sRNAs in bacterial type I and type III toxin-antitoxin systems

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ABSTRACT

Toxin–antitoxin (TA) loci consist of two genes: a stable toxin whose overexpression kills the cell or causes growth stasis and an unstable antitoxin that neutralizes the toxin action. Currently, five TA systems are known. Here, we review type I and type III systems in which the antitoxins are regulatory RNAs. Type I antitoxins act by a base-pairing mechanism on toxin mRNAs. By contrast, type III antitoxins are RNA pseudoknots that bind their cognate toxins directly in an RNA–protein interaction. Whereas for a number of plasmid-encoded systems detailed information on structural requirements, kinetics of interaction with their targets, factors that regulate their expression, RNases that degrade them and the sRNA/target RNA complexes and, finally, their biological roles is available, the investigation of chromosomal systems is still in its infancy. Here, we summarize our current knowledge on that topic. Furthermore, we compare factors and conditions that induce antitoxins or toxins and different mechanisms of toxin action. Finally, we discuss biological roles for chromosome-encoded TA systems.

Keywords: small regulatory RNA; toxin–antitoxin system; RNA antitoxin; type I and type III TA systems; antisense RNA; target RNA interaction; toxic peptide

INTRODUCTION

Toxin–antitoxin (TA) loci encode two-component modules that consist of a stable ‘toxin’ whose ectopic overexpression either kills cells or confers growth stasis, and an unstable ‘antitoxin’ that neutralizes the toxin action (reviewed in Brantl 2012b). Currently, five TA systems are known (summarized in Fig. 1). Whereas in type I and type III TA systems, the antitoxin is a small RNA, and the toxin mRNA encodes a peptide, in type II, IV and V systems, both antitoxin and toxin (TA) are proteins. In type I TA systems, the RNA antitoxin interacts with the toxin mRNA, and in type III systems, it binds the toxin protein directly (see below). In type II systems, the antitoxin inhibits the toxin by a protein–protein interaction, but in type V systems, it is an RNase that cleaves the toxin mRNA thus preventing toxin expression (Wang et al. 2012). By contrast, the antitoxin in type IV TA systems interferes with binding of the toxin protein to its cellular target (Masuda et al. 2012). Biological functions of type I and type III TA systems comprise plasmid maintenance, persistence against antibiotics and abortive phage infection. However, numerous chromosome-encoded systems still await their functional characterization.

Initially, type I TA systems have been discovered on plasmids [e.g. hok/Sok on Escherichia coli plasmid R1 (Gerdes, Rasmussen and Molin 1986); ftsRNAII on Enterococcus faecalis plasmid pAD1 (Weaver and Clewell 1989; Weaver and Tritle 1994)] where they act as post-segregational killing systems (PSK) to prevent
What is known on toxin and antitoxin action?

The majority of type I toxins are small hydrophobic proteins (~60 aa) with transmembrane domains that are thought to act like phage holins, i.e. they induce membrane pores, which impairs ATP synthesis with consequences for replication, transcription and translation. Other type I toxins are enzymes like RNase SymE (Kawano, Aravind and Storz 2007) or the recently identified DNase RaB (Guo et al. 2014). The few currently known type III toxins are endoRNases that process the antitoxin precursors into unit-length molecules and cleave cellular RNAs unspecifically (Short et al. 2013).

Type I antitoxins employ several mechanisms to counteract toxin action irreversibly: they either inhibit toxin translation by different means or promote the degradation of the toxin mRNA or—as we could show recently (Jahn and Brantl 2013)—do both. Additional strategies are used to prevent premature toxin expression. By contrast, type III antitoxins act in a reversible manner by sequestration of their toxin proteins in trimeric complexes (Short et al. 2013).

Whereas plasmid-encoded type I systems are required for plasmid maintenance, the biological function of chromosome-encoded type I TA systems is far less clear. Recently, in E. coli tisB/istR1, a role in persister formation has been found (Dörr, Vulič and Lewis 2010). In Bacillus subtilis, several type I TA systems are located on prophages and suggested to be required for their maintenance (reviewed in Durand et al. 2012). Other reports hypothesize a role in metabolic or stress response or in biofilm formation (Domka, Lee and Wood 2007). By contrast, type III TA systems confer phage resistance by acting as abortive phage infection (abi) modules: they enable a phage-infected cell to commit altruistically suicide to protect the clonal bacterial population (Blower et al. 2011).

In this review, we discuss in detail all currently known mechanisms of action employed by type I and type III antitoxins, structural requirements for their interaction with the cognate targets as well as interaction kinetics and the role of RNases and accessory proteins in TA mRNA degradation. In these aspects, we compare RNA antitoxins with other chromosome-encoded sRNAs involved in control of metabolism, stress response and virulence. Furthermore, we outline expression conditions for toxin and antitoxin genes and summarize our current knowledge on toxins of type I and III, their functions and cellular targets. Finally, we present data and hypotheses on the biological function of type I and type III TA systems.
TA SYSTEMS WITH RNA ANTITOXINS

Two examples for type I TA systems that were discovered in the 1980s and have been studied in great detail are located on plasmids, hok/Sok in E. coli and fst/RNAII in En. faecalis. Later, a plethora of chromosome-encoded type I TA systems have been discovered in a broad variety of bacterial species, among them also hok/Sok or fst/RNAII relatives. In 2009, the first type III TA system has been identified, and only two type III systems, in Pectobacterium atrosepticum and in Lactococcus lactis, were investigated in some detail (see below). Table 1 provides an overview of toxin mRNAs and antitoxins of all currently known, experimentally verified/studied type I systems, their genetic organization, mechanism of antitoxin action and regulation of toxin expression.

ANTITOXINS

Type I antitoxins

Mechanism of antitoxin action

Currently, two primary mechanisms of antitoxin action are known: inhibition of translation or promotion of toxin mRNA degradation. However, the first bifunctional antitoxin has been identified recently. B. subtilis SR4 obstructs translation of bsrG RNA and promotes its degradation (Jahn and Brantl 2013).

Inhibition of toxin mRNA translation can be accomplished by different means. The most trivial case is E. coli symE/SymR, where binding of the SymR antitoxin at a region overlapping the toxin RBS (ribosome binding site) inhibits ribosome binding directly (Kawano, Aravind and Storz 2007; Fig. 2A). By contrast, E. coli hok/Sok and ldr/DlD employ inhibition of leader-peptide translation. In hok/Sok, translation of the toxin hok (host killing) is difficult and requires translation of the leader-peptide mok (modulation of killing). The antitoxin Sok (suppression of killing) binds to the Shine Dalgarno (SD) sequence of mok and thereby indirectly prevents hok mRNA translation (Fig. 2B). Likewise, a mok-like open reading frame (ORF) designated ldrX overlaps ldrD, as the ldrD mRNA cannot bind ribosomes directly, and the antitoxin RdlD obstructs leader-peptide ldrX translation (reviewed in Gerdes and Wagner 2007; Kawano 2012). Alternatively, the RNA antitoxin can inhibit translation by blocking a ribosome standby site (RSS; Darfeuille et al. 2007): in E. coli tisb/IstR1, the tisB RBS is sequestered by intramolecular base pairing. The antitoxin IstR1 binds 100 nt upstream of the tisB translation start site. In contrast, the SymR antitoxin at a region overlapping the toxin RBS is sequestered by intramolecular base pairing. The

