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RNA antitoxins

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Recent genomic analyses revealed a surprisingly large number of toxin–antitoxin loci in free-living prokaryotes. The antitoxins are proteins or antisense RNAs that counteract the toxins. Two antisense RNA-regulated toxin–antitoxin gene families, *hok/sok* and *ldr*, are unrelated sequence-wise but have strikingly similar properties at the level of gene and RNA organization. Recently, two SOS-induced toxins were found to be regulated by RNA antitoxins. One such toxin, SymE, exhibits similarity with MazE antitoxin and, surprisingly, inhibits translation. Thus, it is possible that an ancestral antitoxin gene evolved into the present toxin gene (*symE*) whose translation is repressed by an RNA antitoxin (SymR).

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Introduction

Prokaryotic plasmids and chromosomes encode toxin–antitoxin (TA) loci, often in a few or even many copies [1–4]. TA loci are two-component systems that code for a stable 'toxin', whose ectopic overexpression either kills the cells or confers growth stasis, and an unstable 'antitoxin'. TA loci are of two types: those that are regulated by antisense RNAs ('RNA antitoxins') and those that are regulated by protein antitoxins [5–7]. Antisense RNA regulated TA loci were reviewed a decade ago [5], and an update therefore seems timely. Here, we review the RNA antitoxin field, with an emphasis on recently discovered *cis*-encoded and *trans*-encoded regulatory RNAs.

