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Type I toxin-antitoxin systems in *Bacillus subtilis*

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Type I toxin-antitoxin (TA) systems are widespread in bacteria and consist of a toxin-encoding mRNA and a partially overlapping antisense RNA that blocks expression of the toxin, either at the level of translation or by mRNA degradation. Four type I toxin families have so far been proposed in *B. subtilis* based on sequence similarity: TxpA/BsrG, BsrH/BsrE, YonT and YhzE and two (T xpA and BsrG) have been studied in some detail. Here we review what is known about these confirmed and putative toxin-antitoxin families in *B. subtilis*, their regulatory mechanisms, their potential roles and how they may link to the physiology of the cell.

Introduction

Toxin-antitoxin systems are widespread in bacteria and have been classified according to three types I, II and III. Type I toxin loci typically encode a small hydrophobic toxic protein (less than 60 aa) containing a potential transmembrane domain. The antitoxin is an antisense RNA, which alters the stability and/or translation of the toxin mRNA (Fig. 1). In type II systems, the toxin proteins are generally longer (around 100 amino acids) and have endoribonuclease, protein kinase or phosphotransferase activity (for recent reviews see refs. 1 and 2). They can also generate dou-

bacteria, the first type I toxin-antitoxin system was identified on plasmid pAD1 of *Enterococcus faecalis*. This toxin belongs to the *par* family. The toxin (Fst) is encoded by RNA I and the antitoxin by RNA II. RNA I and RNA II overlap by 35 nucleotides at their 3' ends and have complementary direct repeats at their 5' ends. Interaction between these direct repeats and their complementary sequences is important for translation repression. Moreover, RNA II is less stable than RNA I, which allows Fst to act as a post-segregational killing system (PSK), ensuring mainte-

ance of pAD1 in cells (for more details, see K. Weaver, this issue). Recently, Fst-like toxin-antitoxin systems were also found on plasmids from *Enterococcus faecalis*, *Lactobacillus curvatus* and *Staphylococcus aureus*, the chromosome of *Enterococcus faecalis*, *Lactobacillus casei* and *Staphylococcus saprophyticus* and a phage from *Lactobacillus gasseri*.

The first type I toxin-antitoxin system identified on the chro-

some of *B. subtilis* was *txpA-ratA* in 2005. Five years later, 13 additional potential type I toxin-antitoxin cassettes were identified on the chromosome of *B. subtilis* by deep sequencing and bioinformatics analyses. These systems thus appear to be even more highly represented than type II toxin-antitoxin systems in this organism, underlining their importance. Toxins from *B. subtilis* range from 28 to 60 amino acids and are mostly located in prophage elements, except for those belonging to the YhzE family (see below). Three of the 14 have been shown to be toxic upon overexpression in either *B. subtilis* (T xpA, BsrG) or *E. coli* (YonT) and the others are proposed to behave similarly. Based on an alignment of the different toxins of *B. subtilis* (Fig. 2), four toxin families can be proposed: T xpA/BsrG, BsrH/BsrE, YonT and YheZ. BsrG is about 80% similar to the N-terminal half of T xpA, for example, while BsrE is about 90% similar to BsrH. An alignment of the YheZ family is shown in Figure 3. In contrast to type II toxins, there is no shared type I toxin-antitoxin fam-

ily between *B. subtilis* and Gram-negative bacteria, despite the fact that all of them are predicted to affect membrane integrity. We detail here recent work done to characterize the type I toxin-

antitoxin systems of *B. subtilis* and show how some of them are important for cell physiology.

Type I Toxins in *B. subtilis*: The TxpA/BsrG Family

T xpA/RatA. The toxin-antitoxin module *txpA-ratA* is located on the skin prophage of *B. subtilis* (Fig. 1). The *txpA* gene encodes a toxic protein of 59 amino acids. This protein has a predicted transmembrane domain at its N-terminus and charged amino acids at the C-terminus (Fig. 2).
Although the N- and C-termini are variable in size and sequence, TxpA homologs have a similar hydrophobic domain followed by charged amino acids.

**BsrG/Sr4.** The toxin-antitoxin module *bsrG-sr4* is located on the SPβ prophage of the *B. subtilis* chromosome (Fig. 1). The *bsrG* RNA is 294 nt long and encodes a toxic peptide of 38 amino acids with a predicted transmembrane domain in the central part and charged amino acids at the C-terminus (Fig. 2).