<p>| Table 1. Overview of currently known Type I TA systems: occurrence, characteristics, mechanism of action and regulation of expression. |</p>
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<th>Length of toxin RNA</th>
<th>Mode of AT action</th>
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<td>Convergent (64)</td>
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<td>372</td>
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<td>292/250/186</td>
<td>TI</td>
<td>Convergent (21)</td>
<td>Toxin SOS induced Multiple loci</td>
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<tr>
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<td>Convergent (118)</td>
<td>bsrG RNA temperature sensitive</td>
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<td>&gt; 450</td>
<td>?</td>
<td>Convergent (111)</td>
<td></td>
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<tr>
<td>B. subtilis</td>
<td></td>
<td>200</td>
<td>285</td>
<td>RD</td>
<td>Convergent (140)</td>
<td>bsrH RNA degraded by RNase J1</td>
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<td>fst/RNAII</td>
<td>66</td>
<td>214</td>
<td>TI, RS</td>
<td>Convergent (8, 12, ≈ 35)</td>
<td></td>
</tr>
<tr>
<td>S. aureus chromosome/plasmids</td>
<td>sprA1/SprA1AS</td>
<td>60</td>
<td>208</td>
<td>TI</td>
<td>Convergent (10, 16)</td>
<td>fst/RNAII homologs</td>
</tr>
<tr>
<td>S. aureus</td>
<td>sprF/SprG</td>
<td>141</td>
<td>439/312</td>
<td>TI</td>
<td>Convergent (134)</td>
<td>tpxA/RatA homolog multiple loci</td>
</tr>
</tbody>
</table>

TI, translational inhibition; RD, promotion of toxin mRNA degradation; RS, RNA stabilization; ?, regulation proposed but not experimentally shown. For further details see text.
initiation region (TIR) at an unstructured RSS, thereby restraining ribosomes from binding there and sliding into the TIR, when the stem-loop region unfolds incidentally (Fig. 2C, reviewed in Wagner and Unoson 2012). In E. coli zorO/OrzO, the antitoxin also binds within the 180 nt long 5′ UTR, and it is not yet clear, if this region comprises a small translated ORF as in hok/Sok or an RSS as in tisB/IstR1 (Wen, Won and Fozo 2014). Alternatively, cleavage sites detected in a 16S RNA strain within the complementary region and downstream of it, but upstream of the zorO RSS, do not exclude that OrzO promotes RNA degradation by employing a so far unidentified E. coli endoribonuclease (the existence of which was proposed by Opdyke et al. 2011).

In the B. subtilis type I TA systems investigated so far [txpA/RatA (Silvaggi, Perkins and Losick 2005); bsrG/SR4 (Jahn et al. 2012)], the RNA antitoxin binds at the 3′ end of the toxin mRNA and supports its degradation (Fig. 2D). Both RNAs share a long complementary stretch of ≈120 bp. For bsrG/SR4, we have found recently that SR4 does not only promote degradation of bsrG mRNA but induces a conformational change around the bsrG RBS that further obstructs ribosome binding, thus, additionally impeding toxin translation (Jahn and Brantl 2013; Fig. 2D) making SR4 the first bifunctional antitoxin. Such a conformational change was neither observed upon RatA binding to txpA mRNA (Durand, Gilet and Condon 2012) nor for antitoxin SR5 in another B. subtilis type I TA system, bsrE/SR5 [formerly bsrE/as-bsrE (Meißner, Jahn and Brantl unpublished)], suggesting that RatA and SR5 do solely cause toxin degradation. It remains to be seen if this also holds true for yonT/as-yonT, a system, for which evidence that it is a type I TA system is so far only indirect, as deletion of as-yonT has not been investigated in vivo in B. subtilis (Durand, Gilet and Condon 2012).
In all the cases discussed above, TA RNA binding involves only one complementary region.

By contrast, En. faecalis RNAI and fst toxin mRNA (RNAI) interact at different regions located apart: binding starts at the 3’ end of both RNAs at the loops of a bidirectional transcription terminator that comprises ≈35 bp, and later, two direct repeat (DR) regions located further upstream interact yielding a partial duplex that suffices to block fst translation (Greenfield et al. 2001; Fig. 2F). Mutations in either the terminator or simultaneously in both DR regions abrogated the ability of the antitoxin to neutralize toxicity in vivo (Greenfield et al. 2000). In the RNAI/RNAII complex, the toxin start codon is located in a double-stranded region. Interestingly, TA RNA interaction does not result in fst toxin mRNA degradation, but in accumulation of a complex with higher stability of both the toxin mRNA (40 instead of 9 min, measured with a mutant that lacked the 5’ stabilizing helix), and the antitoxin RNAII (16 instead of 4 min). For the recently published Staphylococcus aureus TA system sprA1/SprA1B (Sayed, Jousselin and Felden 2011; Sayed et al. 2012), a chromosomal member of the fst/RNAII family, toeprinting and in vitro translation in the presence and absence of the antitoxin SprA1B indicate that it inhibits translation. However, as RNA half-lives have not yet been determined, it cannot be excluded that translation inhibition is accompanied by RNA stabilization as in fst/RNAII or by RNA degradation as in bsrG/Sl4. Similar to En. faecalis RNAII, the E. coli Sib antitoxins use two target recognition domains, TRD1 within the ibs ORF and TRD2 in the ibs RBS (Han et al. 2010; reviewed in Fozo 2012), to inhibit ibs translation. The interaction of TRD1 with its complement within the ibs ORF is critical for the discrimination between different, highly similar target toxins (Han et al. 2010).

Another S. aureus TA system, sprG1/SprF1 (Pinel-Marie, Brielle and Felden 2014), bears similarity to B. subtilis tpxA/RatA or bsrG/Sl4, as both genes overlap at their 3’ ends in a long complementary region (134 nt) which would suggest an RNA degradation mechanism. However, since no influence of SprF1 on sprG1-mRNA levels has been investigated (neither in wild-type nor in RNase III knockout strains), it is not clear, if the SprF1-mediated translation inhibition observed in vitro is caused by induction of structure alterations at the toxin RBS as in B. subtilis bsrG/Sl4 followed by RNA degradation in vivo.

Interaction kinetics and binding pathway of RNA antitoxin and toxin mRNA

For many cis-encoded antisense/sense RNA pairs, binding pathways have been studied in detail and binding kinetics measured (reviewed in Brantl 2007). Initial pairing rate constants (k_{app}) are usually in the range of 1 × 10^{5} to 1 × 10^{6} M^{-1} s^{-1}. Similar values have been also calculated for several type I TA pairs: for hok/Sok, 1 × 10^{5} M^{-1} s^{-1} (Thisted et al. 1994) and in both fst/RNAI (Greenfield et al. 2001) and bsrG/Sl4 (Jahn and Brantl 2013) k_{app} values of 10^{5} M^{-1} s^{-1} were reported. For the two interaction sites between ibs RNA and Sib, 2 × 10^{5} M^{-1} s^{-1} and 5 × 10^{5} M^{-1} s^{-1} were determined, respectively (Han et al. 2010). For E. coli tisB/IsnB, 2 × 10^{5} M^{-1} s^{-1} have been measured (F. Darfeuille and E. G. Wagner unpublished data).

Initial contacts between sRNA and target mRNA can either occur between two complementary loops (as found for a number of replication control systems) or between a loop and a single-stranded region. The latter has been found for hok/Sok, where the 5’ single-stranded tail of Sok recognizes a loop of the hok target RNA that tops a stem comprising the mok SD sequence (Thisted and Gerdes 1992; Fig. 2B), and binding most probably follows a one-step scheme (Fig. 3A). Independent of a
one- or multi-step pathway, the final result of the interaction is a complete duplex that is degraded by the double-strand-specific RNase III (see below).

Although a time-course experiment for fst/RNAI showed that both terminator and DR regions of toxin and antitoxin RNAs might interact simultaneously, the use of a series of loop mutations allowed to ascertain that interaction starts at the two terminator loops, afterwards involves the DRa region, reaches the Drb region and from there, progresses into the final complex, which is, however, only a partial duplex (Greenfield et al. 2001; Fig. 3B). In the case of bsrG/SR4, binding initiates between the terminator stem loop (SL4) of SR4 and loop 3 of bsrG RNA, followed by an interaction between SL3 of SR4 and a region within the main helix of bsrG RNA. Finally, SL2 of SR4 and the bsrG terminator loop interact, but this last step is not required for inhibition (Jahn and Brantl 2013; Fig. 3C). By contrast, in E. coli ibs/Sib, a simultaneous interaction between TRD1 and TRD2 of Sib antitoxin and their complementary domains in ibs RNA was observed (Han et al. 2010; Fig. 3D). This is reminiscent of the simultaneous interaction of two loop pairs in RNAII/RNAII, the replication control system of plasmid pIP501 (Heidrich and Brantl 2007).