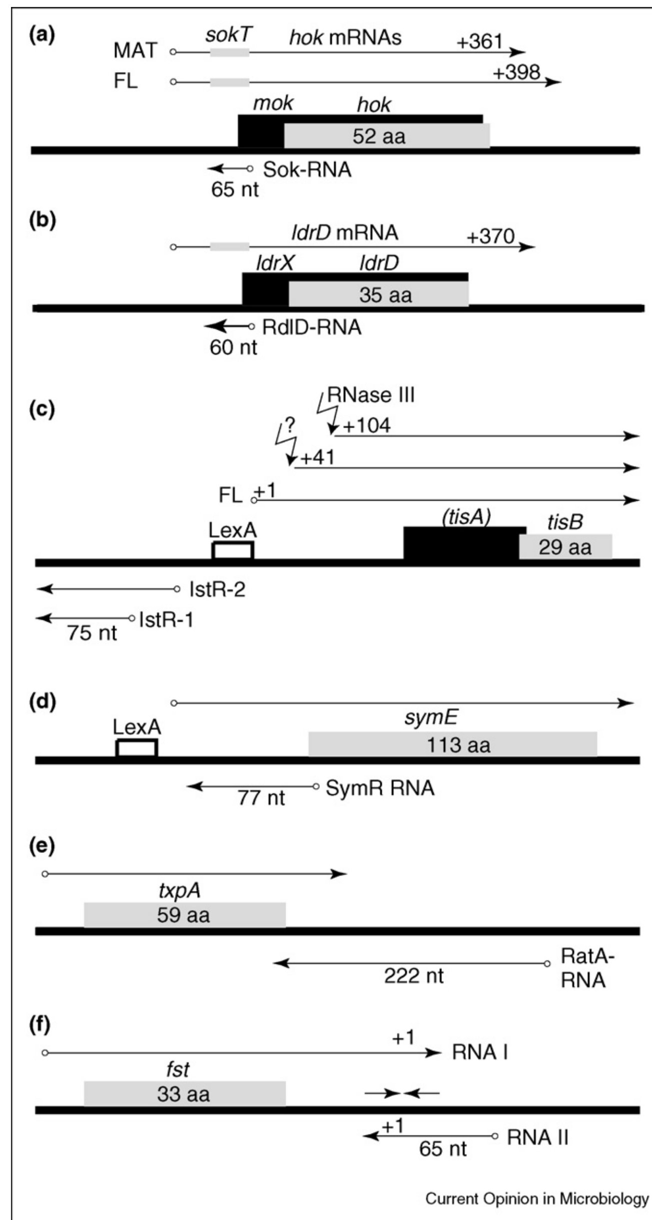
The *hok/sok* locus of plasmid R1

Antisense RNAs have pivotal roles in the life of many mobile genetic elements (plasmids, phages and transposons). Importantly, antisense RNAs are key regulators of replication in many plasmids, including those with high (pMB1, ColE1) and low (R1, pLS1) copy numbers. The extensive analysis of these model systems have provided a profound understanding of the control loops that govern the initiation of plasmid replication [8–10]. Antisense RNAs also control plasmid conjugation and, in some cases, also plasmid maintenance [5,11].

The *hok/sok* locus of plasmid R1 was identified in a screen for genes that can stabilize plasmids [12]. Plasmid stabilization by *hok/sok* is as a result of a phenotype called post-segregational killing (PSK), a phenomenon even exerted by some protein-regulated TA loci [13,14]. As shown in Figure 1a, this locus has three genes: 'host killing' (*hok*) encodes a highly toxic transmembrane protein that irreversibly damages the cell membrane [15]; 'modulation of killing' (*mok*) overlaps with *hok* and is required for *hok* translation. Finally, 'suppression of killing' (*sok*) specifies a small *cis*-encoded antisense RNA that blocks translation of *mok*. Because translation of *hok* depends on *mok* translation, Sok RNA indirectly inhibits *hok* translation by inhibiting *mok* translation [16]. Sok RNA is driven by a strong promoter but is very unstable (half-life in the order of 30 seconds). By contrast, *hok* mRNA is synthesized from a weak promoter but is very stable (half-life of ~20 minutes) [17]. During steady-state cell growth, Sok RNA is in considerable molar excess over *hok* mRNA [18]. However, newborn plasmid-free cells experience rapid depletion of Sok RNA, leaving behind a pool of *hok* mRNA that can be translated. Hence, Hok toxin is synthesized specifically in plasmid-free cells, and *hok/sok* therefore increases plasmid maintenance by reducing the survival of these cells.