The antisense RNA SR4 is 180 nucleotides long and overlaps the 3’ end of *bsrG* by 123 nucleotides (Fig. 5). The interaction of both RNAs at their 3’ ends promotes degradation of *bsrG* RNA. RNase III cleaves the *bsrG* RNA/SR4 duplex at position 185 of *bsrG* RNA, 8 nt downstream from the stop codon, but is not involved in the degradation of either *bsrG* RNA or SR4 alone. Endonuclease Y and the 3’-5’ exoribonuclease R are responsible for further degradation of both RNAs. PNPase processes SR4 precursors into the mature RNA. The essential function of RNase III in *B. subtilis* was shown not to be related to the *bsrG-sr4* TA system, since a Δrnc suppressor strain neither lysed on agar plates nor had mutations in the *bsrG* ORF. This suppressor strain is now known to have a deletion of the skin prophage and a tendency to lose SPβ, to reduce TxpA and YonT toxicity (Durand et al., submitted). It is not excluded that SR4 not only directs *bsrG* RNA into the degradation pathway, but additionally inhibits *bsrG* translation. Preliminary secondary structure analyses of *bsrG* RNA and the *bsrG-RNA/SR4* complex indicate structural alterations around the *bsrG*-ribosome binding site in the presence of SR4 (Jahn and Brantl, unpublished). These data have to be corroborated by toe-printing studies. Complex formation assays with wild-type *bsrG* RNA/SR4 yielded an apparent binding constant $k_{app}$ of 7 × 10⁵ M⁻¹ s⁻¹ (Jahn, unpublished), which is the same order of magnitude as those of other cis-encoded sense/antisense RNA pairs.

The *sr4* promoter is about 6- to 10-fold stronger than the *bsrG* promoter, which should result in an excess of the antibiotic over the toxin, as was seen for *txpA*-RatA. A potential ResD site is situated upstream of the *bsrG* promoter. ResD is a transcription factor induced upon oxygen stress in *B. subtilis*.13

### Type I Toxins in *B. subtilis*: The YonT Family

YonT/as-YonT. The toxin gene *yonT* and its antidote *as-yonT* were identified by bioinformatic analysis and are located on the SPβ prophage (Fig. 1). YonT encodes a protein of 58 amino acids that is toxic when overexpressed in *E. coli*. This system differs from the other toxins previously described. Indeed, northern blot analysis of *yonT* shows a band of ~1 kb, suggesting that *yonT* is transcribed in an operon with the downstream genes *yoyJ* and *yonU* (Durand et al., submitted). A shorter band, likely to be a processed species of ~200 nucleotides, codes for just the YonT toxin (Durand et al., submitted).
Northern blots to detect the antisense RNA of the yonT transcript (as-yonT) revealed a band around 100 nucleotides in size, consistent with transcription from a putative sigma A promoter that ends at the rho independent terminator found in this region (Fig. 5). This antisense RNA is half as long as those described previously. The as-yonT RNA is complementary to both the 3' end of yonT and the 5' end of yojf, raising the question of the impact of this antisense RNA on the expression of the downstream yojf and yonU genes and whether these might also encode toxins. Like tspA, the yonT mRNA is stabilized upon RNase III depletion. Deletion of these two toxin mRNAs is sufficient to render the RNase III dispensable in B. subtilis (Durand et al., submitted).

**Putative Type I Toxins in B. subtilis: the BsrH/E Family**

BsrH/as-BsrH. The bsrH locus was originally identified by Saito et al. by encoding a small regulatory RNA. Following a deep-sequencing analysis, Irnov et al. subsequently proposed that this locus encodes a type I toxin-antitoxin system (bsrH/as-bsrH). It is located in the same intergenic region as tspA/ratA in the skin prophage (Fig. 1). The bsrH mRNA encodes a small 29 amino acid peptide. Interestingly, like bsrG, a potential ResD binding site has been found, located around 100 nucleotides upstream of the predicted transcription start site of bsrH (Fig. 5).

