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**A Staphylococcus aureus Small RNA Is Required for Bacterial Virulence and Regulates the Expression of an Immune-Evasion Molecule**

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**Abstract**

*Staphylococcus aureus*, a pathogen responsible for hospital and community-acquired infections, expresses many virulence factors under the control of numerous regulatory systems. Here we show that one of the small pathogenicity island RNAs, named SprD, contributes significantly to causing disease in an animal model of infection. We have identified one of the targets of SprD and our in vivo data demonstrate that SprD negatively regulates the expression of the Sbi immune-evasion molecule, impairing both the adaptive and innate host immune responses. SprD interacts with the S’ part of the sbi mRNA and structural mapping of SprD, its mRNA target, and the ‘SprD-mRNA’ duplex, in combination with mutational analysis, reveals the molecular details of the regulation. It demonstrates that the accessible SprD central region interacts with the sbi mRNA translational start site. We show by toeprint experiments that SprD prevents translation initiation of sbi mRNA by an antisense mechanism. SprD is a small regulatory RNA required for *S. aureus* pathogenicity with an identified function, although the mechanism of virulence control by the RNA is yet to be elucidated.

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**Introduction**

*Staphylococcus aureus* is a member of the commensal flora that can be an opportunistic pathogen and a cause of nosocomial and community-acquired infections [1]. With the widespread use of antimicrobials, the incidence and spread of highly antibiotic-resistant *S. aureus* strains have increased rapidly in recent years and constitute a clinical and epidemiological challenge in hospitals all over the world. In order to survive and to establish an infection, *S. aureus* inhibits the attack of the host immune system, utilizing diverse escape mechanisms [2]. The staphylococcal protein A (SpA) recognizes the Fc domain of immunoglobulins which results in inverted tagging and blocking the C1q and Fcγ receptor binding sites [3]. *S. aureus* IgG binding protein (Sbi) is another immunoglobulin-binding protein expressed by *S. aureus* [4]. Sbi acts also as a complement inhibitor and forms a tripartite complex with host complement factors H and C3b [5].

*S. aureus* modulates the expression of virulence genes in response to environmental changes thanks to global regulatory elements. They are either two-component regulatory systems as the *agr* (accessory gene regulator) regulon which is a sensor of the population density [6], or transcription factors as the SarA family of DNA binding-proteins [7]. These pathways allow the expression of virulence factor regulation during host colonization and dissemination. In addition to protein-mediated regulations, ribonucleic acids also possess regulatory functions in many bacterial pathogens [8]. Until now, RNAIII is the only *S. aureus* regulatory RNA with a demonstrated function. It is the effector of the global *agr* regulon that controls the synthesis of several virulence factors [9,10]. RNAIII regulates the expression of numerous mRNA targets at the translational and/or transcriptional levels [11] and also acts as an mRNA, containing a small ORF encoding the delta-hemolysin.

Additional regulatory RNAs are expressed by *S. aureus* [12–14]. Their expression profiles vary among clinical strains and many of them, called *Spr* for ‘small pathogenicity island RNAs’, are expressed from genomic pathogenicity islands containing virulence and antibiotic resistance genes. Their functions are so far unknown. This study was aimed at elucidating the role of one of them, SprD. The Shi immune evasion protein was identified as a molecular target of SprD. We show that SprD interacts with the sbi mRNA by an antisense mechanism, occluding the Shine-Dalgarno (SD) sequence and the initiation codon. Moreover, we show that a small regulatory RNA SprD has a major implication during the intravenous (i.v.) infection of mice by a *S. aureus* clinical strain.

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**Results**

**SprD expression profile during growth**

The expression of SprD was monitored during the growth of N315 (*agr−*) [15,16] and MRSA252 (*agr+*) [17], two *S. aureus* clinical isolates. SprD is already expressed during the early exponential (E) phase (**Figure 1**), in contrast to RNAIII that is transcribed at late exponential and stationary (S) growth phases.
SprD expression increases during the E phase, up to the end of the E phase for N315 and MRSA252 strains. To evaluate the implications of the RNAIII in SprD expression during growth, an RNAIII deletion mutant (ΔRNAIII) was constructed in strain RN1 (agr+) [18]. In contrast to N315 and MRSA252, the SprD expression levels remain almost identical during cell growth in strain RN1 (Figure 1C–D). Also, during growth SprD expression is similar in RN1wt and in RN1-ΔRNAIII isogenic strains (Figure 1C–D), suggesting that the expression pattern of SprD is not influenced by the RNAIII. Therefore, the expression of SprD is independent of the presence of absence of RNAIII.

SprD regulates the expression of an immune-evasion protein at translational level

SprD is expressed from the genome of a converting phage containing virulence factors [12]. In most S. aureus strains, sprD is situated in-between scn and chp, within the 8 kb innate immune evasion cluster (IEC) that contains the genes for modulation of the early immune response. Such a genomic localization, as well as its growth phase dependent expression, suggest that this RNA may regulate the expression of virulence factor(s). In order to identify the target(s) of SprD, we analyzed whether SprD modifies the expression of extracellular proteins that contain many virulence factors. For this purpose, a sprD deletion mutant (ΔsprD) was constructed. We determined sprD 5’-end by RACE (rapid

**Author Summary**

Bacteria possess numerous and diverse means of gene regulation using RNA molecules, including small RNAs (sRNAs). Here we show that one sRNA is essential for a major human bacterial pathogen, *Staphylococcus aureus*, to cause a disease in an animal model of infection. Our study provides evidence that this RNA regulates the expression of an immune evasion molecule secreted by the bacterium to impair the host immune responses, and we have solved the mechanism of the RNA-based regulation at molecular level. So far, the mechanism of bacterial virulence controlled by SprD is unrevealed, but that small RNA has a huge impact in the course of a bacterial infection. It implies possible new strategies in fighting against that major human and animal bacterial pathogen in preventing the expression of this regulatory RNA.