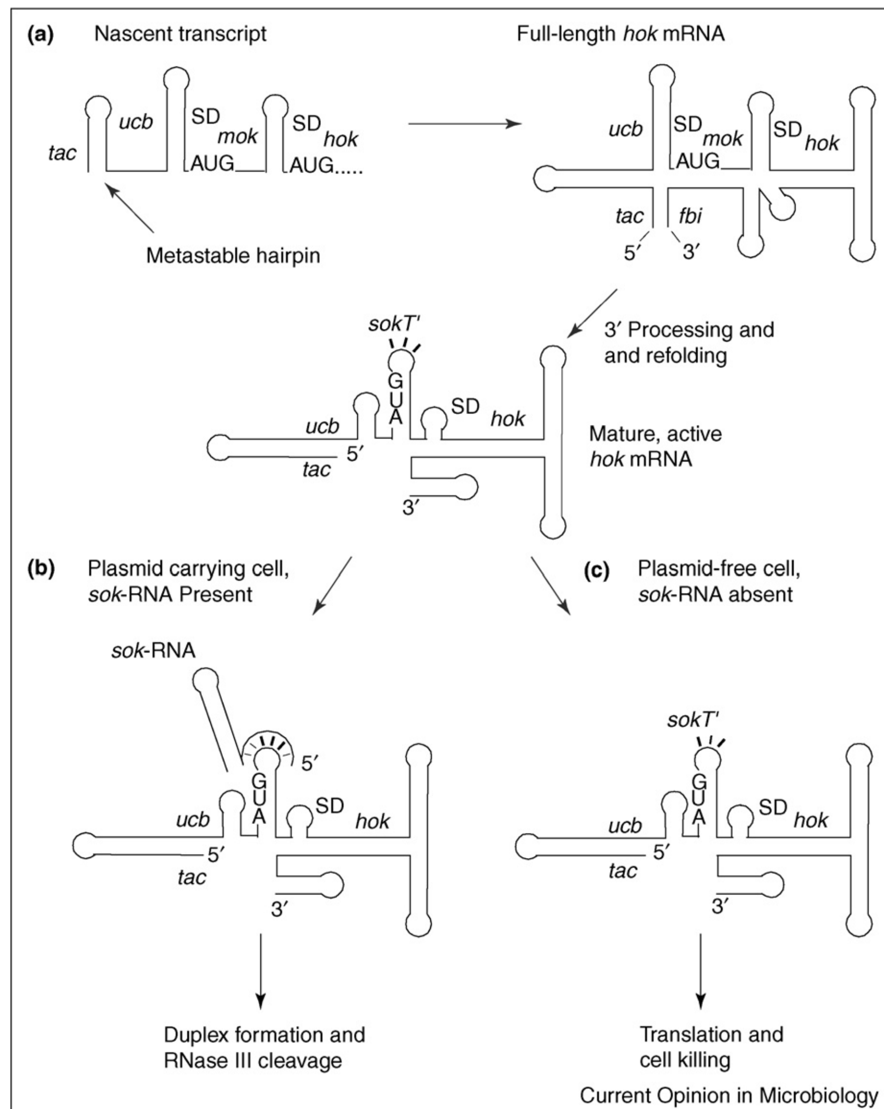
Sok RNA and *hok* mRNA form a complete RNA duplex that is rapidly cleaved by RNase III [19]. This observation raises the obvious and nontrivial question of how *hok* mRNA translation is activated in plasmid-free cells. It turns out that the primary *hok* mRNA is stable and inactive but can be activated by 3′ processing [by RNase II and polyribonucleotide nucleotidyltransferase (PNPase)]. Thus, the *hok* full-length (FL) transcript is stable and adopts an inert conformation that is inaccessible to both initiating ribosomes and Sok RNA. By contrast, the mature, 3′-processed isoform is translatable and capable of binding to Sok RNA (MAT in Figure 1a). In cells with a *hok/sok*-carrying plasmid (which produces Sok RNA), Sok RNA binds to the mature form of *hok* mRNA and represses translation by preventing ribosome entry. In plasmid-free cells, the Sok RNA pool is depleted owing to rapid decay of the antisense RNA, the mature and translatable form of *hok* mRNA accumulates, and Hok protein synthesis results in killing of the plasmid-free cells.

Figure 1



Genetic organization of RNA antitixon-regulated gene loci. **(a)** *hok/sok* of plasmid R1. *sokT* is the Sok-RNA target in *hok* mRNA (indicated by a small grey box). The *mok* reading frame is required for *hok* to be translated and the gene product itself has no known function. The *mok* reading frame is out-of-frame with *hok* and terminates 1 nt downstream of *hok* (i.e. there is one nt between the two stop codons). FL denotes the full-length, inactive *hok* mRNA; MAT denotes the 3′-truncated, active *hok* mRNA. **(b)**, *ldrD* locus of *E*. *coli* K-12. *ldrX* denotes an open reading frame that starts with UUG and overlaps with *ldrD*. *ldrX* and *ldrD* are 'in-frame' and they therefore share the same translational termination codon. We predict that RdlD RNA regulates *ldrD* translation by regulating *ldrX* translation. Numbers indicate the lengths of the RNAs in nucleotides. Small grey boxes symbolize the regions in the mRNAs that are complementary to the respective antisense RNAs. **(c)** *tisAB* locus of *E*. *coli* K-12. A parenthesis around *tisA* indicates that the gene is not translation and has no known function. FL denotes the inactive full-length *tisAB* mRNA that is processed by an unknown RNase at +41 to yield the active, translatable isoform. This mRNA, in turn, is bound by the IstR-1 antisense RNA that has a patch of 21 nt that is complementary to 21 nt in the *tisAB* mRNA. IstR-1 is thus a *trans*-regulatory antisense RNA [52]. The resulting duplex is cleaved by RNase III to yield the +104 isoform that is also translationally inactive. LexA indicates a LexA binding site in the *tisAB* promoter that enables SOS-regulation of *tisAB* transcription. IstR-2 is not involved in *tisAB* regulation and has a target elsewhere. **(d)** The *symE*/*symR* locus of *E*. *coli* K-12. The *symE* gene encodes a 113 aa toxin that exhibits similarity with MazE antitoxin. SymR antisense RNA inhibits translation of *symE* mRNA. A LexA box is shown to indicate that the *symE* promoter is under SOS regulon control. **(e)** *ratA*–*txpA* locus of *B*. *subtilis*. RatA antisense RNA inhibits expression of *txpA*. The 3′-ends of RatA-RNA and *txpA* RNAs overlap by approximately 75 nt and RatA most probably induces degradation of *txpA* mRNA and thereby reduces TxpA expression. **(f)** The *par* locus of *E*. *faecalis* plasmid pAD1 encoding RNA I and RNA II. RNA I encodes the Fst toxin and is stable while RNA II antisense is unstable but inhibits translation of *fst* encoded by RNA I. RNA I and RNA II form a stable duplex from which RNA I can be activated and kill plasmid-free cells analogous to *hok*/*sok* of plasmid R1. The

