**In Vitro Characterization of the Type I Toxin-Antitoxin System bsrE/SR5 from Bacillus subtilis**

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BsrE/SR5 is a new type I toxin/antitoxin system located on the prophage-like region P6 of the *Bacillus subtilis* chromosome. The *bsrE* gene encoding a 30-amino acid hydrophobic toxin and the antitoxin gene *sr5* overlap at their 3′ ends by 112 bp. Overexpression of *bsrE* causes cell lysis on agar plates. Here, we present a detailed in vitro analysis of *bsrE/SR5*. The secondary structures of SR5, *bsrE* mRNA, and the SR5/*bsrE* RNA complex were determined. Apparent binding rate constants (*k*~app~) of wild-type and mutated SR5 species with wild-type *bsrE* mRNA were calculated, and SR5 regions required for efficient inhibition of *bsrE* mRNA narrowed down. In vivo studies confirmed the in vitro data but indicated that a so far unknown RNA binding protein might exist in *B. subtilis* that can promote antitoxin/toxin RNA interaction. Using time course experiments, the binding pathway of SR5 and *bsrE* RNA was elucidated. A comparison with the previously well characterized type I TA system from the *B. subtilis* chromosome, *bsrG/SR4*, reveals similarities but also significant differences.

Small regulatory RNAs (sRNAs) are key players in bacterial post-transcriptional gene regulation and have been discovered in a plethora of species (reviewed in Refs. 1–3). They employ either RNA/RNA base pairing or protein binding to inhibit or activate target gene expression. A special case of base pairing sRNAs is type I antitoxins that interact with complementary mRNAs encoding small toxic peptides (reviewed in Refs. 4 and 5).

Originally, type I toxin-antitoxin (TA) systems were discovered on plasmids (e.g., *hok/Sok* on *Escherichia coli* plasmid R1 (6, 7) or *fst/RNAII* on *Enterococcus faecalis* plasmid pAD1 (reviewed in Refs. 8 and 9)), in which they act as postsegregational killing systems. Subsequently, many chromosome-encoded type I TA systems were found and investigated, e.g., in *E. coli* tisB/IstR1 (reviewed in Ref. 10); *symE/SymR* (reviewed in Ref. 11); in *ibs/Sib*, *shoB/OhsC*, and *zor/Orz* (reviewed in Ref. 12); and in *B. subtilis* tcpA/RatA (13) and *bsrG/SR4* (14). They are arranged as overlapping, convergently transcribed gene pairs or as divergently transcribed gene pairs located apart. The interaction between RNA antitoxin and toxin mRNA either inhibits translation or facilitates degradation of the toxin mRNA (5). One exception is *bsrG/SR4*, whose antitoxin SR4 is bifunctional: it promotes degradation of the toxin mRNA and inhibits toxin translation by inducing a structural alteration around the *bsrG* ribosome binding site (RBS) (15). Another exception is *fst/RNAII* from *E. faecalis*, in which antitoxin binding yields a complex that stabilizes both RNAs but prevents toxin translation (16).

