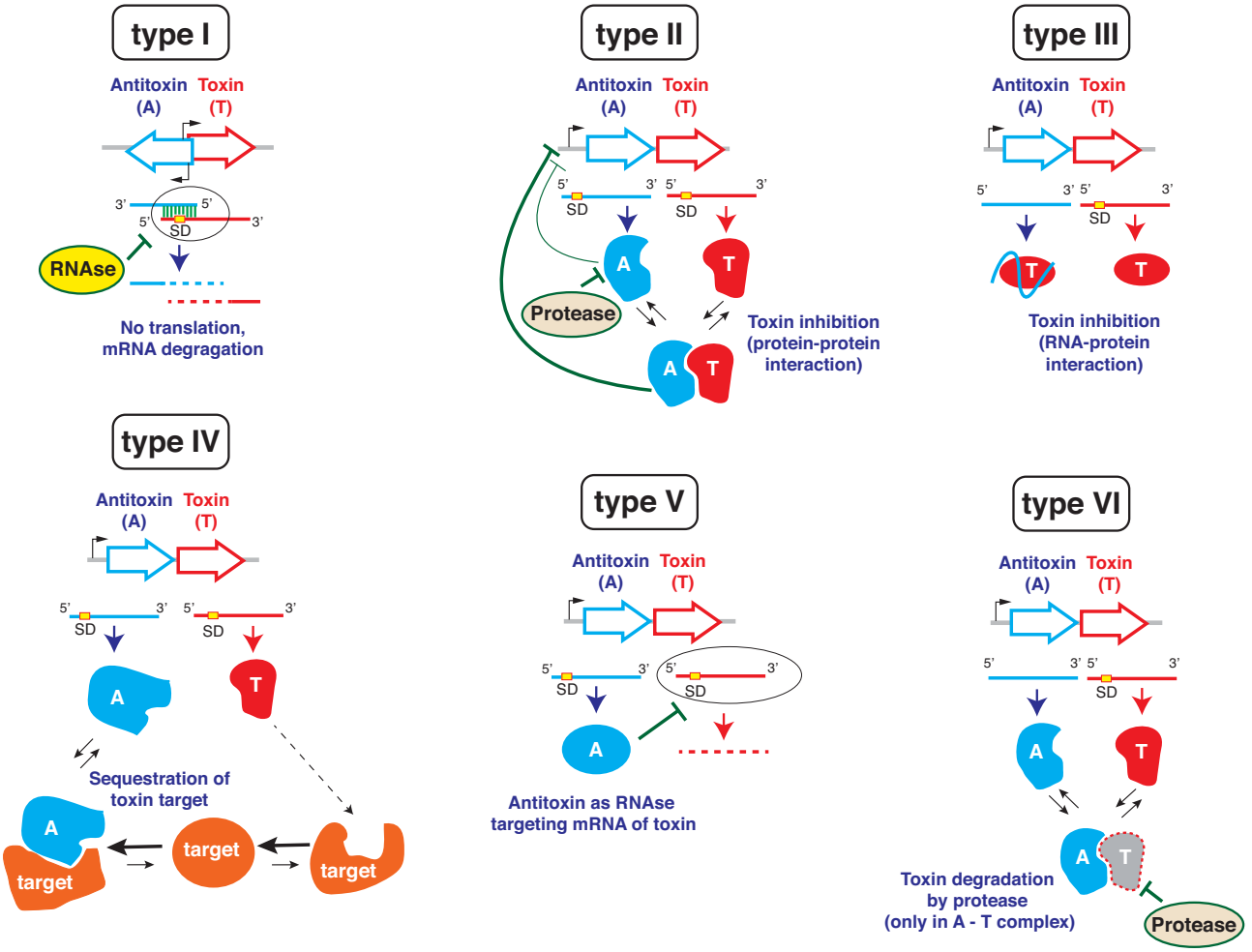

Figure 1. Types of TA systems characterized to date. Toxins (T) and antitoxins (A) are indicated in red and blue, respectively. The schemes depict the main features of each system. Type I: the antitoxin is an antisense RNA molecule that pairs with the toxin-coding mRNA. The duplex is targeted by RNAses preventing in this manner translation of the toxin. Type II: the antitoxin is a protein that blocks toxin activity by direct protein–protein interaction. In most cases, the T-A complex binds to the promoter to efficiently repress transcription of both elements. Antitoxin alone can also bind to and repress its own protomer, but less efficiently than in combination with the toxin. Levels of the antitoxin are accurately controlled by proteases. Type III: the antitoxin is an RNA that blocks toxin activity by direct interaction with the toxin. Type IV: both toxin and antitoxin binds the same target. The antitoxin inhibits toxin activity by competing for binding to a common substrate. Type V: the antitoxin has RNAse activity and targets the toxin transcript. Type VI: the antitoxin favors toxin degradation upon formation of the T-A complex; in this conformation, the toxin is selectively targeted by proteases. SD, Shine-Dalgarno sequence.

that chromosomal TA systems could induce programmed cell death in part of the bacterial population as an altruistic strategy to survive adverse situations (Yarmolinsky 1995; Aizenman, Engelberg-Kulka and Glaser 1996). Bioinformatic analyses have shown that many chromosomally integrated mobile genetic elements (MGE), such as pathogenic islands and conjugative or mobilizable integrons, contain TA systems (Makarova, Wolf and Koonin 2009; Cambray, Guerout and Mazel 2010). Like plasmidic TA modules, those TA encoded in MGE have the potential to contribute to the maintenance of these genetic elements in the genome, thereby contributing to spread MGE-encoded resistance or virulence determinants (Hernandez-Arriaga *et al.* 2014). The differential stability of the toxin and its antitoxin is a simple but effective strategy that can be exploited by the bacteria for different purposes. These include plasmid maintenance, bacterial adaptation to hostile environments, stabilization of chromosomal regions and bacterial survival during infection. A compilation of functional and structural data of the main TA systems found in prokaryotic organisms has been published recently (Gerdes 2013). In this review, we focus on the most recent evidences that connect TA systems with bacterial pathogenesis.

Classification of TA systems

TA systems are currently classified into six types depending on the nature and activity of the antitoxins (Page and Peti 2016): antitoxins of proteinaceous nature in type II, IV, V and VI TA systems, and small regulatory RNAs in types I and III (Fig. 1). In the extensively studied type II systems, antitoxins interact with and neutralize their cognate toxins (Yamaguchi and Inouye 2011). Type II antitoxins have a structured DNA-binding motif, usually at the N-terminal region and a disorganized distal C-terminal region involved in toxin interaction and neutralization. In the absence of the toxin, the C-terminus of antitoxins is exposed and cleaved by ATP-dependent proteases determining the differential stability between toxins and antitoxins (Chan, Espinosa and Yeo 2016). Interaction of the antitoxin with DNA represses transcription of these systems. Moreover, in type II TA modules toxins act as corepressors when interacting

with its cognate antitoxin. This transcriptional regulation is in many type II systems dependent on the toxin:antitoxin ratio. An excess of antitoxin results in TA complexes that are efficient repressors. However, when the number of molecules of toxin increases, the stoichiometry of the complex changes and repression is relieved. This regulation feature, named 'conditional cooperativity', is proposed to modulate a bistable situation dominated either by the toxin or the antitoxin that favors either entrance or the exit from a bacteriostatic status (quiescence or persistence) induced by the toxin (Cataudella *et al.* 2012; Maisonneuve and Gerdes 2014; Page and Peti 2016). Bistability is a bifurcation of bacterial cultures in two different populations in which particular genes are differently expressed. This phenotypic heterogeneity can be triggered by reversible switchers and represent a strategy that favors bacterial survival in response to particular stresses (Dubnau and Losick 2006).

In type IV TA modules, the proteinaceous antitoxin neutralizes its cognate toxin via toxin-target blockage instead of a direct protein–protein interaction. The prototype of type IV systems is *yeeUV*. The antitoxin YeeU permits bundling of cytoskeletal proteins MerB and FtsZ protecting their polymerization from the inhibitory action of the YeeV (CbtA) toxin (Masuda *et al.* 2012a,b). In the only type V TA module reported to date, *ghoST,* the toxin GhoT is a small hydrophobic peptide that disrupts membrane potential and therefore ATP synthesis (Wang *et al.* 2012). GhoS, the antitoxin of this system, is an RNase that cleaves *ghoT*-encoding mRNA thus preventing synthesis of the toxin (Wang *et al.* 2012). This is the only case described in which the antitoxin has an enzymatic activity. The regulation of *ghoST* system goes beyond the antitoxin, as a second TA locus, *mqsRA*, controls *ghoS* transcript levels. *ghoS* mRNA is specifically cleaved by the RNase activity of the MqsR toxin (Wang *et al.* 2013). Thus, the *msqAR* and *gohST* systems act coordinately to regulate the activity of a transmembrane toxin, GhoT.

socAB constitutes a new type (VI) of TA system (Aakre *et al.* 2013). SocA antitoxin is an adaptor protein that binds SocB toxin. Unlike type II TA modules, SocA does not neutralize SocB directly but promotes its degradation by a specific protease.

Type I and III TA loci have antitoxins that are RNAs. In type I modules, antitoxins are small antisense RNAs that interact at the 5′ or 3′ regions of toxin mRNAs preventing their translation (Gerdes and Wagner 2007; Fozo *et al.* 2008b; Wen and Fozo 2014) (Fig. 1). The best studied type III TA systems is *toxIN* (Fineran *et al.* 2009). Antitoxin ToxI is a short RNA generated by the RNase activity of the ToxN. *toxN* mRNA is preceded by a transcription termination signal and before by a tandem of repeats. Processing of these repeats by the RNase activity of ToxN originates ToxI antitoxin, composed of small RNA pseudonots that bind ToxN and neutralize its RNase activity (Short *et al.* 2013).