In many sense/antisense systems, 5’ YUNR motifs present in either the antisense or the target mRNA were found to be important to provide a scaffold for rapid RNA-RNA interaction (reviewed in Franch and Gerdes 2000). Interestingly, the first of these 5’ YUNR motifs was discovered in hok mRNA, and this motif was experimentally shown to form a U-turn (Franch et al. 1999; Fig. 3A). Surprisingly, in the bsrG/SR4 system, two potentially important 5’ YUNR motifs were detected. However, whereas in loop L3 of bsrG RNA was involved in the initial contact with loop L4 of SR4 (see Fig. 3C), the motif in loop L2 of SR4, which interacts with the bsrg terminator loop, was not required, as the entire SL2 was not needed for the inhibitory function of SR4 (see above, Jahn and Brantl 2013). Preliminary data suggest that in B. subtilis bsrE/SR5 the initial rapid interaction occurs between two loops each containing a U-turn motif (Meißner, Jahn and Brantl unpublished). In fst/RNAII, the fst terminator loop contains a 5’ YUNR motif, and, as stated above, it is involved in the primary interaction with the RNAII terminator loop (Fig. 3B). In the case of ibs/Sib, a 5’ YUNR motif is found in the decisive loop within the ibr mRNA ORF (Han et al. 2010), whose interaction is important for the discrimination between the different ibs/Sib systems (see Fig. 3D). In the S. aureus fst homolog sprA1/SprA1as (Sayed, Josselin and Felden 2011), the antitoxin loop L1 carries a potential 5’ YUNR motif, whose ‘R’ was, however, mapped in the double-stranded stem region which makes it unlikely to form a U-turn.

However, except for hok/Sok, no experimental proof for the formation of U-turns at the 5’ YUNR positions in other TA systems was provided, so far. In tspA/RatA, neither of the interacting loops carries a 5’ YUNR motif (see secondary structures in Durand, Gilet and Condon 2012). For the other type I TA systems, no experimentally probed RNA secondary structures are available to ascertain, if potential 5’ YUNR motifs in either antitoxin or toxin mRNA are present in loop regions. Recently, for zor/OrzO and zorP/OrzP, it has been shown that the immediate 5’ end of the RNA antitoxin is required for specific pairing and discrimination between different zor/Orz systems (Wen, Won and Fozo 2014). However, although this end contains a 5’ YUNR, it cannot form a loop.

Many antisense RNAs mediate inhibition by forming complexes that involve limited numbers of base pairs with their targets, and full duplex formation, which would be too slow to account for the observed biological effects, is not required for control (reviewed in Wagner and Brantl 1998). Whereas e.g. in hok/Sok, symE/SymR or bsrg/SR4, toxin-antitoxin binding finally leads to a complete duplex, other systems such as fst/RNAII, tisB/IstR, zor/OrzO or ralR/RalA employ only short complementary regions whose interaction results in a partial duplexes.

Additional modes to prevent premature toxin expression
Although toxin inhibition by the antitoxin is the main regulatory principle, additional strategies are employed to ensure tight regulation of toxin expression. All these strategies use the high capacity of RNA to fold and refold into complex structures.

One strategy is the requirement for a processing event that converts an inactive long toxin mRNA into a shorter translationally competent molecule: in E. coli hok/Sok, the 3’ end (termed fbi = fold-back inhibition element) of the 398 nt long hok mRNA interacts with its 5’ end to block the mok TIR (Thisted, Sørensen and Gerdes 1995; Fig. 2B). The long hok species is slowly degraded by 3’-5’exoribonucleases RNase I and PNPase into a shorter (361 nt) species that is translationally competent (Fig. 2B). After processing, Sok binds very efficiently to the mok SD in the short hok mRNA to inhibit its translation (Thisted et al. 1994; Thisted, Sørensen and Gerdes 1995). Rapid binding of Sok to full-length hok mRNA would be detrimental, as the hok RNA/Sok duplex is rapidly cleaved by RNase III (Gerdes et al. 1992) and thus, the activatable pool of hok mRNA would be depleted too quickly.

Similarly, in E. coli tisB/IstR1 (Vogel et al. 2004; Darfeuille et al. 2007), the primary tisB transcript (+1) is translationally inactive and has to be processed within its 5’ end into a shorter (+2) translationally competent (lowly abundant) molecule (Fig. 2C). Only the +2 transcript has an unstructured 5’ segment containing an RSS to which, at the same time, the antitoxin IstR1 can bind to prevent binding of the 30S subunit. After IstR1 binding, RNase III cleaves the +2 transcript into another translationally inactive +166 tisB RNA molecule.

In E. coli shoB/OhsC, different 5’ ends of shoB (+1, +2) had been mapped (Kawano et al. 2005). The longer of two shoB mRNA species (~320 nt) was translationally inactive, whereas the shorter (~280 nt) showed low activity and could be ~2-fold inhibited by OhsC (Fozo et al. 2008), indicating another case of toxin–mRNA processing.

Alternative approaches were found in Gram-positive bacteria: in B. subtilis, tspA, bsrg and bsre sequester their SD sequences in double-stranded regions comprising 4 or 5 GC base pairs (reviewed in Durand et al. 2012), which suffice to obstruct ribosome binding. For bsrg, neither toeprint nor detectable amounts of in vitro translation product could be obtained from wild-type mRNA unless mutations were introduced that opened the double-stranded region (Jahn and Brantl 2013). In En. faecalis, the fst SD sequence is also sequestered in a double-stranded region of four adjacent base pairs in a stem loop termed 5’ SL (Greenfield et al. 2000) and postulated to temporarily inhibit ribosome binding until the terminator loop can be transcribed (Shokeen et al. 2008). A processing event was neither observed in fst/RNAII nor in the B. subtilis systems.

Furthermore, tspA and yonT have perfect RBS (≥11 bp complementarity to anti-SD in 16S rRNA), which are predicted to efficiently recruit, but slowly release ribosomes (reviewed in Durand et al. 2012).

Beyond that, in some cases—E. coli dinQ, B. subtilis yonT and En. faecalis fst—the rare start codon GUG is used instead of AUG, which is also expected to decrease the efficiency of translation, as it is used in only 14% and 9% of all genes in E. coli and B. subtilis, respectively (Rocha, Danchin and Viari 1999).
Interestingly, for S. aureus sprA1 mRNA, it was suggested that the compact secondary structure comprising RNA pseudoknots flanked by stable stem loops would obstruct internal ribosome binding (Sayed, Jousselin and Felden 2011). Recently, the same pseudoknots were proposed for the structurally similar En. faecalis fst mRNA (Weaver 2014).

Role of Hfq

Surprisingly, the ralR/RalA TA system of E. coli prophage rac requires Hfq for antitoxin function (Guo et al. 2014). This might be due to the rather short (16 nt) sequence complementarity between ralR andRalA. At high concentrations,RalA binds Hfq, but it is still unknown if Hfq promotes ralR/RalA complex formation or if it affects the stability of either RNA or perhaps translation of ralR (Guo et al. 2014). The latter would resemble another sRNA/target RNA system, SR1/ahrC, where Hfq was needed for ahrC mRNA translation (Heidrich, Moll and Brantl 2007). Currently, ralR/RalA is the only type I TA system, where a function for Hfq has been found. Neither the other E. coli antitoxins with relatively short (18 to 19 nt) complementary regions to their toxin mRNA like IstRI (Darfeuille et al. 2007), OrzO (Wen, Won and Fozo 2014), OhsC or the Sbs (E. Fozo, personal communication) nor the B. subtilis antitoxins like SR4 (Jahn et al. 2012) that use \( \approx \)120 nt of complementarity need Hfq. En. faecalis fst/RNAII system does not even encode Hfq. However, it is not excluded that other, still unidentified RNA binding proteins might play a role that other short base-pairing regions.

