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Dual Toxic-Peptide-Coding *Staphylococcus aureus* RNA under Antisense Regulation Targets Host Cells and Bacterial Rivals Unequally

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**SUMMARY**

Produced from the pathogenicity islands of *Staphylococcus aureus* clinical isolates, stable SprG1 RNA encodes two peptides from a single internal reading frame. These two peptides accumulate at the membrane, and inducing their expression triggers *S. aureus* death. Replacement of the two initiation codons by termination signals reverses this toxicity. During growth, cis-antisense RNA SprF1 is expressed, preventing mortality by reducing SprG1 RNA and peptide levels. The peptides are secreted extracellularly, where they lyse human host erythrocytes, a process performed more efficiently by the longer peptide. The two peptides also inactivate Gram-negative and -positive bacteria, with the shorter peptide more effective against *S. aureus* rivals. Two peptides are secreted from an individual RNA containing two functional initiation codons. Thus, we present an unconventional type I toxin-antitoxin system expressed from a human pathogen producing two hemolytic and antibacterial peptides from a dual-coding RNA, negatively regulated by a dual-acting antisense RNA.

**INTRODUCTION**

The *Staphylococcus aureus* bacterium is a serious human pathogen that causes life-threatening nosocomial and community-associated infections (Anstead et al., 2014). *S. aureus* generates virulence factors (Zecconi and Scali, 2013) whose timing and expression levels are tuned by regulatory proteins and small RNAs (sRNAs). Bacterial sRNAs control the expression of targets such as virulence and stress response genes directly or indirectly through regulatory cascades (Caldelari et al., 2013). Direct regulations are achieved through pairing with mRNA targets by sharing limited or extended complementarity or by modulating protein activity (Storz et al., 2011). As for *Listeria monocytogenes* (Mellin and Cossart, 2012) or *Streptococcus pneumoniae* (Mann et al., 2012), *S. aureus* expresses hundreds of sRNAs, making it a model organism for sRNA studies in Gram-positive bacteria (Guillet et al., 2013; Tomasini et al., 2014). In *S. aureus*, several sRNAs are expressed from small pathogenicity islands (SaPIs) containing virulence and antibiotic resistance genes; thus, these sRNAs have been named “Spr” (small pathogenicity island RNAs). Interestingly, one of these, SprD, contributes to disease induction in a mouse model of infection and reduces the expression of a host immune evasion molecule (Chabelskaya et al., 2010). Recent reports suggest that some sRNAs, originally termed “noncoding regulatory RNAs,” encode peptides or small proteins from internal reading frames (Vanderpool et al., 2011; Sayed et al., 2012a).

This study investigated the functions of SprF and SprG, RNA present in multiple copies in the staphylococcal genome and initially detected by computer searches and transcriptomic analysis (Pichon and Felden, 2005). Sequence comparisons suggested that the SprF1/SprG1 pair might be a type I toxin-antitoxin (TA) system (Fozo et al., 2010). TA systems are classified into five types depending on the nature and mode of action of the antitoxin. In types I and III, the antitoxins are RNA that either inhibit toxin synthesis or that sequester the toxins. In types II, IV, and V, the antitoxins are proteins that sequester toxins, counterbalance toxin activity, or inhibit toxin synthesis. Type I TA systems are abundant in the eubacterial and archaeal genomes and are made of overlapping gene pairs that allow toxic peptide expression, which is then neutralized by an antisense RNA antitoxin (Brantl, 2012). They permit plasmid maintenance or act as stability modules for their genomes (Holecik and Iyer, 1997; Van Melderen, 2010). When overexpressed, some of these pairs trigger cell death, whereas others contribute to formation of persisters, thus inducing a dormant stage tolerant to certain antibiotics (Dörr et al., 2010). However, the precise roles of these TA systems remain obscure. We report here that the SprG1/SprF1 pair is an unconventional type I TA system expressed in *S. aureus*. SprG1 uses two in-frame initiation codons to produce two toxic peptides. Peptide expression is negatively regulated by SprF1, a dual-acting cis-antisense RNA. The two secreted peptides are able to lyse host cells and competing bacteria with dissimilar strengths.