Usually TA loci are bicistronic operons but occasionally they can encode a third proteinaceous component involved in (i) transcriptional regulation of the operon (e.g. *ωεζ* or *paaR-paaA-parE* TA systems) or (ii) acting as chaperon that allows proper folding of the antitoxin (*higBA* TA system of *Mycobacterium tuberculosis*) (Zielenkiewicz and Ceglowski 2005; Hallez *et al.* 2010; Bordes *et al.* 2011). A recent comprehensive survey of the TA systems can be found in Gerdes (2013).

Toxin-mediated regulation of bacterial physiology

Toxins of TA loci target essential physiological processes, including protein synthesis, DNA replication, cytoskeletal proteins assembly during cell division, cell-wall synthesis, ATP synthesis driven by membrane potential and, RNA or DNA integrity (Gerdes 2013; Guo *et al.* 2014). Depending on the doses and on the specific TA system, these toxins can inhibit cell growth or induce cell death (Yamaguchi and Inouye 2011). In general, protein translation inhibition by toxins of type II modules reflects a reversible cell growth arrest (Gerdes 2013). However, depolarization of cell membrane potential by hydrophobic peptides of type I TA loci (Gerdes, Rasmussen and Molin 1986) or poisoning of DNA gyrase by type II toxins, such as CcdB or ParE, causes cell death. CcdB or ParE activity results in severe disturbance of chromosomal topology, inhibition of DNA synthesis, induction of breaks and nicks in the DNA, and finally cell death (Jaffe, Ogura and Hiraga 1985; Bernard and Couturier 1992; Jensen and Gerdes 1995; Jiang *et al.* 2002). Additionally, the toxin of the chromosomal type II TA system *mazEF* is thought to induce cell death in part of the bacterial population. Under particular cell growth conditions, this altruistic behavior would liberate nutrients that would allow survival of the rest of the population (Yarmolinsky 1995; Aizenman, Engelberg-Kulka and Glaser 1996). Regardless of the individual action of a defined toxin, most organisms have different TA systems, which opens the possibility of crosstalk between diverse toxins and antitoxins to ultimately modulate cell growth and viability (Goeders and Van Melderen 2014). Recent studies have shown interaction between toxins and antitoxins of either type II only or type I–type II TA modules (Zhu *et al.* 2010; Smith *et al.* 2012; Wang *et al.* 2013; Wessner *et al.* 2015). These interactions provide a strong support to the idea of a coordinated action for some of these TA modules.

Induction of TA systems relies on a highly controlled inactivation of antitoxins, either by RNases or proteases. These are complex phenomena whose mechanisms are still poorly understood. Most studies have focused in type II TA modules (Brzozowska and Zielenkiewicz 2013). These studies show that, in general, a defined antitoxin is degraded by a protease such as Lon or, alternatively, by two-component proteases such as ClpAP, ClpXP or ClpCP. Alternatives to this general scheme are also known. Thus, more than one protease can inactivate a particular antitoxin. For instance, MazE is cleaved by Lon in fast growing cultures of *Escherichia coli* and by the ClpAP protease in stationary phase cultures (Aizenman, Engelberg-Kulka and Glaser 1996; Christensen *et al.* 2003). A single protease, such as ClpCP, activates multiple TA systems in *Staphylococcus aureus* (Donegan *et al.* 2010). In the *kis-kid* system of plasmid R1, the antitoxin Kis is cleaved by the ClpAP protease and this cleavage is modulated by the relative toxin:antitoxin ratio (Diago-Navarro *et al.* 2013). Stimuli that ultimately control these proteases or RNases are also poorly understood. The alarmone (p)ppGpp, which is synthesized under aminoacid starvation, is required for Lon protease-dependent activation in some type II TA modules and for persistence (Maisonneuve, Castro-Camargo and Gerdes 2013). More recently, (p)ppGpp was also shown to be required for Obg GTPase-dependent transcription of *hok*, the toxin gene of type I *hok-sok* TA system (Verstraeten *et al.* 2015). Although the action of proteases and RNases are mechanistically well established, the signals that regulate their activities in response to stresses, some of which probably encountered by pathogens inside their hosts (Fig. 2), is a topic that attracts growing interest (see later section, 'Responsiveness of TA systems to host signals').

Methods for the identification of TA modules

The unsolved question of why bacteria possess so many TA systems could have an answer in the capacity of many bacteria to adapt to diverse adverse conditions. This plethora of

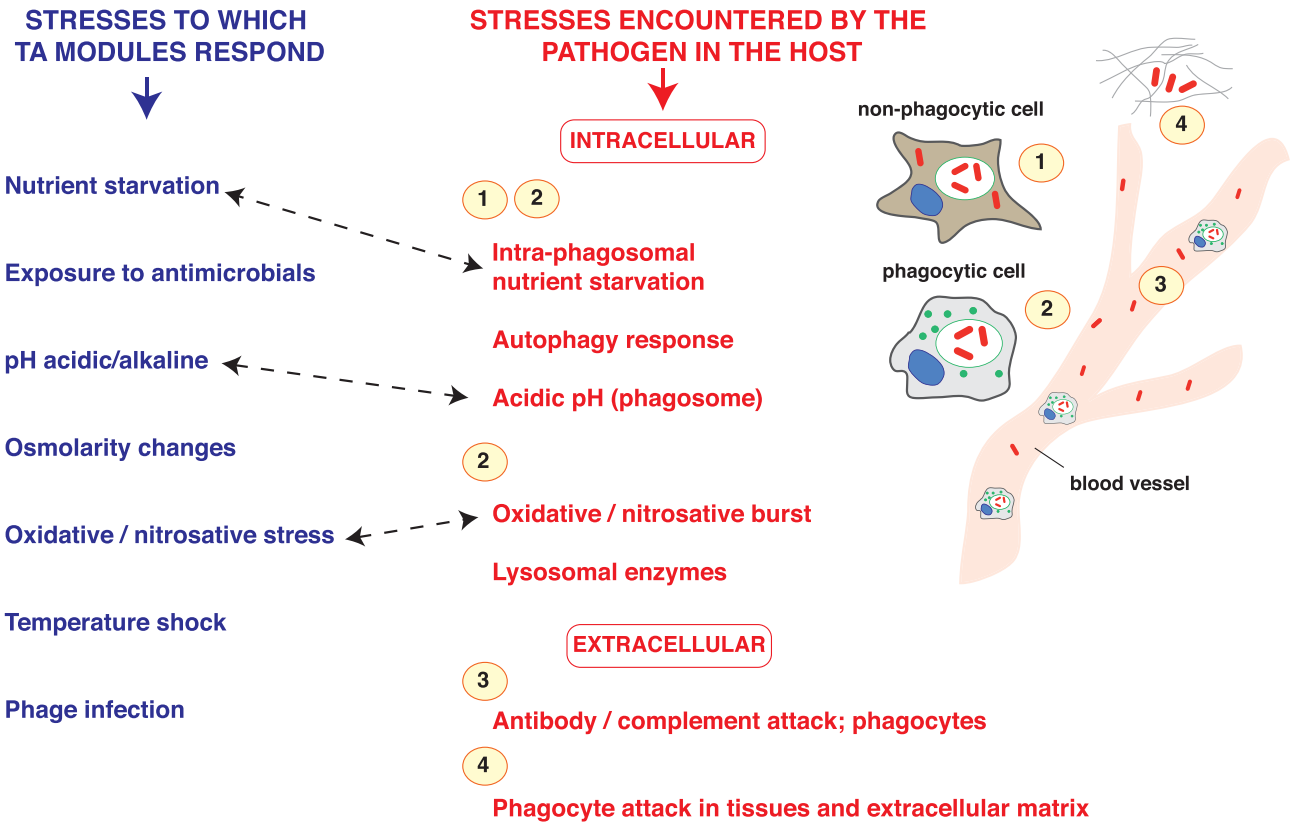

Figure 2. Parallelisms between host stress responses and stimuli to which TA systems are responsive. Indicated are stresses to which TA systems are known to respond (left side) and stresses faced by pathogen following encounter with the host (right side). Numbers refer to stresses encountered in different host environments: **(1)** the intracellular niche of a non-phagocytic cell (fibroblast, epithelial cells); **(2)** the phagosomal compartment of phagocytic immune cells (macrophages, dendritic cells); **(3)** complement proteins and circulating antibodies in fluids such as blood; and **(4)** exposure to antimicrobial molecules and attack of phagocytes in intercellular sites (matrix) of tissues and organs.

TA modules requires global approaches capable of unraveling their identity to further investigate how they combine to fine-tune bacterial responses to stress. This is relevant in bacterial pathogens, which cope with host-derived stresses during infection. In this section, we briefly describe the most common tools and databases used to identify TA systems (Table 1), which could be easily applicable to the model pathogen of interest.

The first massive search of TA systems in prokaryotes was based on sequence homology of toxins and antitoxins (Pandey and Gerdes 2005). Using BLASTP and TBLASTN, this study identified members of diverse TA families not only in plasmids but also in prokaryotic chromosomes (Table 1). Nucleotide and amino acid sequence similarities restrict however TA identification to those genes for which a clear homolog exists. New search methodologies were introduced to solve this drawback. The software named 'Rapid Automated Scan for Toxins and Antitoxins' in Bacteria (RASTA-Bacteria) is the most common tool for type II TA system discovery (Sevin and Barloy-Hubler 2007). This free online application (http://genoweb.univ-rennes1.fr/duals/RASTA-Bacteria/index.php?page=home) permits the screening of genome sequences using RPSBLAST, a 'Reverse PSI-BLAST' that searches for conserved domains (Altschul *et al.* 1997). RASTA-Bacteria algorithm filters hits using three features of TA systems: (i) presence of common TA domains, (ii) genes of small size and (iii) pairing of an ORF with another (Table 1).