Figure 2



The *hok* mRNA folding pathway **(a)** and the two fates of activated *hok* mRNA **(b,c)**. *tac* denotes the translational activator sequence that sequentially base pairs with three different sequence elements within *hok* mRNA: in the metastable hairpin; with the upstream complementary box (*ucb*) and with fold-back inhibition (*fbi*) element. The *ucb* element is complementary to both *tac* and SD of *mok*, see text for a further detailed explanation. The everted bases of the U-turn in the refolded *hok* mRNA are indicated with dashes. SD: Shine & Dalgarno elements.

The inactive FL *hok* mRNA differs from the active 3′-truncated mRNA by only 39 nucleotides (nt). What, then, confers such different properties to these RNAs? In a genetic analysis, we showed that FL *hok* mRNA is unusually highly structured, and that the 5′-ends and 3′-ends form a blunt-end pairing (*fbi–tac*) interaction (Figure 2a, right). These features contribute to stability (slow 3′ processing [20]) and render the mRNA inactive. A comprehensive phylogenetic and experimental analysis of the *hok* mRNA folding pathway showed that the slow 3′ processing triggers refolding of the mRNA into an active isoform (Figure 2a, middle) that can either bind to Sok RNA and be degraded by RNase III (in plasmid-carrying cells; Figure 2b) or bind to ribosomes and be translated (in plasmid-free cells; Figure 2c). A genetic algorithm that calculates RNA structures co-transcriptionally predicted the *hok* mRNA folding pathway, as shown in Figure 2a [21]. The folding pathway was additionally supported by coupled nucleotide co-variations that reflect alternative pairings of the RNA elements involved [22] and

(Figure 1 Legend Continued) mechanism of activation of translation of RNA I is not yet known. Small opposing arrows indicate a bi-directional transcriptional terminator. Small open circles symbolize transcription start points (promoters).

confirmed by chemical probing and by mutational and functional analyses [17].