**Figure 1. SprD RNA expression profiles in several S. aureus strains.** The expression of SprD during a 24-hour growth of *S. aureus* N315 (A), MRSA252 (B), RN1 (C) and RN1 ΔRNAIII (D) strains by Northern blots using labeled DNA probes for SprD and for the RNAIII. As loading controls, the blots were also probed for 5S rRNAs. The growth curves of N315 (A), MRSA252 (B), RN1 (C) and RN1 ΔRNAIII (D) strains are presented, with the quantification of the SprD levels in the four strains relative to the amount of 5S rRNAs from the same RNA extraction, the maximum value of SprD expression for each strain was normalized to 100. (AU, arbitrary units).

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amplification of cDNA ends) at position C2007178 from the S. aureus N315 sequence [16]. Based on (i) 5’-end mapping, (ii) transcript size derived from Northern blot analysis [12] and (iii) transcription terminator prediction (Figure S1), sprD 3’-end was assigned at position G2007037, implying that SprD has 142 nts. In S. aureus N315, the sprD gene was substituted for an erythromycin resistance cassette by homologous recombination, abolishing the SprD expression (Figure 2A). Complementation of ΔsprD was achieved with a pCN38ΔsprD plasmid expressing SprD from its endogenous promoter (strain ΔsprD+SprD). In S. aureus strains RN4220 [19] and SH1000 [20] naturally lacking the sprD gene (Figure 2A). SprD was expressed with the pCN38ΔsprD plasmid (Figure 2A). In all three strains, levels of a ~45kD protein decrease in the presence of SprD (Figure 2B). In the RN4220 strain, proteins from that band were eluted, a tryptic digest was prepared and the fragments analyzed by MALDI-TOF mass spectrometry. Twenty-five peptides were identified, all matching the sequence of the Sbi protein (Table S1). A confirmation of the decrease of the Sbi levels by SprD within the extracellular proteins was obtained by monitoring the Sbi protein by Western blots (Figure 2C). Interestingly, the SprD-dependent downregulation of the Sbi protein was also observable within the intracellular proteins (Figure 2C), indicating that the regulation does not affect Sbi protein export but the overall Sbi protein expression levels. Complementation of ΔsprD with the pCN38ΔsprD vector reduces the Sbi protein levels in vivo (Figure 2, panels B and C), demonstrating that SprD by itself regulates the expression of Sbi protein. These results were also obtained in strain RN1 and its isogenic ΔsprD mutant (data not shown).

Wild-type N315 and ΔsprD strains growth curves are superimposable in rich broth (Figure 2D), demonstrating that the SprD does not influence S. aureus proliferation. The complemented strain leads to lower Sbi protein levels compared to the wild-type N315 strain (Figure 2, panels B and C) because the expression of SprD from pCN38ΔsprD is higher than its endogenous expression levels in wild-type N315 strain (Figure 2A). In the N315 strain, the highest expression of the Sbi protein during growth is at mid-exponential phase and goes to zero at early stationary phase and beyond (Figure 2D). In its isogenic ΔsprD mutant, the Sbi protein levels are higher during growth but the expression profile remains similar (Figure 2D). Taken together, these data establish a functional link between the Sbi protein levels and the expression of SprD demonstrating that, in different S. aureus genetic backgrounds, SprD represses Sbi expression in vivo.

To test whether the regulation of Sbi by SprD is at transcriptional and/or at translational level(s), the sbi mRNA levels were measured. The expression of Sbi increases during growth in strains N315 (WT) and S. aureus SprD. In its isogenic ΔsprD mutant (data not shown). SprD by itself represses Sbi expression in vivo (Figure 2D). However, the results obtained in some strains do not influence S. aureus proliferation. The complemented strain leads to lower Sbi protein levels compared to the wild-type N315 strain (Figure 2, panels B and C) because the expression of SprD from pCN38ΔsprD is higher than its endogenous expression levels in wild-type N315 strain (Figure 2A). In the N315 strain, the highest expression of the Sbi protein during growth is at mid-exponential phase and goes to zero at early stationary phase and beyond (Figure 2D). In its isogenic ΔsprD mutant, the Sbi protein levels are higher during growth but the expression profile remains similar (Figure 2D). Taken together, these data establish a functional link between the Sbi protein levels and the expression of SprD demonstrating that, in different S. aureus genetic backgrounds, SprD represses Sbi expression in vivo.

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levels were monitored by Northern blots in wt N315 and ΔsprD strains. The \( sbi \) mRNA expression profiles are similar in both strains (Figure 2E), with a gradual increase of the mRNA expression up to the early-exponential phase and a sharp decrease to basal levels later on. When the \( sbi \) mRNA strongly decreases at the stationary phase, SprD is not more expressed, indicating that the Sbi repression at the S phase is ‘SprD-independent’. Also, the \( sbi \) mRNA expression profile does not follow the protein synthesis pattern, probably meaning that the Sbi protein is stable and accumulates during growth. Therefore, in strain N315, the expression of the Sbi protein is dictated by its transcription profile during bacterial growth. In addition, SprD does not modify the steady state level of the \( sbi \) mRNA. Taken together, these results show that SprD downregulates Sbi expression at translational level.