Type III antitoxins

In contrast to type I antitoxins that use a base-pairing mechanism to inhibit their corresponding toxins, type III antitoxins act by RNA-protein binding. In this regard, they resemble regulatory sRNAs that sequester proteins like e.g. CsrB from E. coli that binds CsrA, 6S RNA that interacts with RNAP or Rcd from plasmid ColE1 that binds tryptophanase (reviewed in Brantl 2009).

The first type III TA system, toxN/ToxI, was discovered on a cryptic plasmid from Erwinia carotovora subsp. atroseptica, later renamed P. atrosepticum subsp. atroseptica (Fineran et al. 2009). Whereas type I toxins and antitoxins are transcribed from independent promoters on complementary DNA strands, type III antitoxins and toxins are cotranscribed from a single promoter upstream of the antitoxin gene (see Fig. 1C). Infrequent (10%) read-through of a Rhino-dependent transcriptional terminator separating both genes warrants an excess of antitoxin over toxin. Full-length antitoxin ToxI (200 nt) consists of 5.5 sequence repeats of 36 nt, but a single repeat suffices to repress ToxN toxicity (Fineran et al. 2009; Blower et al. 2011). ToxI monomers are generated from the 200 nt precursor via sequence-specific processing by the ToxN endorRNAse. Structure analysis revealed that ToxI folds into an RNA pseudoknot. Three ToxI monomers bind three ToxN proteins in a trimeric Toxl/ToxN complex resulting in inhibition of ToxN function. An extensive set of RNA-protein contacts is used. ToxN functions by a reversible growth-inhibiting (bacteriostatic) mechanism (Fineran et al. 2009).

The second currently known type III TA system, abiQ/antiQ, was reported in 2013 in L. lactis and the toxin structure solved (Samson et al. 2013). As toxN/ToxI, it is located on a plasmid and required for abortive phage infection. Although the primary sequences of both type III loci show only 31% similarity, the antitoxin gene is always located 5′ of the toxin gene, separated from it by a weak terminator and comprises several (here 2.8 instead of 5.5) 35 nt repeats. The AbiQ toxin is also an RNAse that cleaves the antitoxin repeats, and its 3D structure is similar to that of ToxN. AntiQ repeats also form pseudoknots, and, upon AbiQ binding, the RNAse activity of the toxin is inhibited.

A recent bioinformatics approach predicted a variety of type III TA systems in the chromosomes and plasmids of different bacterial species. In addition to toxN/ToxI, two novel type III TA families, ctn/CptI and tenp/TenpI, with slightly longer antitoxin repeats (40–48 and 39–57, respectively) and toxins of approximately the same size as ToxN (148 to 160 aa) were found. Among them, CptI is only chromosome encoded and—so far— not detected on plasmids (Blower et al. 2012).

Role of RNase III and other RNases involved in antitoxin/toxin-mRNA degradation

Already 25 years ago, it had been shown for many cis-encoded regulatory sRNAs (antisense RNAs) that the ubiquitous double-strand-specific RNAse III cleaves the antisense/sense RNA duplex thereby initiating degradation of the target mRNA and, consequently, inhibiting target gene expression. The first cases analysed in detail were plasmid-, transposon- or phage-encoded antisense RNAs like e.g. CopA regulating replication of E. coli plasmid R1 (Blomberg, Wagner and Nordström 1990), RNA-OUT controlling transposition of IS10 (Case, Simons and Simons 1990) and phase \( \lambda \) OOP RNA regulating cII mRNA, an activator of \( \lambda \) cI transcription (Krinke and Wulf 1987, 1990). In type I TA systems, a role of RNAse III was also found for hok/Sok of plasmid R1 (Gerdes et al. 1992) and 20 years later for bsrG/SR4 (Jahn et al. 2012) and txpA/RatA (Durand, Gilet and Condor 2012) from the B. subtilis chromosome. However, in some cases—\( \lambda \) OOP, RatA, as-yonT (Durand, Gilet and Condor 2012) and Orzo (Wen, Won and Fozo 2014)—RNAse III is essential for target inhibition, whereas it is not required by CopA, RNA-OUT and the type I antitoxin Sok (reviewed in Brantl 2007), SymR (Kawano, Aravid and Storz 2007), IstRI (Wagner and Unson 2012), Sib (Fozo 2012), B. subtilis SR4 (Jahn et al. 2012) and most probably, En. faecalis fst (Weaver 2014). RNAse III seems to be needed in all cases, where target control is exerted exclusively via mRNA degradation, whereas in those cases, where sRNAs/antitoxins regulate steps preceding degradation, e.g. translation (Sok, SymR, IstRI), RNAse III is dispensable and merely employed to cleave subsequently the sRNA/target RNA duplex. Interestingly, for zorO/Orzo, so far no mechanism of antitoxin action has been elucidated, and it is tempting to speculate that this antitoxin that binds to a region 73 to 91 nt upstream of the zorO start codon within the 5′ UTR might induce a processing event to trigger toxin mRNA degradation. Indeed, in the presence of RNAse III, 19 and 20 nt downstream from the end of the zorO/Orz complementary region, two cleavage sites, most probably due to action of a single-strand-specific endoribonuclease, have been mapped (Wen, Won and Fozo 2014).

The above hypothesis is corroborated by a comparison between bsrG/SR4 and txpA/RatA from B. subtilis: SR4 is a bifunctional antitoxin that does not only promote degradation but also inhibits translation of bsrG mRNA (Jahn and Brantl 2013). The fact that neither cell lysis nor mutations in the bsrG ORF were observed in a \( \Delta \)rcn strain (Jahn et al. 2012) confirms that RNAse III is not essential for toxin inhibition. RNAse III cleaves bsrG mRNA at position 185 i.e. 14 nt downstream of the 3′ end of complementarity with SR4. Half-lives of both SR4 and bsrG RNA were \( \approx \)2.5-fold increased in the absence of RNAse III, whereas degradation of SR4 or bsrG RNA alone were not affected (Jahn et al. 2012). By contrast, a recent analysis of txpA/RatA revealed that RNAse III cleavage of txpA RNA in the txpA/RatA duplex
prevents toxin expression and is essential (Durand, Gilet and Condon 2012). RatA acts only on target mRNA stability and does not induce a tpxA mRNA conformation that further inhibits translation (shown by in vitro secondary structure probing in Durand, Gilet and Condon 2012). In a ∆rec strain, tpxA (toxin) RNA levels were 6-fold increased, whereas those of RatA were unaffected. Instead, RatA degradation is initiated by the major B. subtilis endonuclease RNase Y reflected by higher RatA levels in an rny strain (see Fig. 2D). After initial cleavage by RNase Y, the 5′ segment of RatA alone is degraded by 3′-5′-exonuclease PnpA and the 3′ segment by 5′-3′ exoribonuclease J1 (Even et al. 2005). The RNase III cleavage products of tpxA mRNA are further processed by the 3′-5′ exoribonuclease PnpA (Durand, Gilet and Condon 2012).

The role of RNase III for antitoxin stability differs between the various type I TA systems analysed so far: two other B. subtilis type I antitoxins, as-yonT (Durand, Gilet and Condon 2012) and SR5 that regulates bsrE RNA (Ring, Jahn and Brantl unpublished data), behave like RatA in that their half-lives are not affected by RNase III. Most probably, the relative amount of TA mRNA is decisive: both RatA (Durand, Gilet and Condon 2012) and SR5 (Ring, Jahn and Brantl unpublished) are in excess over their target RNAs, whereas for SR4, an excess over bsrG RNA is only apparent under certain growth conditions (Jahn and Brantl unpublished).

