Nucleic Acids Research Advance Access published February 7, 2014

Nucleic Acids Research, 2014, 1–12
doi:10.1093/nar/gku119

Dual RNA regulatory control of a *Staphylococcus aureus* virulence factor

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Received July 8, 2013; Revised December 28, 2013; Accepted January 17, 2014

ABSTRACT

In pathogens, the accurate programming of virulence gene expression is essential for infection. It is achieved by sophisticated arrays of regulatory proteins and ribonucleic acids (sRNAs), but in many cases their contributions and connections are not yet known. Based on genetic, biochemical and structural evidence, we report that the expression pattern of a *Staphylococcus aureus* host immune evasion protein is enabled by the collaborative actions of RNAIII and small pathogenicity island RNA D (SprD). Their combined expression profiles during bacterial growth permit early and transient synthesis of Sbi to avoid host immune responses. Together, these two sRNAs use antisense mechanisms to monitor Sbi expression at the translational level. Deletion analysis combined with structural analysis of RNAIII in complex with its novel messenger RNA (mRNA) target indicate that three distant RNAIII domains interact with distinct sites of the *sbi* mRNA and that two locations are deep in the *sbi* coding region. Through distinct domains, RNAIII lowers production of two proteins required for avoiding innate host immunity, staphylococcal protein A and Sbi. Toeprints and *in vivo* mutational analysis reveal a novel regulatory module within RNAIII essential for attenuation of Sbi translation. The sophisticated translational control of mRNA by two differentially expressed sRNAs ensures supervision of host immune escape by a major pathogen.

INTRODUCTION

Staphylococcus aureus is a commensal flora, but it can also be an opportunistic pathogen and the cause of nosocomial and community-acquired infections (1). *Staphylococcus aureus* produces a large number of virulence determinants to survive and to establish an infection. These factors include surface proteins for adherence to host cells and tissues, haemolytic toxins that contribute to tissue damage and dissemination and exoproteins for host immune avoidance (2). For instance, staphylococcal protein A (SpA) recognizes the immunoglobulin Fc domain, which results in inverted tagging and in turn blocks C1q and Fcγ receptor binding sites (3). Another immunoglobulin-binding protein (Sbi) is also expressed by *S. aureus* (4), and it impairs the host immune response. Sbi acts as a complement inhibitor and forms a tripartite complex with host complement factors H and C3b (5). Recent data indicate that Sbi and extracellular fibrinogen-binding protein recruit human plasmin to degrade complement C3 and C3b (6).

A coordinated and timely expression of multiple virulence factors is essential for *S. aureus* infection. The process involves two-component sensory transduction systems and global regulatory proteins (7). In addition to protein-mediated gene controls, ribonucleic acids (RNAs) have regulatory functions in many bacterial pathogens (8,9), including *S. aureus* (10). In *S. aureus*, ~250 regulatory RNAs are expressed (11–17), but until now few have demonstrated physiological functions. One is the multifunctional RNAIII, which combines quorum sensing with virulence regulation (18). RNAIII acts as a messenger RNA (mRNA), encoding the 26-aa delta-haemolysin peptide. As a *trans*-acting RNA, it has multiple regulatory functions (7), including the regulation of the expression of numerous mRNAs at the translational and/or transcriptional levels (19,20) (Figure 1A). The RNAIII 5′-domain activates translation of α-haemolysin by disrupting the inhibitory translation initiation site (TIS) hairpin of *hla* mRNA (21). The RNAIII 3′-domain represses the translation of several virulence factors and of the transcriptional repressor of toxins (Rot), together reducing early expressed virulence factors (19,20,22), thus facilitating *S. aureus* infection (19). One RNAIII target is the SpA immune evasion molecule, whose expression is downregulated at both transcriptional (18) and translational (23) levels.

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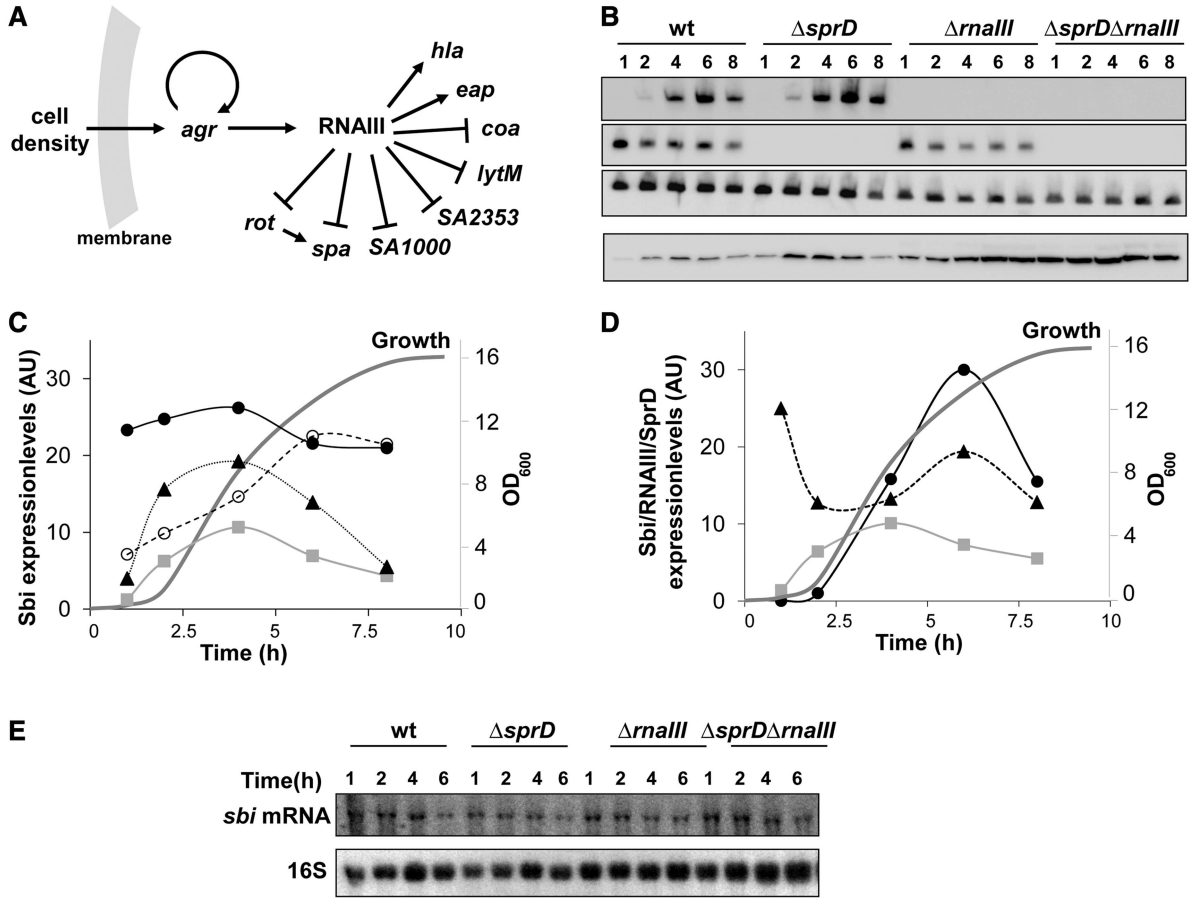