Some chromosome-encoded type I TA systems are involved in persister formation (17–20), whereas others are involved in recycling of damaged RNA (21), DNA recombination (22), or antibiotic resistance (23). Of 14 predicted *B. subtilis* type I systems, 5 are located on prophages and might be required for their maintenance or overcoming metabolic or environmental stress (24). Recently, we published the first temperature-dependent type I TA system *bsrG/SR4* and investigated it both in vivo and in vitro (14, 15). The 38-amino acid hydrophobic toxin BsrG causes membrane invaginations that dislocate the cell-wall synthesis machinery, which finally leads to cell death in the absence of the antitoxin SR4 (25). SR4 (180 nt) is complementary to the 3′ end of *bsrG* mRNA (294 nt) and promotes its degradation by an RNase III-dependent mechanism. In addition, it impedes *bsrG* translation (Ref. 15 and see above). The amount of *bsrG* RNA, but not SR4, decreases drastically upon heat shock (48 °C) because of faster degradation at high temperatures (14). Similar to *fst/RNAII* (8) but unlike *hok/Sok* (26) or *sib/lbs* (27), *bsrG* mRNA/SR4 binding occurs by three progressive interactions between sets of complementary regions (15). *bsrE/anti-bsrE* in *B. subtilis* was proposed to be a type I TA system (24, 28), and we renamed it *bsrE/SR5*. We demonstrated that *bsrE/SR5* is a type I TA system in vivo in *B. subtilis*, i.e. *bsrE* overexpression causes cell lysis. Intracellular amounts, expression profiles, and half-lives of SR5 and *bsrE* RNA were determined, and their decay by different RNases was investigated. RNase J1 is the major player in degradation of both RNAs, and RNase III cleaves the *bsrE/SR5* duplex. Interestingly, SR5 and/or *bsrE* responds to multiple stresses, e.g., anoxia, Fe2⁺ limitation, ethanol, and pH alterations. In addition, *bsrE* RNA is heat shock-sensitive but, unlike *bsrG*, only in the presence of SR5 (4).

Here, we provide a detailed in vitro characterization of SR5, *bsrE* RNA, and the SR5/*bsrE* complex. Secondary structures of both RNAs, as well as that of the complex, were determined, and the region of initial contact between antitoxin and toxin interaction were supported by Grant BR1552/10-1 from the Deutsche Forschungsgemeinschaft (to S.B.). The authors declare that they have no conflicts of interest with the contents of this article.

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mRNA was identified. The SR5/bsrE RNA binding pathway was elucidated employing EMSAs and time course experiments. Eventually, in vivo experiments with plasmids expressing bsrE and SR5 derivatives suggest that an unknown RNA binding protein could overcome the deficiency of truncated SR5 species to interact with bsrE RNA. A comparison between bsrE/SR5 and the structurally highly similar bsrG/SR4 type I TA system revealed commonalities but also interesting differences.

**Experimental Procedures**

**Enzymes, Media, and Strains**—The chemicals used were of the highest purity available. Taq DNA polymerase from Roche, Firepol polymerase from Solis Biodyne, RNAses T1 and V1 from AMBION, RNase T2 from Sigma and nuclease S1 from Fermentas were used. T7 RNA polymerase, calf-intestinal phosphatase, and T4 nucleotide kinase were from New England Enzymes, Media, and Strains—

**Construction of Plasmids for in Vivo Assays**—High copy number plasmids were constructed to overexpress wild-type bsrE and truncated sr5 variants in B. subtilis under control of their own promoters. Plasmid pUCBES4 was generated as follows: a PCR on chromosomal DNA from strain DB104 with primer pair SB2045/SB2265 yielded a fragment comprising the sequence for truncated sr5_1–50 followed by the heterologous bsrE terminator (32). It was digested with BamHI and PstI and inserted into pUC19. After confirmation of the sequence, the fragment was recloned into BamHI/PstI digested pUCBES2 carrying the wild-type bsrE gene. The resulting plasmid pUCBES4 that comprises wild-type bsrE, and truncated sr5 was used for transformation of B. subtilis DB104(ΔbsrE/Δsr5::cat). Plasmids pUCBES7, 8, and 9 containing wild-type bsrE and mutated sr5 variants were constructed likewise using the primer pairs listed in supplemental Table S1. Table 1 summarizes all plasmids. Plasmid pUCBES6 was constructed using three subsequent PCR steps. First, three parallel PCRs were performed on plasmid pUCBES2 as template using primer pairs SB2045/SB2418 resulting in fragment F4. Finally, F1 and F4 were combined and amplified with primer pair SB2045/2400. The resulting fragment, F5, was digested with BamHI and PstI and inserted into pUCBES2 as described above.