The antisense RNA (as-bsrH) is 200 nucleotides long and overlaps bsrH at its 3' end by approximately 120 nucleotides. In contrast to tspA, bsrH is insensitive to RNase III and Y, but increased levels of bsrH mRNA are detected in RNase J1 and RNase R mutant strains and as-bsrH in an RNase PH mutant strain, suggesting that these RNAs are degraded principally by an exoribonucleolytic mechanism (Durand et al. unpublished result). Although the TxpA and BsrH type I toxins are both encoded in the same intergenic region, the regulation of their turnover is clearly different. This may indicate that the structure of the bsrH/as-bsrH duplex is different from that of other TA systems in B. subtilis. It remains to be seen whether as-BsrH affects bsrH stability, or translation, as observed for the Fst toxin of E. coli.

BsrE/as-BsrE. The BsrE sRNA was also originally identified by Saito et al. Subsequent deep-sequencing analysis confirmed the detection of the BsrE RNA and a partially complementary RNA, which encodes a potential toxin, based on sequence homology. To avoid confusion and keep a consistent nomenclature, we propose that the toxin-encoding strand be renamed bsrE and its putative regulatory antisense RNA as-BSrE. The bsrE toxin gene is located in the P6 prophage and encodes a peptide of 30 amino acids (Fig. 1). The amino acid sequence of BsrE is highly similar to BsrH (Fig. 2) and, as for the bsrH and bsrG genes, a potential ResD binding site has been detected at position -54 (Fig. 5).

A northern blot of the regulatory antisense RNA (as-bsrE) reveals two bands at ~190 and 280 nucleotides. The band at 190 nucleotides corresponds to the predicted distance between a putative rho independent terminator and a sigma A promoter (Fig. 5). The minor band at 280 nucleotides could be a read-through product from the upstream gene or due to a termination defect. The two RNAs overlap by ~100 nucleotides at their 3' ends.

**Putative Type I Toxins in B. subtilis: The YhzE (SscA) Family**

By bioinformatic analysis, two genes located in the same intergenic region, yhzE (sscA) and yhzE-2, have been proposed to encode toxin proteins. They are under the control of a sporulation specific sigma K promoter and have a putative binding site for the transcription factor GerE, necessary for the expression of late spore coat genes.

An antisense RNA of ~120 nucleotides to the 3' end of yhzE-2 gene was detected by northern blotting. YhzE-2 does not seem to be toxic in E. coli or B. subtilis, at least under the conditions of overexpression tested by Fozo et al. More recently, a study from Kodoma et al. showed that yhzE (sscA) is involved in spore germination and spore coat assembly.

Interestingly, B. subtilis encodes 7 other paralogs of the YhzE proteins encoded by the yoaA, yjcZ, yoyG, yczM, yczN, yuzF and ykzV genes. Some of them are duplicated in the same intergenic region, such as yhzE and yhzE-2 and yczN and yczM. These proteins have a conserved hydrophobic region and variable C-terminal and N-terminal domains. The N-terminus is always rich in glycines and aromatic residues (Fig. 3). All these genes have a putative sigma K promoter and a recent tiling array study shows an increase of their expression during sporulation. Only yhzE-2 and possibly ykzV, yjcZ and yoaA, have an antisense RNA detectable by tiling array. Therefore, it remains to be seen whether these are true type I toxin-antitoxin systems or whether they encode simply related but non-toxic peptides with a constructive role in sporulation. Other proteins of the same family have also been detected by BLAST in several bacteria; all of them are spore-forming bacteria belonging to the genus Bacillus or Clostridium.
Figure 4. Secondary structures of txpA, RatA and the txpA/RatA duplex. (A) Secondary structure of individual txpA and RatA RNAs, showing the potential loop-loop (kissing) interaction between stem-loop (SL) 6 of txpA and the transcription terminator (ter) of RatA and between the terminator of txpA and SL3 of RatA (Durand et al., submitted). The start and stop codons of txpA are underlined and the Shine-Dalgarno (SD) sequence is indicated. (B) Secondary structure of extended duplex between txpA and RatA. Major sites of RNase III cleavage are indicated.
B. subtilis preferentially target the 3' ends of toxin mRNAs? One explanation could be the fact that the degradation machinery in Gram-positives is quite different from that of Gram-negative bacteria. Indeed, B. subtilis possesses a 5'-3' exoribonuclease activity, which plays a major role in mRNA degradation. Blocking this 5'-3' mRNA degradation pathway by a pairing with an antisense RNA could be detrimental in some cases.