Figure 1. Translational control of Sbi during *S. aureus* growth is shared by RNAIII and SprD. (**A**) Simplified overview of the RNAIII regulon. RNAIII is the effector of the accessory gene regulator (*agr*) quorum sensing system in *S. aureus*. RNAIII positively regulates *hla* and *eap* expression levels (21,22), whereas RNAIII represses the expression of *spa*, *rot*, *coa*, *SA2353*, *SA1000* and *lytM* by translation initiation inhibition (19,23,24). (**B**) RNAIII and SprD expression profiles were monitored in wt, Δ*sprD,* Δ*rnaIII* and *sprD-ΔrnaIII* HG001 isogenic strains by northern blots using labelled DNA probes specific for each RNA (upper panels). Immunoblot analysis with anti-Sbi antibodies of the intracellular and membrane proteins in the four strains during *S. aureus* growth (lower panel). The 5S ribosomal RNAs (rRNAs) were used as loading controls. (**C**) Quantifications of the Sbi protein levels, during *S. aureus* growth, in strains expressing, or not, SprD and/or RNAIII. The amounts of Sbi proteins are provided in arbitrary units (AU) and were calculated relative to the quantity of total protein amounts per tracks, evidenced by Coomassie staining in Supplementary Figure S5A. The graphs indicate the Sbi protein levels in wt (grey squares), Δ*sprD* (triangles), Δ*rnaIII* (empty circles) and Δ*sprD-ΔrnaIII* (filled circles) *S. aureus* strains. The data are representative of at least three independent experiments. The growth curve of strain wt is presented. (**D**) Expression levels of the Sbi protein (grey squares), RNAIII (circles) and SprD (triangles) in *S. aureus* strain wt during growth. Quantifications of the Sbi protein was performed as for panel C. The amounts of RNAIII (circles) and SprD (triangles) are shown in arbitrary units (AU) and were calculated relative to the 5S rRNA. (**E**) Northern blot analysis of the steady-state *sbi* mRNA levels in wt strain HG001, Δ*sprD,* Δ*rnaIII* and Δ*sprD*-Δ*rnaIII* mutants during bacterial growth. The 16S rRNAs were used as internal loading controls.

Small pathogenicity island RNA D (SprD) is among the few *S. aureus* RNAs with identified functions. SprD is expressed from the genome of a converting phage (11), a horizontally acquired pathogenicity island that is a repository for many toxins, adherence and invasion factors, superantigens and secretion systems (25). Using an antisense mechanism, SprD downregulates the translational expression of the Sbi immune evasion molecule (26). Interestingly, SprD significantly contributes to disease incidence in a mouse model of staphylococcal infection, although this effect is not linked solely to deregulation of Sbi production.

In this article, *in vitro* and *in vivo* genetic, biochemical and structural approaches were used to newly identify the Sbi immune evasion protein as a target for RNAIII. RNAIII operates as an antisense RNA annealing *sbi* mRNA at multiple sites, including at the TIS, thus downregulating the expression of Sbi. We have also identified a new unconventional single-stranded zone in RNAIII that is essential for attenuating Sbi translation. Experimental evidence shows that RNAIII cooperates with a second RNA, SprD, to achieve accurate monitoring of *sbi* expression during *S. aureus* growth. Such a sophisticated translational control of virulence factor-encoding mRNA by two differentially expressed RNAs ensures that immune evasion molecules are fabricated early, temporarily and at the appropriate levels to defeat host immune defences.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

The strains used are listed in Supplementary Table S1. The *sprD* gene was deleted in strain HG001 using pBT2Δ*sprD* vector as described for strain N315 (26). Inactivation of

the *rnaIII* gene using pBT2Δ*rnaIII* was performed as described previously by the authors (26), yielding the strain HG001Δ*rnaIII*a. Isogenic strains HG001Δ*rnaIII* and HG001Δ*sprD*Δ*rnaIII* were constructed by transducting the *cat*-tagged *rnaIII* mutation from WA400 (27) into strains HG001 and HG001Δ*sprD,* respectively, and by selecting for chloramphenicol resistance. The *S. aureus* strains were cultured at 37°C in brain–heart infusion broth (Oxoid). When necessary, erythromycin was added at a concentration of 10 µg/ml. In pCN38-*rnaIII*, RNAIII was expressed from its endogenous promoter. The *rnaIII* sequence was polymerase chain reaction (PCR)-amplified from HG001 genomic DNA as a 1063-bp fragment with flanking *Pst*I and *Eco*RI restriction sites, then cloned into the vector pCN38 (28). We used mutagenized oligonucleotides to produce RNAIII mutants (Supplementary Table S2).

Protein isolations, immunoblots, DNA and RNA manipulations

Protein extract preparations and detection of the protein Sbi were performed as previously described (26). Culture pellets were washed with Tris-EDTA (TE) buffer (50 mM Tris, pH 7.5, 50 mM EDTA) and suspended in 0.2 ml of this solution supplemented with 0.1 mg/ml lysostaphin. Following 37°C incubation for 10 min, samples were boiled 5 min and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. For the immunoblots, proteins were transferred to PolyVinyliDene Fluoride (PVDF) membrane (Immobilon-P, Millipore). Sbi was visualized by anti-Sbi antibodies (Dr van den Elsen, University of Bath, UK), anti-rabbit IgG, peroxidase-conjugated, secondary antibodies (Jackson) and the 'Amersham ECL' system (Amersham Pharmacia Biotech). Total *S. aureus* RNA was prepared as previously described (25). Northern blots targeting SprD and RNAIII were performed with 5 µg of total RNAs (26). RNAs used for probing, gel-shift and toeprint assays were transcribed from PCR fragments amplified from purified genomic DNA using the primers listed in Supplementary Table S2. To produce the template-encoding RNAIII mutants, mutagenized oligonucleotides were used (Supplementary Table S2). RNAs were produced by *in vitro* transcription using MEGAscript (Ambion). The 5′-labelling of RNAs was performed as described previously (29). RNAs were purified by 8% polyacrylamide gel electrophoresis, eluted, ethanol-precipitated and then stored at −80°C until needed.

RNA probing, gel-shift and toeprint assays

Gel retardation assays were performed (26), and 0.4 pmol of labelled *sbi* mRNA or RNAIII were incubated with various concentrations (from 1.6 to 50 pmols) of unlabelled RNAs. The toeprint assays were performed as previously described (26). The annealing mixtures used contained 0.2 pmol of *sbi* mRNA and 1 pmol of labelled SBIrevTR primer in a reaction buffer of 10 mM Tris-acetate, pH 7.5, 60 mM NH_4Cl and 1 mM dithiothreitol (DTT). For the competition assays, various concentrations of SprD, RNAIII or RNAIII mutants were added before the purified *Escherichia coli* 70S ribosomes. Ribosomes were activated for 15 min at 37°C and diluted in the same reaction buffer in the presence of 1 mM $MgCl_2$. Next, 4 pmols of ribosomes were added and incubated for 5 min, then the $MgCl_2$ was adjusted to 10 mM and the reactions were incubated for 5 min. In all, 10 pmols of uncharged transfer RNA^{fMet} ($tRNA^{fMet}$) were then added for 15 min. Complementary DNAs were synthesized with 2 UI of AMV RT (Biolabs) for 15 min at 37°C. Reactions were ended by the addition of 10 µl of loading buffer II (Ambion). The complementary DNAs were separated in 8% denaturating polyacrylamide gel electrophoresis. Gels were dried and visualized using a STORM 840 Phosphorimager (Molecular Dynamics).