**Results**

**Secondary Structures of SR5 and bsrE mRNA**—Computer-predicted sRNA structures often differ from experimentally determined ones (15, 33, 34). Therefore, we conducted limited digestions with structure-specific ribonucleases to determine the secondary structures of SR5 and bsrE RNA. Full-length SR5 (163 nt), as well as 5′ truncated SR5, were carried out as described previously (15), RNases T1 and T2 were used at 10⁻² U and 2.2 × 10⁻² units, respectively. Preparation of Total RNA and Northern Blotting—Preparation of total RNA and Northern blotting were carried out as described (14).

**TABLE 1**

Plasmids used in this work

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<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
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<td>Ref. 29</td>
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<tr>
<td>pUCB2</td>
<td>pUC19 pUB10 shuttle vector, Neo&lt;sup&gt;+&lt;/sup&gt;, Pheo&lt;sup&gt;+&lt;/sup&gt;, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pUCB2 with bsrE gene under own promoter and 96 bp upstream of ~35 box of pucer</td>
<td>Footnote 4</td>
</tr>
<tr>
<td>pUCB5.1</td>
<td>pUCB2 with sr5 gene under own promoter and 87 bp upstream of ~35 box of pucer</td>
<td>Footnote 4</td>
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<td>This study</td>
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</tr>
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In Vitro Characterization of Type I TA System bsrE/SR5

**In Vitro Transcription and Secondary Structure Analysis**—In vitro transcription and partial digests of in vitro synthesized 5′ end-labeled SR5 and bsrE RNA species with RNAses T1 (1 unit/µl), T2 (22 units/µl), and V (0.1 unit/µl) and nuclease S1 (100 units/µl) were carried out as described (15). Additionally, bsrE RNA was 3′ end-labeled with [³²P]pCP and RNA ligase for 30 min at 37 °C. For the analysis of SR5/bsrE complexes with T1 and T2, either SR5 or bsrE RNA was 5′ end-labeled, and either an equimolar amount or a 10- or 100-fold excess of unlabeled complementary RNA was added prior to RNase digestion.

**Analysis of RNA-RNA Complex Formation and Time Course Experiments**—Both bsrE and SR5 were synthesized in vitro either on PCR-generated template fragments or on double strands generated by hybridizing complementary oligodeoxyribonucleotides (supplemental Table S1). SR5/bsrE complex formation studies and time course experiments with in vitro synthesized 5′-labeled SR5 (0.025 µM) and a 2-fold excess of unlabeled bsrE RNA were performed as described previously (15). RNases T1 and T2 were used at 10⁻² U and 2.2 × 10⁻² units, respectively.

**Preparation of Total RNA and Northern Blotting**—Preparation of total RNA and Northern blotting were carried out as described (14).
The secondary structure of full-length \textit{bsrE} mRNA (255 nt) was determined by enzymatic probing of 5′ and 3′ end-labeled RNA species using nucleases T1, T2, V1, and S1 as described under “Experimental Procedures.” Representative gels are shown in Fig. 2A–D, the schematic representation of the \textit{bsrE} structure in Fig. 2E. Similar to \textit{bsrG} RNA (15), \textit{bsrE} RNA is...
highly structured and barely contains single-stranded regions: the 5’ nt 1–36 and the 3’ nt 196–226 base pair in the long helix P1, which is divided by bulged out nt into five subhelices. P1 branches on top into stem loop structure SL1 and a short paired region that itself branches into helix P2 and a large internal loop J1/J2 that is topped by stem loop SL4. On top of P2, an internal loop branches into SL2 and SL3. Alternatively, T2 signals at positions 192/193 could indicate that nt 57–59 might interact with nt 131–133 in J1 instead of with nt 192–194; however, this would result in 3 bp—among them one GU bp—instead of 4 bp, which would decrease the stability of this structure. The start codon is situated in the short paired region upstream of P2 and the stop codon is located in L4 (Fig. 2E). The detection of weak T1 cuts additionally to V1 cuts indicates certain breathing
around the AUG start codon. The terminator stem loop SL4 comprises nt 232–254 and is separated from P1 by 5 unpaired nt. The sizes of loops L2, L3, and L4, as well as the length of single-stranded regions J1 and J2, were confirmed by using single-strand specific RNases and DMS-modified bsrE RNA (not shown).