**RESULTS**

SprF1 and SprG1 End Mapping, Expression, Relative Amounts, and Stabilities  
SprF and sprG read in opposite directions, with sequence overlaps predicted at their 3’ ends. Using probes specific for
RACE mapping was done to determine the 5'0 S. aureus with strain N315 because it is a clinical, Methicillin-resistant strain (MRSA) that expresses all the gene copies. sprG2 and sprG4, probably due to their sequence similarities (Figure S1). We chose to work with strain N315 because it is a clinical, Methicillin-resistant strain of S. aureus strain (MRSA) that expresses all the gene copies. RACE mapping was done to determine the 5' and 3' ends of the four sprF/G RNA pairs present in this strain (Table S1). This report focuses the SprF1/SprG1 pair located within the Sa3 PI, which is the genome of a virulence factor-containing converting phage (Pichon and Felden, 2005), compared to the other copies located in the core genome. SprF1 and SprG1 3' ends overlapped, excluding only seven nucleotides (nt) at the SprF1 5' end (Figure 1A). Northern blot and RACE mapping of SprG1 indicated that it is expressed as two transscripts differing at their 5' ends: a SprG1312 major form, and a SprG1439 minor form (Figure S2). Long and short SprG1 have conserved -10TATTA-6 and -9TATAAT-4 boxes, respectively, as predicted promoters (Figure S3, red). The SprF1 5' end was assigned at position A2101270 downstream of a predicted conserved -30TATATTA-15 promoter sequence (Figure S4, red). SprG1 and SprF1 expression were monitored during S. aureus growth (Figure 1B). During growth, the RNAs are constitutively and concomitantly expressed. Quantitative estimations of SprF1, SprG1439, and SprG1312 were done by comparing their in vivo expression to a concentration range of purified synthetic RNA (Figure 1C). At all times, there was about twice the amount of SprG1312 as of SprF1, whereas the expression of SprG1439 was about 20-fold less than that of SprG1312. Half-life determinations of the RNAs (Figures 1D and 1E) showed about a 12-fold shorter half-life for SprF1 (~10 min) than for SprG1312 (>120 min). In summary, unstable SprF1 and stable SprG1 are constitutively expressed, and their RNA sequences overlap.

SprG1 Ribosome Loading at Two Internal Initiation Codons Generates Two Peptides

SprF1 and sprG1 phylogenetic distribution is restricted to the Bacillales (staphylococcaceae, genus staphylococcus), with an increased sequence identity between the strains (Figures S3 and S4). In all identified sprG1 sequences, an internal open reading frame (ORF) was identified with two predicted Shine-Dalgarno (SD) sequences ~8 nt upstream from two potential initiation codons (Figure S3, underlined). SprG1 may therefore express two polypeptides: one containing 44 amino acids with a predicted molecular weight (MW) of ~5 kDa; and a shorter peptide of ~3.5 kDa theoretical MW that lacks 13 residues at the N terminus (Figure 2A). A probable ORF was also detected in SprF1 (Figure S4). To determine whether SprF1 and/or SprG1 express peptides, in vitro translation assays were performed (Figure 2B). No translation was detected with SprF1. Synthetic SprG1312 and SprG1439 RNA, however, produce peptides of ~3–5 kDa apparent MW, which is in agreement with the peptide lengths inferred from the internal coding sequence. To investigate whether translation initiates at one or both predicted methionine codons, they were replaced by two termination codons (mutant SprG1439-STOP1,14), but this interrupted translation. Next, to test whether the short peptide encoded by SprG1 arises from initiation at the second in-frame methionine codon (M14), it was mutated into Alanine (mutant SprG1439-Ala14). Mutating M14 into Ala14 caused a strong inhibition of the short peptide...
translation, although translation of the long peptide was maintained. This suggests that the shorter peptide was produced from ribosome initiation at M\textsubscript{14}. When SprF1 is added to the reaction at a 2.5-fold molar excess as compared to SprG1, SprG1\textsubscript{312}, and SprG1\textsubscript{439} translations were reduced about 3- and 2-fold, respectively. The direct interaction of the ribosomes with the predicted translation initiation signals was evaluated using toeprint assays on ternary initiation complexes that included purified ribosomes, initiator tRNA\textsubscript{fMet}, and SprG1\textsubscript{312} (Figure 2 C). Two ribosome toeprints were detected at positions A\textsubscript{89} and G\textsubscript{135} on SprG1, 15 and 23 nt downstream, respectively, from A\textsubscript{+1} of the first and second predicted initiation codons in the SprG1 ORF (Figure 2D).