TA systems are prone to horizontal gene transfer and intragenomic recombination (Pandey and Gerdes 2005; Sevin and Barloy-Hubler 2007; Makarova, Wolf and Koonin 2009). Keeping in mind the high interchange rate of genetic elements in prokaryotes, a new study utilized phyletic patterns of 'Clusters of Orthologous Genes' (COGs) to develop a search strategy for predicted bicistronic operons not uniformly distributed among prokaryotic genomes (Makarova, Wolf and Koonin 2009). Using COGs candidates and already known TA families, these operons were used as queries for PSI-BLAST analysis (Table 1). PSI-BLAST allows the identification of proteins with similar function but with no or very low sequence homology (Schaffer *et al.* 2001). Complementary to these two approaches, a 'guilt by association' principle was also implemented (Aravind 2000; Galperin and Koonin 2000; Gabaldon and Huynen 2004). In the context of TA systems, this principle states that a gene flanking another gene encoding an antitoxin or a toxin may encode its toxin or antitoxin partner, respectively. In 2011, a similar report used experimentally validated TA pairs, iterative PSI-BLAST and the 'guilt by association' principle to analyze an extensive collection of bacterial, plasmidic and phage genomes (Leplae *et al.* 2011). The aforementioned procedures and databases have been integrated into a free online software denominated 'Toxin-Antitoxin Data Base'—TADB—(http://202.120.12.135/TADB2/tools.html), which allows the identification of new type II TA systems (Table 1). TADB also includes new validated TA systems absent in previous databases (Shao

Table 1. Methodologies employed for the *in silico* identification of putative TA systems.

Type of TA system detected	Resource	Possibility of analyzing new genomes	Approach	Reference
II	Database	No	BLASTP and TBLASTN	Pandey and Gerdes (2005)
II	On-line free software (RASTA Bacteria) (http://genoweb.univ-rennes1.fr/duals/RASTA-Bacteria/index.php?page = home)	Yes	RPSBLAST, presence of common TA domains, genes of small size and pairing of an ORF with another.	Sevin and Barloy-Hubler (2007)
II	Database	No	Phyletic patterns of COGs, PSI-BLAST, 'guilt by association' principle	Makarova, Wolf and Koonin (2009)
II	Database	No	PSI-BLAST, 'guilt by association' principle	Leplae *et al.* (2011)
II	Online free software (TADB) (http://202.120.12.135/TADB2/tools.html)	Yes	BLAST against TADB. RPSBLAST against NCBI. Includes data from RASTA, Pandey and Gerdes (2005) and Makarova, Wolf and Koonin (2009)	Shao *et al.* (2011)
II	Database	No	Shotgun cloning and Sanger-based sequencing	Sberro *et al.* (2013)
I	Database	No	PSI-BLAST and TBLASTN, small genes encoding proteins (<70 amino acids) with transmembrane domains, tandemly repeated genes	Fozo *et al.* (2010)
III	Database	No	Structure-based homology searches, toxin-encoding gene preceded by a short palindromic repeat, in turn, preceded by a tandem repeat constituting the antitoxin, iterative BLAST	Blower *et al.* (2012)

et al. 2011). From our point of view, TADB website is to the date the most comprehensive and powerful tool to identify type II TA systems.

Shotgun cloning and massive sequencing were recently used to identify novel members of the type II TA family (Sberro *et al.* 2013). This work exploited the fact that a toxin can only be cloned when its cognate antitoxin is present (Table 1). Several drawbacks should be however considered to this study. First, TA operons encoding very small antitoxins were not detected. Second, only TA systems active in *E. coli* can be detected by this approach. Third, false TA pairs were also detected, that is, a gene whose expression is toxic to the cell in the absence of the neighbor gene which apparently acts as an antitoxin. This last case has been previously documented for *E. coli* replication proteins DnaB and DnaC. Unbalanced overproduction of DnaC results toxic because of helicase activity inhibition of DnaB. However, simultaneous synthesis of DnaC and DnaB blocks growth inhibition mediated by DnaC overproduction (Allen and Kornberg 1991). The main function of the toxin in a bona fide TA module must be directly related to cell growth inhibition or death (Gerdes 2013).

Massive searches for type I and type III TA systems are also known. Identification of type I TA modules has two main disadvantages: the toxins of these systems are small hydrophobic proteins (less than 70 amino acid in length) and the antitoxins are small antisense RNAs. Methodological problems associated with a similarity search for small proteins are well recognized (Koonin and Galperin 2003). To overcome these problems, Fozo *et al.* (2010) developed a new algorithm to identify type I TA modules based on the following: (i) PSI-BLAST and TBLASTN searches using experimentally characterized toxins of type I modules; (ii) the search of genes encoding transmembrane proteins of less than 70 amino acids; (iii) the selection of candidates with intergenic regions higher than 400 nt from the upstream gene and lower than 250 nt from the downstream gene (data empirically obtained from previous observations); (iv) the exclusion of pseudogenes; and (v) the tendency of type I TA loci to be tandemly duplicated (Kawano *et al.* 2002; Fozo, Hemm and Storz 2008a) (Table 1).

New type III TA modules were identified following the discovery of the *toxIN* TA system (Fineran *et al.* 2009). Protein structure-based homology searches against bacterial and archaeal proteomes were performed based on the crystallized toxin ToxN (Blower *et al.* 2012). This analysis was subsequently cured using known features of type III TA systems (Blower *et al.* 2009, 2011; Fineran *et al.* 2009). Positive hits were used as templates for iterative BLAST searches leading to the discovery of 125 type III TA systems distributed among a wide number of bacterial and archaeal species (Blower *et al.* 2012) (Table 1).

TA SYSTEMS IN THE HOST–PATHOGEN ENCOUNTER

To establish a successful infection, bacterial pathogens withstand multiple stresses and changing environments encountered during host colonization (Ribet and Cossart 2015). During the journey from the external non-host environment to the host, the invading microbe deals with multiple host defenses. For instance, enteric pathogens encounter acidic pH in the stomach (De Biase and Lund 2015) and then further compete with the intestinal microbiota. Other important host defenses in the intestinal lumen are the cationic antimicrobial peptides (CAMP) and immunoglobulins secreted by intestinal cells (Santaolalla and Abreu 2012; Kagnoff 2014).

Activated phagocytes (macrophages, dendritic cells) constitute the main cellular defense barrier of the host. Following

phagocytosis, these cell types force the mobilization of the ingested microbe to acidified phagolysosomal compartments (Weiss and Schaible 2015). These compartments are a harmful niche avoided by successful intracellular bacterial pathogens that subvert the vesicular trafficking machinery of the host cell (Baxt, Garza-Mayers and Goldberg 2013). Additional stresses imposed by the host in response to the infection include reactive oxygen and nitrosative intermediates (Green, Rolfe and Smith 2014) and deprivation of nutrients such as iron in both extracellular and intracellular locations (Silva-Gomes *et al.* 2013; Becker and Skaar 2014). Other highly reactive metabolites, such as formaldehyde, are documented as damaging molecules for the invading microbe (Chen *et al.* 2016). Autophagy is another important cellular defense against pathogens that inhabit intracellular locations (Huang and Brumell 2014).

Bacterial pathogens need to adjust its virulence gene expression in a proper spatial and temporal manner to ensure successful host colonization (Green, Rolfe and Smith 2014; Moorthy, Keklak and Klein 2016). This transcriptional readjustment is directed by host-specific signals and is crucial to ensure viability in the harsh conditions imposed by the new niche to be colonized. Bacterial systems that sense molecular oxygen or nitric oxide, acidic pH, nutrient limitation or CAMP are representative examples (Dalebroux and Miller 2014; Green, Rolfe and Smith 2014). For instance, the intracellular pathogen *Salmonella enterica* uses the two-component systems PhoP-PhoQ to sense low pH, nutrient starvation and CAMP in the intraphagosomal environment stimulating the expression of many virulence factors (Fass and Groisman 2009; Dalebroux and Miller 2014). Other consequence linked to perception of host-derived signals by the PhoP-PhoQ system is the change of the outer membrane composition, resulting in decreased susceptibility to CAMP and less recognition by the innate immune system (Ernst, Guina and Miller 2001). These cases exemplify how signals and stresses imposed by the host act in the invading pathogen driving changes in its physiology that will ultimately favor progression of the infection.

TA loci in genomes of pathogenic and non-pathogenic bacteria

The first genomic comparative analysis of TA loci performed by Pandey and Gerdes (2005) noticed two major findings: (i) the absence of TA loci in the genomes of some obligate intracellular bacteria (both pathogenic and non-pathogenic) and (ii) the presence of TA loci in all archaea and free-living or facultative intracellular bacteria. Subsequent analyses involving a much larger number of genomes have partially changed those initial conclusions. For example, a study performed with 24 genomes showed that TA loci are more abundant in genomes of pathogens associated with severe or chronic human infections compared to closely related bacteria, either non-pathogenic or causing only mild diseases (Georgiades and Raoult 2011). This study also showed that there is no apparent correlation between the presence of TA loci and the extent of the genome undergoing degradation, denoted by a relative abundance of pseudogenes. A remarkable example is that of *Rickettsiae*. These are obligate intracellular pathogens for which now several studies demonstrate that they do have TA loci (Audoly *et al.* 2011; Socolovschi, Audoly and Raoult 2013; Merhej *et al.* 2014) despite having genomes in which gene loss has been prominent, with an average of ~1.1–1.3 Mb in size. TA loci distribution in *Rickettsia* genomes reveals a positive correlation between their presence and the capacity of the pathogen to be vertically transmitted in arthropod hosts and to cause inoculation eschar in human and other animal hosts (Socolovschi, Audoly and Raoult 2013). TA loci of *Rickettsiae* have been postulated to control basic metabolism of these obligate intracellular bacteria and/or contribute to partial renewal of the bacterial populations during periods of stress and starvation (Merhej *et al.* 2014).