Secondary Structure of the SR5/bsrE RNA Complex—SR5 and bsrE RNA are complementary over 112 nt and therefore expected to form a stable complex. To investigate conformational alterations in the secondary structures of both RNAs upon pairing, the secondary structure of the SR5/bsrE RNA complex was determined. To ascertain alterations in the bsrE RNA structure, 5′/H32P-labeled bsrE RNA was incubated with an excess of unlabeled SR4, and the complex was allowed to form for 5 min at 37 °C and subsequently partially digested with T1, T2 and S1 (Fig. 3A). On the other hand, 5′/H32P-labeled SR5 was incubated with an excess of unlabeled bsrE RNA and treated likewise (Fig. 3B). In Fig. 4, the schematic representation of the complex is displayed. As expected, no alterations in SL1 of SR5 were observed, because this stem loop is not part of the region complementary to bsrE RNA. By contrast, the entire region of SR5 complementary to bsrE (nt 51–163) was found to be double-stranded in the complex, visible by a significant reduction of T1 and T2 specific signals already upon addition of equimolar amounts of the toxin mRNA.

In the bsrE/SR5 complex, the 5′ end of bsrE RNA until nt 36—in the absence of SR5 paired with the 3′ end forming helix P1—is single-stranded, as demonstrated by a multitude of binding-induced S1, T1, and T2 cuts. In the complex, helix P2 is extended at its bottom by 3 bp, because during complex formation nt 192–195 are no longer available for interaction with nt 56–59, and instead, nt 57–59 can base pair with nt 130–132. Further structural changes in the region complementary to SR5 were observed in SL4, SL5, and J2 in bsrE RNA and in the short single-stranded region that connects P1 and SL5. Interestingly, bsrE SL1 comprising the SD sequence was not subject to structural changes. Taken together, experimental probing of the bsrE/SR5 complex demonstrated that the complementary regions of both interaction partners form a perfect duplex.

Binding Assays of Wild-type and Truncated SR5/bsrE mRNA Pairs—To analyze the kinetics of stable complex formation in vitro, we first studied binding between labeled full-length SR5 and unlabeled full-length bsrE RNA and vice versa by gel shift assays (EMSAs) as described under “Experimental Procedures.” Fig. 5A summarizes the calculated second order binding rate constants (k_{app}), and Fig. 5B shows representative EMSAs. For the full-length SR5/bsrE pair, a k_{app} value of ~1.4 × 10^{-6} M^{-1} s^{-1} was determined, independent of which of the interacting RNAs was labeled and which was provided unlabeled in excess. To investigate which structural elements are required for stable complex formation, different truncations were introduced into SR5. bsrE RNA could not be truncated, because this altered its secondary structure (not shown). SR547–163 lacking only SL1 and J1 that are not complementary to bsrE RNA showed an even higher k_{app} value than full-length SR5, whereas SR51–50 comprising only SL1 and J1 was not able to bind bsrE RNA.
Likewise, very short species comprising only SL2 (SR5_{47–79}) or the terminator stem loop SL4 (SR5_{127–163}) proved to be unable to form a stable complex with \textit{bsrE} RNA. However, individual deletions of either SL2 (SR5_{1–48, 79–163}) or SL4 (SR5_{1–126}) in an otherwise wild-type context decreased \(k_{\text{app}}\) to 14 or 16\%, respectively, revealing that both loops contribute equally to stable pairing. That at least one stem loop of SR5 is required for complex formation was corroborated by an SR5 species composed of only single-stranded regions J2 and J3 (SR5_{80–102, 102–126}), which was unable to form a duplex.