### SprD interacts with the \( sbi \) mRNA by an antisense mechanism

We focused our next investigations on Sbi to elucidate the mechanism of its regulation by SprD. A substantial fraction of bacterial regulatory RNAs for which a function was identified interacts with target mRNAs to regulate gene expression [21]. Putative interactions between SprD and the 5’-portion of \( sbi \) mRNA were detected \textit{in silico} (Figure 3A). We first determined the \( sbi \) mRNA transcriptional start site by RACE at position G\textsubscript{2474939} from the N315 genomic sequence [16]. Therefore, \( sbi \) mRNA 5’-end is located 41 nts upstream of the AUG initiation codon (Figure 3A). Duplex formation between SprD and a 179 nts-long \( sbi \) mRNA fragment containing its 5’ UTR sequence followed by 46 codons was analyzed by gel retardation assays. A ‘SprD-\( sbi \) mRNA’ duplex was detected at a 1:4 molar ratio and nearly all \( sbi \) mRNA was in complex with SprD at a 1:20 molar ratio (Figure 3B). The binding is specific since a 100- to 2,000-fold molar excess of total tRNAs do not displace the \( sbi \) mRNA from a preformed ‘SprD-\( sbi \) mRNA’ complex. A \( sbi \) mRNA deletion mutant lacking 61 nts at its 5’-end (\( sbi\Delta61 \), Figure 3A, brackets), predicted to be part of the interaction, does not bind SprD (Figure 3B), demonstrating that these nucleotides are required to interact with SprD. Reciprocally, the deletion of 36 nucleotides (U35 to U70) from SprD (SprD\textsubscript{Δ36}, Figure 3A, brackets) abolishes complex formation (Figure 3B), showing that these nucleotides are also required for the ‘SprD-\( sbi \) mRNA’ interaction.

To provide a direct evidence \textit{in vivo} of the interaction between SprD and the \( sbi \) mRNA, we have expressed the SprD\textsubscript{Δ36} RNA in the ΔsprD strain. Western blots indicate that SprD\textsubscript{Δ36} RNA is unable to downregulate the Sbi protein levels \textit{in vivo}, in contrast to full-length SprD (Figure 3C). Northern blot indicates that the SprD\textsubscript{Δ36} mutant RNA is expressed at similar levels than SprD wt, demonstrating that the absence of Sbi downregulation by the SprD\textsubscript{Δ36} mutant RNA is not due to its instability \textit{in vivo}. Therefore, this result is a strong evidence of a direct interaction between SprD and the \( sbi \) mRNA \textit{in vivo}, as illustrated in Figure 3A. The interaction between the \( sbi \) mRNA and SprD forms \textit{in vitro} without the contribution of a helper molecule.

\begin{figure}
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\caption{The regulation of Sbi by SprD involves a direct interaction between SprD and the \( sbi \) mRNA. (A) \textit{In silico} prediction of an interaction between SprD and the \( sbi \) mRNA. The free energy of the SprD-\( sbi \) mRNA pairing is provided. The nucleotides bordered by two brackets were deleted in SprD\textsubscript{Δ36} and in \( sbi\Delta61 \). In the \( sbi \) mRNA sequence, the grey nucleotides are the putative SD (5’-GAAAAAGG-3’) and the start codon. (B) Complex formation between SprD and the \( sbi \) mRNA. Native gel retardation assays of purified labeled \( sbi \) mRNAs (the \( sbi \) mRNA contains 179 nts at the mRNA 5’-end and \( sbi\Delta61 \) contains 118 nts) with increasing amounts of either unlabeled SprD, mutant SprD lacking nts 35–70 (SprD\textsubscript{Δ36}) or of a 100 to 2,000-fold excess of unlabeled yeast total tRNAs. (C) Monitoring \textit{in vivo} the expression levels of the Sbi protein in strain N315 ΔsprD (−) complemented by either pCN38ΔSprD (+SprD), or by pCN38ΔSprD\textsubscript{Δ36} (+SprD\textsubscript{Δ36}) at E phase. Bottom panel: Northern blot analysis of SprD\textsubscript{Δ36} and SprD RNAs, 5S tRNAs are the loading controls. (D) Monitoring the expression levels of the Sbi protein in the RN4220 WT and RN4220 Δhfq isogenic strains, in the presence and absence of the SprD, by immunoblots with anti-Sbi antibodies. doi:10.1371/journal.ppat.1000927.g003}
\end{figure}
(Figure 3B), as the Sm-like Hfq protein. To test the contribution of the Hfq protein in vivo, we have monitored the SprD-mediated regulation of Sbi in an hfq deletion strain versus an isogenic wild-type strain. As shown in Figure 3D, the in vivo regulation of Sbi by SprD takes place independently of the presence or absence of Hfq. These results demonstrate that SprD forms a stable complex with the sbi mRNA in vitro and in vivo, as well as deletions altering the complementarities between the two RNAs impair complex formation.