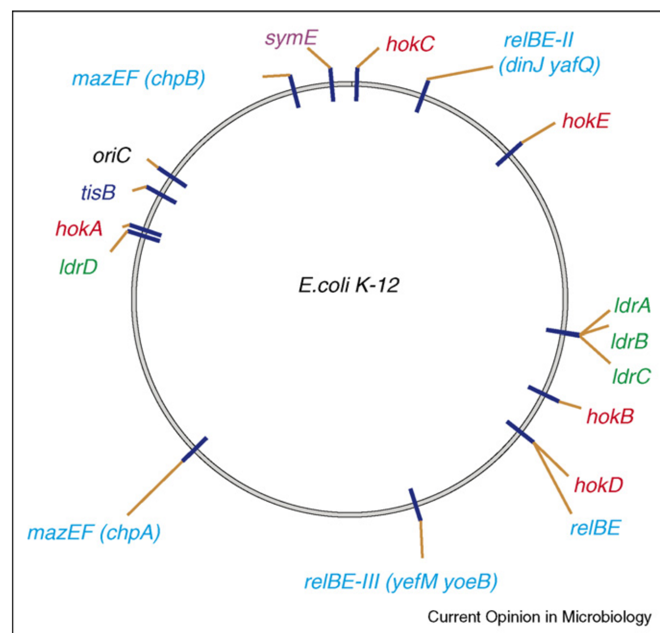
The crucial activation step following removal of the 3′ 39 nt of FL *hok* mRNA is a triggered refolding of the 5′-end to create the target stem–loop structure for Sok RNA binding (Figure 2a, middle). Sok RNA recognizes the target loop in *hok* mRNA through its 5′-single-stranded tail (Figure 2b) [17,21]. Subsequently, the initial pairing proceeds to formation of an RNA duplex that is rapidly cleaved by RNase III [19,23]. Interestingly, the target loop in *hok* mRNA contains a U-turn structure that increases the rate of Sok RNA binding by ~10-fold. The U-turn everts three bases of the target loop in a half α-helical configuration, thus lowering the ΔG of the initial binding reaction [24].

The formation of the target stem–loop in the mature *hok* mRNA depends on the long *tac*-stem present in the very 5′-end of the *hok* mRNA (Figure 2a, middle). Why does the *tac*-stem not form in the nascent transcript, in spite of its high predicted local stability ($\Delta G = -22$ kcal mol^{-1})? If formed during transcription, the *tac*-stem would lead to premature antisense binding and/or translation and, hence, would prevent accumulation of the inert FL mRNA — a key feature of the mechanism. Genetic algorithm calculations predicted a local, metastable hairpin at the very 5′-end of the nascent *hok* transcript that precludes *tac*-stem formation (Figure 2a, left) [21]. The metastable hairpin was strongly supported by co-variation analysis [21] and further confirmed by a structural probing comparison between native, and denatured/renatured *hok* mRNA [25]; only the native RNA exhibited a probing pattern consistent with the presence of the local metastable hairpin. Moreover, mutations that stabilized this hairpin displayed the expected phenotypes of reduced translation and antisense-binding rates. Thus, subtle conformational changes at the very 5′-end of the *hok* mRNA make this system functional: (i) in the nascent transcript, the metastable hairpin prevents the formation of the *tac*-stem, thereby reducing Sok RNA binding and translation during transcription; (ii) in the completed FL mRNA, the very 5′-end pairs with the very 3′-end to form the slowly processed inert isoform of *hok* mRNA; and (iii) in the mature *hok* mRNA, the very 5′-end refolds into the *tac*-stem that favors formation of the antisense RNA target stem–loop (Figure 2a). It is attractive to view the complicated (and conserved) folding pathway of *hok* mRNA as a means to enable activation of *hok* translation in a situation in which Sok RNA is depleted, such as in plasmid-free progeny.

hok/sok loci are present on the chromosomes of enteric bacteria

Database searches indicate that *hok/sok* loci are confined to enteric bacteria and closely related species [2]. The *Escherichia coli* resistance plasmid R1 carries one *hok/sok* locus, whereas the F plasmid carries two *hok/sok* homologous loci (*flm* and *srnB*) [26]. The chromosome of *E. coli* K-12 has five *hok/sok*-homologous loci (Figure 3) [27]. All five loci seem to be inactivated, by insertion sequences, point mutations or larger rearrangements [27]. It should

Figure 3



Localization of 16 TA loci on the *E. coli* K-12 chromosome. Protein-regulated TA (blue), *hok/sok* (red), *ldr* (green), IstR–*tisAB* (dark blue) and SymR–*symE* (magenta) loci are shown.