Mycobacteria have also been extensively studied at the genome level for the presence of TA loci. Comparative genome studies in mycobacteria reflect two interesting features. First, these bacteria have a plethora of TA loci in all genomes analyzed belonging to different families and types (Sala, Bordes and Genevaux 2014). Second, the number of TA loci was found higher in pathogenic *Mycobacterium tuberculosis* than in non-pathogenic environmental mycobacteria (Ramage, Connolly and Cox 2009; Georgiades and Raoult 2011; Sala *et al.* 2014). Of interest, a recent comparative analysis of 173 *M. tuberculosis* genomes showed single nucleotide polymorphisms in some type II TA systems that correlated to distinct pathotypes (Zaychikova *et al.* 2015). Taken together, all these studies suggest the idea that profusion of TA loci could reflect an evolutionary trait linked to acquisition of pathogenicity in defined bacterial species. This hypothesis is supported by genomic comparative studies of clones from the same species isolated from different sources.

Differences in the number of TA loci are particularly evident when clinical isolates are examined. For instance, clinical isolates of *Pseudomonas putida* have more TA modules than environmental isolates (Molina *et al.* 2016). Variable numbers of TA systems have been also reported for carriage (non-pathogenic) and disease (clones causing sepsis) isolates of *Streptococcus pneumoniae* (Cleary *et al.* 2016). Genome comparison in distinct *Klebsiella pneumoniae* isolates showed variable TA loci numbers, from 13 to 29 (Wei *et al.* 2015). Variable repertoires of TA systems have also been shown in different *Escherichia coli* phylogenetic groups, which was tentatively linked to distinct virulence potential and lifestyles (Norton and Mulvey 2012; Fiedoruk *et al.* 2015). These genome comparisons could provide insights into which TA modules have specifically evolved in connection to virulence.

Responsiveness of TA systems to host signals

To date, few studies have focused specifically on responsiveness of TA systems to host signals (Westermann, Gorski and Vogel 2012; Hammarlof, Canals and Hinton 2013). Some stresses known to occur in the host attack are, in essence, similar to those to which some TA systems have been reported to be responsive (Fig. 2). Thus, TA systems have been linked to responses to oxidative and nitrosative stresses, nutrient deprivation, acid/alkaline pH and bile acids (Norton and Mulvey 2012; Tiwari *et al.* 2015; Chowdhury *et al.* 2016; Muller *et al.* 2016). In this sense, some studies have reported data in infection models demonstrating the expression and upregulation of defined TA loci. Thus, *Vibrio cholerae* induces all its seven *relBE* type II TA modules in virulence-inducing conditions, with two of them required for efficient host colonization (Wang *et al.* 2015). *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) induces the expression of TA systems inside macrophages, fibroblasts and epithelial cells (Helaine *et al.* 2014; Lobato-Márquez *et al.* 2015; Silva-Herzog *et al.* 2015). Of interest, *S.* Typhimurium upregulates distinct TA modules attending to the infected cell type (Lobato-Márquez *et al.* 2015; Silva-Herzog *et al.* 2015). Globally, these data suggest that the distinct microenvironment found inside different cell types could trigger disparate repertoires of TA systems.

Studies in other bacterial pathogens have shown production of components of TA systems in bacteria located inside host cells. The toxins ChpK and MazF of the zoonotic pathogen *Leptospira interrogans,* but not their cognate antitoxins ChpI and MazE, were identified in the cytosol of infected macrophages (Komi *et al.* 2015). Dual RNA-seq has revealed the induction of TA loci of non-typable *Haemophilus influenzae* after infection of mucosal intestinal epithelia (Baddal *et al.* 2015). Six of the multiple type II TA systems identified in the genome of *M. tuberculosis* (i.e. three *vapBC* and three *relBE* modules) also respond to the intracellular niche of macrophages (Korch, Contreras and Clark-Curtiss 2009; Ramage, Connolly and Cox 2009). Another example of induction of TA loci by stresses found in the host was reported for the type II TA system BrnT/BrnA of the intracellular bacterial pathogen *Brucella abortus*, which is strongly activated by oxidative stress and acidic pH 4.0 (Heaton *et al.* 2012). TA responsiveness to stresses present in the host is not restricted to human pathogens. Thus, the *vapBC* module of the plant pathogen *Acidovorax citrulli* was reported to be induced during infection (Shavit *et al.* 2015).

Despite these studies showing the expression of TA loci in infection models, the host signals to which TA systems respond selectively remain elusive. Analyzing the response triggered by host stresses in laboratory media has proved to be difficult, especially regarding the attempt for reproducing in the test tube the intracellular environment of the infected cell (Shen and Fang 2012). To the date, we can only speculate on the idea of distinct factors (or a combination of them) needed to activate different TA modules. Moreover, key components regulating TA systems activation such as are proteases or RNases and the proper antitoxins that coordinately regulate toxin levels have not been studied along the host colonization process. These questions must be addressed to better understand TA regulation during infection.

Involvement of TA systems in biofilm formation

Biofilms are complex communities of microorganisms that attach to and develop on biotic and abiotic surfaces. They constitute a serious problem in clinics since these communities contaminate catheters and medical implants causing recurrent diseases in humans (Joo and Otto 2012). Among the bacterial pathogens prone to cause biofilm-associated infections are *Staphylococcus aureus* and *Pseudomonas aeruginosa*, the latter being particularly important for causing severe chronic infection in patients with cystic fibrosis (Hoiby 1974; Otto 2008). Biofilms show decreased sensitivity to antibiotic treatments and host immune defenses (Costerton, Stewart and Greenberg 1999). This increased resistance has been linked to a different physiological status of sessile bacteria within the biofilm matrix, which represents a diffusion barrier against damaging molecules (Joo and Otto 2012), and the different protein and gene expression profiles of sessile cells compared to planktonic cells (Jackson *et al.* 2002; Sauer *et al.* 2002). Among the biofilm-associated genes are those encoding quorum-sensing molecules and TA systems (Singh *et al.* 2000; Ren *et al.* 2004). These molecules could act in an integrated network controlling the development of the biofilm. Thus, deletion of the type II TA module *mqsRA* of *E. coli* reduces biofilm formation (González-Barrios *et al.* 2006; Soo and Wood 2013). Surprisingly, this TA locus is influenced by the quorum-sensing signal autoinducer-2 (AI-2), which thereby regulates biofilm formation (Gonzalez Barrios *et al.* 2006). Additional TA loci have been recently linked to the ability of some pathogenic bacteria to form biofilms (Wen, Behiels and Devreese 2014; Wood and Wood 2016). Together, these lines of evidence indicate that TA system can indirectly affect pathogenicity through the formation of resistant bacterial communities that can be especially important during chronic and recurrent infections (Table 2).

Contribution of plasmidic TA systems in bacterial infection

The role of plasmidic TA systems has been associated with stabilization of these episomal elements. Some pathogenic bacteria contain virulence plasmids that carry virulence factors. Thus, the stabilization of such virulence plasmids by TA modules could benefit host infection. This is the case for some plasmids of *Shigella flexneri*, *Enterococcus faecalis*, enteroinvasive *E. coli* or *Salmonella* spp. (Sansonetti, Kopecko and Formal 1982; Makino, Sasakawa and Yoshikawa 1988; Gulig *et al.* 1993; Sayeed *et al.* 2005; Clewell 2007). All these episomal elements are low-copy-number plasmids in which the TA loci contribute to their stabilization in the cell and, therefore, ensure maintenance of virulence genes (Table 2).

In addition to this putative relationship between plasmidic TA loci and virulence, more direct connections have recently emerged. Thus, the TA system $vapBC2_{ST}$ encoded in the virulence plasmid of *S.* Typhimurium plays a main role in host cells infection. $vapBC2_{ST}$ contributes to the intracellular survival of *S.* Typhimurium in non-phagocytic cells such as fibroblasts and epithelial cells (Lobato-Márquez *et al.* 2015). Remarkably, the *S.* Typhimurium $vapBC2_{ST}$ is highly homologous to the TA system *mvpAT*, involved in stability of the large virulence plasmid of *S. flexneri* (Sayeed *et al.* 2005). It could be of interest to test in future studies whether this *mvpAT* module might also play an important role in the intracellular lifestyle of this pathogen.

Table 2. Indirect contribution of TA systems to bacterial virulence.

Location of TA loci	Role of TA	TA contribution to virulence	Examples	Reference
Mobile genetic element (MGE)	Stabilization of pathogenicity islands	MGE containing virulence genes and/or antibiotic resistance genes	Integrative and conjugative element SXT of *V. cholerae*	Wozniak and Waldor (2009)
Plasmids	Plasmid stabilization	Virulence plasmids containing virulence factors and/or antibiotic resistance genes	Virulence plasmids of some Enterobacteria such as *S. flexneri* or *S.* Typhimurium	Sansonetti, Kopecko and Formal (1982); Gulig *et al.* (1993)
Chromosome	Biofilm formation	Establishment of bacterial communities more tolerant to host or antibiotics attacks	*relBE7* of *V. cholerae* superintegron, *higBA* of *P. aeruginosa*	Wang *et al.* (2015); Wood and Wood (2016)

EXAMPLES OF TA SYSTEMS CONTRIBUTION TO BACTERIAL VIRULENCE

The previous section describes clear lines of evidence connecting TA systems with bacterial pathogenesis. Complementary to these findings, mounting evidence suggests a direct causal role of TA systems in promoting virulence. In this section, we describe examples in which using classical deletion mutants and varied infection models, TA modules of human bacterial pathogens were shown to directly contribute to the infection process. While some of the TA systems are exploited by the pathogens mainly for their own survival purpose, there are also exceptions in which the toxins of these modules inflict damage to the host. These two facets are discussed in this section.