The ribosome binding site of the sbi mRNA is sequestered by SprD

Next, we analyzed in detail complex formation between SprD and the sbi mRNA. As a prerequisite to this study, conformations of the free SprD (nt 1–142) and of the 5'-sbi mRNA (nt 1–179) were investigated using chemical and enzymatic probes. Both transcripts were end-labeled and their solution structures were probed by RNase V1, which cleaves double-stranded (ds) RNAs or stacked nucleotides, and by nuclease S1 and lead, which both cleave accessible single-stranded (ss) RNAs. The reactivity toward these structural probes were monitored for each nucleotide (Figure S2 for SprD and Figure S3 for the 5'-sbi mRNA).

The data are summarized onto SprD and sbi mRNA 5'-end models that they support (Figure 4A). Out of the 142 nts of SprD, 96 are involved in intramolecular pairings, implying structural stability. SprD has two folded ends (H1 and H3–H4) flanking a 54 nt-long accessible domain made of an unstable stem (H2) capped by a loop (L2), bordered by two ss (H1/H2 and H2/H3) junctions. For the 5'-end of the sbi mRNA, the data support the existence of two folded stem-loops (S1-B1 and S2-B2) flanking a...
9 nt-long accessible domain (S1/S2 junction) that contains the predicted SD (Shine-Dalgarno) sequence. The AUG initiation codon is located in loop B2.

The pairing prediction and structural changes induced by complex formation between the two RNAs were examined by subjecting a ‘SprD-sbi mRNA’ complex to statistical nuclease S1 and RNases V1 cleavages. Binding of sbi mRNA induced structural changes in a restricted region of SprD (from U21 to G76), covering the H1/H2 junction, H2, L2, and the H2/H3 junction (Figure 4B).

The structural data that supports the interaction within each helix, as drawn in Figure 4D, are the following: in the presence of the sbi mRNA, S1 cleavages at U23–U30 (H1/H2 junction and H2), U37–G39 and U60–U61 (H2), U46–U47 (loop L2) and A69-U70 (H2/H3 junction) disappeared within the SprD structure, whereas S1 cuts at G51-A54 (H2) appear. Upon duplex formation, V1 cuts appeared at A32 (H2) and at U45–U46 (L2). The binding of SprD led to correlated structural changes in 5’-end of sbi mRNA (from G1 to G62, Figure 4C), encompassing the predicted SD and AUG initiation codon. In the ‘sbi mRNA-SprD’ duplex, S1 cleavages appeared at positions A35-C59 (S2) and disappeared at positions U43-A48 (B2) within the sbi mRNA sequence. Also, RNase V1 cuts appeared at positions A16 and U50-A52 appeared, supporting the complex formation as drawn at Figure 4D.

Therefore, these data are consistent with the deletion analysis of the ‘SprD-sbi mRNA’ complex and support a bipartite helical interaction between the two RNAs (Figure 4D). Structural probing of the RNA duplex indicates that a discontinuous helical domain forms between the two RNAs (22–48SprD/28–53sbi mRNA including the SD and AUG codon and 56–75SprD/1–19sbi mRNA). This helical domain is interrupted by an accessible ss RNA (49–55SprD/22–27sbi mRNA). Nucleotides from the region of mRNAs covered by the ribosomes during translation initiation [22], SprD should prevent ribosome loading on the sbi mRNA. To test this, toeprint assays were performed on ternary initiation complexes including purified 70S ribosomes, initiator tRNAMet and the sbi mRNA. Two ribosome toeprints were detected onto the sbi mRNA, at 15 and 17 nts downstream from the initiation codon respectively (Figure 5A, lane 4), supporting the location of the sbi mRNA start codon as drawn on Figure 4. SprD reduced ribosome loading onto the sbi mRNA in a concentration-dependent manner (Figure 5A, lanes 5–7). Increasing amounts of SprDA36, that cannot form a complex with the sbi mRNA (Figure 5B), did not prevent ribosome loading onto the sbi mRNA (Figure 5A, lanes 8–10). It is concluded that SprD inhibits sbi mRNA translation by preventing ribosome binding by antissequence pairings with the sbi mRNA 5'-end. These results are in agreement with data obtained in vivo, showing that SprD inhibits Sbi expression at translational level.

SprD enhances the virulence of S. aureus

Since one SprD target is the Sbi immune-evasion molecule that was proposed to be involved in S. aureus pathogenicity [4,5], this RNA may play (a) role(s) during staphylococcal infections. This suggestion is in agreement with its co-location with virulence factors [12]. Therefore, we tested the importance of the SprD RNA during staphylococcal infections on an animal infection model. Using a murine i.v. sepsis model with an inoculum of 10^9 S. aureus per mouse, we showed that the virulence of the ΔsprD mutant is abolished (100% survival at day 21 of infection), whereas all animals infected with the parental wild-type strain die (Figure 6A). The virulence of the trans-complemented ΔsprD+SprD strain is partially restored as compared to the wild type (50% survival at day 21, P<0.02). In a different i.v. infection experiment with a 5×10^9 CFU inoculum per mouse in which animals were sacrificed at day 6, the kidneys of mice inoculated with the ΔsprD mutant are small and homogenous red-brown, whereas those of mice inoculated with the wild-type strain are substantially swollen and displayed mottled discoloration suggesting numerous abscesses (Figure 6B). Kidneys of mice infected with the ΔsprD+SprD strain are slightly less swollen than the latter, but display homogenous discoloration with no distinct abscesses (Figure 6B). Results of the macroscopic observation are confirmed in the same experiment by viable bacteria counts, as
A Small RNA Required for *S. aureus* Pathogenicity