Because SL2 alone was unable to form a stable complex, a species containing additionally J2 and SL3 was tested (SR5_{47–104}). For this species, very weak binding was detected with a \(k_{\text{app}}\) value 3 orders of magnitude lower than that of full-length SR5. This could not be explained simply by the lack of SL4. Therefore, we added the long single-stranded region J3 (SR5_{47–126}) (not shown), and binding increased to 20\% of the wild-type value, demonstrating that J3 might contribute significantly to stable pairing. Apparently, J3 of SR5 is important for efficient pairing. To find out whether SL3 is needed, we first deleted this stem...
loop (SR5_{47-90,115-126}). Indeed, there was no difference between the $k_{app}$ value of this species and SR5_{47-126}. Subsequently, we further shortened the SR5-J3 region to only the 5' part (SR5_{47-90,105-114}), which decreased the $k_{app}$ value ≈50-fold. Therefore, the entire region single-stranded region J3 is crucial, because it interacts with a single-stranded bulge region in the complementary bsrE RNA. A full-length SR5_{163} with simultaneous nt exchanges in both loops L2 and L4 was significantly (≈14-fold) impaired in binding (Fig. 5).

In conclusion, SL2 and SL4 are fundamental for the formation of a stable duplex with bsrE RNA, because the lack of both SLs hardly allowed binding, and deletion of either of them or of both loop sequences decreased binding 6–7-fold. Furthermore, J3 of SR5 is essential for stable complex formation.

**Binding Pathway of SR5 and bsrE mRNA—In vitro binding assays do not allow conclusions regarding whether binding between both RNAs initiates with one or two simultaneous loop-loop contact(s). Therefore, a time course experiment (see “Experimental Procedures”) using 5'-labeled SR5 and unlabeled bsrE RNA was performed to assay the sequential pairing between antisense and sense (toxin) RNA. As shown in Fig. 6 (A and B), T1 and T2 cleavage signals specific for SR5 loop L4 decreased 2-fold already 40 s after addition of bsrE RNA, indicating that the initial interaction occurs between this loop and bsrE L4. This result is consistent with the data from the binding assays (Fig. 5) and not unexpected, because both loops contain 5' YUNR motifs known to provide a scaffold for rapid RNA/RNA interactions (35, 36). The next contact occurs between loop L3 and the 3' part of helix P1 of the bsrE RNA, because a 2-fold reduction of the L3 signals was observed 75 s after addition of bsrE RNA, indicating a conversion to a double-stranded region. Finally, intermolecular helix progression reached SR5 loop L2 and the 3' part of J3 that binds to the terminator loop and J2 of

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**FIGURE 5. Binding assays of wild-type and truncated SR5/bsrE RNA pairs.** Binding experiments were performed as described under “Experimental Procedures.” A, summary of pairing rate constants ($k_{app}$). The conformation of the used SR5 species is shown schematically in the left column, and sequence alterations are indicated. The $k_{app}$ values were calculated as described previously (15). B, representative binding assays with wild-type and mutated SR5 derivatives. SR5 species were 5'-labeled with [γ-32P]ATP and used in at least 10-fold lower equimolar amounts compared with full-length bsrE RNA. The concentrations of unlabeled wild-type bsrE RNA species are indicated. F, free labeled RNA; D, SR5/bsrE RNA duplex.
bsrE RNA, respectively (50% bound after 120 s). After addition of bsrE RNA, duplex formation in the J3 region of SR5 was slowest. In direct comparison, the interaction with L2 was faster in the beginning, but went down to 50% also after 120 s. As expected, no alterations in the cleavage pattern were observed over 600 s for SR5-L1 that is not complementary to bsrE RNA. Fig. 6B shows the quantification of the gel in Fig. 6A, and Fig. 6C represents the binding pathway schematically.