be noted that the chromosomal *hok/sok* loci produce Sok-homologous antisense RNAs that regulate translation of the *hok*-homologous mRNAs. Moreover, these chromosomal mRNAs contain all the regulatory elements described above, and thus the *hok* mRNA folding pathway is conserved [21]. The Sok-homologous antisense RNAs are unstable. Hence, it is possible that translation of the chromosomal *hok* mRNAs can be activated by as-yet unknown signals. Interestingly, some enteric bacteria have a particularly large number of *hok/sok* loci (e.g. *E. coli* O157 has 13).

What might be a putative function for a chromosomally encoded *hok/sok* locus? One interesting view argues that these loci could ensure the maintenance of the chromosomal regions in which they reside because a deletion would entail killing the cells that have lost this locus – in analogy to the plasmid case [28]. A similar role has been suggested for the protein-regulated TA loci in the super-integron of *Vibrio cholerae* [29,30]. However, it is also possible that as-yet unknown cues trigger activation of the chromosomal *hok/sok* loci. We speculate that RNase E, which inactivates Sok RNA by 5′ cleavage [31], could be involved in such regulation.

A *hok/sok*-like gene family

The *E. coli* K-12 chromosome contains four 'long direct repeat' (*ldr*) loci located symmetrically on the genetic map (Figure 3) [32]. These loci encode small open reading frames of 35 codons and have a shared genetic organization highly reminiscent of that of *hok/sok* (Figure 1b). The *ldrD* locus has been analyzed in detail [32]: (i) the *ldrD* mRNA encodes a small toxin whose overexpression is lethal to host cells; (ii) the *ldrD* mRNA is stable, and its translation is repressed by an unstable antisense RNA (RdlD RNA); (iii) all *ldr* loci contain an additional *mok*-like reading frame that overlaps with both the 5′-end of the antisense RNA gene and part of, or the entire, *ldr* toxin reading frame (Figure 1b; K Gerdes and EGH Wagner, unpublished); and (iv) *ldrD* loci are present in multiple copies in many enteric bacteria [2,32]. So far, different isoforms of *ldrD* mRNA have not yet been identified, and it is still unclear how translation of the stable *ldrD* mRNA is activated. A phylogenetic analysis (i.e. sequence alignment) of the *ldrD* mRNAs might, as in the case of *hok/sok* loci, be helpful to reveal regulatory elements involved in regulation.

The SOS-induced toxin TisB is regulated by the sRNA IstR-1

A first antisense RNA-regulated SOS-induced toxin was recently described in *E. coli*. The *istR–tisAB* locus (istR: inhibition of SOS-induced toxicity by RNA; tis: toxicity induced by SOS) [33] is divergently transcribed into two small RNAs (sRNAs; different promoters, same terminator) and a toxin-encoding mRNA (Figure 1c). IstR-1 is constitutively transcribed and carries in its 5′-unstructured tail a stretch of 21 nt with complementarity to a sequence in the *tisAB* mRNA — suggesting antisense control. IstR-2, the second sRNA, does not take part in *tisAB* regulation (data not shown). Previous work showed that *tisAB* transcription is under LexA control. During normal growth, very little *tisAB* mRNA is synthesized, and IstR-1 base-pairs rapidly to its target site. This entails translational inhibition, RNase III-dependent cleavage of the interacting RNAs ([33]; Darfeuille *et al.*, submitted), and inhibition of toxin production. Following SOS challenge, *tisAB* transcription is strongly induced. Excess of the *tisAB* mRNA out-titrates IstR-1, and cells cease to grow. Mutational analyses showed that toxicity is conferred by TisB. Recent data additionally show that the *tisA* reading frame is not translated. Thus, this control system is set up so that the antisense RNA normally prevents inadvertent toxicity but that severe DNA damage leads to an override — probably resulting in a reversible slowdown or inhibition of macromolecular synthesis (Unoson *et al.*, in preparation).