RESULTS

The immune evasion protein Sbi is a newly discovered RNAIII molecular target

Sbi is an immune evasion protein expressed by *S. aureus,* an expression that must be tightly controlled during staphylococcal infection. The inactivation of the accessory gene regulator *(agr)* acting as a global virulence regulator increases Sbi abundance *in vivo* (30), indicating that *agr* is a negative regulator of *sbi* expression. Because RNAIII is the effector of the *agr* regulon, we tested to see whether RNAIII by itself could influence *sbi* expression in *S. aureus* cells. We analysed the Sbi protein levels during growth by comparing western blots in wild-type (wt) strain HG001 and in an isogenic Δ*rnaIII* mutant (Figure 1B). The absence of RNAIII increased Sbi protein levels throughout *S. aureus* growth, demonstrating that endogenous RNAIII reduces the expression of Sbi *in vivo*. RNAIII has a specific expression profile during *S. aureus* growth: low levels are detected early but its expression significantly increases up to the pre-stationary growth phase (Figure 1, panels B and D). In the absence of RNAIII (strain Δ*rnaIII*), the Sbi levels significantly increase during the post-exponential phase of growth (Figure 1, panels B and C), when RNAIII expression are the highest in strain wt (Figure 1, panels B and D). Therefore, the absence of RNAIII alters the overall expression pattern of Sbi, indicating RNAIII expression is necessary to ensure the temporal control of Sbi expression. Therefore, RNAIII is required for adjustment of Sbi expression during *S. aureus* growth.

Joint action of two RNAs to adjust Sbi levels during *S. aureus* growth

SprD controls Sbi expression by antisense pairings, preventing translation initiation in the *agr*-negative strain N315 (26). The deletion of SprD in the *agr*-positive HG001 strain (Δ*sprD*) results in elevated Sbi protein levels, although its overall expression profile during growth remains similar (Figure 1, panels B and C) to what has been reported for strain N315 (26). Because Sbi expression is monitored by at least two sRNAs in *S. aureus*, we constructed a Δ*sprD*Δ*rnaIII* double mutant in strain HG001. In this HG001Δ*sprD*Δ*rnaIII*

strain, we detected a considerable increase in Sbi levels throughout growth (Figure 1, panels B and C). This demonstrates that RNAIII and SprD are necessary for lowering and adjusting Sbi protein expression *in vivo*.

These sRNAs possess specific distinct expression profiles during growth (Figure 1, panels B and D). SprD expression is high at beginning of *S. aureus* growth and decreases later (Figure 1, panels B and D). RNAIII expression, however, begins later, then gradually increases during growth. SprD and RNAIII act in concert to shape the expression profile of Sbi, which is highest at early- to mid-exponential (E) phase (Figure 1, panels B and D). There is thus a tight functional relationship between Sbi protein levels and the expression profiles of these two RNA regulators during *S. aureus* growth.

To determine whether *sbi* regulation by RNAIII occurs at the transcriptional level, we examined the effect of RNAIII on the steady-state levels of *sbi* mRNA. In wt strain, *sbi* levels were detected early during growth and decreased during the stationary (S) phase (Figure 1E). Northern blots (Figure 1E) and quantitative PCR (Supplementary Figure S1A) showed that in strains deleted for *rnaIII*, *sprD* or for both, the *sbi* mRNA levels were similar. The implications of RNAIII and SprD on the stability of the *sbi* mRNA were investigated. In the presence of rifampicin, *sbi* mRNA half-life was measured in strain HG001 and in an isogenic strain deleted for both sRNAs (HG001*ΔsprDΔrnaIII*, Supplementary Figure S1B). In both strains, the *sbi* mRNA half-life was estimated ~4 min, indicating that neither SprD nor RNAIII affect *sbi* mRNA stability. It implies that, as previously reported in strain N315 for SprD (26), RNAIII and SprD do not influence *sbi* expression at the RNA level. Together, these data show that RNAIII negatively controls *sbi* expression and that the regulation occurs at the post-transcriptional level.

RNAIII 5′- and 3′-domains interact at distinct sites on *sbi* mRNA

The *in vivo* data prompted us to test for the existence of a direct interaction between RNAIII and *sbi* mRNA. We did gel retardation assays to analyse the duplex formation between RNAIII and a 179 nt-long *sbi* mRNA fragment ($sbi_{1\text{-}179}$) containing its 5′ UTR sequence followed by the first 46 codons. RNAIII binds $sbi_{1\text{-}179}$mRNA, and this interaction was specific because a 1000-fold molar excess of total tRNAs displaced neither the *sbi* mRNA nor the RNAIII from a preformed RNAIII-*sbi* mRNA complex (Figure 2A and Supplementary Figure S2A). Therefore, *in vitro* and without the help of additional molecules, RNAIII forms a specific complex with *sbi* mRNA, thus demonstrating that *sbi* mRNA is a newly discovered direct target of RNAIII.

RNAIII has an intricate structure made of 14 stem loops (31). To analyse complex formation between RNAIII and the *sbi* mRNA and to identify the RNAIII domain or domains involved in the interaction, we made three RNAIII deletion mutants (Figure 2B and Supplementary Figure S2B). Each retained either 73 nt at the RNAIII 5′-end (5′RNAIII), 165 nt at the 3′-end (3′RNAIII) or the stem-loops 3–11 (middle RNAIII). We found that middle RNAIII did not interact with *sbi* mRNA (Supplementary Figure S2C). On the other hand, complex formation between the labelled *sbi* mRNA and

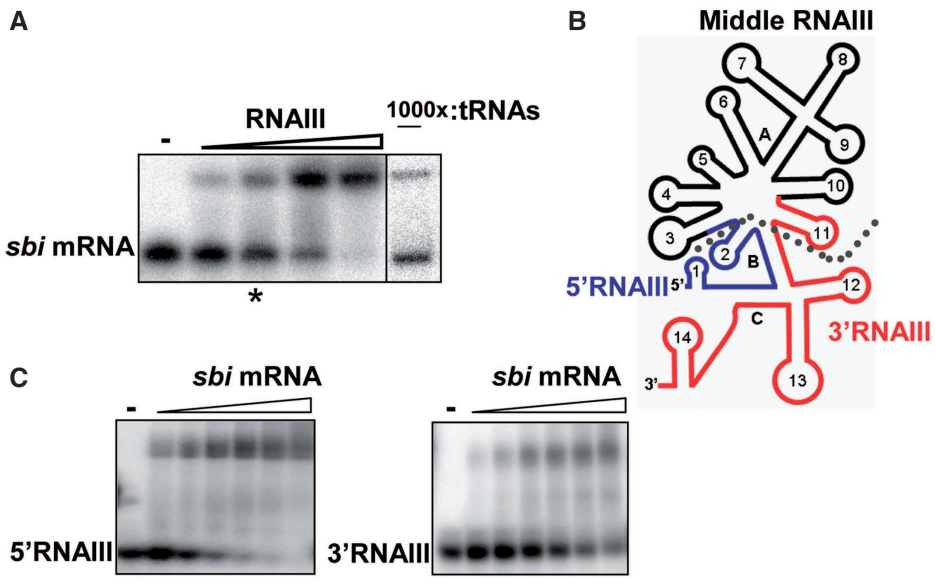

Figure 2. RNAIII directly interacts with *sbi* mRNA through its 5′- and 3′-domains. (**A**) Native gel retardation assays of purified labelled $sbi_{1\text{–}179}$ with increasing amounts of unlabelled RNAIII (0.1, 0.5, 1 and 2 μM). The asterisk indicates the *sbi* mRNA/RNAIII molar ratio used to perform gel-shift competition assays with a 1000-fold molar excess of total yeast tRNAs. (**B**) Schematic presentation of RNAIII deletion mutants constructed and produced in this study: Middle RNAIII is indicated by a dotted line and corresponds to stem-loops 3–11, 5′RNAIII is blue and contains stem-loops 1–2 with half B and half C and 3′RNAIII is red and encloses stem-loops 11–14. (**C**) RNAIII has 5′ and 3′ binding sites that interact with *sbi* mRNA. Complex formation between increasing concentrations of *sbi* mRNA (0.1, 0.2, 0.5, 1, 2 and 4 μM) with labelled 5′- or 3′-RNAIII are shown as detected by native gel retardation assays.

the other two constructs was detected (Figure 2C and Supplementary Figure S2C).