**In Vivo Studies**—To corroborate the in vitro data in vivo, both bsrE RNA and wild-type or mutated sr5 were expressed under their own promoters from plasmid pUCB2 (∼50 copies/cell) in B. subtilis DB104(Δsr5/ΔbsrE). Transformants were analyzed for lysis on agar plates after overnight incubation at 37 °C followed by 24 h at 24 °C (summarized in Fig. 6A). As expected, DB104(Δsr5/ΔbsrE) containing pUCBE1 lysed on agar plates. Likewise, DB104(Δsr5/ΔbsrE:cat) with pUCBES4 encoding bsrE and the noncomplementary 5’ part of sr5 (SL1 and J1) displayed a lysis phenotype. By contrast, the additional expression of full-length sr5 (plasmid pUCBES2) could perfectly compensate the lysis effect (Fig. 7B). Surprisingly, sr5 variants lacking SL2 (pUCBES8) or composed of only SL2 and single-stranded regions J2 and J3 (pUCBES7), which were still able to form a duplex with bsrE RNA in vitro, albeit with 7- or 4-fold reduced $k_{app}$ values, respectively, could compensate lysis. Most surprisingly, however, an sr5 mutant comprising only SL2, J2, and SL3 (pUCBES9) that displayed a 1000-fold reduced $k_{app}$ value in vitro (see SR547–104 in Fig. 5) did not lyse either. Similarly, both behaved like pUCBES6 carrying altered L2 and L4 loop sequences, which were >100-fold impaired in complex formation in vitro. This unexpected result could be either due to reduced expression of bsrE RNA or altered expression or stability of mutated SR5 species in the Δsr5/ΔbsrE strain. Alternatively, an RNA chaperone might be present in vivo that facilitates the interaction between bsrE RNA and the severely truncated SR5 species.

First, we analyzed expression of bsrE and sr5 in the corresponding strains by Northern blotting (Fig. 7C) and quantified the relative amount of bsrE (Fig. 7D). In strains containing
pUCBES2 and pUCBES8, the amount of bsrE RNA was \( \approx 30 \)-fold lower than in the strain with pUCBES4, which showed lysis. This suggests that a small stretch of complementarity on an SR5 variant is sufficient to direct bsrE RNA into the degradation pathway \textit{in vivo}.

For pUCBES7 and pUCBES9, a 5–8-fold higher amount of bsrE RNA was calculated compared with pUCBES2 and 8, although it was still 4–6-fold lower than in the case of pUCBES4, again indicative for the ability of the severely truncated SR5 variants to promote bsrE degradation. Despite these differences, for all strains (with either pUCBES2, 6, 7, 8, or 9) no lysis was observed. The amount of bsrE seems to be a more sensitive measure for the inhibitory effect of SR5 than cell lysis. Apparently, as soon as a small region of SR5 complementary to bsrE is expressed, it can promote its degradation \textit{in vivo}.

Truncated SR5 species might display altered stabilities \textit{in vivo}. However, SR5 species of the expected size were expressed from pUCBES2, 7, and 8 (Fig. 7C). The only exception was pUCBES9, in which the expected 60-nt-long species was not visible. However, this species was able to compensate lysis.

As shown in a recent paper, the RNA chaperone Hfq stabilized SR5 but not bsrE RNA. Furthermore, Dambach et al. (37) published an Hfq peak in their co-immunoprecipitation experiments to bsrE/SR5. Although \textit{cis}-encoded sRNAs with a long stretch of complementarity do usually not need Hfq to promote the efficient interaction with their target RNA, it could not be excluded that SR5/bsrE is an exception. Therefore, we constructed a \( \Delta hfq \)/cat, \( \Delta sr5/\Delta bsrE \)/cat strain, transformed it with all pUCBES derivatives and analyzed lysis on agar plates. Furthermore, as \textit{csrA} is the only other protein known in \textit{B. subtilis} to bind mRNAs and thus might be able to affect antisense/target RNA interaction \textit{in vivo}, a \( \Delta csrA/\text{spec} \Delta sr5/\Delta bsrE/\text{cat} \) strain was also generated and assayed with all pUCBES derivatives.
tives. However, both knock-out strains behaved like the iso-
gen wild-type strains, i.e. no lysis was observed when SR5 species even with a very short region complementary to bsrE were expressed, whereas the control without SR5 revealed lysis (not shown). Based on these results, we conclude that a so far unidentified RNA binding protein exists in B. subtilis that can overcome the inefficient interaction of severely truncated SR5 species with wild-type bsrE RNA.