The SprD RNA enhances the virulence of a *S. aureus* clinical isolate on infected mice. (A) Survival of mice infected with *S. aureus* wild-type strain N315 (black square), its isogenic ΔsprD mutant (black circle) and ΔsprD mutant complemented with pCN38ΔsprD (black triangle). Groups of eight-week old Swiss mice were inoculated i.v. with 1.5×10⁸ bacteria and monitored daily for 3 weeks. (B) Macrophscopic aspect of kidneys after i.v. infection with *S. aureus* wild-type strain N315 (black square), its isogenic ΔsprD mutant (Δ) and ΔsprD mutant complemented with pCN38ΔsprD (Δ+SprD). Increased size, discoloration and multiple abscesses (black arrow) caused by the wild-type strain was not observed with the ΔsprD mutant, while the ΔsprD complemented strain yielded diffuse discoloration instead of focal abscesses (white arrow). Eight-week old Swiss mice were inoculated with 1.5×10⁸ bacteria and sacrificed after six days. (C) Recovery of *S. aureus* strains from the kidneys of infected mice six days after bacterial challenge. Groups of five mice were inoculated i.v. with 1.5×10⁸ CFU of wild-type strain N315, ΔsprD mutant and ΔsprD mutant complemented with pCN38ΔsprD, respectively. Each individual animal is indicated by a circle symbol with mean bacterial titres represented as a line. doi:10.1371/journal.ppat.1000927.g006

Figure 6. The SprD RNA enhances the virulence of a *S. aureus* clinical isolate on infected mice. The survival of mice infected with *S. aureus* wild-type strain N315 (black square), its isogenic ΔsprD mutant (black circle) and ΔsprD mutant complemented with pCN38ΔsprD (black triangle). Groups of eight-week old Swiss mice were inoculated i.v. with ca. 1.5×10⁸ bacteria and monitored daily for 3 weeks. (B) Macroscopic aspect of kidneys after i.v. infection with *S. aureus* wild-type strain N315 (black square), its isogenic ΔsprD mutant (Δ) and ΔsprD mutant complemented with pCN38ΔsprD (Δ+SprD). Increased size, discoloration and multiple abscesses (black arrow) caused by the wild-type strain was not observed with the ΔsprD mutant, while the ΔsprD complemented strain yielded diffuse discoloration instead of focal abscesses (white arrow). Eight-week old Swiss mice were inoculated with ca. 1.5×10⁸ bacteria and sacrificed after six days. (C) Recovery of *S. aureus* strains from the kidneys of infected mice six days after bacterial challenge. Groups of five mice were inoculated i.v. with ca. 1.5×10⁸ CFU of wild-type strain N315, ΔsprD mutant and ΔsprD mutant complemented with pCN38ΔsprD, respectively. Each individual animal is indicated by a circle symbol with mean bacterial titres represented as a line. doi:10.1371/journal.ppat.1000927.g006

the mean kidney titres (± SD) were 7.2±0.3, 4.9±1.0, 8.5±0.6 log₁₀ CFU per pair of kidneys for the wild-type, ΔsprD, and ΔsprD+SprD strains, respectively (Figure 6C). After 6 days of infection, the *in vivo* persistence of plasmid pCN38ΔsprD in the ΔsprD+SprD strain was verified in 100 randomly selected colonies obtained from kidney homogenates. All of them have retained resistance to chloramphenicol, a specific marker of pCN38. The virulence defect of a SprD-deletion strain, compared to an isogenic wild-type strain, was also observed in the *agr* positive RN1 strain (data not shown). Altogether, these results demonstrate the importance of SprD during bacterial infections triggered by *S. aureus* clinical isolates. Using the same murine i.v. sepsis model, we also tested the implications of Sbi in *S. aureus* virulence. For this purpose, a Δsbi deletion strain (Δsbi) and a strain overexpressing *sbi* under its endogenous promoter from the pCN38ΔsprD plasmid (Δsbi*) were constructed (Figure panels S6A and S6B). We showed that the virulence of the two Δsbi and Δsbi* mutants is similar to that of the isogenic wild-type strains (Figure S6C). These results indicate that only varying the expression levels of the Sbi protein is insufficient to account for the SprD virulence phenotype in our animal infection model and imply that SprD has additional target(s) involved in staphylococcal virulence. Taken together, our findings indicate that SprD plays a major role in the virulence of *S. aureus*.

Discussion

In this report, we show that a small regulatory RNA expressed by *S. aureus* clinical strains plays an essential role in bacterial virulence during the infection of mice in a model of septicemia. After RNAIII, SprD is the second regulatory RNA that plays a major role in *S. aureus* virulence. RNAs are emerging as regulators that enable bacterial pathogens to express virulence genes when required during infection, illustrating their essential roles in pathogenesis [23]. Numerous sRNAs are implicated in the infections caused by Gram-positive and negative bacteria [23]. Some sRNAs are expressed from pathogenicity islands [12], and such horizontally acquired post-transcriptional regulators can regulate the expression of genes encoded by the core genome [24 and this report]. Some sRNAs regulate the expression of virulence factors [10] or are expressed when bacteria multiplies within mammalian cells [25]. Their implication in bacterial pathogenesis, however, was not demonstrated in animal models of infection. Recent studies have shown that several sRNAs expressed from various bacteria including *V. cholerae, L. monocytogenes* and *S. typhimurium* modulate or are involved in virulence on mice infection models [26–28].