The SprG1-Encoded Peptides Originate from Two Internal In-Frame Methionine Codons
To investigate whether SprG1 produced the two peptide(s) in vivo during bacterial growth, a reporter construct was designed by adding an \textasciitilde 2.75 kDa theoretical MW 3 x FLAG (FLAG) sequence in-frame ahead of the ORF’s predicted termination codon (Figure S3). In addition to endogenous SprG1\textsubscript{312}, northern blots validated the expression of SprG1\textsubscript{312-FLAG} during growth (Figure 3 A). Using anti-FLAG, immunoblotting revealed that SprG1 fusion peptides are expressed in vivo during S. aureus growth (Figure 3B). Two peptides were detected: a major abundant form and a minor shorter product, both of \textasciitilde 5–7.5 kDa apparent MWs, in agreement with the predicted 31+22- and 44+22-amino-acid-long fusion peptides inferred from the SprG1 sequence (Figure S3). The peptides were expressed early, accumulated during the early phase, and decreased later on. This drop in peptide expression was not caused by a toxicity-induced cell plasmid number reduction (see below) because the SprG1\textsubscript{312-FLAG} RNA levels were stable throughout growth (Figure 3 A). Compared to S. aureus cells with an empty vector, isogenic bacteria expressing SprG1\textsubscript{312-FLAG} had reproducible minor growth defects (Figure 3 C). To determine whether SprF1 effects SprG1-encoded peptide expression in vivo, an additional construct was built to produce the flagged peptides in the presence of excess SprF1. Northern blots showed that inducing about a 3-fold increase in SprF1 expression reduced the amount of SprG1\textsubscript{312-FLAG} and SprG1\textsubscript{312} endogenous RNA (Figure 3 A), as well as reducing the number of flagged peptides (Figure 3B), thus complementing the growth defect (Figure 3 C). To test whether the peptides encoded by SprG1 resulted from translation initiation at two
internal AUG codon (M1 and M14) or at a second in-frame valine codon (V2), they were mutated into a UAA termination codon (Figure 2A). When compared with the fusion peptides expressed by SprG1, immunoblots revealed the lack of expression of the longer peptide in the STOP1 and STOP2 mutants, together with a significant increase of expression of the short peptide predicted to start at M14 (Figure 3D). In the STOP14 mutant used as a control, no peptides were produced, because the truncated 13 residue form of the long peptide is not flagged and thus not detected. More surprising was the absence of peptides produced by the STOP1,2 mutant, suggesting that the sequence at and around M1 influences production of the shorter peptide, possibly requiring an initial ribosome loading further upstream. Mutating M14 into Ala14 caused the disappearance of the shorter peptide while maintaining the expression of the longer one (Figure 3D). Altogether, the mutational analysis of the initiation codons of the SprG1 coding sequence indicated that the long and short peptides were produced from ribosome initiation at M1 and M14, respectively. In addition, it was determined that specific mutations could lead to the exclusive synthesis of the long (Ala14) or the short (STOPi) peptides. Therefore, SprG1 stable RNA contains a dual-coding ORF.

**Both SprG1-Expressed Peptides Are Detected at the *S. aureus* Membrane**

The amino acid sequences of the peptides encoded by SprG1 and produced by strain N315 are conserved in all sequenced *S. aureus* genomes, with minor sequence variations leading to one to two amino acid changes in the 44 residues (Figure S5). The second methionine codon was lacking in three species containing *sprG1* and *sprF1*. Sequence alignments and the amino acid composition of the SprG1-encoded peptides suggested the existence of a transmembrane hydrophobic domain and two cationic ones. Subcellular localization of the two peptides was performed within the *S. aureus* N315 cells. Membrane and cytosolic fractions were prepared, and the cell fractionation