Surprisingly, in B. subtilis bsrH/as-bsrH, the levels of toxin mRNA are neither influenced by RNase III nor RNase Y, but by RNase J1 and degradation is proposed to be rather exonucleolytically (Durand, Gilet and Condon 2012). This is reminiscent of some targets of trans-encoded sRNAs in E. coli, whose degradation is not initiated by RNase III but by endonuclease E as a consequence of sRNA binding (e.g. RyhB/soDB, Massé, Escorcia and Gottesman 2003; Prévost et al. 2011). It remains to be seen if other type I toxin mRNAs are substrates for degradation by alternative RNases and not by RNase III. Recently, we also observed an effect of RNase J1 on the stability of B. subtilis antitoxin SR5 that regulates bsrE (Ring, Jahn and Brantl unpublished data).

For bsrG/SR4, an about 2-fold influence of RNase Y on the degradation of both RNAs was found. Presumably, RNase Y cleaves bsrG RNA to yield ≈213 nt and ≈256 nt long species (Jahn et al. 2012). The main 3′-5′ exonucleolyse involved in the degradation of both bsrG mRNA and SR4 is RNase K, whereas RNases J1, Rph and YhaM did not influence the stability of either RNA. The 3′-5′ exonuclease PnpA trims three SR4 precursors from their 3′ ends to the mature 180 nt SR4, but only marginally contributes to SR4 or bsrG RNA degradation. In hok/Sok from E. coli, in addition to the role of RNase III for hok mRNA cleavage, contributions of the main endonuclease RNase E (the E. coli equivalent to RNase Y) and polyA polymerase PAPI have been found for Sok degradation (Mikkelsen and Gerdes 1997). By contrast, degradation of full-length hok mRNA (398 nt) is initiated by the 3′ exonuclease RNase II and PnpA yielding the translationally active 361 nt long species (reviewed in Gerdes and Wagner 2007).

Although in the case of ∆srrfsRNAII, no RNases have been discovered so far that influence the stability of antitoxin or toxin mRNA; an upstream helix designated 5′ UH has been analysed that comprises the immediate 5′ end and nt 133–139 in the central part of ∆srrfs RNA (Shokeen et al. 2009). 5′ UH protects the 5′ part of ∆srrfs RNA from RNases and is at least partially responsible for the greater stability of ∆srrfs RNA compared to its antitoxin, RNAII. As described above, TA RNA interaction results in accumulation of a complex with higher stability of the toxin mRNA, namely 40 instead of 9 min, and of antitoxin RNAII from 4 to 16 min (Weaver et al. 2004).

In summary, in all but one (bsrH) cases analysed to date, toxin mRNAs are, when in duplex with the antitoxin, cleaved by RNase III, but this is only essential for their inhibition in cases, where antitoxins exclusively promote RNA degradation. By contrast, effects of RNase III on RNA antitoxins are only evident when they are in large excess over their target toxin RNAs. A combination of other endo- and exoribonucleases—so far only investigated in detail for E. coli hok/Sok and some B. subtilis TA systems—is involved in regulating TA mRNA stability.

Regulation of toxin and antitoxin expression—threshold-based mechanisms

Toxin expression has to be tightly controlled. A number of type I toxin genes from E. coli are under SOS control: symE, tisB and dinQ have Lex boxes. By contrast, B. subtilis bsrG is temperature sensitive: bsrG RNA has a 3- to 4-fold shorter half-life at high temperatures (Jahn et al. 2012). This might be explained by refolding of bsrG mRNA at 55°C that makes it accessible to single-strand-specific RNases (Jahn and Brantl unpublished). In other cases, transcriptional regulation depending on metabolic or stress conditions has been proposed but not yet experimentally confirmed: for instance, upstream of bsrG, bsrH and bsrE, hypothetical ResD sites have been found. In the case of bsrG, however, first analyses have shown that this toxin is not regulated by ResD (Jahn and Brantl unpublished). Staphylococcus aureus sprA1 expression decreases 2-fold at lower pH, while it increases 3-fold under oxidative stress (Sayed, Jousselin and Felden 2011). For these alterations, responsible factors are not yet known. Interestingly, E. coli rulR was found to be higher expressed in later stages of biofilm development (Domka, Lee and Wood 2007). In the case of SymE, an additional post-translational regulation by Lon protease was discovered.

Much less is known on regulation of antitoxin expression. Usually, antitoxins are expressed constitutively. For instance, E. coli Sib RNAs (Fozo et al. 2008; Fozo, Hemm and Storz 2008), B. subtilis SR4 (Jahn et al. 2012) are expressed equally well in both rich and minimal media. Exceptions are En. faecalis RNAII and S. aureus SprA1ΔS (Sayed, Jousselin and Felden 2011) that peak at mid-log phase.

For the type III TA system toxI/ToxN, an autoregulation of the toxIN promoter by the trimeric toxI/ToxN complex was observed (Blower et al. 2009).

The balance between the levels of TA mRNA is critical for the conditions under which the toxin is expressed. Genetically identical bacterial populations exhibit heterogeneity, with e.g. both actively growing and dormant cells. This is a great advantage upon rapid changes of environmental conditions. Under conditions where antitoxin levels are higher than toxin RNA levels, toxin expression is successfully prevented and the cell survives. By contrast, when toxin RNA levels significantly exceed antitoxin levels, the toxin escapes repression and causes cell death or growth arrest. However, when toxin and antitoxin levels are close, some cells will have an imbalance in the toxin-antitoxin ratio and might become persisters (see below). A detailed mathematical analysis of persister formation was recently published (Rotem et al. 2010).

Comparison between antitoxins and sRNAs involved in control of metabolism, stress response and virulence

So far, only different modes of translational inhibition or promotion of RNA degradation have been found as regulatory mechanisms used by type I antitoxins. The only exception is ∆srrfRNAII, where translation inhibition is accompanied by stabilization
of both interacting RNAs. By contrast, cis- and trans-encoded sRNAs from plasmids or chromosomes that act by base pairing on their target RNAs can employ a much broader variety of regulatory mechanisms: some plasmid-encoded antisense RNAs exert target control by transcriptional attenuation (plasmids pT181 and pIP501; reviewed in Brantl 2014), translational attenuation (plasmid pSK41, Kwong, Skurray and Firth 2006) or inhibition of pseudoknot formation (In1u/IncB plasmids, Asano and Mizobuchi 1998). Other chromosome-encoded sRNAs employ transcriptional interference (Clostridium acetobutylicum ubiC operon, André et al. 2008) or mRNA stabilization owing to a processing event (E. coli Gdy/gadWX, Opdyke et al. 2011). Therefore, it remains to be seen in the future if type I TA systems will be discovered that use other, so far unprecedented mechanisms of antitoxin action.

For many chromosomally trans-encoded sRNAs, in particular from Gram-negative bacteria, the abundant RNA chaperone Hfq is required for either stabilization of sRNA or target mRNA or to promote complex formation between them. By contrast, only for one type I TA system, ralR/RalA, a role of Hfq has been reported (Guo et al. 2014). However, it is not excluded that other—perhaps still unknown—RNA binding proteins are involved in the stabilization of the rather short complementary regions between TA mRNA in vivo. Candidates could be the small RNA binding protein FbpA in B. subtilis, which is required for the regulation of the lutABC operon by the sRNA FsaA (Smaldone et al. 2012; reviewed in Brantl and Brückner, 2014) or E. coli CarA, which was recently found to bind, additionally to Hfq, to the sRNA McaS (Jørgensen et al. 2013).

The majority of type I antitoxins are transcribed constitutively, except SprA180S from S. aureus that peaks at mid-log phase (Sayed, Joussetin and Felden 2011) and the chromosomal En. faecalis fst homolog, encoded by parF/parO (Weaver 2014). By contrast, the majority of chromosome-encoded sRNAs are only expressed under certain environmental or stress conditions (e.g. OxyS under oxidative stress, Altuvia et al. 1997). Consequently, their amount varies significantly. It is in the nature of type I antitoxins to be short-lived, with half-lives between 30 s (Sok, Gerdes and Wagner 2007), 3–4 min (SR4, Jahn et al. 2012) and 8–9 min (RNAII, Weaver et al. 2004). By contrast, half-lives of chromosomal-encoded sRNAs display much broader variations from 2 to 32 min (Vogel et al. 2003) or even 55 min (Preis et al. 2009).