Discussion

So far, 14 putative type I TA systems were predicted in B. subtilis (24), and three of them were confirmed in vivo and analyzed in vitro: txpA/RatA (13, 38), bsrG/SR4 (14, 15), and bsrE/SR5 (this report). These three systems show commonalities but also interesting differences. In all cases, the toxin is a small hydrophobic peptide that causes cell lysis on agar plates. However, both knock-out strains behaved like the iso-
gen wild-type strains, i.e. no lysis was observed when SR5 species even with a very short region complementary to bsrE were expressed, whereas the control without SR5 revealed lysis (not shown). Based on these results, we conclude that a so far unidentified RNA binding protein exists in B. subtilis that can overcome the inefficient interaction of severely truncated SR5 species with wild-type bsrE RNA.

In Vitro Characterization of Type I TA System bsrE/SR5

The first applies to both bsrE/SR5 (Fig. 5) and bsrG/SR4 (15). Binding starts with one loop/loop interaction between toxin L3 and antitoxin terminator loop L4, the so-called recognition loops. Subsequently, it progresses toward the 3’ end of both complementary molecules via L3 and later the single-stranded region J3 and L3 of the antitoxin. However, both binding pathways reveal a few differences. Although antitoxin loop L2 is bound last by both bsrG and bsrE RNA, both L4 and L2 of SR5 are important for efficient interaction with bsrE RNA (Fig. 5), whereas in bsrG/SR4, a shorter antitoxin in complex formation (Fig. 5) and pairing in vivo (Fig. 7). Because bsrE and bsrG mRNA have a highly similar secondary structure (Fig. 2 and Ref. 15), it is not surprising that loop L3 of bsrG RNA carrying a 5’ YUNR motif is also required for recognition by SR4. However, in bsrG/SR4, only the toxin RNA but not the complementary antitoxin loop L4 displays such a motif. Another potential U-turn motif in L2 of SR4 was neither required for efficient pairing with bsrG RNA in vitro nor for the neutralizing activity of SR4 in vivo (15). Taken together, in all but one (txpA/RatA) type I TA system, U-turn motifs play an important role in rapid and efficient primary contacts between RNA antitoxin and toxin mRNA. However, only in hok mRNA has the predicted U-turn been corroborated experimentally (44).