![Figure 3. SprG1 Expresses Two Membrane Peptides from Two Internal Methionine Codons Whose Expression Is Inhibited by SprF1 during *S. aureus* Growth](image-url)
Figure 4. SprF1 Reduces SprG1 Expression by a Direct cis Interaction that Involves the Sequence Overlap between the RNAs
(A) Native gel retardation assays of purified labeled SprF1 (A) with unlabeled SprG1$_{312}$. The complexes were revealed with a Phosphoimager for the radioactive assay. The competition assays were performed with a 2,000-fold molar excess of polyU RNAs or with a 10-fold molar excess of unlabeled SprF1. The apparent binding constant ($K_d$) between SprG1 and SprF1 was inferred from three independent experiments: $K_d = 1.3 \pm 0.4$ nM.
(B) Native gel retardation assays of SprF1 with SprG1$_{312}$, the 5' and ORF sequences of SprG1$_{312}$ (SprG1-5'-ORF), and the 3' sequence of SprG1$_{312}$ (SprG1-3'). The complexes were revealed by ethidium bromide staining.
(C) Northern blot analysis of SprF1 and SprG1 expression levels during growth in a S. aureus N315 strain carrying an empty pCN35 vector and in an isogenic strain containing pCN35U SprF1. The 5S rRNAs are the internal controls.

SprF1 Reduces SprG1 RNA and Peptide Levels by a Direct cis Interaction Involving Its Sequence Overlap
SprF1 prevented SprG1$_{312}$ and SprG1$_{439}$ translation in vitro (Figure 2B) and in vivo (Figure 3B) and the RNAs possess nucleotide complementarities involving 134 nt at their 3' ends (Figure 1A). Native gel retardation assays performed between SprF1 and SprG1$_{312}$ detected an RNA complex (Figure 4A) with an apparent binding constant of about 1.3 ± 0.4 nM. Binding is specific, because a 2,000-fold molar excess of unrelated RNA does not remove SprF1 from its preformed SprF1-SprG1$_{312}$ complex, whereas a 10-fold excess of SprF1 does. Antisense RNA pairs do not necessarily depend on their overlapping sequences for interaction and regulation (Sayed et al., 2012a). SprG1$_{312}$ was divided into two halves: a SprG1-5' ORF containing its 5' domain and its ORF but without its SprF1 overlap sequence; and SprG1-3' which includes its 3' domain that overlaps with SprF1 (Figure 4B). Gel retardation assays indicated that SprF1 interacts with SprG1 using its 3' domain. In the Figure 3A, we have shown that SprF1 may lower SprG1 translation by affecting SprG1 RNA levels. To confirm this result, a strain overexpressing SprF1 was constructed by cloning the gene with its endogenous promoter into pCN35. Northern blots indicated that, when SprF1 is overexpressed (~20-fold excess), SprG1 was undetectable during bacterial growth (Figure 4C), purity was verified by immunoblotting using anti-ATPase and anti-SarA (staphylococcal accessory regulator) antibodies, respectively (Figure 3E). Immunoblots with anti-FLAG indicated that the SprG1-encoded peptides were only detected in the membrane fractions. As negative controls, no peptides were detected for the S. aureus strains having either an empty vector or a STOP$_{1,14}$ mutant lacking the two internal initiation codons. This indicates that once the peptides were translated, they migrated to the S. aureus membranes. Despite several attempts to monitor the expression of the native untagged peptides, polyclonal antibodies against each of the two chemically synthesized peptides inferred from the SprG1 sequence could not be obtained.

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indicating that SprF1 induction reduced SprG1 RNA levels in vivo.