In *S. aureus*, RNAIII is the paradigm for RNA-controlled expression of virulence genes, being the effector of the *agr* system. RNAIII was the first RNA shown to be involved in bacterial pathogenesis more than fifteen years ago [9] and is the only example in *S. aureus* until now. Compared to the 142 nt-long SprD, the RNAIII (514 nt-long) is almost four times bigger, encodes a small protein, has a complex structure made of 14 stem-loops [29] and regulates the expression of several virulence genes [10]. The importance of *agr* for virulence in animal models has been reported [30–31], but the exact contribution of RNAIII awaits the experimental testing of an RNAIII deletion strain.

This report reveals that a small regulatory RNA expressed by *S. aureus*, SprD, enhances the virulence of the *agr* negative N315 clinical strain (Figure 6) and of the *agr* positive RN1 strain (data not shown). All the mice infected with the *S. aureus* strain that does not express SprD survive three weeks after the inoculation, whereas all mice challenged with the wild type strain expressing SprD die within 16 days following inoculation. The virulence of the trans-complemented strain is half restored, with the mice kidneys containing viable bacteria as for the wild type strain. The partial restoration of the virulence of the complemented strain could be due to partial plasmid loss after day 6 or, on the other
hand, to a negative impact on bacterial virulence of the higher expression of SprD from the plasmid, compared to the wild type strain. The macroscopic aspect of kidneys from mice infected with bacteria expressing, or not, SprD as well as the lower amounts of bacteria detected in the infected kidneys when SprD is not expressed, indicate that this RNA plays a major role in the virulence of S. aureus (Figure 6). The effect of SprD on virulence might be linked to the lower amount of bacteria detected in the infected kidneys in the absence of the RNA.

We tested the ability of SprD to modify gene expression in S. aureus cells and identified the immune evasion Sbi protein as one molecular target of the RNA. The Sbi protein is among the most abundant secreted proteins [32] produced by many S. aureus clinical isolates [4,33]. We have unravelled the mechanism by which SprD regulates Sbi expression. The action of SprD on the sbi mRNA proceeds by antisense pairings, blocking translation initiation. The pairing interaction between SprD and the sbi mRNA and its functional outcome is presented as a model in Figure 5A. A central domain of SprD pairs with the sbi mRNA 5’-end that includes its SD sequence and AUG initiation codon, blocking translation initiation. For SprD, all the structural changes induced by the formation of the duplex are located in stem-loop H2 and single-stranded flanking domains H1/H2 and H2/H3. The pairings between SprD and the sbi mRNA could be divided into three interacting domains that include the very 5’-end of the sbi mRNA, its SD sequence and its AUG initiation codon. The interacting domains that are single-strand in each of the two RNA structures probably pair first (the H2/H3 junctionsSprD with B1sbi, L2SprD with the purine-rich S1/S2 junctions and the H1/H2 junctionsSprD with B2sbi), followed by spreading through their respective secondary structures. In vitro and in vivo, experimental evidences demonstrate that the regulation of Sbi expression by SprD takes place without the need of the Hfq protein, illustrating the facultative requirement of the Hfq protein for sRNA–mRNA duplex formation among bacteria. The ‘SprD-sbi mRNA’ interaction involves 41 base-pairs and, as suggested [34], extended pairings probably overcome the requirement for the Hfq RNA chaperone.

This strategy of gene expression inhibition is frequently used by bacterial regulatory RNAs [reviewed in 21], including the downregulation of another IgG binding protein, Spa, by the RNAIII [35]. Translation inhibition by regulatory RNAs in bacteria is usually sufficient for gene silencing and can occur in the absence of mRNA destabilization [36]. If target mRNA degradation is triggered, as with the double-strand specific RNAse like SpA protein and also controls the expression of additional genes restricted to the regulation of Sbi expression, also suggesting that SprD has additional target(s) that could be involved at various times during the infection. Indeed, RNAs often regulate the expression of more than a single target, as for several E. coli RNAs [reviewed in 44] and for the S. aureus RNAIII [11]. Also, it would not be so surprising that regulatory RNA(s) other than SprD act synergistically to regulate the expression of the sbi mRNA during cell growth, and a reasonable candidate could be the RNAIII. As for SprD that regulates the expression of Sbi and of other putative target(s), the RNAIII represses, by antisense pairings, the expression of the Sbi-like SpA protein and also controls the expression of additional genes either directly or by limiting the expression of the Rot transcriptional regulator [11]. Preliminary data obtained in our laboratory indicate that SprD has at least one mRNA target in S. aureus cells. The identification of SprD additional target(s) and learning how they are regulated by SprD will be required to understand implication of this sRNA in S. aureus virulence. Identification of Sbi as the first target of SprD is an important step in elucidating the complete gene network regulated by this small RNA which has such a major role in virulence.