Uropathogenic *Escherichia coli* (UPEC)

In contrast to the widely accepted concept of pathogenic bacteria possessing more TA modules than non-pathogenic-related species, clinical isolates of extraintestinal pathogenic *E. coli* (ExPEC) have a reduced number of TA systems compared to the non-pathogenic *E. coli* strain MG1655 (Norton and Mulvey 2012). The uropathogenic ExPEC isolate CFT073, isolated from the blood of a patient with pyelonephritis, shares only 6 of the 16 type II TA systems repertoire of *E. coli* strain MG1655. Defective mutants in each of these six TA modules were tested in competition assays with the parental (CFT073) wild-type strain using a well-established CBA/J mouse infection model (Alteri, Smith and Mobley 2009; Blango and Mulvey 2010). Two of these TA loci, *ybaJ-hha* and *yefM-yoeB*, are required for colonization of the bladder and a third, *pasTI*, to colonize the kidneys (Table 3). Intriguingly, the defects observed in competitive assays were manifested in non-competitive assays only for the *pasTI*-defective mutant. This strict correspondence could be related to the impaired growth of this mutant in the presence of reactive oxidative and nitrosative species (Norton and Mulvey 2012), known to be an important host defense against pathogens.

Non-typeable *Haemophilus influenzae*

Non-typeable *H. influenzae* (NTHi) causes respiratory tract infection in humans following colonization of nasopharyngeal cells (Forsgren *et al.* 1994; Bandi *et al.* 2001). This pathogen is the second most common cause of acute otitis media after *Streptococcus pneumoniae* (Murphy 2003). NTHi forms biofilms in middle ears. These multicellular structures are thought to account for the resistance to host immune system and antibiotic treatments, therefore favoring recurrent otitis media (Hall-Stoodley *et al.* 2006; Slinger *et al.* 2006). NTHi is normally studied in two different infection models: (i) an *ex vivo* epithelial cell culture which simulates the human upper respiratory tract and (ii) an *in vivo* otitis media infection in chinchilla (Ehrlich *et al.* 2002; Hong *et al.* 2007). Three type II TA systems (*vapBC-1*, *vapXD* and *toxAvapA*) were interrogated for their putative role in host colonization by NTHi using both *ex vivo* and *in vivo* infection models. Inactivation of *vapBC-1* and *vapXD* TA systems results in attenuation of NTHi survival inside epithelial cells and the ear of infected chinchillas, which demonstrate that these two loci contribute to host colonization (Ren, Walker and Daines 2012). It is worth to note that simultaneous deletion of *vapBC-1* and *vapXD* did not result in reduced survival compared to individual mutants (Ren, Walker and Daines 2012). This finding was interpreted as probable redundant activities at the mechanistic level for these particular TA modules. Another TA of NTHi tested during infection was the *higBA*-like module *toxAvapA* (Table 3). Although *toxAvapA* TA locus contributes to NTHi survival following infection of epithelial cells of the upper respiratory tract, such role was not as pronounced as the observed with *vapBC-1* and *vapXD* modules (Ren *et al.* 2014). By contrast, the *toxAvapA* TA locus plays an important role during chinchilla middle ear infection. Future studies could explore whether the lack of the three TA systems characterized

Table 3. Examples of TA systems directly implicated in bacterial virulence.

Pathogen	TA systems modulating pathogenesis	Type of TA	Infection model	TA contribution to virulence	Reference
Uropathogenic *Escherichia coli* (UPEC)	*ybaJ-hha, yefM-yoeB, pasTI*	II	Mouse CBA/J (bladder, kidney)	Survival inside host	Norton and Mulvey (2012)
Non-typeable *Haemophilus influenzae*	*vapBC-1, vapXD, toxAvapA*	II	Otitis model in chinchilla, respiratory tract epithelial cells	Intracellular survival	Ren, Walker and Daines (2012); Ren *et al.* (2014)
Salmonella Typhimurium	*sehAB*	II	Mouse C57BL/6	Survival inside host	De la Cruz *et al.* (2013)
Salmonella Typhimurium	14 TA systems	II	Macrophages	Persister induction	Helaine *et al.* (2014)
Salmonella Typhimurium	5 TA systems (3 type I and 2 type II)	I, II	Fibroblasts, epithelial cells	Intracellular survival	Lobato-Márquez *et al.* (2015)
Staphylococcus aureus	*sprGF1*	I	Erythrocytes	Induction of hemolysis	Pinel-Marie, Brielle and Felden (2014)
Enterococcus faecalis	*ef0408-0409* (*par*)	I	*Galleria mellonella* larvae, mice, macrophages	Hypervirulence, survival inside host, intracellular survival	Michaux *et al.* (2014)
Neisseria gonorrhoeae	*fitAB*	II	Epithelial cells	Intracellular growth control, epithelial cell crossing speed	Hopper *et al.* (2000b)
Leptospira interrogans	*chpIK, mazEF*	II	Macrophages	Necrosis induction to cell host	Komi *et al.* (2015)
Vibrio cholerae	*relBE4, relBE7*	II	Mice	Host colonization	Wang *et al.* (2015)
Mycobacterium tuberculosis	Combination of three *mazEF*-like systems	II	Macrophages, guinea pig	Intracellular survival, survival inside host	Tiwari *et al.* (2015)

in the context of NTHi pathogenesis could result in highly attenuated strains.

Salmonella enterica

Salmonella enterica is an intracellular bacterial pathogen that causes diseases ranging from self-limiting diarrhea to severe infections in humans and livestock (Grimont and Weill 2007; Baumler *et al.* 2011). The serovar Typhimurium has been extensively studied in murine models in which this pathogen causes acute and chronic infections (Monack *et al.* 2000). In the host, *S.* Typhimurium infects distinct cell types like macrophages or epithelial cells (Sansonetti 2002; Boyle *et al.* 2007). Recent studies have shown that a series of *Salmonella* TA systems contribute to its adaptation to the host. An *in silico* genome analysis performed in *S.* Typhimurium revealed the presence of 27 putative TA loci belonging to types I and II. Only 18 of these 27 TA loci were shown to be fully functional TA operons (Lobato-Márquez *et al.* 2015). Another study analyzed the distribution of 11 type II TA modules of *S.* Typhimurium among pathogenic serovars of *S. enterica* and the non-pathogenic related species *Salmonella bongori* (De la Cruz *et al.* 2013). This study focused on two type II TA loci called *sehAB* and *sehCD* (from *Salmonella enterica* Hig-like), which are present in *S. enterica* serovars infecting warm-blooded animals but absent in *S. enterica* serovars infecting cold-blooded animals or *S. bongori*. Although *sehAB* and *sehCD* were dispensable for intracellular replication of *S.* Typhimurium inside macrophages, the lack of the *sehAB* TA module affected virulence in mice inoculated by the oral route but not in those animals inoculated intraperitoneally (De la Cruz *et al.* 2013). These results indicate that *sehAB* could play a role in the early steps of *S.* Typhimurium infection process, at some stage between the passage though the stomach and penetration of the intestinal epithelium (De la Cruz *et al.* 2013).

S. Typhimurium TA systems have also been studied during infection of cultured fibroblasts and epithelial cells. Fibroblasts act as cellular reservoir during *S.* Typhimurium infection (Boyle *et al.* 2007) and are cells in which *S.* Typhimurium restrains growth and persist for long periods of time (Martinez-Moya *et al.* 1998; Cano *et al.* 2001; Núñez-Hernández *et al.* 2013). Type II toxins $T4_{ST}$, $T5_{ST}$ and $T2_{ST}$ were found differentially expressed by *S.* Typhimurium inside fibroblasts compared to epithelial cells. Survival assays also demonstrated that *S.* Typhimurium uses at least three type I loci (*tisB-istR*$_{ST}$, *hok-sok*$_{ST}$, *ldrA-rdlA*$_{ST}$) and two type II TA loci (*ta4*$_{ST}$ and *vapBC2*$_{ST}$) during adaptation to the intracellular lifestyle inside fibroblasts (Lobato-Márquez *et al.* 2015). Interestingly, among the TA modules studied, only one (*vapBC2*$_{ST}$) was necessary for intracellular survival of *S.* Typhimurium inside epithelial cells (Table 3). It is worth to note that these data do not discard the possibility that other TA systems, not detected, could be exploited by *S.* Typhimurium to adapt inside eukaryotic cells. It is also important to recall probable redundant functions of these TA modules in the context of a particular host–pathogen interaction. The data supporting this evidence comes from phenotypic analysis of a *S.* Typhimurium mutant defective in all five TA modules shown to contribute to fitness inside fibroblasts. This multiple mutant behaved as the respective single mutants (Lobato-Márquez *et al.* 2015).

Staphylococcus aureus

Staphylococcus aureus is a human pathogen that causes important nosocomial infections (Anstead, Cadena and Javeri 2014). This bacterium encodes an atypical type I TA module termed *sprG1/sprF1* composed of two small hydrophobic peptides and one antisense RNA. *sprG1* contains two internal initiation codons that generate two different peptides (Pinel-Marie, Brielle and Felden 2014). Once synthesized, both peptides target the plasma membrane of the bacterium and, if overexpressed, result in *S. aureus* growth inhibition and cell death. Surprisingly, SprG1 peptides are also secreted to the extracellular environment and synthetic SprG1 peptides lysed erythrocytes at low concentrations (Pinel-Marie, Brielle and Felden 2014). Moreover, these toxic peptides lyse other bacteria, including *E. coli* and *P. aeruginosa*. These results were presented as an example in which a secreted toxin of a TA module could contribute to virulence (Table 3). However, no competition assays using Δ*sprG1/sprF1* versus wild-type *S. aureus* have been conducted to the date. This leaves unclear whether, under physiological conditions, secreted SprG1 peptides are capable of lyse neighbor prokaryotic cells. In this same line, it would be interesting to investigate whether physiological levels of these secreted toxins cause hemolysis. It is also remarkable the lack of studies addressing how *S. aureus* protects itself from exogenous SprG1 toxins.