Many trans-encoded sRNAs have more than one target (e.g. S. aureus RNAII or E. coli RybB, reviewed in Brantl 2009), and frequently use different mechanisms to regulate them. Some targets are repressed, while others are activated by the same sRNA (e.g. S. aureus RNAII). In some cases, only translation is affected, in others both translation and RNA degradation (reviewed in Brantl 2009). It is tempting to speculate if some of the type I antitoxins might, as they are usually higher expressed than their toxin mRNAs, moonlight to regulate additional target RNAs.

A comparison between type III antitoxins and sRNAs that act by protein binding like E. coli CsrB/C, Ps. fluorescens RsmX/Y (reviewed in Brantl 2009) or Ps. aeruginosa CrcZ reveals different strategies. Whereas type III antitoxins form small pseudoknots composed of 35–36 nt that sequester their cognate toxins in trimeric complexes using a number of RNA–protein interactions, CsrB and CsrC present minimal 5′ GGA binding motifs—often embedded in an ANGGA context—in the loops of numerous (9 to 22) short stem-loop regions that bind small (61 aa) translational regulator proteins like CsrA or RsmE. Both CarA and Crc proteins are homodimers of intertwined α-strands with protruding α helices that use specific residues in the β-strands to bind two RNA sites simultaneously (CsrA, Schubert et al. 2007; Crc, Wei et al. 2013). However, in both cases, the proteins are bound in a sequence-specific and not a structure-specific manner.

**TYPE I AND TYPE III TOXINS AND THEIR MODE OF ACTION**

The classical characterized type I toxins are—with the exception of SymE and Ral—very small hydrophobic peptides of -60 aa (summarized in Table 2). All currently known hydrophobic type I toxins have an identical predicted or experimentally confirmed secondary structure containing an α-helical transmembrane domain and are supposed to be localized in the membrane (e.g. Fozo et al. 2008; Göbl et al. 2010; Sayed et al. 2012). It is proposed that these toxins can—similar to phage holins—interact with each other to create pore-like structures that destroy the membrane potential and, consequently, inhibit ATP synthesis (e.g. Fozo et al. 2008; Unoson and Wagner 2008). Nevertheless, information about their mode of action and their cellular targets is limited, as in most systems, deletion of the antitoxin does not result in phenotypic alterations making it difficult to analyse toxin effects at cellular levels. Much current information is based on overexpression experiments, in which most of the toxins cause cell death. Several studies have demonstrated that overexpression of hok, relF, srbN, psdA, fst, ibsC, shoB, tisB and dinQ leads to the destruction of membrane potential or membrane itself (Gerdes et al. 1986; Ono et al. 1986; Weaver et al. 2003; Fozo et al. 2008; Unoson and Wagner 2008; Weel-Sneve et al. 2013). Due to the hydrophobic nature of the toxin peptides and their similarity to both phage holins (Wang, Smith and Young 2000) and antimicrobial peptides (Henriques, Melo and Castanho 2006), these effects on the membrane are not surprising (Fozo, Hemm and Storz 2008). However, interactions with other cellular targets cannot be excluded.

The hok/sok module on E. coli plasmid R1 (Gerdes, Larsen and Molin 1985) acts as PSK system (Gerdes, Rasmussen and Molin 1986b). By contrast, most of the Hok-like toxins encoded in the chromosome of E. coli K12 seem to be inactive (Pedersen and Gerdes 1999). It has been demonstrated that overexpression of hok leads to formation of ‘ghost’ cells, which are characterized by condensed cell poles and a centrally located clearing resulting in cell death (Gerdes et al. 1986). For this reason, Hok proteins in general are supposed to kill cells by causing irreversible damage to the cell membrane. In accordance with this, hok overexpression leads to the collapse of membrane potential, respiratory arrest, efflux of small molecules including Mg2+ as well as ATP and influx of extracellular molecules as e.g. ONPG (summarized in Gerdes et al. 1997). The overproduction of the hok homolog, RelF from the E. coli relB operon (Bech et al. 1985), results in the same phenomena: collapse of membrane potential, arrest of respiration, change in cell morphology and cell death (Gerdes et al. 1986).

Overexpression of the pAD1-encoded Fst toxin from En. faecalis (Weaver et al. 1996) is not only toxic in its original host but also in several bacilli, including B. subtilis and in S. aureus (Weaver et al. 2003, 2009; Patel and Weaver 2006). Toxicity can also be detected in E. coli, when the 5′ stem-loop of fst mRNA is deleted (Shokeen et al. 2008). These results indicate that Fst does not have a host-specific target. The primary effect observed in all four species is the condensation of the nucleoid. Whereas in both B. subtilis and E. coli, an elongation of cells could be detected due to the inhibition of cell division at an early time point, the...
division septa in S. aureus can be formed, but not fully completed. Instead, Fst overproduction in En. faecalis leads to misplaced septa resulting in daughter cells with little or without DNA, indicating a segregation defect. The additionally observed increase of membrane permeability turned out to be a secondary effect, probably caused by ongoing division and/or segregation defects (Patel and Weaver 2006). Recently, it was proposed that Fst induces upregulation of energy-requiring membrane transporters (pumps), as an rpoC mutant that was resistant against Fst did not induce these pumps, and chemical inhibition of the pumps rescued the cells from the effects of Fst (Brinkman et al. 2013).

Overproduction of the chromosomally encoded E. coli LdrD, an Fst-homolog from a Gram-negative bacterium (Fozo et al. 2010), evokes a rapid chromosomal condensation as well, and inhibits global translation (Kawano et al. 2002; Kawano 2012). Furthermore, microarray analysis revealed that overexpression results in physiological alterations in the cell, including upregulation of purine metabolism and decrease of intracellular cAMP levels. Recently, it was shown that Ldr may inhibit ATP synthesis, possibly due to its localization in the cell membrane (Yamaguchi et al. 2014).

Transcription of tisB, dinQ and symE RNA is induced by SOS stress response indicating that the toxin proteins might play a role under these stress conditions. TisB targets the inner membrane of E. coli, and its overexpression leads to the decrease of membrane potential resulting in reduced intracellular ATP levels, shut down of macromolecular synthesis including replication, transcription and translation and, consequently, in cell death in a fraction of the population (Unoson and Wagner 2008; Gurnev et al. 2012; Fig. 4A). Synthetic TisB monomers bind rapidly to membranes, and antiparallel dimers were postulated to assemble as an electrostatic charge zipper via a ladder of salt bridges that enables protons to cross the hydrophobic membrane (Steinbrecher et al. 2012). The depolarization of the membrane is probably caused by the formation of small, anion selective pores, which could make the membrane permeable for hydroxyl anions (Gurnev et al. 2012). How ever, all these in vivo effects were only observed upon tisB overexpression, and are, therefore, not conclusive for the biological relevance of TisB during SOS response. Particularly, cell killing is not observed with a single chromosomal tisB copy. Instead, the 1000-fold induced expression of tisB under SOS conditions results in dormant persisters that are highly tolerant to