To analyse the *sbi* mRNA domains involved in the interaction with RNAIII, two shorter fragments were produced: a fragment ($sbi_{1–91}$) containing nucleotides 1–91 at its 5′-end and including the TISs and another ($sbi_{84–179}$) including the 86 nt between 84 and 179 (Supplementary Figure S2D). The 5′RNAIII fragment did not interact with $sbi_{1–91}$ and the 3′RNAIII construct did not recognize $sbi_{84–179}$ (Supplementary Figure S2E). The 5′RNAIII, however, interacted with $sbi_{84–179}$ and 3′RNAIII bound to $sbi_{1–91}$ (Supplementary Figure S2E). Together, these data demonstrate that RNAIII forms a stable complex with the *sbi* mRNA *in vitro* and that this interaction may involve several binding sites.

The interaction between *sbi* mRNA and RNAIII includes the mRNA ribosomal binding site

The interaction between RNAIII and the *sbi* mRNA appears elaborate, as it involves several structural domains from both RNAs. To analyze the molecular basis of complex formation between RNAIII and *sbi* mRNA, we did a structural analysis of such a complex in solution using S1 and V1 probes (Figure 3). The proposed model was based primarily on probing data and represents 238 nt from the 5′-end of *sbi* mRNA in the absence of RNAIII (Supplementary Figure S3 and Figure 3B). The data support the existence of six hairpins (H1–H6) and an accessible J5/6 domain is flanked by an internal branching stem and by a 3′ hairpin (H6). S1 and V1 cleavages were detected in identical positions at several locations within the *sbi* mRNA structure, indicating that this structure is unstable in solution, and probably folds into several ‘fast-exchange’ conformations. S1 and V1 probes and in-line probing of the *sbi* mRNA in the absence or presence of RNAIII were used to identify *sbi* mRNA structural changes induced by RNAIII binding. In the presence of RNAIII, *sbi* mRNA reactivity modifications are concentrated at and around the TIS, but they also appear further downstream from the coding sequence (Figure 3A). The results showed that the presence of wt RNAIII induced cleavage protections within *sbi* mRNA, protections that are restricted to three domains that correspond to those subjected to reactivity changes with S1 and V1 probes (Figure 3A). The areas involved are A29-U37 (TIS), G72-A118 (H3 and H4) and A157-U191 (J5/6, Supplementary Figure S3).

To individually assess where RNAIII binds to *sbi* mRNA, we analysed the complexes between *sbi* mRNA and the RNAIII 5′- and 3′-domains by examining enzymatic cleavages (Figure 3C). Binding to 3′RNAIII induced structural changes in H2, H3 and H4 but not further downstream (Figure 3C and Supplementary Figure S3). The 5′RNAIII fragment, however, induced reactivity changes exclusively at J5/6 (nucleotides 157 to 191), deep in the *sbi* mRNA internal coding sequence (Figure 3C and Supplementary Figure S3). These results are consistent with binding assays (Figure 2C and Supplementary Figure S2B), indicating that 5′RNAIII interacts with $sbi_{84–179}$ but not with $sbi_{1–91}$, and that 3′RNAIII binds to $sbi_{1–91}$ but not to $sbi_{84–179}$. An in-depth analysis was done of the structures of the RNAIII domains involved in the *sbi* mRNA interaction. Binding of *sbi* mRNA onto the RNAIII 5′-domain induced reactivity changes at stems B, C and H2. Binding onto the RNAIII 3′-domain induced reactivity changes at stem B, hairpins H11 and H12, as well as at position C468 located in a single-stranded region between hairpins 13 and 14 (Supplementary Figure S4). Interestingly, the two RNAIII-*sbi* mRNA binding sites are self-complementary domains *via* the B and C stems (Supplementary Figure S4).

The probing data were then used to propose a model of the pairings between RNAIII and *sbi* mRNA (Figure 4A), according to which the interaction uses three sites from RNAIII and three from the mRNA target. In our proposed model, nucleotides U25–U41 from the 5′-end of RNAIII (stems B and C) base pair with A163–A181 from *sbi* mRNA J5/6. Nucleotides U347–A373 from RNAIII, stem H10 and hairpin 11 all pair with U88–G115 from *sbi* mRNA stem-loops H3 and H4. Finally, nucleotides C454–U469 from the 3′-end of RNAIII (domains C and SS, C462-U470; Supplementary Figure S4B) and situated in a single-strand region between hairpins 13 and 14 were paired with nucleotides A31–U43 from the *sbi* mRNA (hairpin H2) where both the Shine–Dalgarno (SD) sequence and initiation codon are located.

To analyse these suggested interactions in details, each of the three domains of RNAIII proposed to interact with the *sbi* mRNA were individually deleted. Three RNAIII mutants were constructed (Figure 4A and B): a mutant lacking 9 nt within the single strand located between hairpins 13 and 14 (*RNAIII-ΔSS*), another missing hairpins 11 and 12 (*RNAIII-Δ11,12*) and the final one lacking 25 nt in stems B and C of the RNAII 5′-domain (*RNAIII-ΔBC*). Labelled *sbi* mRNA was added in complex with each of these mutants, and we then performed statistical V1 cleavages (Figure 4C). In agreement with the proposed model, the RNAIII structural deletions within each mutant led to its inability to induce the *sbi* mRNA structural domain reactivity changes that full-length RNAIII induced. Mutants *RNAIII-ΔSS*, *RNAIII-Δ11* and *RNAIII-ΔBC* did not induce reactivity changes at H2, H3, H4 and J5/6, respectively (Figure 4C), showing that each RNAIII mutant lacks one of the recognition domains for *sbi* mRNA (Figure 4A). Together, these data indicate that the RNAIII 5′- and 3′-domains, linked by intramolecular pairings within the RNAIII structure, interact with the *sbi* mRNA at three distinct sites: one that includes the SD-sequence and initiation codon and two others located further along the *sbi* mRNA internal coding sequence.

The RNAIII 3′-domain is necessary and sufficient for inhibition of *sbi* mRNA translation initiation

The RNAIII 3′-binding site interacts with nucleotides that contain the SD sequence and *sbi* mRNA initiation codon (Figure 4A). It coincides with the mRNA portion covered by the ribosomes for translation initiation (32). This suggests that the 3′-terminal domain of RNAIII prevents initiation of *sbi* mRNA translation. We used toeprint