Even among antitoxins that base-pair with the 3' end of the toxin mRNA, the regulatory outcome is not the same. The primary effect of RNA II on RNA I of pAD1 plasmid of E. faecalis is likely to be more complex than just inhibition of translation. The regulatory mechanisms involving RNA II and RNAs such as RNA I are still not fully understood and warrant further investigation.

**Figure 5.** Nucleotide sequences of type I toxin-antitoxins. Only the toxin-encoding strand is shown. Mapped (txpA and bsrG) and predicted (bsrE and as-bsrH) -10 boxes of the promoters are in bold-type. Transcription start sites are indicated by arrows and transcription termination is indicated by "term". The complementary region between the toxin and antitoxin RNAs is shaded in yellow. Start and stop codons are in red. Putative ResD binding sites are shaded in light brown. The predicted SD sequences are boxed.

**Regulation of Toxin Expression by the Antisense RNA**

In Gram-negative bacteria, the antisense RNA generally base-pairs with the 5' end of the toxin mRNA. The only exception described so far are the Sibs RNAs, which overlap the toxin mRNAs (lbs) completely. Interestingly, in B. subtilis and other Gram-positive bacteria, the antisense RNAs of all known type I toxin-antitoxin systems are complementary to the 3' end of their target RNAs (for review, see refs 12, 19 and 20) (Fig. 1). Why do antitoxins in B. subtilis preferentially target the 3' ends of toxin mRNAs? One explanation could be the fact that the degradation machinery in Gram-positives is quite different from that of Gram-negative bacteria. Indeed, B. subtilis possesses a 5'-3' exoribonuclease activity, which plays a major role in mRNA degradation. Blocking this 5'-3' mRNA degradation pathway by a pairing with an antisense RNA could be detrimental in some cases.

Even among antitoxins that base-pair with the 3' end of the toxin mRNA, the regulatory outcome is not the same. The primary effect of RNA II on RNA I of pAD1 plasmid of E. faecalis...
is inhibition of Fst translation.22 Both RNAs possess complementary direct repeats at their 5' ends, which block ribosome access by pairing. Neither tspA mRNA nor bsrG mRNAs have such repeats and pairing with their cognate antitoxins provokes mRNA degradation through RNase III cleavages (see above; Durand et al., submitted and ref. 11). A study on the regulation of bsrH by as-bsrH may reveal yet another mechanism of regulation, since bsrH is insensitive to the endoribonucleases III and Y and both RNAs are degraded by 3'-5' exoribonucleases (see above). This result raises the question how these RNAs can be attacked by 3'-5' exoribonucleases when their 3' ends are base-paired? These observations highlight the diversity of strategies used by antisense RNA to regulate toxin expression and a careful analysis of toxin-antitoxin sequences may allow elucidated guesses as to the regulatory mechanism of the antitoxin RNA.

B. subtilis TspA, BsrG, BsrE and BsrH toxins also share a common feature with the Fst toxin family. Indeed, they all have a potential secondary structure sequestering the SD sequence that likely reduces translation initiation. It was shown for the Fst toxin that the structure between the SD and its complementary sequence is necessary to allow efficient regulation by the antisense RNA (RNA II).22 YonT does not have this structure to trap the SD sequence but has a GUG start codon, which could sufficiently reduce translation to permit base-pairing with its antitoxin. Furthermore, the SD sequences for tspA and yonT mRNAs have the two most extensive complementarities to the anti-SD of 16S rRNA among all mRNAs in B. subtilis, with 12 and 11 potential base-pairs, respectively.23 Extensive base-pairing between the 30S ribosomal subunit and internal ribosome binding sites has been shown to lead to ribosome pausing.24 In the same manner, these “perfect” SD sequences, which are predicted to be extremely efficient at recruiting ribosomes are likely to have slow ribosome release rates. Thus poor translation initiation may be an important feature for these toxins to allow pairing of the antitoxin RNA to the 3' end.

Deletion of the tspA and bsrG asRNAs is toxic for the cell. Indeed, RatA mutants undergo colony lysis after few days and SR4 mutants lyse overnight on agar plates.8,11 In contrast, deletion of most of the antisense RNAs of type I toxins in Gram-negatives shows no obvious phenotype. It is possible that type I toxins in B. subtilis cause more damage to the cell membrane and/or the basal expression level is higher in these organisms, requiring a tight regulation by the antisense RNA to repress them even in non-stressful environments.