**Both SprG1-Encoded Peptides Inhibit *S. aureus* Growth and Trigger Death**

Sequence comparisons suggested that the SprG1/SprF1 pair belongs to a type I TxpA (Toxic peptide A) TA family (Fozo et al., 2010). To test this hypothesis, native untagged peptides in vivo were produced from the SprG1,439 sequences starting at the upstream transcription initiation site (Figure S3), from the STOP1, Ala14, and STOP1,14 SprG1 mutants. These were all cloned into inducible low copy pALC vectors. After an antibiotic-tetracycline (aTc) induction, northern blots were used to verify the in vivo production of SprG1, SprG1-STOP1, SprG1-Ala14, and SprG1-STOP1,14 (Figure 5A). Without aTc, *S. aureus* cells containing each of the four constructs grew in the same manner as the empty vector cells (data not shown). But in the presence of aTc, SprF1 expression inhibited *S. aureus* growth in liquid cultures (Figure 5B), a process also shown by adding aTc onto a solid media and performing serial dilutions (Figure 5C). The SprG1-STOP1 mutant that only expressed the shorter peptide inhibited *S. aureus* growth, whereas SprG1 and the SprG1-Ala14 mutant only expressed the long peptide (Figures 3D, 5B, and 5C). Mutant SprG1 STOP1,14, however, which does not express either of the two peptides, grows near to wild-type (WT) levels in both liquid and solid cultures. This demonstrates that SprG1 inhibits *S. aureus* growth because it encodes two peptides, and that the production of either of these peptides stops growth. SprG1 also slightly impaired growth independently of its translation products (Figure 5B). To determine whether the SprG1-encoded peptides induce *S. aureus* cell death, bacteria containing an empty pALC, pALC:pSprG1, or the SprG1-STOP1,14 mutant were stained with fluorescent dyes to discriminate between living and dead cells and then viewed by microscopy. For the pALC negative control, bacteria were alive, whereas increasing SprG1 expression levels killed about half the cells (Figure 5D). Up to ~80% of the bacteria carrying SprG1-STOP1,14, a mutant incapable of producing the two peptides, were viable. This shows that it is the peptides, not SprG1 alone, that are required for toxicity.

**The SprG1/SprF1 Pair Is a Type I TA System that Produces Two Toxic Peptides**

Direct detoxification of the SprG1 transcript by SprF1 expression was assayed in vivo. Two sets of plasmids were used: an aTc-inducible pALC with or without SprG1; and a constitutive pCN35 with or without SprF1 expression. Northern blots validated SprF1 induction in Newman strains containing pCN35:SprF1 and SprG1 expression after aTc induction in strains transformed with pALC:pSprG1 (Figure 5E). In the presence of aTc, the strains containing pALC and pCN35:SprF1 (and therefore expressing SprF1) grew in the same way as those containing the two empty vectors (Figure 5F). The cells expressing SprG1 after aTc induction, however, inhibited *S. aureus* growth, and this was rescued by inducing SprF1 expression (Figure 5F). Altogether, these experiments demonstrate that the SprG1/SprF1 pair is a type I toxin/antitoxin system in which toxicity relies upon the internal expression of two peptides, with both being enough on their own to trigger toxicity.

**The SprG1 Peptides Are Secreted, and They Lyse Host Cells and Competing Bacteria with Dissimilar Strengths**

The two peptides were detected at the *S. aureus* membrane (Figure 3E), possibly ready for subsequent release outside the bacteria. To investigate this, total and extracellular proteins were extracted at various times during *S. aureus* growth. The two SprG1-encoded fusion peptides accumulated over time in the extracellular environment in smaller amounts than in the total cell lysates (Figure 6A). The longer peptide was predominantly expressed and secreted, compared to the short peptide. Although cytoplasmic SarA transcription factor was detected in the total protein extracts, its absence within the extracellular fractions rules out the idea that the exterior peptides are due to cell lysis. Immunoblots against Protein A and extracellular adherence protein (Eap) allowed their detection, mostly within the extracellular fractions. This is a result consistent with that for excreted proteins, thus it further supports our protein fractionations. Peptide sequence alignments suggest that they are hydrophobic and contain cationic residues (Figure S5), both attributes of pore-forming peptides (Hancock and Rozek, 2002).