Our work, in combination with what is known about RNAIII, suggests a major role for regulatory RNAs in S. aureus pathogenicity. This study also illustrates how sophisticated the regulations of virulence factors productions are during S. aureus infections. It reinforces the roles of RNAs in regulating numerous biological processes in this bacterium. Further studies will be necessary to identify the complete gene network regulated by SprD, its additional target(s), why SprD has such an important role in staphylococcal virulence and the underlying mechanisms of regulations.
Materials and Methods

Strains and plasmids

Strains and plasmids are listed in Table S2. S. aureus strains were cultured at 37°C in brain heart infusion broth (BHI, Oxoid). When necessary, chloramphenicol and erythromycin were used at a 10 μg/ml concentration. In pCN38ΩsprD and pCN35ΩsprD sprD is expressed from its own promoter. The sprD sequence with 40nts upstream and 35nts downstream was amplified from N315 genomic DNA as a 217-bp fragment, with flanking PstI and EcoRI sites. The PCR product was cloned in pCN38 [45] and pCN35 [45]. For producing the pCN38ΩsprDΔ36, mutagenized oligonucleotides ’T7sprD_delfor’ and ’T7sprD_delre’ were used (Table S3). In pCN35Ωsbi, sbi is expressed from its endogenous promoter. The sbi sequence was PCR amplified from N315 genomic DNA as a 1700-bp fragment with flanking PstI and EcoRI restriction sites.

Construction of the deletion strains

To inactivate the sprD gene, DNA fragments of 1000 bp upstream and 800 bp downstream of sprD were amplified by PCR from genomic DNA and cloned together with the emB from pCN51 [45] into XbaI-EcoRI sites of temperature-sensitive plasmid pBT2 [46]. Primers used for cloning are indicated in Table S3. The resulting plasmid pBT2ΔsprD was transformed into S. aureus strain RN4220 and then into S. aureus N315 to achieve integration of the emB gene into the genome by homologous recombination. Mutants were enriched by cultivation at 42°C. Cells from the stationary-phase culture were plated on TSA plates and incubated at 37°C. Colonies were imprinted on plates supplemented with 10 μg/ml chloramphenicol. Chloramphenicol-sensitive colonies were tested by PCR for replacement of sprD for the erythromycin cassette. The deletion of sprD was confirmed by Northern blot (Figure 2A). Inactivations of the sbi and RNAIII genes were performed by the same method except that no resistance marker was inserted between their 5’ and 3’ DNA sequences. The primers used for constructing pBT2Δsbi and pBT2ΔRNAIII are shown in Table S3.

Animal infection model

Virulence levels of the SprD+ strain N315, its isogenic mutant ΔsprD and complemented strain ΔsprD pCN38ΩsprD were compared using a murine intravenous sepsis model. Groups of 10 female Swiss mice, 6- to 8-weeks old (Charles River Laboratories, L’Arbresle, France) were inoculated i.v. with 10,000 S. aureus. Six to eight-weeks old (Charles River Laboratories) female Swiss mice, 6- to 8-weeks old (Charles River Laboratories) were euthanized with CO2 and their kidneys were homogenized, diluted in 0.9% NaCl and plated on 5% blood agar for determination of bacterial titers, expressed as log10 CFU per pair of kidneys. Morphology observation included swelling, discoloration and presence of macroscopic abscesses. The stability of plasmid pCN38ΩsprDΔsprD (encoding chloramphenicol resistance) in the complemented ΔsprD mutant was assessed by plating randomly selected colonies grown from kidney homogenates on nutrient agar with containing 20 μg/mL chloramphenicol.

Protein isolation, mass spectrometry and immunoblots

For the preparation of protein extracts, bacteria are cultured until the exponential or stationary phases and the cells are pelleted for 10 min at 4°C (8,000g). For purifying the extracellular proteins, the supernatants are collected, filtered (0.45 μm sterilized filter) and precipitated with 10% trichloroacetic acid. The precipitates are washed with ice-cold aceton and loaded onto SDS-PAGE according to [47]. For the total protein extractions, pellets of 2-ml cultures are washed with TE (50 mM EDTA, 50 mM Tris pH 7.5), and suspended in 0.2 ml of the same buffer containing 0.1 mg/ml lysostaphin. Following incubation at 37°C for 10 min, samples are boiled for 5 min, analyzed by SDS-PAGE and stained by Coomassie blue R-250. The proteins of interest are extracted from gel, trypsin digested and the peptides identified by MALDI MS/MS and RP-HPLC/NanoLC/ESI-MS-MS. For the immunoblots, proteins are transferred to PVDF membrane (Immobilon-P, Millipore). Signals are visualized using a STORM 840 Phosphor-Imager (Molecular Dynamics) and quantified using Image-QuantNT 5.2.

RNA isolation, Northern blots, 5’-RACE, transcription and RNA labeling

Total RNAs are prepared as described [48]. For SprD and other sRNAs, Northern are performed with 5 μg of total RNAs, as described [12]. For sbi mRNA, Northern are performed as described [49]. RACE assays are carried out according to 49 with the primers from Table S3. Wild-type and mutant RNAs for probing, gel-shift assays or toeprints are transcribed from PCR fragments generated from genomic DNA with the primers from Table S3. For producing the template-encoding SprDΔ36, mutagenized oligonucleotides (Table S3) were used. The RNAs were produced by in vitro transcription using MEGAscript (Ambion). Adding [α-32P]UTP within the transcription mix produces radioactive transcripts. 5’-RNA labeling is performed as described [49]. The RNAs are purified by 8% PAGE, eluted, ethanol precipitated and stored at −80°C.