The type II TA module *mazEF*$_{Sa}$ has been suggested to be required for *S. aureus* pathogenesis (Zhu *et al.* 2009). MazF$_{Sa}$ toxin recognizes a specific nucleotide sequence, which is overrepresented in genes encoding proteins involved in virulence, such as SraP, an adhesin that promotes interaction of this pathogen with host cells (Zhu *et al.* 2009). No further data sustain this possible correlation between the MazF$_{Sa}$ toxin and the production of virulence-related proteins.

Enterococcus faecalis

Enterococcus faecalis is involved in nosocomial infections such as catheter-associated urinary tract infections, endocarditis or burn wound infections (Jackson *et al.* 2002). In a recent study conducted with this opportunistic pathogen, the *ef0408-0409* (also known as *par*) sRNA encoding the antisense RNA II of a type I TA system was involved in infection (Michaux *et al.* 2014). In *E. faecalis*, *par* is the only chromosomally identified TA module while two TA loci are encoded on plasmids (Weaver *et al.* 2009; Weaver 2012). An Δ*ef0408-0409* mutant strain lacking the antitoxin of the module was analyzed in three infection models: the larvae *Galleria mellonella*, macrophages and urinary tract-infected mice. Surprisingly, the mutant resulted hypervirulent in the two animal models employed with increased larvae killing rates and higher bacterial loads in the kidneys of infected mice (Michaux *et al.* 2014). Moreover, when macrophages were infected with the Δ*ef0408-0409* mutant, this showed an increased survival rate compared to wild-type strain. The hypervirulence phenotype was returned back to wild type in all infection models following complementation of the *ef0408-0409* deletion, demonstrating that the observed phenotype was due to the disruption of the sRNA antitoxin (Michaux *et al.* 2014). To determine further the mechanism explaining Δ*ef0408-0409*-promoted virulence, two stress conditions supposed to happen during normal infection of *E. faecalis* were tested. Similarly to data obtained with the infection models, the antitoxin-deletion mutant appeared more resistant under oxidative stress and low pH (Michaux *et al.* 2014). Additionally, proteomic analysis comparing the profiles of the sRNA in mutant and parental strains showed that oxidative response and pathogenicity genes appeared induced in the deletion mutant (Michaux *et al.* 2014). To our knowledge, this is the only known example in which the lack of an antisense RNA antitoxin encoded by a type I TA locus promotes virulence (Table 3). Moreover, this knowledge sheds light into the signals that can trigger activation of TA systems (Fig. 2).

Neisseria gonorrhoeae

Neisseria gonorrhoeae is a sexually transmitted human pathogen that adheres to and penetrates the urogenital tract epithelium. In this location, the bacterium crosses the epithelial barrier causing a self-limiting local infection. In some cases, this pathogen disseminates to other host tissues provoking arthritis or infertility following infection of fallopian tubes. Gonococcal infection can also lead to the establishment of a carrier state in which the infected individuals are asymptomatic but spread the pathogen to new susceptible hosts. Colonization of the urogenital tract epithelium by gonococci is promoted by the action of multiple virulence factors, including type IV pili and protease IgaA1 (Swanson 1973; Hopper *et al.* 2000a). The type II TA system, named *fitAB* (*fast intracellular trafficker*), was identified by transposon-random mutagenesis as a gonococcal factor regulating intracellular proliferation and epithelial transcytosis (Hopper *et al.* 2000; Mattison *et al.* 2006). Disruption of *fitAB* provokes increased intracellular proliferation associated with augmented ability to cross the epithelial cell layer (Table 3), whereas no effect is observed in normal laboratory media. These results suggest that FitA antitoxin, FitB toxin or their complex could act as intracellular growth regulators in *N. gonorrhoeae*. The FitB toxin has a predicted PIN (PilT-N terminus) domain; proteins bearing this domain have been associated to nucleic acid metabolism, mostly with nuclease activity, in both eukaryotes and prokaryotes. Although the structure of the FitA-FitB complex bound to DNA was resolved, the attempts to demonstrate *in vitro* a nuclease activity for the FitB toxin were unsuccessful (Mattison *et al.* 2006).

Leptospira interrogans

Leptospira species are the causative agents of leptospirosis, a zoonotic infectious disease in humans (Bharti *et al.* 2003). This pathogen invades humans through skin or mucosa causing a disease characterized by fever, myalgia, meningitis and in some cases death of the infected individual (McBride *et al.* 2005). A recent study analyzed the role in infection of two TA modules of *Leptospira interrogans* (Komi *et al.* 2015). The type II TA systems *chpIK* and *mazEF* appeared conserved in pathogenic serovars of *Leptospira* species. Following macrophage infection, *L. interrogans* induces the expression of ChpI and MazE antitoxins and the ChpK and MazF toxins. Using flow cytometry and markers for apoptosis and necrosis, it was shown that Δ*chpIK* and Δ*mazEF* mutants induce less necrotic cell death in macrophages at late post-infection times. The study of the molecular mechanism underlying *chpIK* and *mazEF* cytotoxicity revealed that the toxins ChpK and MazF are secreted into the cell host cytoplasm (Komi *et al.* 2015). This is the first clear report showing active secretion of TA toxins and subsequent interference with cell host viability (Table 3). These results open the question if secreted toxins of TA modules could be a common strategy used by bacterial pathogens to manipulate host cell physiology (Fig. 3).

Vibrio cholerae

Vibrio cholerae is a Gram-negative pathogen responsible for cholera, a devastating watery diarrhea leading to strong dehydration and high rate death in untreated patients (Kaper, Morris and Levine 1995). After ingestion of contaminated food or water, this pathogen reaches the human intestine in which it synthesizes both the cholera toxin, responsible for severe diarrhea, and the toxin-coregulated pilus, required for successful intestinal colonization (Faruque, Albert and Mekalanos 1998). *Vibrio cholerae* genome is composed of two chromosomes. While most essential genes are found in chromosome I, chromosome II contains large number of genes of unknown function and the gene capture and excision system called superintegron (Mazel *et al.* 1998; Heidelberg *et al.* 2000). Interestingly, all 17 out of the 18 type II TA systems identified in *V. cholerae* are encoded within the superintegron (Iqbal *et al.* 2015). In a recent report, all *relBE* systems placed within the superintegron were examined under biofilm and virulence assays (Wang *et al.* 2015). Whereas disruption of *relBE*1 and *relBE*4 modules showed a slight effect in biofilm formation, disruption of *relBE*7 significantly decreased biofilm maturation. Strikingly, *relBE*2, whose deletion mutant does not impact formation of biofilm, and *relBE7* have the same sequence. This result emphasizes the idea that TA systems with high-sequence homology may be used selectively in response to different stimuli. Consistently, the viability of all seven mutants lacking *relBE* modules did not differ from that of wild-type bacteria when were exposed to stresses that occur during *V. cholerae* infection, including reactive oxygen species or bile salts (Wang *et al.* 2015). On the other hand, when *V. cholerae* was grown in a medium that induces its virulence genes (Iwanaga *et al.* 1986), all *relBE* systems appeared induced. Additionally, under competition assays Δ*relBE4* and Δ*relBE7* strains show lower colonization rates in a mouse infection model (Table 3). Future work should investigate whether *relBE*4 and *relBE*7 modules act coordinately and decipher the signals that are specifically recognized by each TA system.

Mycobacterium tuberculosis

Mycobacterium tuberculosis is one of the most successful and dreadful human pathogens, infecting one-third of the world population (North and Jung 2004). This pathogen still claims for two million deaths every year. *M. tuberculosis* persists in the infected individual establishing long asymptomatic infections with the potential to be reactivated any time during life (Stewart, Robertson and Young 2003). Thus, latency in the host is a major issue in *M. tuberculosis* pathogenesis. One of the proposed latency-inducing factors are TA loci. The *M. tuberculosis* genome harbors more than 80 type II TA modules mainly belonging to *relBE, parDE, ccdAB, higAB, vapBC* and *mazEF* families (Ramage, Connolly and Cox 2009). *M. tuberculosis* possesses nine different *mazEF*-like loci of which only three (*mazEF3*, *mazEF6* and *mazEF9*) seem to encode an active toxin (Tiwari *et al.* 2015). These three *mazEF* TA systems have been extensively studied under different stresses that mimic host defenses that the pathogen copes during human infection, i.e. oxidative, nitrosative, nutrient-limiting and low-oxygen stresses. Interestingly, these TA systems show different expression patterns when bacteria are exposed to these stresses: (i) toxin gene *mazF9* is only induced under oxidative stress, (ii) genes *mazF6* and *mazF9* appeared induced under nitrosative stress and (iii) all these three genes are induced when bacteria expose to either starvation or hypoxia. In all the cases, the transcripts of toxin-encoding genes are presented at higher levels than those of the antitoxins (Tiwari *et al.* 2015). Unfortunately, no data at the protein level were reported in this study. On the other hand, this study analyzed survival of mutants in all the three MazF toxins and combined versions under different stress conditions. The simultaneous deficiency of MazF3, MazF6 and MazF9 toxins (Δ*mazF3*Δ6Δ9) reduced survival rates following exposure to oxidative stress, while no effect was observed for any mutant bacteria exposed to hydrogen peroxide. Of interest, following nutrient deprivation, *M. tuberculosis* lacking MazF3, MazF6 and MazF9 toxins displayed in an additive