An unexpected twist is evident from recent mechanistic studies. *In vivo*, three *tisAB* mRNA species are observed (Figure 1c). The major species (primary transcript, FL mRNA; +1) becomes endonucleolytically processed 41 nt from its 5′-end into a minor mRNA species (+41). A third RNA results from IstR-1 binding and subsequent RNase III cleavage (+104). *In vitro*, we found that only the processed +41 RNA is translated, whereas the other two are translationally inert. When present, IstR-1 binds rapidly to +41 RNA to prevent TisB translation at a distance — the antisense target region is located >80 nt upstream of the *tisB* translation initiation region (TIR). Structure determinations, toeprinting assays and mutational studies suggest that the *tisB* TIR region is sequestered in a stable structure in all three *tisAB* mRNAs. However, +41 mRNA, but not +1 and +104, displays an unstructured region far upstream that could serve as a 'standby' site for 30S ribosomes [34,35]. Standby binding does not involve initiation complex formation but rather weak association with unstructured stretches of RNA. Thus, initiation would be facilitated on +41 RNA by bound ribosomes sliding into place following breathing of the *tisB* TIR structure. This standby site is structurally not available in +1, +104 or +41 when IstR-1 is bound (Darfeuille *et al.*, submitted). Thus, this control mechanism makes use of inactive and active mRNAs, reminiscent of *hok/sok*, but does so by an unrelated mechanism of control.

SymE, a second SOS-induced toxin, is regulated by SymR antisense RNA

The SOS-induced toxin SymE (SOS-induced yjiW gene with similarity to MazE) was recently discovered [36]. During steady-state cell growth, *symE* transcription is repressed by LexA, and translation of residual *symE* transcripts is repressed by an abundant, *cis*-encoded antisense RNA, SymR (Figure 1d). Furthermore, SymE toxin

is cleaved by Lon protease, a feature shared with other SOS-induced proteins [36,37]. Thus, *symE* expression is repressed at three levels: transcriptionally by LexA, translationally by *SymR* RNA, and post-translationally by Lon. Interestingly, a large number of SymE homologs were identified in free-living proteobacteria [36].

Unexpectedly, ectopic expression of SymE inhibited global translation by cleavage of mRNA. Some non-translated RNAs, but not *SymR* RNA, were also cleaved. As in the cases of other mRNA-cleaving toxins, such as RelE, SymE co-purified with ribosomes [36,38]. These results are surprising because SymE exhibits similarity with MazE, the antitoxin encoded by the *mazEF* locus. MazF, the mRNA interferase encoded by *mazEF*, is activated by nutritional stress [39]. Purified MazF cleaves naked RNA *in vitro*, and ectopic production of MazF leads to RNA cleavage outside mRNA coding regions *in vivo* [40]. MazE, the antitoxin that neutralizes MazF, has no known RNA cleavage activity. The MazEF complex binds to the *mazEF* promoter region through the AbrB-fold in MazE and represses *mazEF* transcription [41,42]. SymE toxin, astonishingly, is similar to MazE and also exhibits an AbrB fold. In addition, the SymE family proteins have conserved amino acid (aa) residues predicted to be involved in nucleic acid interaction. This suggests that SymE proteins have undergone a functional shift from a transcription factor or antitoxin to an RNA-associating protein with toxin-like properties. This conjecture is further supported by the observation that many *symE* toxin homologs are located next to potential antitoxin genes – that is, some *symE* genes might be antagonized by antisense RNAs, whereas others might be antagonized by protein antitoxins [36].