Table 2. Overview of type I and type III toxins.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Peptide length (aa)</th>
<th>Organism plasmid</th>
<th>Localization</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hok∗</td>
<td>52</td>
<td>E. coli plasmid R1</td>
<td>Inner membrane</td>
<td>Loss of membrane potential → arrest of respiration, efflux/influx of small molecules, ghost cells</td>
</tr>
<tr>
<td>RelF∗</td>
<td>51</td>
<td>E. coli</td>
<td>Inner membrane</td>
<td>Loss of membrane potential → arrest of respiration, ghost cells</td>
</tr>
<tr>
<td>PndA∗</td>
<td>50</td>
<td>E. coli plasmid R483</td>
<td>Inner membrane</td>
<td>Increase of membrane permeability</td>
</tr>
<tr>
<td>SmB∗</td>
<td>68</td>
<td>E. coli plasmid F</td>
<td>Inner membrane</td>
<td>Increase membrane permeability</td>
</tr>
<tr>
<td>IbsC</td>
<td>18-19</td>
<td>E. coli</td>
<td>Inner membrane</td>
<td>Loss of membrane potential</td>
</tr>
<tr>
<td>ShoB</td>
<td>26</td>
<td>E. coli</td>
<td>Inner membrane</td>
<td>Loss of membrane potential</td>
</tr>
<tr>
<td>LdrD</td>
<td>35</td>
<td>E. coli</td>
<td>Inner membrane</td>
<td>Nucleoid condensation, inhibition of translation, loss of cell viability, physiological alteration (purin metabolism, cellular cAMP levels)</td>
</tr>
<tr>
<td>TisB</td>
<td>29</td>
<td>E. coli</td>
<td>Inner membrane</td>
<td>Loss of membrane potential/disruption by pore → decrease in intracellular ATP levels → inhibition of macromolecular synthesis; single copy: persister formation</td>
</tr>
<tr>
<td>DinQ</td>
<td>27</td>
<td>E. coli</td>
<td>Inner membrane</td>
<td>Loss of membrane potential → decrease in intracellular ATP levels, modulates recombination and nucleoid compaction</td>
</tr>
<tr>
<td>SymE</td>
<td>113</td>
<td>E. coli</td>
<td>Cytoplasm?</td>
<td>Degradation of selected RNAs</td>
</tr>
<tr>
<td>RalR</td>
<td>64</td>
<td>E. coli</td>
<td>Cytoplasm?</td>
<td>Non-specific endoRNase</td>
</tr>
<tr>
<td>Fst</td>
<td>33</td>
<td>En. faecalis plasmid pAD1</td>
<td>Membrane</td>
<td>Cell anomalies: nucleoid condensation, division and segregation defects, secondary effect: increase of cell permeability → inhibition of macromolecular synthesis</td>
</tr>
<tr>
<td>Fst-Sm</td>
<td>32</td>
<td>St. mutans</td>
<td>Membrane?</td>
<td>Mild overexpression of Fst-Sm/srSm system: decrease of persister levels</td>
</tr>
<tr>
<td>SprA1</td>
<td>30–33</td>
<td>S. aureus</td>
<td>Membrane?</td>
<td>Antimicrobial activity against Gram-negative and Gram-positive bacteria; cytolytic for human cells</td>
</tr>
<tr>
<td>SprF1</td>
<td>31, 44</td>
<td>S. aureus</td>
<td>Membrane</td>
<td>Two peptides are secreted in small amounts; antimicrobial activity against Gram-negative and Gram-positive bacteria; cytolytic for human cells</td>
</tr>
<tr>
<td>ToxN</td>
<td>171</td>
<td>P. atrosepticum plasmid pECA1039</td>
<td>Cytoplasm?</td>
<td>EndoRNase activity, abi system</td>
</tr>
<tr>
<td>AbiQ</td>
<td>172</td>
<td>L. lactis plasmid pSRQ900</td>
<td>Cytoplasm?</td>
<td>EndoRNase activity, abi system</td>
</tr>
</tbody>
</table>

∗hok gene family; ?, suggested, but not experimentally corroborated.
E. coli

Figure 4. Mode of action of toxins. A Gram-negative bacterium with inner and outer membrane, chromosomal DNA and, if present, plasmids are shown. Black and white bars denote -35 and -10 boxes of promoters, respectively. The LexA binding site is represented by a yellow box. Toxins are drawn in blue and antitoxin RNA in red. Toxin ORFs are shown as light blue bars. Grey boxes indicate SD sequences. (A) TisB (chromosomal type I TA system tisB/istR1). TisB is an SOS-induced, hydrophobic toxic peptide that arrests cell growth by decreasing the membrane potential, which results in decreasing ATP levels and shut-off of macromolecule synthesis. (B) SymE (chromosomal type I TA system symE/SymR). SymE is activated by the SOS response system (LexA) and acts as endoribonuclease that degrades mRNAs and non-coding RNAs, but not SymR. The SymE toxin itself can be degraded by Lon protease. (C) ToxN (plasmid-encoded type III TA system toxN/toxI). Transcriptional read through from the toxI promoter into toxN allows the synthesis of toxN mRNA and, consequently, the expression of the toxin ToxN. ToxN acts as a ribonuclease directed against both general cellular targets and its own antitoxin ToxI. (D) RaI (chromosomal type I TA system raI/RaI). The expression of raI is induced under unknown conditions. RaI functions as a non-specific endodeoxyribonuclease that cleaves both methylated and unmethylated DNA resulting in cell death.

unrelated antibiotics (Unoson and Wagner 2008; Dörr, Vulic and Lewis 2010). These effects can be brought into line with the effects of TisB on membrane integrity and reduced intracellular ATP levels (Unoson and Wagner 2008), since the growth arrest would explain the antibiotic-resistant phenotype of persisters (Unoson and Wagner 2008; Dörr, Vulic and Lewis 2010).

The recently identified chromosomally encoded dinQ/Agr module of E. coli shares many features with the tisB/istR1 locus. DinQ is also localized in the inner membrane and its overexpression leads to membrane depolarization and, consequently, to the decrease of intracellular ATP levels (Weel-Sneve et al. 2013). Furthermore, it has been shown that it affects membrane-dependent activities like nucleoid compaction and recombination.

A number of type I toxins evoke other effects in the cell aside from disruption of membrane potential or membrane damage: The SOS-induced SymE from E. coli with a length of 113 aa is not hydrophobic, but an RNase suggested to play a role in recycling of damaged RNAs under SOS conditions (Kawano, Aravind and Storz 2007). This protein does not show functional homology to other type I toxin proteins, but instead has homology to the AbrB-fold superfamily proteins, which act as transcriptional factors and antitoxins in different type II TA systems like mazEF (Kawano, Aravind and Storz 2007). On the other hand, SymE shares properties with type II toxins such as MazF that cleaves RNAs ribosomally independent, since it enhances the degradation of both mRNAs and non-coding RNAs but not its antitoxin SymR (Kawano, Aravind and Storz 2007; Fig 4B).

This mode of action was also found in type III toxins (Blower et al. 2011; Samson et al. 2013). The two type III toxins ToxN and AbiQ known so far have sequence-specific, ribosome-independent endorNase activities which, on the one hand, process their antitoxins from a precursor and, on the other hand, degrade phage RNA acting as abi (abortive phage infection) systems (Blower et al. 2011; Samson et al. 2013). In the case of ToxN, it was demonstrated that it does not only protect its host against multiple phages but also cleaves within host RNA (Short et al. 2013; Fig. 4C). In both ToxN and AbiQ, a serine residue present at position 51 or 52, respectively, is involved in catalysis.

The recently discovered type I toxin RalR of the cryptic E. coli prophage rac is the first known toxin that acts as a non-specific endoDNase (Guo et al. 2014; Fig. 4D). It is probably located in the cytoplasm and has been shown to cleave methylated and unmethylated DNA equally well.

For years it was speculated about extracellular effects of type I toxins, similar to those of antimicrobial peptides. S. aureus toxin SprA1 that belongs to the fts family (Fozo et al. 2010) was reported to behave not only as a type I toxin in staphylococci but also as a toxin that kills bacterial rivals in a mixed-cell population and as a cytolsin that lyzes human erythrocytes (Sayed, Jousselin and Felden 2011). This would indicate that it might outcompete other S. aureus strains or other species like E. coli or B. subtilis and that it is—upon infection—released from the membrane and could act on human host cells. All these properties would be contradictory to a toxin of a bona fide TA system, which, by definition, is only directed against its own cell. A previous study with a chemically synthesized En. faecalis Fst suggested that it can neither lyse human erythrocytes nor could it act against B. subtilis or E. coli as potential bacterial rivals (Göbl et al. 2010). Likewise, it was demonstrated that the exogenous application of Hok does not significantly kill other bacterial strains (Pecota et al. 2003). A comparison of the aa sequences of Fst and SprA1 reveals only structural, but no...
sequence similarity. In particular, SprA1 contains a central cysteine residue that is lacking in Fst. NMR studies revealed that this cysteine is located at a flexible hinge, and the authors hypothesize that it might trigger dimerization (Sayed et al. 2012). Therefore, it cannot be excluded that differences in the primary sequences might be responsible for the diverging properties of both toxins.