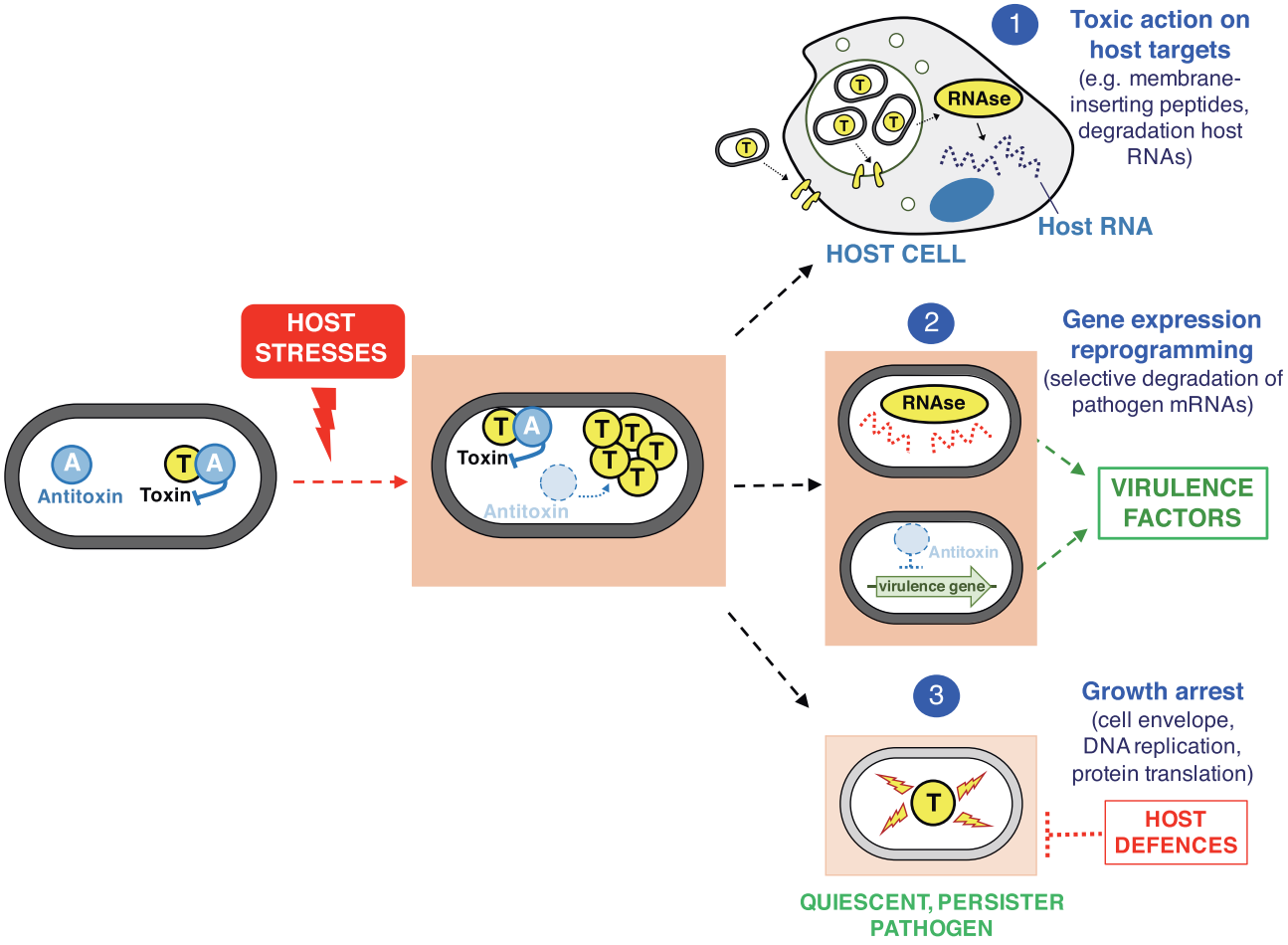

Figure 3. Scheme denoting potential modes of intervention of TA systems in bacterial virulence. Following contact with the host, the pathogen encounters adverse conditions that activate TA modules via antitoxin degradation, subsequently unbalancing toxin:antitoxin ratios. Distinct scenarios involving a direct contribution of TA modules in the host–pathogen interaction include **(1)** toxins of TA modules secreted by the pathogen may target host functions in extracellular or intracellular locations. Some examples are as follows. (i) Hydrophobic peptides of type I TA modules that insert into membranes to disrupt either membrane integrity or function of membranes proteins and (ii) pathogen RNAse toxins targeting host RNAs. **(2)** TA systems may also alter bacterial transcriptome following host encounter, favoring the induction of virulence genes. Two alternative mechanisms are considered: (i) action of RNase toxins in increasing turnover rate of defined RNA transcripts and (ii) the relief of expression in a virulence gene that is normally repressed by an antitoxin in non-host environments. **(3)** Pathogen growth arrest driven by toxins of TA loci. Essential bacterial processes such as DNA replication, protein translation and cell envelope homeostasis are targeted. The entry of the pathogen in a persistence or quiescent state could facilitate evasion of host immune defenses.

manner lower survival compared to wild-type strain (Tiwari *et al.* 2015). This effect was consistent with the high expression levels of the three *mazEF* modules when nutrients become limited. The lack of MazF3, MazF6 and MazF9 toxins has also consequences in *M. tuberculosis* virulence in *ex vivo* (macrophages) and *in vivo* (guinea pig) infection models. In both models, the Δ*mazF3*Δ6Δ9 mutant showed decreased survival compared to wild type (Table 3). Concretely, the mutant exhibited reduced bacillary loads in lungs and spleen of the guinea pig, which is an unequivocal proof for the role of these three TA modules in *M. tuberculosis* pathogenesis (Tiwari *et al.* 2015). Although growth of all mutants was tested in laboratory media, it is important to note that *mazE* antitoxins-encoding RNAs were upregulated in *mazF* deletion mutants, especially in the triple knockout. Elevated expression of these antitoxins could interfere with *M. tuberculosis* physiology under specific environmental conditions. *M. tuberculosis mazEF* TA systems are, therefore, an excellent example of how TA modules can be integrated in different metabolic networks facilitating the adaptation of the recipient bacterium to varied environments.

DISCUSSION AND CONCLUDING REMARKS

For a long time, it was speculated whether TA modules could play additional roles in bacterial physiology apart from their selfish maintenance in the cell (Gerdes, Christensen and Lobner-Olesen 2005; Van Melderen and Saavedra De Bast 2009). In the last decade, a large number of laboratories have demonstrated that, besides their own maintenance, TA loci modulate important functions in the cell contributing to central metabolism following exposure to different stresses. This is why TA modules are currently considered as 'stress response managers'. These findings explain in part TA systems abundance in many bacterial species. In evolutionary terms, TA modules could have been initially acquired by horizontal gene transfer and maintained in the cell because of their 'addiction' property. Some TA loci could have been evolved and integrated into physiological networks acquiring important functions in the bacterial life (Hayes and Van Melderen 2011). When considering bacterial pathogens, one could envision subsets of TA loci that might have selectively evolved to promote successful interactions with the

Table 4. Future directions for studies involving TA systems in bacterial pathogens.

New concept to be analyzed	Experimental approaches*
Expression of TA modules in space and time during infection	(1) Isolation of host cell/pathogen extracts (*in vitro*). Detection of toxins/antitoxin proteins of interest by western blot assays and/or proteomics. (2) Genome expression profiling–microarray, RNA-seq in bacteria isolated from infected cells and tissues (*in vitro* and *in vivo*) (3) Real-time microscopy with gene expression reporters coupled to TA promoters (*in vitro* and *in vivo*)
Single cell analyses of TA module expression and dynamics in bacterial populations	(1) Cytometry assays using gene reporters and/or toxin- or antitoxin-specific antibodies. (2) Sorting of bacterial populations inducing or not specific TA systems to monitor dynamics of TA gene expression
Action TA toxins in the host	(1) Identification of TA toxins in culture supernatant or membrane vesicles released by the pathogen. (2) Cytotoxicity assays in cultured eukaryotic cells incubated with purified TA toxins (3) Genome expression profiling in eukaryotic cells exposed to the TA toxin.
Responsiveness of TA modules to host defenses	(1) TA loci reported monitored in presence of host insults (e.g. antimicrobial peptides, ROI and RNI generators). (2) Expression of TA loci in conditions known to occur in the host (e.g. iron deprivation, acidic pH, exposure to bile salts).

**In vivo* refers to infection in animal models; *in vitro,* to assays involving cultured eukaryotic cells.
RNI, reactive nitrogen intermediates; ROI, reactive oxidative intermediates.

host. The most recent data, obtained in a still relatively scarce number of infection models, favor this idea since some TA loci contribute to the infection whereas others are dispensable.

TA systems were found to be more abundant in some pathogenic bacteria compared to non-pathogenic-related species (Georgiades and Raoult 2011). Although some exceptions exist to this rule, as that of certain pathogenic *Escherichia coli* isolates (Norton and Mulvey 2012), the comparisons in other pathogens, like mycobacteria (Ramage, Connolly and Cox 2009; Leplae *et al.* 2011), certainly prompt to pursuing the participation of more TA modules in pathogenesis. The work of many laboratories now demonstrates that a few TA modules play an important role in bacterial infection during host colonization. In this regard, this specialized subset of TA systems fits the definition and could be considered as 'new virulence factors'. Nonetheless, there are unresolved questions that need to be addressed to fully understand the contribution of TA modules of pathogens to host adaptation (Table 4). Despite inferring such a role by phenotypic analyses of mutants lacking defined TA systems, the molecular mechanism(s) is dissected in only few cases and, even in these cases, the process it is not fully understood. Moreover, no study has yet explored expression of TA loci in the context of an infection in a spatial and temporal manner. We only found a study in which expression of the *vapBC* operon from the plant pathogen *Acidovorax citrulli* was measured during infection (Shavit *et al.* 2015). However, this study focused in a TA system not contributing to virulence and its expression was only monitored at defined time points. It is also worth to note the heterogeneity in behavior is observed for pathogens during infection. This phenomenon is documented in tissue culture infection models (García-del Portillo 2008) and *in vivo*, in which evidence was found for the involvement of TA modules in the generation of persister cells (Helaine *et al.* 2010, 2014; Maisonneuve *et al.* 2011; Claudi *et al.* 2014). These observations open the possibility of differential expression of TA loci at the single cell level during host colonization. *In vivo* time-lapse imaging could be implemented in infection settings, as it was previously shown for the study of TA system expression during formation of persister cells (Maisonneuve, Castro-Camargo and Gerdes 2013). These are certainly promising studies that could shed new insights into how TA loci are activated inside the host (Table 4).