### Potential Role of Helper Proteins

In Gram-negative bacteria, a number of trans-encoded base-pairing sRNAs require the abundant RNA chaperone Hfq either for their stability or the interaction with their target RNAs.25 No cis-encoded bacterial sRNA has been found that needs Hfq for its function. In Gram-positive bacteria, there is so far only one case where a role of Hfq for the promotion of sense/antisense RNA interaction was found.26 Although neither bsrG-srA nor tspA-ratA seem to depend on Hfq for the control mechanism,8,11 the hybridization region between these two RNAs co-immunoprecipitates with Hfq (W. Winkler personal communication). Indeed this observation can be extended to the bsrElas-bsrE and bsrHtas-bsrH TA systems. However, the functional consequences of these interactions are not known. It cannot be entirely excluded that Hfq might be needed to open up secondary structures that prevent translation of some of the toxin mRNAs, as has been found for abrC RNA regulated by SR1 in B. subtilis.27

### Functions of Type I Toxin-Antitoxin Systems

Type I toxin-antitoxins are widespread in bacteria but their function is still unclear. Two type I toxins in E. coli, TisB and SymE, have been shown to be induced by the SOS response28,29 and TisB to play a role in persistence.30

In B. subtilis, the YhzE protein family seems to be important during sporulation but as mentioned above, it remains to be seen whether these are true type I toxins. The other toxins described previously (TspA, BsrG, BsrE, BsrH and YonT) are all located in prophages. They contain N-terminal transmembrane domains and charged amino acids at their C-termini (Fig. 2). They are probably localized to the membrane and could act similarly to phage holins, which may have been their primary function when the prophage was still functional.

Why maintain toxin genes on the chromosome? It has been suggested that TspA helps to maintain the skin element in B. subtilis genome. The skin prophage is excised from the chromosome by a DNA rearrangement during sporulation.31,32 This excision allows reconstitution of a functional sigK gene, a transcription factor controlling mother-cell-specific gene expression at a late stage of sporulation. In this case, TspA would have a similar function to the post-segregational killing (PSK) system of pAD1 (Fst toxin) of E. faecalis. Curiously, however, the PBSX prophage appears to be maintained on the chromosome of B. subtilis without a type I toxin-antitoxin system. Indeed, the PBSX prophage of B. subtilis is highly similar to the skin element,33 but the intergenic region encoding the TspA and BsrH toxins in skin is absent in PBSX.

It is also possible that toxins bring advantages to the cell during stress conditions, as observed for some type II toxin-antitoxins or TisB. All toxin-antitoxin modules encoded in B. subtilis prophages are under the control of a sigma A promoter, allowing rapid expression of toxins under defined conditions. One way to express toxins efficiently is to perturb the balance between toxin and antitoxin synthesis through transcriptional activators. Identification of these factors could give some clues about each toxin’s function. For example, the bsrG, bsrE and bsrH genes have putative ResD binding sites upstream, suggesting expression of the toxin under conditions of oxygen limitation. In agreement with this hypothesis, a recent tiling array study by Nicolas et al.16 shows increased expression of the three toxin mRNAs in cultures where oxygen is limiting (cultures without shaking) and a reduction of expression upon oxidative stress following paraquat, diamide or H$_2$O$_2$ treatment. One function of these toxins could therefore be to cause bacteriostasis to limit oxygen consumption during oxygen limitation. In the same study, expression of...
the txpA mRNA seems to be phosphate dependent. Moreover, the mRNA levels of txpA, bsrE, bsrG, bsrH and yonT increase once glucose is exhausted in the medium, linking these toxins to the metabolic state of the cell.11,16 Similarly, a heat shock at 48°C results in 3- to 4-fold faster degradation of bsrG RNA,11 whereas SR4 is not affected. Upon increasing temperature, the toxin RNA might refold into a structure that is more accessible to certain endonucleases. The biological significance of the heat-induced degradation of bsrG RNA is still unclear, but once again there appears to be strong link between toxin expression and cell physiology.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Note Added in Proof
The paper cited as “Durand et al., submitted” has been accepted: Durand, S, Gilet, L, Condron, C. The essential function of B. subtilis Rnase III is to silence foreign toxin genes. PLoS Genet 2012; 8:e1003181; PMID:23300471; http://dx.doi.org/10.1371/journal.pgen.1003181.

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