Different strategies exist that prevent premature toxin expression in type I TA systems. Although in some cases, processing events are required to generate shorter, translatable toxin mRNA (reviewed in Ref. 5), in others, the RBS is seques-
tered by complementary base pairing. The latter applies to E. faecalis ftsRNAII (41) and also to the three B. subtilis systems; the SD sequences of txpA (38), bsrG (15), and bsrE (Fig. 2) are located in a 4- or 5-bp GC-rich stems making them inaccessible to 30 S ribosomal subunits, reflected by our failure to detect a toeprint or in vitro translation product from bsrG mRNA unless mutations unclosed the double-stranded region (15). However, whereas antitoxin SR4 induces a conformational change around the bsrG SD that extends the double-stranded region to 8 bp and further impedes translation, this was the case neither for bsrE (Fig. 3) nor for txpA (38). Therefore, the corresponding antitoxins RatA and SR5 act—as stated above—solely by promoting toxin RNA degradation. In addition, txpA has a perfect RBS (≥11 bp complementarity to anti-SD in 16S rRNA), which is proposed to efficiently recruit but slowly release ribosomes (47).
In many trans-encoded sense/antisense RNA systems from Gram-negative bacteria, Hfq plays an important role in either stabilizing the antisense RNA or promoting its interaction with the target RNA (reviewed in Refs. 1, 48, and 49). The only type I TA system in which a role for Hfq has been discovered is E. coli ralA/RalR (23). This might be due to the rather short (16 nt) sequence complementarity between ralA and RalA. RalA binds Hfq at high concentrations, but it is unclear whether Hfq promotes ralA/RalA complex formation and prevents degradation of either RNA or ralA translation (23). By contrast, in Gram-positives, only one case—LhrA from Listeria monocytogenes (50)—has been discovered so far in which Hfq was needed for sense/antisense RNA interaction. cis-encoded antisense RNAs display a long stretch of complementarity to their targets and generally do not depend on RNA chaperones to stabilize complementary base pairing. This does also apply for txpA/RatA, bsrG/SR4, and bsrE/SR5. Whereas Δhfq strains did not lyse on agar plates, indicating that all three antitoxins could neutralize toxin activity in the absence of Hfq in vivo (13, 14),4 Hfq stabilized SR5 but not SR4 and neither of the toxin RNAs bsrE or bsrG (14).4 Whereas in bsrG/SR4, we found a good correlation between in vitro and in vivo data for the functionality of truncated antitoxin species (15), this was, unexpectedly, not always the case for bsrE/SR5 (Figs. 5 and 7): SR5 species barely able to base pair with bsrE RNA could complement BsrE-induced lysis on agar plates, indicating that an RNA binding protein might help overcome this pairing deficiency in vivo. Since we could exclude Hfq (see above), we tested CsrA, which is known to bind sequence specifically to mRNAs and to be sequestered by sRNAs in Gram-negative bacteria (reviewed in Ref. 51). E. coli CsrA was recently found to be titrated by an sRNA, McaS, which could also bind a complementary target RNA and Hfq (52), indicating that both Hfq and CsrA might affect regulation by base pairing sRNAs. B. subtilis CsrA was shown to bind hag mRNA, but also FLiW protein, thereby establishing a checkpoint in flagellum synthesis (53). However, a B. subtilis ΔcsrA strain could still compensate lysis induced by truncated SR5. Therefore, we hypothesize that a so far unknown RNA binding protein exists in B. subtilis that is responsible for the observed effects. Currently, experiments are underway to identify this protein.

In summary, all three B. subtilis type I TA systems, bsrE/5R, bsrG/5R4, and txpA/RatA, are regulated by RNA antitoxins that recruit the double-strand specific RNase III for toxin mRNA degradation. A peculiarity is bsrG/5R4, whose antisense SR4 has a second function; it additionally induces a structural change at the toxin SD that further impedes translation. In all cases, antitoxin/toxin RNA pairing occurs progressively, starting with one loop/contact followed by helix progression in the 3′ direction. Binding pathways have been elucidated for bsrG/5R4 and bsrE/5R5. The \( k_{\text{app}} \) values for complex formation are with \( \approx 10^5 \text{ M}^{-1} \text{ s}^{-1} \) in the range of other antisense/sense RNA systems. Premature toxin translation is not prevented by toxin RNA processing, but by sequestration of the RBS in a GC-rich 4–5-bp double-stranded region. In addition, txpA has an almost perfect RBS that might efficiently bind but slowly release ribosomes, which further obstructs translation. Similar to most other type I TA systems (e.g. hok/Sok, lds/Sib, and fst/RNAII), the initial antitoxin/toxin RNA interaction in bsrG/SR4 and bsrE/5R5 involves a potential U-turn in the toxin-RNA loop, with the latter system even using a U-turn/U-turn interaction. This makes txpA/RatA, in which no U-turn was involved, an exception. Hfq is not required in either system for the inhibitory action of the antitoxin. Interestingly, in contrast to bsrG/5R4, in bsrE/5R5, a presently unknown RNA chaperone allows for efficient antitoxin/toxin-RNA base pairing when the antitoxin is severely corrupted.

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