The mechanisms by which TA systems contribute to host colonization are largely unknown. The data obtained in several pathogens demonstrate that some TA modules are required for survival in different tissues or eukaryotic cells. The distinct nature and strength of stresses inherent to different tissues colonized by bacterial pathogens could result in a selective activation of one or more TA systems. Signaling to TA modules by host alarms is certainly a major area in this field for which, unfortunately, is lacking precise information. Antitoxin degradation by RNases or proteases is the only known mechanism leading to TA operon derepression. However, how proteases activity is regulated during infection remains undefined. Hypothetically, varied stresses could activate distinct proteases which, consequently, would degrade different antitoxins and, as a result, activate their cognate toxins.

Attending to the types of TA modules discussed in this review and the data accumulated to date, it is tempting to speculate on several hypotheses to explain how toxins or antitoxins may contribute to pathogenesis. In type I TA modules, toxins are small hydrophobic peptides that insert into membranes. SprG1 peptides of a type I TA system of *S. aureus* induce hemolysis (Pinel-Marie, Brielle and Felden 2014). *S.* Typhimurium *hok-sok*$_{ST}$, *ldrA-rdlA*$_{ST}$ y *tisB-istR*$_{ST}$ type I TA systems contribute to the intracellular lifestyle of this pathogen (Lobato-Márquez *et al.* 2015). Like SprG1, *S.* Typhimurium toxins Hok$_{ST}$, LdrA$_{ST}$ and TisB$_{ST}$ could interfere with host membranes (Fig. 3). Several lines of evidence show that *S.* Typhimurium secretes virulence factors independently of type III secretion systems via outer membrane vesicles (Yoon *et al.* 2011). Hydrophobic peptides of type I TA systems could also regulate the function of other membrane

proteins, as shown for some small hydrophobic peptides (Alix and Blanc-Potard 2009). Representative examples are the MgtR peptide, which controls the S. Typhimurium MgtC virulence factor levels via FtsH protease degradation (Alix and Blanc-Potard 2008); the peptide SpoVM of *Bacillus subtilis* that inhibit FtsH protease indirectly inhibiting the degradation of many other unknown proteins (Cutting *et al.* 1997); and YneN in *E. coli*, which connects two different two-component systems (Eguchi *et al.* 2007).

In type II TA modules, most of their toxins are endoribonucleases. The length and sequence of nucleotides and the secondary structure of RNA determine the spectrum of RNA targets that a single toxin can process. This suggests that bacterial toxins could degrade defined RNA targets causing important changes in transcriptome readjustment (Christensen *et al.* 2001; Kim *et al.* 2010). Specific selection of RNA targets by endoribonuclease toxins is exemplified in the *higBA* TA locus of *P. aeruginosa*, in which the toxin HigB regulates transcription levels of several genes involved in the virulence of this pathogen (Wood and Wood 2016). Another example is the *vapBC* operon of *Mycobacterium smegmatis*. This system is supposed to control at the post-transcriptional level metabolism and uptake of sugars (McKenzie *et al.* 2012).

Some toxins of type II TA systems elicit toxic effects when produced or injected into eukaryotic cells (Yamamoto, Gerdes and Tunnacliffe 2002; de la Cueva-Mendez *et al.* 2003; Audoly *et al.* 2011). Moreover, when *M. tuberculosis* is deprived of nutrients, it secretes to the extracellular environment some toxins of type II TA loci (Albrethsen *et al.* 2013). Strikingly, a recent study using the pathogen *L. interrogans* demonstrates that the two toxins ChpK and MazF, belonging to type II TA systems, are secreted to the infected host cell, inducing necrotic cell death (Komi *et al.* 2015). The mechanism(s) by which these toxins are secreted remain unknown. As demonstrated for other bacterial virulence factors, bacterial toxins of TA modules could be secreted to the host cell subverting host immune defenses or inducing host cell death, allowing in this manner intracellular survival or persistence and dissemination of the infecting pathogen (Fig. 3).

Most antitoxins of type II modules only act as transcriptional repressors for their own TA operons. However, there are few examples (e.g. *mqsRA* and *dinJ-yafQ*) in which the antitoxin also represses the expression of other genes affecting the general stress response (Kim *et al.* 2010; Wang *et al.* 2011; Hu, Benedik and Wood 2012). Using a similar mechanism, some antitoxins could control expression of other virulence factors or metabolic routes needed during infection (Fig. 3). Although antisense antitoxin of type I TA modules have not yet proved to regulate the expression of any other gene apart from its cognate toxin, there are many examples of regulatory small RNAs (Ortega *et al.* 2014). The case of *E. faecalis* sRNA ef0408-0409 described here illustrates this hypothesis. When this antisense RNA was deleted, multiple genes belonging to different metabolic pathways are overproduced and this results in hypervirulence of the defective strain (Michaux *et al.* 2014). Finally, there are cases in which the target of the toxin involved in bacterial pathogenesis remains unknown (Lobato-Márquez *et al.* 2015). Unprecedented regulatory functions for newly discovered toxins will be expected.

Another concept explaining the role of TA systems during infection, especially in long-term chronic infections, is that of toxins inducing a 'quiescent status' in the bacterium allowing its survival within the host (Fig. 3). This idea is supported by a novel study in which the effect of type II TA modules of S. Typhimurium was analyzed during macrophage infection. *Salmonella* internalization by the macrophage favors the development of non-replicating persisters (Helaine *et al.* 2014). Generation of non-replicating S. Typhimurium persisters inside macrophages depends on TA systems. These persisters could more easily cope with the adverse intracellular environment. This model is supported by the fact that (i) S. Typhimurium persisters transiently resist more stresses besides that of antibiotics, including oxidative stress, low pH and low iron (Silva-Herzog *et al.* 2015); (ii) most TA systems target processes essential to growth (DNA replication and transcription, envelope biosynthesis and protein translation, among others), therefore inducing mainly bacteriostasis (Gerdes 2013); and (iii) TA loci expression is also activated in phagosome-related stress conditions (Hautefort *et al.* 2008; Helaine *et al.* 2014). The beneficial condition of quiescence is also supported by a study using *M. smegmatis*, in which it was shown that the *vapBC* type II TA locus induces dormancy under potassium-limiting conditions (Demidenok, Kaprelyants and Goncharenko 2014). Three excellent and recent review articles address the phenomenon of persistence, which we recommend for further reading (Helaine and Kugelberg 2014; Maisonneuve and Gerdes 2014; Page and Peti 2016).

Some final considerations must be taken into account when studying the role in pathogenesis of TA systems: (i) TA genes encode a toxic protein coupled to its antitoxin, so random mutagenesis using transposon libraries could be bias to only-antitoxin mutants (Sberro *et al.* 2013); (ii) TA modules are tightly regulated operons at the transcriptional and translational level, meaning that only-antitoxin deletion mutants could cause increased toxin-producing strains whose viability is already compromised. Partial deletion of TA operons (e.g. only antitoxin or toxin deletions) could lead to false-positive phenotypes or results difficult to interpret. For example, an exhaustive deletion mutant library was generated in the human pathogen *Yersinia pestis* and screened for mutants with attenuated virulence in a mouse model of bubonic plague (Pradel *et al.* 2014). A gene, named *ympt1.66c*, further identified as the antitoxin-coding gene *hicB3*, resulted important for full virulence (Pradel *et al.* 2014). Surprisingly, a second study in which the 'entire' TA *hicB3A3* locus of *Y. pestis* was deleted did not show any significant phenotype (Bibi-Triki *et al.* 2014). In another example, three TA modules were claimed necessary for S. Typhimurium virulence after using a transposon-mutagenized bank of mutants in three distinct animal models (Hall-Stoodley and Stoodley 2002). Unfortunately, no further data were reported for these TA modules. These observations do not preclude an antitoxin as a component contributing to virulence. Moreover, some antitoxins such as MqsA and DinJ have been shown to regulate transcription of other loci besides its own operon (Kim *et al.* 2010; Wang *et al.* 2011; Hu, Benedik and Wood 2012). On the other hand, antitoxins, at least in type II TA modules, are the transcriptional repressor of the module so, once deleted, toxin-coding genes become upregulated. Additionally, in some type II TA loci such as the $\omega\varepsilon\zeta$ of *Streptococcus pyogenes* pSM19035 plasmid or, the *paaR-paaA-parE* of *E. coli*, there is a third component conforming the TA operon responsible for the transcriptional regulation of the system (Zielenkiewicz and Ceglowski 2005; Hallez *et al.* 2010). Apart from controlling $\varepsilon\zeta$ transcription, the ω protein also functions as a global regulator in the cell (Zielenkiewicz and Ceglowski 2005). In most of the cases described in this review, only complete TA loci mutations were generated, and usually the resulting attenuated phenotype in virulence was attributed to the toxin of the system. Future studies should differentiate the role of both elements and decipher whether toxins, antitoxins or additional regulatory elements (or all of them) are the main 'players' in the host–pathogen interface.

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