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

Svetlana Chabelskaya, Valérie Bordeau, and Brice Felden.

*Author affiliation:* Université de Rennes 1, Inserm U835-UPRES EA 2311, Biochimie Pharmaceutique, 2 avenue du Prof. Léon Bernard, 35043 Rennes, France.

*Corresponding author:* Brice Felden, Université de Rennes 1, Inserm U835-UPRES EA 2311, Biochimie Pharmaceutique, 2 avenue du Prof. Léon Bernard, 35043 Rennes, France. E-mail: bFelden@univ-rennes1.fr.

**Supplementary data**
**Supplementary Figure 1. RNAIII and SprD RNAs do not control Sbi expression at the RNA level.** (A) Quantification by PCR of the *sbi* mRNA levels at the E phase (OD$_{600nm}$: 3) in wild-type (wt) and in ΔsprD, ΔrnaIII and ΔsprD-ΔrnaIII *S. aureus* HG001 isogenic deletion strains. Both, SprD and RNAIII, are expressed at this growth point (Figures 1B and 1D). The *sbi* mRNA expression levels of the three mutants were calculated relative to the value measured for the wt strain. The error bars indicate the mean values derived from three independent experiments. For the quantitative real-time PCR (qRT–PCR), cDNAs were prepared using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT–PCR experiments were performed using RealMasterMix SYBR ROX (5 PRIME) with the primers listed in Supplementary Table 2. Three independent experiments were performed, with independent RNA purifications. The *hu* and *ssrA* genes were used for normalization. (B) Evaluation of the half-life of *sbi* mRNA in the strains HG001 (WT) and HG001ΔsprDΔrnaIII (ΔsprDΔrnaIII).
$sbi$ mRNA half-life was measured in the presence of rifampicin (200 mg/ml) at mid-exponential growth phase (after 4h of growth). qRT-PCR conditions were as for panel (A). The half-life was given as the time where 50\% of the RNA was degraded. Four independent experiments were performed, with independent RNA purifications.
Supplementary Figure 2. RNAIII 5’ and 3’ domains interact at distinct sites on sbi mRNA. (A) Native gel retardation assays of labeled RNAIII with increasing amounts of unlabeled sbi₁₋₁₇₉ mRNA (0.1, 0.2, 0.5, 1 and 2 µM). The asterisk indicates the sbi mRNA/RNAIII molar ratio used to perform the competition assays with a 1000-fold molar excess of total yeast tRNAs. (B) Deletion mutants constructed from and presented on the RNAIII secondary structure. ‘middle RNAIII’ is gray and corresponds to stem-loops 3-11 (grey), 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) Both its 5’- and 3’-domains are involved in RNAIII’s interaction with sbi mRNA. Native gel retardation assays were used to show complex formation between labeled sbi mRNA and: full-length RNAIII (RNAIII; 0.1, 0.5 and 2 µM,
left to right); 3’-domain (3’RNAIII; 0.1, 0.5 and 2 µM); central domain (Middle RNAIII; 0.1, 0.5 and 2 µM); or 5’-domain (5’RNAIII; 0.1, 0.2, 0.5, 1 and 2.5 µM). (D) Schematic representation and location of the sbi1-91 and sbi84-179 constructs on sbi mRNA. Only sbi1-91 contains the translation initiation signals. (E) RNAIII 3’-domain binds at the 5’-end of the sbi mRNA, while the 5’-domain of RNAIII interacts with the 3’-end of the sbi mRNA fragment. Native gel retardation assays were used to show complex formation between purified labeled 1-91 nts sbi mRNA (sbi1-91*) or 84-179 nts sbi mRNA (sbi84-179*) constructs with increasing amounts (0.2, 1.0 and 2.5 µM) of unlabeled full-length RNAIII, 5’-RNAIII or 3’-RNAIII.
Supplementary Figure 3. The 5'-secondary structure of sbi mRNA. Secondary structure model of the sbi mRNA 5’-end (nts 1-238) of *S. aureus* strain N315. Solution probing data provided experimental support for the proposed structure. A conformation of only 62 nucleotides from the sbi mRNA 5’-end was already reported (Chabelskaya *et al.*, 2010). Since the RNAIII-sbi mRNA interactions are extensive and complicated, the probing data were collected for a longer sbi mRNA fragment. Triangles indicate V1 cuts; arrows capped by circles indicate S1 cuts. Shown are the structural changes induced by sbi mRNA binding of 5’RNAIII (blue), 3’RNAIII (red), and full-length RNAIII. The boxed nucleotides are the sbi mRNA TIS.
Supplementary Figure 4. Structural analysis of the conformational changes within the structure of RNAIII when in complex with *sbi* mRNA. (A) Conformational changes induced by the *sbi* mRNA on 5’RNAIII (left) or 3’RNAIII (right), detected by structural probes in solution. Shown are autoradiograms of the cleavage products of 5’-labeled RNAIII fragments by Nuclease S1 and RNase V1 in the presence (+) or absence (−) of *sbi* mRNA. Track C, incubation controls; track Gt, RNase T₁ hydrolysis ladders; track A₂, RNase U₂ hydrolysis ladders; track AH, alkaline hydrolysis ladders. (B) The RNAIII secondary structure (Benito *et al.* 2000) supported by the probing data presented in this report, showing the structural changes induced by complex formation with *sbi* mRNA. Triangles indicate V1 cuts; arrows capped by circles indicate S1 cuts. The structural changes induced by *sbi* mRNA binding with RNAIII are red, and the outlined nucleotides are those that are subjected to reactivity changes. Complex formation between *sbi* mRNA and 5’RNAIII induced structural changes located 22-44 nucleotides from the RNAIII 5’-end (part of stems B and C and H2). Binding between
the *sbi* mRNA and 3’RNAIII induced structural changes located at loop 11, stem-loop 12, part of stem 13, and at SS.
Supplementary Figure 5. A RNAIII-ΔBC mutant is still able for governance of Sbi protein expression in vivo. The expression of protein Sbi at the exponential phase of growth in strain HG001ΔrnaIII complemented with pCN38ΔRNAIII (RNAIII) or pCN38ΔRNAIII-ΔBC (RNAIII-ΔBC). Total protein amounts loaded and stained by Coomassie are shown. The lower panel depicts the Northern blot expression levels of RNAIII and mutant.
Supplementary Figure 6. Internal protein controls. Coomassie staining of the samples presented in Figures 1A (A), 6B (B) and 6C (C).
Supplementary Figure 7. Proposed pairings between the *sbi* mRNA translation initiation signals, SprD, and RNAIII. Pairings are based on the experimental data provided in this report and from our previous work (18). The structural domains of the two RNAs are indicated and the gray highlighting corresponds to the *sbi* mRNA TIS.
**Supplementary Table 1.** Strains used in and constructed for this study