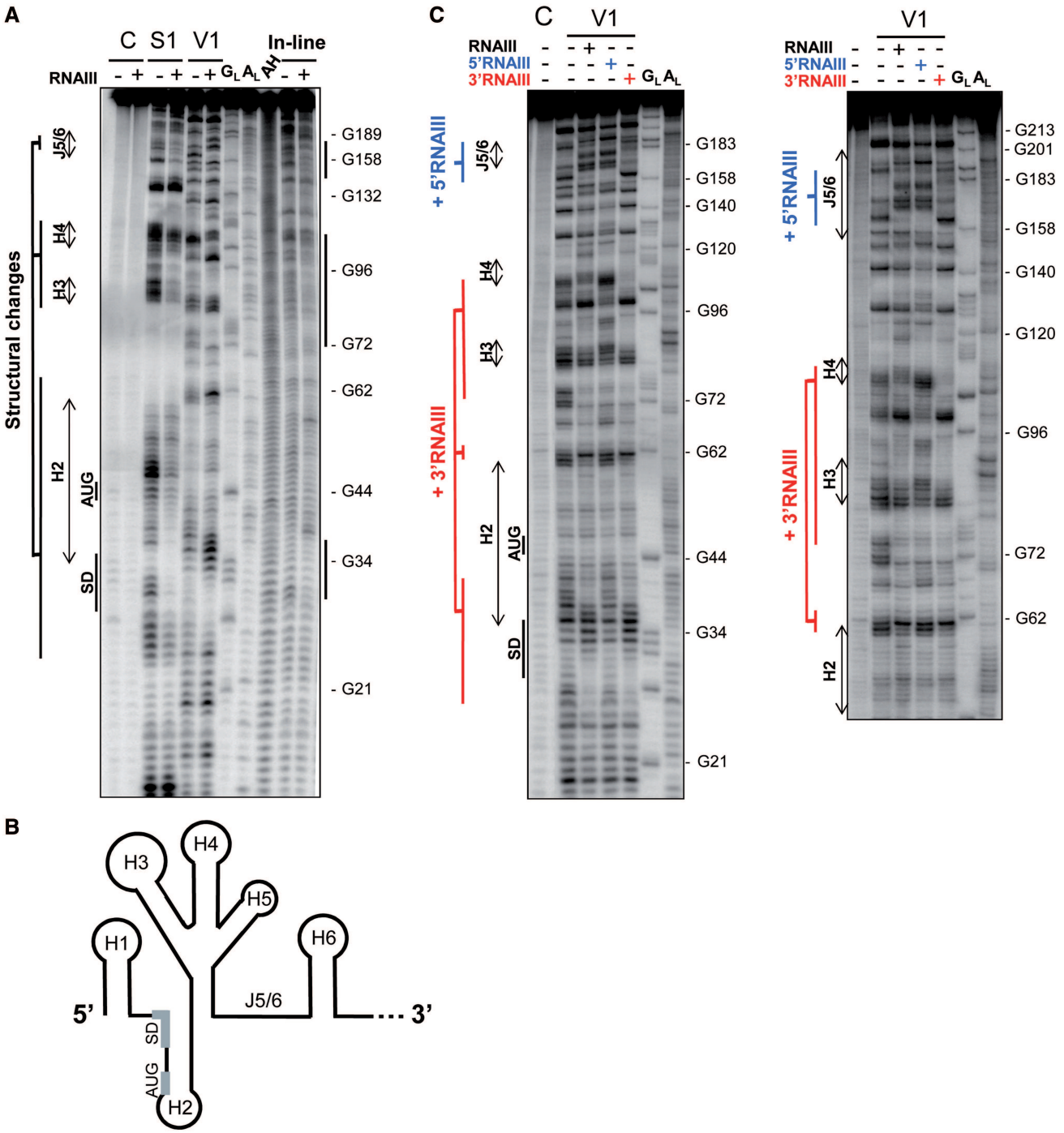

Figure 3. Structural analysis of *sbi* mRNA conformational changes induced by complex formation with RNAIII, 5′RNAIII and 3′RNAIII. (**A**) Conformational changes of *sbi* mRNA when in complex with RNAIII, as detected by structural probes. Autoradiograms of cleavage products of 5′-end-labelled *sbi* mRNA by RNases S1 and V1 in the presence (+) or absence (−) of RNAIII or obtained by 'in-line' probing. Track C, incubation controls; track G_L, RNase T_1 hydrolysis ladders; track A_L, RNase U_2 hydrolysis ladders; track AH, alkaline hydrolysis ladders. The *sbi* mRNA sequence is indexed on the right sides of the panels. On RNAIII binding, the location of the structural changes within the *sbi* mRNA is indicated on the left side of the panel. (**B**) Schematic presentation of the structural domains from *sbi* mRNA 5′-end (1–238 nt) from *S. aureus* based on the solution probing data. (**C**) Conformational changes of the sbi_{238} mRNA induced by either wt RNAIII (blue and red) or by its 5′- (blue) or 3′-domains (red). Short (left) and long (right) runs are presented; the other indications are as in (A).

assays to determine whether full-length RNAIII or its independent 5′- or 3′-domains could prevent ribosomes from loading onto *sbi* mRNA. Ternary initiation complexes made of purified ribosomes, initiator $tRNA^{fMet}$ and $sbi_{1–179}$ mRNA were assembled. A ribosome toeprint was detected on the *sbi* mRNA 15–17 nt downstream from the initiation codon, as previously reported (26). The toeprint was reduced by full-length RNAIII in a concentration-