The lytic activity of the SprG1-expressed peptides was seen when adding increasing concentrations of each of the two synthesized formylated peptides to human erythrocytes. The longer peptide has significantly higher (~5-fold) activity toward human erythrocytes than that of the shorter peptide, and is more active (~3-fold) than δ-hemolysin, the positive control (Figure 6B). As the peptides encoded by SprG1 are secreted, they might also inhibit the growth of competing bacteria during colonization and infection. Their antibacterial activity against representative Gram-positive and -negative bacteria was measured by a growth inhibition assay in liquid cultures, using serial peptide dilution and recording the minimal inhibitory concentration (MIC) values (Figure 6C). Compared to nisin (a Gram-positive antibacterial peptide) and cecropin (a Gram-negative antibacterial peptide), these peptides possess moderate antibacterial activities against *S. aureus* as well as against *E. coli* and (to a lesser extent) *Pseudomonas aeruginosa*, two bacteria frequently associated with staphylococcal infections. Interestingly, the short peptide has a lower MIC value against *S. aureus* than the longer peptide. Both peptides, however, have similar MICs against *E. coli* and *P. aeruginosa*, and both are more active against the former. To evaluate the antibacterial activity of the SprG1-encoded peptides directly produced by *S. aureus* during growth, *S. aureus* membrane extracts were prepared from bacteria deleted in the sprG1/sprF1 locus (negative control). These either expressed SprG1 and therefore the two peptides, or a STOP1,14 mutant unable to produce the two peptides, and were then incubated with intact *S. aureus* cells. Like nisin, the membrane extracts containing the two peptides encoded by SprG1 prevent *S. aureus* growth (Figure 6D). Interestingly, similar amounts of membrane extracts from strain jsprG1/sprF1 and from isogenic strain pALC SprG1-STOP1,14 (both strains not expressing the SprG1 peptides) allow bacterial growth in the same manner as an isogenic WT strain. Therefore, peptides produced by growing bacteria can prevent the growth of competing *S. aureus* cells.
Figure 5. SprG1 Overexpression Triggers S. aureus Death Caused by Either Encoded Peptide and Reversed by SprF1

(A) Northern blot analysis of the expression levels of SprG1 and three SprG1 mutants (STOP1, Ala14, STOP1,14) after induction by anhydrotetracycline (aTc).
(B and C) (B) Growths in liquid cultures and (C) serial dilutions of exponentially grown N315 bacteria containing pALC (black), pALC-SprG1 (pink), pALC-SprG1-STOP1 (green), pALC-SprG1-Ala14 (yellow), and pALC-SprG1-STOP1,14 (blue) RNA after aTc induction. The error bars in (B) indicate variations between three replicates.
(D) Cells counts of fluorescent viable S. aureus cells after LIVE/DEAD experiments. Data were expressed as the mean ± SD of four independent experiments. *p < 0.05; ***p < 0.001.
(E) Northern blot analysis of SprG1 and SprF1 expression levels in isogenic S. aureus Newman strains containing pALC and pCN35, pALC and pCN35-SprF1, pALC-SprG1 and pCN35, and pALC-SprG1 and pCN35-SprF1 plasmids. The internal loading control is 5S rRNA.
(F) Growths of Newman strains containing pALC and pCN35 (black), pALC and pCN35-SprF1 (gray), pALC-SprG1 and pCN35 (pink), and pALC-SprG1 and pCN35-SprF1 (blue) plasmids after aTc induction. The error bars indicate variations between three replicates.
DISCUSSION

We report on an unconventional type I TA system expressed by S. aureus: the SprG1/SprF1 pair. Type I TA systems consist of a stable toxin whose expression kills the cells or confers growth stasis, regulated by unstable RNA antitoxins. SprG1 is a dual-coding RNA sequence that produces two membranes and secretes toxic peptides by utilizing two internal in-frame initiation codons. The expression of the toxic peptides is negatively regulated by SprF1, a dual-acting cis-antisense RNA. Compared to the short (~5 min) half-lives of the majority of the S. aureus mRNAs, SprG1 is a stable RNA (Roberts et al., 2006). It is expressed as two transcripts differing at their 5′ ends, which can both produce the two peptides. SprG1312 prevails at all times during growth, whereas SprG1149 is mainly detected at later stages, suggesting that environmental or metabolic signals modulate promoter usage. In S. aureus, the major transcriptional regulator SarA is also transcribed from distinct widely spaced promoters that yield three overlapping transcripts that vary at their 5′ ends (Bayer et al., 1996) for optimal expression regulation.