| Strains | Description | References |
|---------|-------------|------------|
| **E. coli** | | |
| DH5α | F·φ80 ΔlacZ ΔM15 Δ(lacZA-argF)U169 deoR recA1 endA1 hsdR17 (rK− mK−) phoA supE44 λ− thi-1 gyrA96 relA1 | 3 |
| **S. aureus** | | |
| RN4220 | Restriction-defective derivative of 8325-4 | 4 |
| HG001 | rsbU restored strain 8325, lysogenic for phages φ11, φ12, and φ13 | 5 |
| HG001 ΔrnaIIIa | HG001 deleted for rnaIII by homologous recombination | 2 |
| HG001 ΔrnaIII | HG001 deleted for rnaIII; rnaIII::cat86 | This work |
| HG001 ΔsprD | HG001 deleted for sprD | This work |
| HG001 ΔsprDΔrnaIII | HG001 deleted for sprD and rnaIII; rnaIII::cat86 | This work |
**Supplementary Table 2.** DNA primers used in this study.

| DNAs         | Sequences                                                                 | Purposes                                                                 |
|--------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Anti1640     | GGCGCTCCTTGAAAAACGCC                                                         | SprD Northern                                                            |
| NB3’RNAIII   | TCAATAATGATAAATCGATGTTG                                                    | RNAIII Northern                                                           |
| T7RNAIIIfor  | TAATACGACTCTATTAGGCCTAGTCACTAGAGATGATG                                    | RNAIII transcription                                                    |
| T7RNAIIIrev  | CAAAAGGCCGAGCCTGGGA                                                        |                                                                         |
| SBIforTR     | TAATACGACTCTACTATAGGCATAAAATTTAATATGAA                                    | sbi mRNA transcription, toepript                                          |
| SBIrevTR179  | GTCGTTTGTGTTGTTGTTGTTG                                                      |                                                                         |
| SbiT7rev238  | TCTGTGATACTCTTTAGATGTA                                                     |                                                                         |
| T7rev_Sbi_5’90 | GTCGTTTGTGTTGTTGTTGTTG                                                      | sbi, s1 transcript                                                      |
| SBIT7delta84 | TAATACGACTCTACTATAGGCGCTAGTCACTAGGATGATG                                   | sbi, s1 transcript                                                      |
| RNAIII1_2rev | CTTCCTTAATTAAGATAAAAATTC                                                    | 5’RNAIII transcription                                                  |
| RNAIII11_14for | TAATACGACTCTACTATAGGCCTAGTCACTAGGATGATG                                   | 3’RNAIII transcription                                                  |
| RNAIII2_10for | TAATACGACTCTACTATAGGCCTAGTCACTAGGATGATG                                   | ‘middle’ RNAIII transcription                                           |
| RNAIII3_11rev | GATAGCTTACATGCTAGAAATAT                                                    |                                                                         |
| Revdelta14   | CAGTTATTTTTCTCACTTATT                                                     | RNAIII-ASS & 14 mutation, for in vitro transcription                    |
| D14_mut_tb14rev | CAAAAGGCCGAGCCCTTCTTTGTCACGACCCTACTTTATTA                                  | RNAIII-luT14 mutation, for in vitro transcription                       |
| D14ssfor     | ATAGATGGAAAAAAATAACTTGAABILITYATAAGTATGCTGTTG                            | RNAIII-ASS mutation, for in vitro transcription and in vivo expression  |
| D14ssrev     | TCACGACCACTACTTATTATTTGCTATTGTTTATTTTATCACTTAT                              |                                                                         |
| RNAIIIdeltaBC | TAATACGACTCTACTATAGGCGCTAGTCACTAGGATGATG                                   | ‘RNAIII-ΔABC’ RNAIII transcription                                     |
| rnaIII Delta11_12_for | AATTATCATACGATAATTATAAACACACATCTTTCATTACAATAACACACACATCTTTC                 | ‘RNAIII-Δ11.12’, RNAIII transcription                                   |
| rnaIII Delta11_12_rev | TGTATTATAATGAAAAAGAGTGTGTGTTTATAATTATCTGATGATAATT                         |                                                                         |
| RNAIII3EcoRI | AGTAGGAATTCCACGGAAAATATACCTGTAT                                             | RNAIII plasmid                                                          |
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