RatA RNA prevents expression of cell lysis protein TxpA in *Bacillus subtilis*

RNA antitoxin A (RatA) was identified in a hunt for RNAs encoded by intergenic regions of *Bacillus subtilis* [43•]. RatA RNA is a 222 nt transcript whose 3′-end overlaps with the 3′-end of toxic peptide A (*txpA*) mRNA (Figure 1e). This mRNA encodes a 59 aa toxic peptide that mediates cell lysis. Similarly to Hok, TxpA has a predicted N-terminal membrane-spanning domain. Thus, it is possible that cell lysis occurs by TxpA-dependent cell membrane damage. The genes encoding RatA RNA and TxpA are convergently transcribed, and the 3′-ends of their transcripts overlap by ~75 nt (Figure 1e). Deletion of the 3′-end of RatA RNA resulted in significant accumulation of *txpA* mRNA. Therefore, the overlap between the RNAs is required for repression of *txpA* expression. It was speculated that RNA duplex formation results in mRNA degradation and, ultimately, shutdown of TxpA synthesis [43•].

The *txpA–ratA* module is located within the *skin* element, a cryptic remnant of a temperate phage. The *skin* element interrupts the coding sequence for the mother cell-specific regulatory protein σ^K [44]. During sporulation, *skin* is excised from the chromosome of the mother-cell compartment to generate the intact coding sequence for σ^K. The mother cell undergoes terminal differentiation and ultimately lyses. The *skin* element is not excised from the chromosome of the forespore, the precursor of the spore. Thus, if *txpA* expression was induced in cells that experience excision of *skin*, then these cells would be killed. In this scenario, *txpA–ratA* functions as a selfish genetic element that secures the maintenance of the *skin* element by a mechanism reminiscent of how *hok/sok* loci increase the maintenance of plasmids [43•].

An RNA antitoxin from an *Enterococcus faecalis* plasmid

The conjugative low-copy-number plasmid pAD1 from *Enterococcus faecalis* is stabilized by a region termed *par* (Figure 1f). This locus encodes two small RNAs, RNA I and RNA II, of 210 nt and 65 nt, respectively [45]. RNA I encodes a 33 aa toxic peptide, *E. faecalis* plasmid stabilization toxin (Fst), whose synthesis affects cell division and promotes nucleoid condensation, membrane permeabilization and cell death [45–47]. RNA II prevents translation of RNA I by an unusual mechanism [48–50]. The 3′-ends of the RNA I and RNA II genes overlap by 75 base pairs, resulting in complementary transcriptional terminator hairpins (Figure 1f). In addition, two additional patches of complementarity are located in single-stranded 5′-parts of both RNA I and II. *In vitro*, RNA I and II form a stable complex in which RNA I cannot be translated. Structural and mutational analyses showed that the RNA I–RNA II interaction depends on both regions of complementarity. Toeprint analysis revealed that RNA II prevents ribosomes from binding to RNA I. Unlike in several other antisense-target systems, full RNA duplex formation seems not to be required for the function of *par*.

As for *hok/sok*, the RNA II antitoxin is more unstable than the toxin-encoding RNA I [49], suggesting that *par* of pAD1 stabilizes plasmids by post-segregational killing. RNA I has not been observed in two forms, and the steps leading to activation of translation of RNA I are still elusive. Because the putative complex between RNA I and RNA II is unlikely to be a substrate for RNase III, the downstream events in plasmid-free cells could be different from the *hok/sok* case. The intrinsic instability of the RNA I–RNA II complex might enable slow dissociation. The instability of RNA II would then eventually enable RNA I accumulation, which, in the absence of the inhibitor, would be translated and kill the plasmid-free cells. However, the RNA I–RNA II complex *in vitro* dissociation rate is low, perhaps suggesting a mechanism that actively disrupts this complex *in vivo* [51].

Conclusions

It is discussed here that the number of RNA antitoxins known is steadily increasing. The recent identification of

two SOS-regulated and RNA antitoxin-regulated TA loci supports the notion that RNA antitoxins might function as stress-response regulators, as has been described for protein antitoxins. Future analyses might reveal whether the *hok/sok* and *ldr* loci likewise have roles in bacterial stress responses.

Acknowledgements

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- • of special interest
- •• of outstanding interest

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