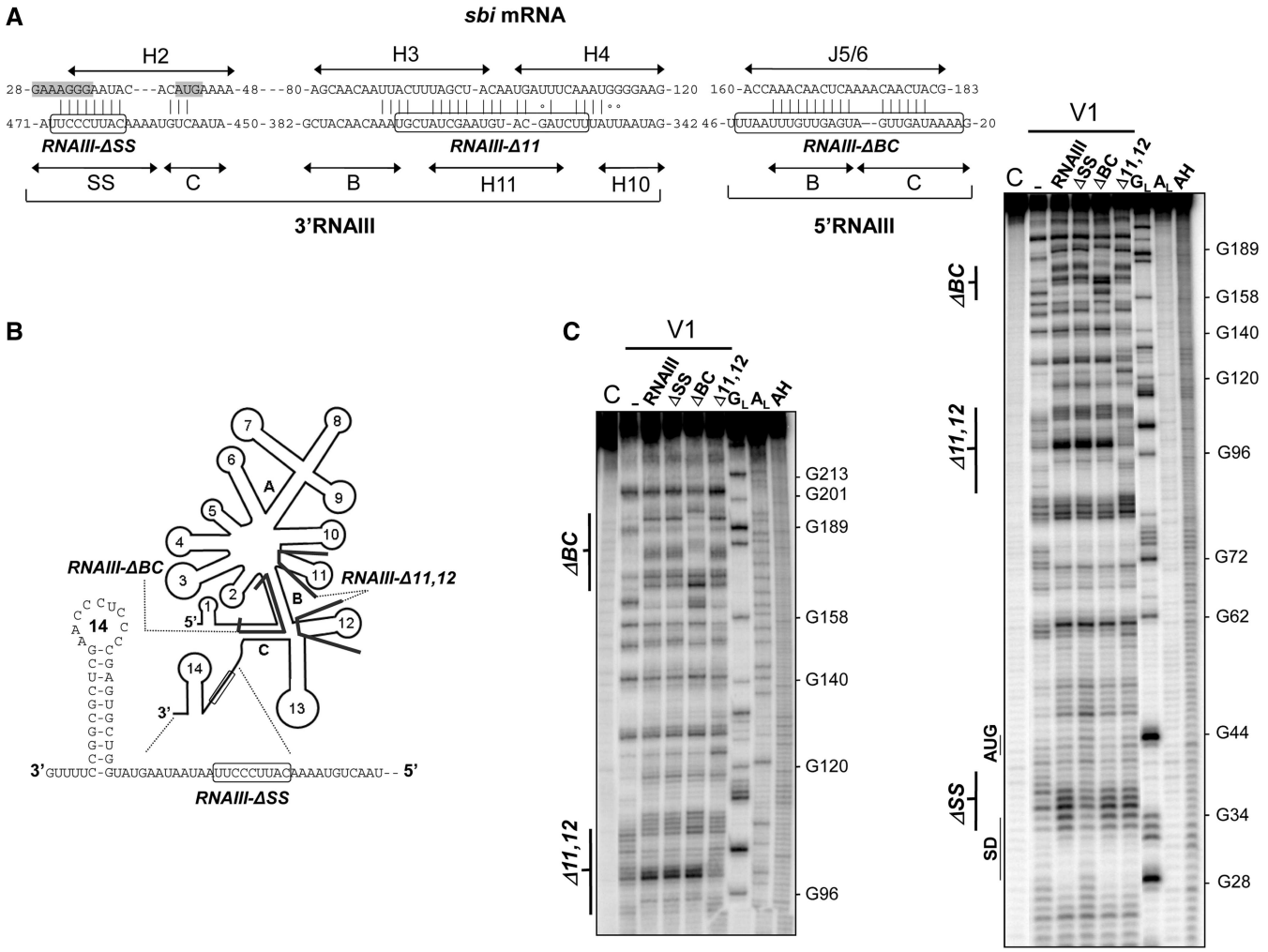

Figure 4. The interaction between *sbi* mRNA and RNAIII involves three structural domains from the two RNAs and covers the mRNA ribosome binding site. (**A**) Proposed pairings between RNAIII and the *sbi* mRNA inducing *sbi* mRNA ribosome binding site sequestration, highlighted in grey. The RNAIII–*sbi* mRNA interactions are based on native gel retardation assays, deletion analysis and structural mapping of *sbi* mRNA–RNAIII complexes that included the RNAIII deletion mutants. RNAIII deletions (*RNAIII-ΔSS*, *RNAIII-Δ11* and *RNAIII-ΔBC*) are circled. (**B**) Location of three RNAIII mutants on a schematic representation of the RNAIII structure. Inset: the *RNAIII-ΔSS* mutant is made of nine nucleotides (circled) within the single-strand connecting hairpins 13–14. *RNAIII-ΔBC* is deleted of one strand in stem B and one in stem C, and *RNAIII-Δ11,12* is deleted from hairpins 11 and 12. (**C**) Structural probing of *sbi* mRNA in complex with each of the three RNAIII deletion mutants and compared with full-length RNAIII. These additional structural data support the proposed interaction between *sbi* mRNA and RNAIII as shown in (A). Track C, incubation controls; track G_L, RNase T_1 hydrolysis ladders; track A_L, RNase U_2 hydrolysis ladders; track AH, alkaline hydrolysis ladders. The *sbi* mRNA sequence is indexed on the right sides of the panels.

dependent manner (Figure 5A). Therefore, our results demonstrate that RNAIII prevents *sbi* mRNA translation initiation.

Because *sbi* translation is controlled by two regulatory RNAs, we tested the ribosome loading onto the *sbi* mRNA by adding SprD and RNAIII simultaneously, and we compared this with the contribution of each RNA. When SprD and RNAIII were added individually, the toeprint was also reduced in a concentration-dependent manner (Figure 5B). The effect of either RNAIII or SprD on initiation of *sbi* translation was similar for each concentration tested. In fact, the decrease in *sbi* translation caused by an equimolar SprD-RNAIII mix was equivalent to the decrease caused by the same concentration of each RNA on its own (Figure 5B). Therefore, RNAIII reduces *sbi* translation initiation independently of SprD and both RNAs possess a similar ability to lower *sbi* translation *in vitro*.

Next, we tested the involvement of different parts of RNAIII in the inhibition of *sbi* translation. As anticipated, increasing amounts of middle RNAIII, which was unable to interact with *sbi* mRNA (Figure 2C), did not prevent ribosome loading (Figure 5A). Increasing amounts of 5′RNAIII had no influence on ribosome loading (Figure 5A), indicating that this site is, by itself, insufficient for control of *sbi* translation. On the other hand, increasing amounts of 3′RNAIII, which interacts with *sbi* mRNA domains containing the TIS (Figure 4A), prevented ribosome loading in a concentration-dependent manner

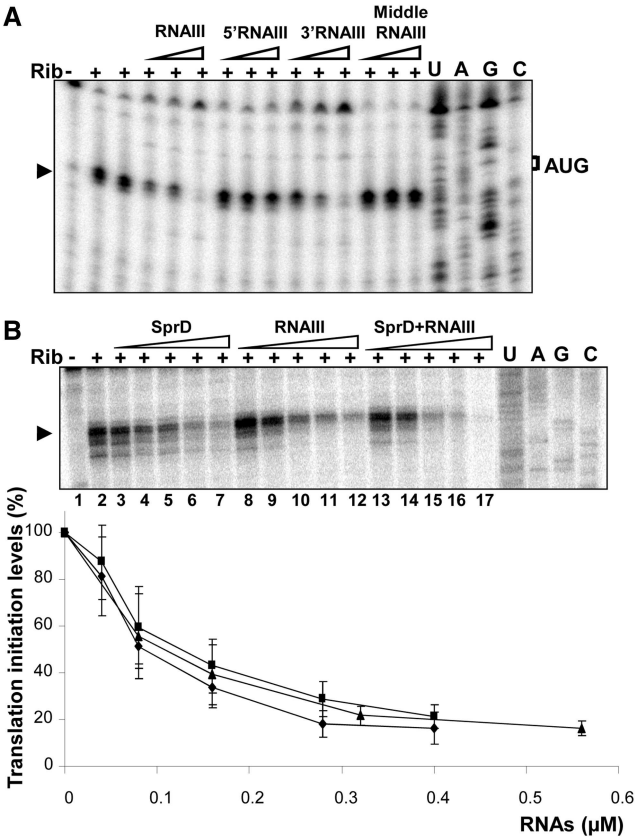

Figure 5. RNAIII regulates *sbi* translation *in vitro* (**A**) Toeprint assays showing that the RNAIII 3′-domain prevents ribosome loading and translation initiation onto the *sbi* mRNA, whereas the other domains from RNAIII do not. 'Plus/minus' indicates the 'presence/absence' of purified ribosomes added to RNAIII, 5′RNAIII, 3′RNAIII or middle RNAIII (at concentrations of 0.4, 2 and 10 μM, respectively). The black arrow indicates the toeprints; 'U', 'A', 'G' and 'C' refer to the *sbi* mRNA sequence. (**B**) Similar inhibitory effects of RNAIII and SprD on initiation of *sbi* mRNA translation. (Upper panel) Ribosome toeprint assays of the *sbi* mRNA with increasing amounts of SprD, RNAIII or both RNAs added simultaneously. The presence/absence of purified ribosomes with SprD (tracks 2–7), RNAIII (tracks 8–12) or SprD and RNAIII at identical concentrations (equimolar mixture, tracks 13–17) is indicated with plus/minus. Concentrations of SprD, RNAIII or of an equal mix of SprD/RNAIII were 0.4 μM (tracks 3 and 8), 0.8 μM (tracks 4 and 9), 1.6 μM (tracks 5 and 10), 2.8 μM (tracks 6 and 11) and 4 μM (tracks 7 and 12). Concentrations of an equal SprD/RNAIII mix were 0.8, 1.6, 3.2, 5.6 and 8 μM (tracks 13–17). The arrow points to the toeprints. U, A, G and C refer to the *sbi* mRNA sequence. (Lower panel) Quantification of the inhibition of *sbi* mRNA translation by increasing concentrations of SprD (diamonds), RNAIII (squares) and by an equimolar amount of the two RNAs (triangles).

(Figure 5A). Taken together, our results demonstrate that RNAIII prevents initiation of *sbi* mRNA translation through antisense pairings at the TIS, and that the RNAIII 3′-domain is required and sufficient for this regulation *in vitro*.

A novel regulatory element within RNAIII prevents *sbi* mRNA translation

According to our proposed RNAIII-*sbi* mRNA interaction model (Figure 4A), the region of RNAIII that interacts with the *sbi* mRNA TIS is situated in the single strand between hairpins 13 and 14. The involvement of this newly-discovered RNAIII element in the control of *sbi* translation was investigated. We first tested to see whether a 9-nt C462-U470 deletion mutant found between hairpins 13 and 14 (*RNAIII-ΔSS*, Figure 4) and which is unable to interact with the *sbi* mRNA TIS region might reduce translation initiation. Toeprint assays showed that unlike wt RNAIII, *RNAIII-ΔSS* cannot control *sbi* mRNA translation initiation (Figure 6A). This indicates that the 9 nt at the 3′-end of RNAIII are actually necessary for regulation of *sbi* translation. To demonstrate the necessity of the RNAIII SS domain in the lowering of Sbi protein levels *in vivo*, full-length RNAIII, 3′RNAIII, 5′RNAIII and the RNAIII-ΔSS mutants were expressed in a HG001*ΔrnaIII*a strain. As evidenced *in vitro*, western blots showed that both RNAIII and 3′RNAIII repressed *sbi* translation (Figure 6B), indicating that the expression of the 3′-domain of RNAIII is sufficient to decrease the Sbi protein levels. In accordance with these results, an RNAIII mutant lacking 25 nt in stems B and C from its 5′-domain (*RNAIII-ΔBC*) was able to decrease the Sbi expression levels *in vivo* (Supplementary Figure S5). Unexpectedly, expressing the 5′-domain of RNAIII on its own increased the levels of Sbi protein *in vivo* (Figure 6C). The mechanism of such effect is unknown and will require further investigations. Nevertheless, *in vitro* data indicate that RNAIII 5′ does not affect Sbi translation initiation suggests that this positive effect probably occurs at the mRNA level.