It will be exciting to see which other modes of action of type I toxins will be identified in the future. The most common target of these toxins is the membrane, with two exceptions: SymE and RaiR. In contrast to type I toxins, type II toxins typically act as endorNases, either as free enzymes (Jørgensen et al. 2009) or ribosome-associated ones (Neubauer et al. 2009). Other mechanisms elucidated for type II toxins are inhibition of DNA gyrase, of protease or phosphotransferase activity (Jiang et al. 2002; Meinhart et al. 2003; Yamamoto et al. 2009).

**BIOLICAL ROLES OF TYPE I AND TYPE III TA SYSTEMS**

The role of plasmid-encoded type I TA systems is evident: they are PSK systems, i.e. in plasmid-free cells, the short-lived antitoxin is rapidly degraded and thus cannot prevent toxin expression anymore, whereas the stable toxin mRNA is translated, eventually causing cell death. This scenario has been verified first for *E. coli* hok/Sok (reviewed in Gerdes and Wagner 2007) and later for *En. faecalis* fst/RNAII (reviewed in Weaver 2012, 2014).

By contrast, the function of chromosome-encoded type I TA systems, among them homologs of hok/Sok (Gerdes et al. 1997; Pedersen and Gerdes 1999) or fst/RNAII (Weaver et al. 2009) is largely unknown. For chromosome-encoded type II TA systems, a role in persister formation has been well established: whereas individual deletions of the 10 known type II TA systems comprising mRNase toxins from the *E. coli* genome had only minor effects, the simultaneous deletion of all 10 currently known type II loci dramatically reduced persistence (Maisonneuve et al. 2011). Persisters cells are a subset of a bacterial population that has stochastically entered a dormant state and thus became refractory to the action of antibiotics, whereas their isogenic, rapidly growing siblings remain sensitive. After long periods of time, persisters may be resuscitated and resume growth, re-establishing the population of antibiotics-susceptible cells. The mechanism of persister formation is still unknown. So far, only for one type I TA system, a role in persister formation has clearly demonstrated: in *E. coli* tisB/IstR1, SOS-dependent induction of tisB expression increased the level of persister cells resistant to the antibiotic ciprofloxacin—a gyrase inhibitor—significantly, whereas deletion of the entire tisB/IstR locus caused a sharp decrease of persisters (Dör, Lewis and Vulic 2009 and Dör, Vulic and Lewis 2010). This finding can be reconciled with the effect of TisB on membrane integrity (see above).

Recently, fst-Sm/SrSm, a chromosomal fst/RNAII homolog in *Streptococcus mutans* (Tables 1 and 2) has been also linked to persister formation (Koyanagi and Levesque 2013): however, mild overproduction of the entire fst-Sm/srSm locus from a multi-copy plasmid surprisingly decreased the levels of persister cells tolerant to cell-wall synthesis inhibitors like vancomycin. Perhaps, placing toxin and antitoxin genes out of their chromosomal context on a plasmid resulted in an imbalance between toxin and antitoxin levels.

Although *E. coli* dinQ/AgrB is similar to tisB/IstR1, persister formation has not been studied, but this TA system was found to be important for chromosome stability: a ΔagrB strain showed 2-fold elevated DinQ levels and displayed a 400-fold reduced recombination frequency, suggesting that the toxin interferes with homologous recombination (Weel-Sneve et al. 2013). No effect on general DNA uptake during conjugation was found.

The recently discovered ral/RalA TA system from *E. coli* was also shown to be beneficial for resistance against a cell-wall-inhibiting antibiotic, fosfomycin (Guo et al. 2014). However, persister formation has not been investigated so far.

The RNA-cleaveing *E. coli* toxin SymE has been suggested to recycle damaged RNAs produced under SOS stress conditions or to prevent infection with RNA phages (reviewed in Kawano 2012).

On the contrary, many *B. subtilis* toxin genes (*txpA, yonT, bsrG, bsrE, bsrH*) are located on prophages and prophage-like regions and were proposed to be required for maintenance of these elements, reminiscent of the role of plasmid-encoded PSK systems. For instance, txpA/RatA was suggested to sustain the skin element, which is excised by a DNA rearrangement during sporulation from the *B. subtilis* genome (reviewed in Durand et al. 2012). This excision ensures reconstitution of a functional sigK gene encoding a transcription factor that controls mother-cell-specific gene expression late in sporulation. All TA modules in *B. subtilis* are under control of α^+^ promoters allowing rapid toxin expression under defined conditions. Therefore, the identification of transcriptional activators that might perturb the balance between toxin and antitoxin expression could provide us with clues about the function of the individual toxins. Although putative resD sites have been predicted upstream of bsrG, bsrE and bsrH, it remains to be seen if increased expression under oxygen-limiting conditions as suggested (Nicolas et al. 2012) is indeed due to ResD binding to its cognate site.

Some TA systems are induced under certain environmental conditions like metabolic stress (e.g. glucose starvation) or oxygen stress, and the function of the corresponding toxins could be to cause bacteriostasis to limit nutrient consumption or oxygen exhaustion (see Durand et al. 2012), i.e. these systems would be involved in metabolic or stress adaptation. In accordance with this hypothesis, txpA mRNA expression seems to be phosphate dependent, and txpA, bsrG, bsrH and yonT RNA levels were proposed to increase when glucose is exhausted, linking these toxins to the metabolic state of the cell (based on Nicolas et al. 2012). Interestingly, bsrG could play a role in response to temperature changes, as its half-life is temperature dependent, and the mRNA is rapidly degraded upon temperature shock at 48 or 55 °C (Jahn et al. 2012, see above).

The two known type III TA systems, toxN/ToxI and abiQ/AntiQ, are located on plasmids, but they are abi modules, i.e. they are not required for propage maintenance, but for defence against phage invasion (e.g. Samson et al. 2013). The role of chromosome-encoded type III systems is as elusive as that of type I systems (Blower et al. 2012).

**PERSPECTIVES/CONCLUSION**

Despite all data accumulated over the past years, our knowledge on type I TA systems is still limited. It seems that all bacteria that do not live as intracellular parasites encode a variety of TA modules in their genomes. Notwithstanding the variation between the type I systems discovered and studied so far, they seem to constitute a conserved family with similar regulatory properties. Although some of these systems have taught us new modes by which a small regulatory RNA can inhibit expression of its target mRNA (e.g. IstR1—blockage of an RSS), we can expect that other still unprecedented regulatory mechanisms will come to
our attention in which both RNA antitoxins and other sRNAs are involved.

Furthermore, it is tempting to speculate if, after the discovery of two classes of RNA antitoxins that either bind toxin mRNA (type I) or toxin peptide directly (type III), another class of RNA antitoxins might exist that is able to bind both the corresponding toxin mRNA and the toxin peptide.

As it is possible that stochastic expression of toxins, and consequently, effects on cells of only a subpopulation, might explain why such modules are broadly distributed in genomes of all bacterial species that do not live as parasites, future research will have to focus on two aspects. Firstly, single-cell techniques and reporter gene fusions should be employed to detect endogenously expressed toxin proteins under various conditions. This would allow for a better insight into population heterogeneity. Secondly, the investigation of differences between wild-type strains and strains deleted for all known type I TA systems under stress conditions like phage infection, depletion of nutrients, harmful environments as presence of antibiotics etc. would help to elucidate the still enigmatic biological role of these numerous systems.

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