Gel-shift assays and RNA probing

Gel retardation assays are performed as described [49], 0.4 pmol of labeled wt or sbiΔ61 mRNAs are incubated with various concentrations (from 1.6 to 20 pmol) of unlabeled wt SprD or SprDΔ36. For structural analysis duplexes between sbi mRNA and SprD are prepared by incubating 0.4 pmol of labeled RNA and 1.6 pmol of unlabeled RNA in a buffer containing 10mM Tris-HCl (pH 7.5), 60 mM NaCl, 10mM EDTA and 5 mM DTT for 15 min at 25°C. Structural assays are performed as described [49]. Digestions are at 25°C for 15 min with 2.5 μg of yeast tRNAs with 0.2 or 1 unit of S1 and 10− or 3.10−5 units of V1. Lead(II) cleavages are performed with 0.2 or 0.4 mM PbAc in 25 mM Hepes (pH 7.5), 7 mM Mg acetate and 55 mM K acetate for 10 min at 25°C. The reactions are precipitated, the pellets dissolved in loading buffer (Ambion). The samples are denatured for 5 min at 65°C prior to separation on 8% polyacrylamide/8M urea gels. Gels are dried and visualized (STORM 840 Phosphor-Imager).

Toeprints

The toeprints are as described [30] with modifications. Annealing mixtures contain 0.2 pmol of sbi mRNA and 1 pmol of labeled ‘SBrevTR’ primer in a buffer containing 10 mM Tris-acetate (pH 7.5), 60 mM NH₄Cl, and 1 mM DTT. For the assays in the presence of SprD, various concentrations of wt or SprDΔ36 are added prior to the purified E. coli 70S ribosomes. The ribosomes are reactivated for 15 min at 37°C and diluted in the reaction buffer in the presence of 1 mM MgCl₂. 4 pmols of 70S are added in each assay, incubated for 5 min and MgCl₂ is
adjusted to 10 mM. After 5 min, 10 pmols of uncharged tRNA\textsuperscript{5\textprime} are added and incubated for 15 min. cDNA is synthesized with 2 UI of AMV RT (Biolabs) for 15 min. Reactions are ended by 10 µl of loading buffer II (Ambion). The cDNAs are loaded and separated onto 8% PAGE. The toeprints are located on the sbi mRNA sequence by sequencing the DNA.

**Ethics statement**

All animal experiments were performed in accordance to European guidelines and recommendation of the French Agricultural Office for the care of animals subjects. Experiments were carried out in the accredited research animal facility of Institut Pasteur de Lille (accreditation number, A59107). All animal protocols were approved by the locally appointed investigational review board (Institut Pasteur de Lille, accreditation number, A59107).

**List of accession numbers of genes and proteins mentioned in the text**

- *S. aureus* Immunoglobulin G binding protein A: GenBank ID: BAB41326.1
- *S. aureus* Sbi protein: Genbank ID: AF027155
- *S. aureus* RNAIII (nt 1260 to 1571): GenBank accession number: X52543
- *S. aureus* Hfq: PDB code 1Kq1A

**Supporting Information**

**Figure S1** Sequence alignments of SprD from several *S. aureus* strains. The bolded nucleotides are the 5\textprime; ends derived from N315 RACE mapping and the underlined nucleotides are the sequence variations. The stars are the sequence identities. SprD has a 9-base pair helix (H4) ending by a U\textsubscript{6} stretch, acting as a transcription terminator.

**Figure S2** Monitoring of SprD conformation by structural probes. SprD conformation was probed by RNase V1, that cleaves accessible single-strands (ss). Autoradiograms of cleavage products of 5\textprime;-labeled SprD by lead, nuclease S1 and lead. For the details, please refer to Figure S3 legend.

**Figure S3** Monitoring the conformation of the sbi mRNA 5\textprime;-end (179 nts) by structural probes. Autoradiograms of cleavage products of 5\textprime;-labeled sbi mRNA by RNase V1, nuclease S1 and lead. For the details, please refer to Figure S3 legend.

**Figure S4** Human IgGs from serum increase Sbi protein levels in the presence (+) and absence (−) of SprD. Immunoblot analysis with anti-Sbi antibodies of total intracellular proteins in *S. aureus* SH1000 strain in the presence (+) or absence (−) of 10% human serum.

**Figure S5** Coomassie staining of the samples presented on Fig. 2, panels B and C (A) and on Fig. 2D (B) indicates that identical amounts of proteins were loaded for strains ‘wt’, ‘Δ’ and ΔsprD’. Immunoblot analysis with anti-Sbi antibodies.

**Figure S6** Deleting or overproducing the Sbi protein have no detectable effect on the virulence of the N315 *S. aureus* clinical isolate on infected mice. Monitoring the expression of the Sbi protein in strains N315 Δsbi (A) and in the sbi overproducing strain pCN35-Δsbi (B), compared to a strain carrying the empty plasmid vector (pCN35) and to the wild-type strain (wt) by immunoblots with anti-Sbi antibodies. (C) Survival of mice infected with *S. aureus* wild-type strain N315 (square), its isogenic Δsbi mutant (circle) and wild-type strain transformed with pCN35Δsbi (triangle). Groups of 5 seven-week old Swiss mice were inoculated i.v. with 2,109 bacteria and monitored daily for 2 weeks.

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**Author Contributions**

Conceived and designed the experiments: SC OG BF. Performed the experiments: SC OG. Analyzed the data: SC OG BF. Wrote the paper: SC BF.

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