Unlike full-length RNAIII, mutant *RNAIII-ΔSS* was unable to lower Sbi protein levels *in vivo* (Figure 6B), in agreement with *in vitro* data. Northern blots indicated that *RNAIII-ΔSS* and full-length RNAIII are expressed at similar levels (Figure 6B), ruling out the possibility that the absence of *RNAIII-ΔSS* regulation might be due to its lower expression or stability when compared with wt RNAIII. Altogether, our *in vitro* and *in vivo* results highlight an elaborate and direct interaction between RNAIII and *sbi* mRNA, bringing to light a novel regulatory function module within the RNAIII structure.

DISCUSSION

RNAIII is a useful paradigm for studying RNA-mediated virulence gene regulations in bacteria. As the effector of the accessory gene regulator (*agr*) two-component system, RNAIII governs the expression of a large number of virulence factors including cell wall-associated proteins, exoproteins and global regulators. Therefore, RNAIII is a multitasking RNA, coupling quorum sensing with virulence regulation (7,8). In this report, we identified a novel direct RNAIII target: the immune evasion protein Sbi that traps host IgGs and has a region triggering complement C3 activation (37). We recently reported the major implication of another sRNA, SprD, in the control of *sbi* expression levels (26). Here, we provide experimental evidence to show that SprD collaborates with RNAIII to achieve an optimal and efficient monitoring of *sbi*

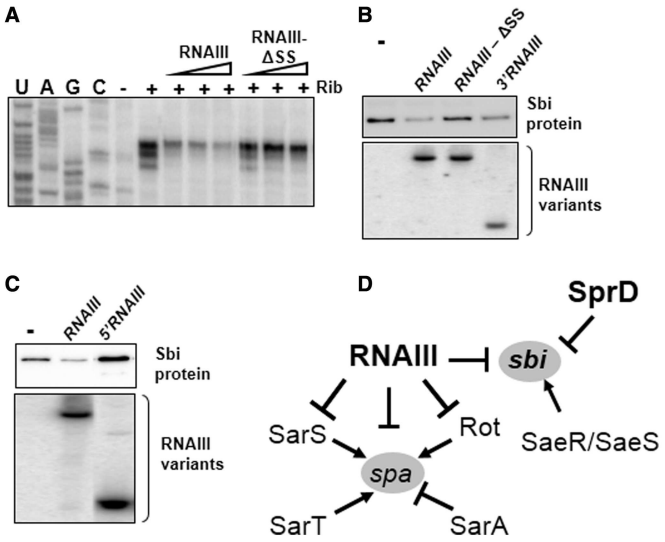

Figure 6. RNAIII adjusts *sbi* translation through a novel regulatory element located at its 3′-end. (**A**) Ribosome toeprint assays of the *sbi* mRNA in the presence of increasing amounts of full-length RNAIII or *RNAIII-ΔSS* mutant. A 9-nt single-strand at the RNAIII 3′-end is required to control Sbi translation initiation. The concentrations of RNAIII and its variant were 0.4, 1.2 and 2.5 μM. The other indications are as in (A). (**B**) A 9-nt single strand at the RNAIII 3′-end is indispensable for governance of Sbi protein expression *in vivo*. Here, we show the *in vivo* expression of protein Sbi at the exponential phase of growth in strain HG001*ΔrnaIII* (in complement with pCN38Ω*RNAIII* (RNAIII), pCN38Ω*RNAIII-ΔSS* (RNAIII-ΔSS) or pCN38Ω3′*RNAIII* (3′RNAIII). Total protein amounts loaded per track are shown in Supplementary Figure S5B. The lower panel depicts the northern blot expression levels of RNAIII and its mutants. (**C**) Stimulatory effect of the 5′-domain from RNAIII onto the expression of the Sbi proteins *in vivo*. Sbi expression of HG001*ΔrnaIII* transformed with pCN38Ω*RNAIII* (RNAIII) or with pCN38Ω5′*RNAIII* (5′RNAIII). The total protein amounts loaded per track are shown in Supplementary Figure S5C. The lower panel depicts the expression levels of RNAIII and the RNAIII 5′ domain by northern blots. (**D**) Model depicting the regulatory network controlling the expression of two *S. aureus* immune evasion molecules, Sbi and SpA. The *sbi* is positively regulated at the transcriptional level by the two-component system SaeR/SaeS (33) and repressed at the translational level by the two sRNAs, SprD and RNAIII. The *spa* transcription is negatively regulated by SarA and positively regulated by Rot, SarS and SarT (34–36). The *rot* and *spa* translations are inhibited by RNAIII, by a direct interaction at translational level (19,23).

expression during *S. aureus* growth. The regulation of *sbi* expression profiling needs at least two sRNAs (RNAIII and SprD), as the absence of one of these causes strong alterations in Sbi levels and expression profiles during bacterial growth (Figure 1). We propose that the two sRNAs RNAIII and SprD reduce *sbi* expression according to their individual expression profiles. During *S. aureus* growth, SprD is continually expressed with a drop at the beginning of the E phase, whereas RNAIII is undetectable at first and slowly accumulates during growth (Figure 1, panels B and D). In a Δ*sprD* mutant, the Sbi protein levels are higher during growth while maintaining an overall expression profile similar than isogenic wt (Figure 1, panels B and C). SprD probably sets the basal levels of Sbi expression through growth, whereas RNAIII intervenes at later stages to restrict protein expression when immune evasion is no longer needed. *In vivo*, the sum of their actions permits the early expression of an immune evasion molecule and its repression at later stages of the infectious process.

Such collaboration between these two sRNAs to control the expression of a virulence gene is a new vision of *S. aureus* physiology. However, the use of several sRNAs to control the expression of a target gene has been identified in other bacteria. For instance, in enterobacteria, expression-level monitoring of a single mRNA that encodes outer membrane proteins is performed by multiple RNAs (38–40). In *E. coli*, translation of the RpoS alternative sigma factor is regulated by at least four sRNAs. DsrA, RprA and ArcZ sRNAs all stimulate RpoS translation (4,41–44), whereas a fourth sRNA, OxyS, negatively regulates RpoS expression (45,46). Another remarkable case recently reported is that of at least five sRNAs that work together to control the expression of the CsgD transcription factor, essential in *E. coli* adhesion and biofilm production (47).

The two regulatory RNAs SprD and RNAIII control the expression of a common target through a shared mechanism. They both prevent translation initiation by antisense pairings: exclusively at the mRNA TIS for SprD (26), and at three distinct sites, including the mRNA TIS, for RNAIII. The SprD-*sbi* mRNA interaction involves the first 41 nt at the *sbi* mRNA 5′-end that includes its SD sequence and AUG initiation codon [(26) and Supplementary Figure S7]. The RNAIII-*sbi* mRNA pairing positioned deep in the *sbi* mRNA coding sequence also includes the SD sequence and AUG codon. There is a 13 nt overlap between RNAIII and SprD at the mRNA TIS (Supplementary Figure S7), implying that each sRNA acts independently to reduce *sbi* translation and that their regulations are mutually exclusive. This is in agreement with our *in vitro* results, which demonstrate that RNAIII reduces *sbi* translation initiation independently of SprD (Figure 5B). Moreover, it is the first evidence to our knowledge that RNAIII controls the expression of a target exclusively at translational level.

In this report, we propose that RNAIII uses three binding sites to control *sbi* expression. One of these is an imperfect duplex that sequesters the mRNA TIS, whereas the two others involve *sbi* mRNA nucleotides from deep in its coding sequence (Figure 4A). The RNAIII-*sbi* mRNA pairing interactions extend to the 46th codon of the target mRNA, far downstream from the initiation signals, a distance which is unprecedented. The involvement of a novel regulatory module within RNAIII structure that interacts with the *sbi* mRNA TIS to repress Sbi translation was demonstrated *in vivo*. The roles of the other two interacting domains from RNAIII in the regulation of Sbi expression are, however, unknown. Remarkably, the 5′domain of RNAIII increases the Sbi expression levels *in vivo* (Figure 6B). Expressing the RNAIII 5′ domain *in vivo* increases the *sbi* mRNA levels (data not shown), and additional investigations will be required to understand the mechanism, enhancing mRNA stability and/or transcription. The use of multiple RNA binding sites is probably essential for such a multifunctional RNAIII to achieve accurate and coordinated regulations of its numerous direct molecular targets and to increase

specificity for binding its molecular targets. Several other mRNA targets of RNAIII use two distant domains for the interaction. The first, RNAIII-dependent *rot* mRNA translational repression, involves two domains remote from the *rot* mRNA 5′-leader region and recognized by the loop–loop pairings involving the RNAIII H7 and H14 hairpins (19). RNAIII also arrests translation of staphylocoagulase (*coa*), a virulence factor promoting human plasma clotting, by interacting at two sites on *coa* mRNA: the TIS and further along at the 13th codon (20). Multiple mRNA target regulations by RNAIII are achieved by specific interactions outside the TIS of each mRNA, which can surprisingly extend >130 nt downstream from the start codon, as we have shown here. An extreme case was also documented in *E. coli,* where *trans*-acting DsrA interacts with the *hns* mRNA initiation and termination codons located 400 nt away (48). Another spectacular example of sRNA regulation was reported in *Bacillus subtilis*. The SR1 sRNA inhibits translation initiation of the *ahrC* mRNA and induces structural changes downstream from the TIS of its mRNA target (49).

The molecular mechanisms of RNAIII-mediated downregulation of several early virulence factors (such as coagulase, SpA, the peptidoglycan hydrolase LytM, Rot and Sbi) are strikingly similar to each other, with antisense pairings preventing translation initiations. The structural domains of RNAIII involved in these regulations, however, are variable (50), thus allowing a single RNAIII molecule to regulate them all. For the RNAIII-*sbi* mRNA interaction, a remarkable array of RNAIII domains is involved, including its 5′- and 3′-domains. This contrasts with previous reports in which only three hairpins from RNAIII (H7, H13 and H14) were reported to be needed to arrest translation of several virulence factors (19–20,23). Moreover, in this work we have uncovered a novel 'CU-rich' regulatory module within RNAIII, in a single strand between hairpins 13 and 14 and essential for Sbi translation control. Alignment of RNAIII gene sequences from various staphylococcal species (51) indicates that this 9-nt stretch is strictly conserved but its flanking sequences are not (Figure 7), suggesting positive selection pressure. This novel regulatory module within RNAIII is predicted to control the expression of other target genes.

Interestingly, the RNAIII 'B' and 'C' self-complement domains are subjected to reactivity changes on complex formation with the *sbi* mRNA. The unfolding of RNAIII 5′ and 3′-ends will provide access to these interacting sequences located next one another in RNAIII tertiary structure, resulting in an efficient interaction between RNAIII and the *sbi* mRNA. RNAIII unfolding of its 5′- and 3′-ends allow the concomitant recognition and pairing with the interspaced *sbi* mRNA binding sites, for regulation.

At both transcriptional and translational levels, RNAIII reduces the expression of a second immune evasion molecule (18,23), SpA (Figure 6D). SpA cloaks the bacteria with IgGs, blocking interactions with neutrophil Fc receptors thus hindering phagocytosis (52). The Sbi and SpA immune evasion molecules are expressed early on, when RNAIII is not yet transcribed, and they then switch off when RNAIII becomes expressed during growth. Concurrent monitoring of the expression of two immune evasion molecules by a single regulatory RNA is an elegant way of regulating the expression of functionally linked molecules during *S. aureus* growth to achieve a united physiological impact. The expression levels of both the immune evasion molecules are also positively and negatively controlled by additional transcription regulators (Figure 6D), some of which (SarS and Rot) are also directly downregulated by RNAIII, a 'double-check' mechanism that probably ensures the regulation process. From the results reported here, we anticipate the existence of additional joint regulators of Sbi and SpA expression.

CONCLUSION

Our report reveals an unprecedented case in *S. aureus*: the direct control of a single mRNA by two sRNAs. It further reinforces the essential role of the RNome in regulating virulence gene expression. In Gram-positive bacteria, *S. aureus* becomes a model organism for identifying the targets and regulatory mechanisms of the nearly 250 sRNAs expressed (53), most of which have unknown biological functions. The next challenge will be to understand how these sRNAs are integrated into the intertwined protein-based regulatory networks that are involved in stress adaptation and virulence. At present, the few known examples of regulatory RNA actions on shared

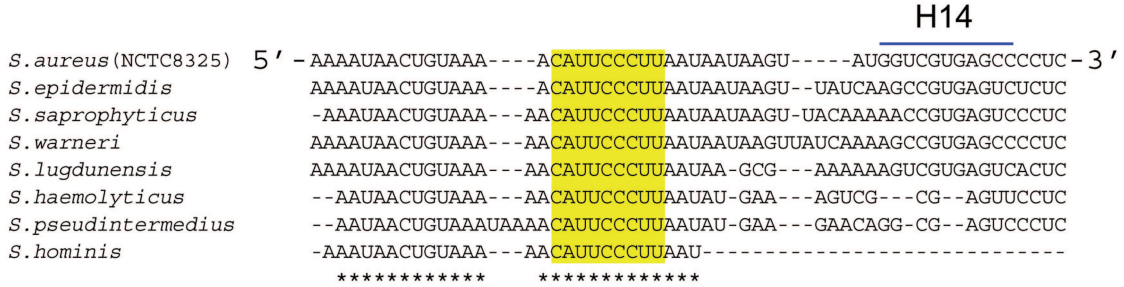

Figure 7. Sequences conservations of the novel regulatory element at RNAIII 3′-end. Sequence alignments of the 3′-end of RNAIII from various species of the *Staphylococcus* genus illustrating nucleotide conservation in its additional regulatory domain (in grey). For internal positioning within the RNAIII sequence, the helix H14 is indicated. The asterisks indicate the conserved nucleotides.

targets probably correspond to the tip of the iceberg. We anticipate that similar sRNA collaborations will turn out to be a widespread phenomenon in living organisms.

SUPPLEMENTARY DATA

Supplementary Data are available Online.

ACKNOWLEDGEMENTS

The authors are grateful to Dr J. van den Elsen (University of Bath, UK) for the anti-Sbi antibodies and to M. Hallier and N. Amiot from our laboratory for the purified ribosomes.

FUNDING

Salary support was provided by the Institut National de la Santé et de la Recherche Medicale and through the regional 'Fonds de maturation' of Bretagne (to S.C.). Agence Nationale de la Recherche [ANR-09-MIEN-030-01 to B.F.]; Funding for open access charge: Inserm dotation.

Conflict of interest statement. None declared.

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