Figure 6E shows SprG1 regulation by SprF1, SprG1’s expression products, and the outcomes of this for the bacterial physiology. SprG1 expression is toxic when stimulated and is tightly protected by the unstable SprF1 antitoxin. SprF1 counteracts SprG1 toxicity at both the RNA and peptide levels by direct interaction in cis, which includes the SprG1-overlapping 3′ domain. In type I TA systems, antitoxins either inhibit toxic mRNA translation or promote mRNA degradation (Brantl, 2012). SprF1 performs at both of these regulatory levels, perhaps interfering with SprG1 transcription, triggering its degradation by dedicated RNases (Romilly et al., 2012), or by remodeling SprG1 confirmation to prevent toxic peptide translation. Surprisingly, during S. aureus growth there is twice as much SprG1 as SprF1. The dual-acting control of SprG1 by SprF1, and perhaps the involvement of other SprF copies, is enough to counteract SprG1 toxicity during growth. The molecular mechanisms of these regulations remain to be elucidated. Dual-acting antitoxin has been detected in Bacillus subtilis, with the SR4 antitoxin that controls bsRG toxin mRNA decay and translation (Jahn and Brantl, 2013). Moreover, the lysC riboswitch also controls translation initiation and mRNA decay in E. coli (Caron et al., 2012).

SprG1 is an unconventional type I toxin that produces two toxic peptides from two in-frame initiation codons. Such usage of multiple initiation codons contributes to diversity through expression of several peptide isoforms from a single transcript. In bacteria, dual-coding mRNAs have already been reported. For instance, in E. coli ClpA encodes the ATPase subunit of a protease and uses dual translational initiation sites to produce two proteins from a single reading frame (Seol et al., 1994). E. coli dnaX expresses two peptides from a single mRNA through ribosomal frameshifting that produces distinct DNA polymerase subunits (Tsuchihashi and Kornberg, 1990). In S. enterica, a leader region controls the expression of an ion transporter by responding to proline levels via translation of a proline codon-rich ORF located in that region (Park et al., 2010). Some RNAs also possess dual functions, expressing a protein from an internal ORF as well as regulating mRNA targets (Vanderpool et al., 2011). In S. aureus, at least two sRNAs encode a peptide: RNAIII encodes h-emolysin (Novick and Geisinger, 2008), and SprA1 encodes a 31 amino acid-long type I toxin whose expression is prevented by an antitoxin (Sayed et al., 2012a). If it controls target gene expression, SprG1 may also belong to this group. Preventing the expression of the longer peptide by mutations increases the translation of the shorter peptide. This suggests a possible conflict between the elongating and initiating ribosomes at the second, downstream AUG codon, because they are only separated by 12 codons. In monocistronic RNAs with two adjacent SD sequences, ribosome binding interferences have been reported (Nishizawa et al., 2010), and this may also occur with SprG1. SD modules located within coding sequences cause pervasive translational pausing (Li et al., 2012), providing opportunities for heterologous peptide expression in bacteria.

Inducing the expression of the two membrane peptides triggers S. aureus death, possibly by generating pores. Except for SymE, all known type I toxins are small hydrophobic peptides acting as phage holins, forming pores in cell membranes and impairing ATP synthesis (Brantl, 2012). The TisB toxin produces clusters of anion-selective pores in lipid bilayers (Gurnev et al., 2012). The SprA1 type I toxin, expressed by S. aureus, gets inserted into the membranes (Sayed et al., 2012b). In enterobacteria, hydrophobic peptides have other roles, acting as modulators, transporter stabilizers, or sensor kinase regulators (Alix and Blanc-Potard, 2009). The peptides expressed by SprG1 could possess other regulatory functions at the membrane level. Between 5% and 20% of the two membrane peptides, varying according to the time selected during growth, are excreted in the extracellular medium, where they can lyse host cells and competing bacteria with dissimilar strengths. The longer SprG1-encoded peptide is the one primarily expressed and secreted. When compared to its shorter version, it is about three times more active against human erythrocytes and about half as active against competing S. aureus cells. The smaller size of a peptide could facilitate its passage through the thick cell wall of Gram-positive bacteria, which could account efficacy toward S. aureus but not against Gram-negative bacteria. As opposed to the short peptide, the longer version has a second N-terminus domain containing four additional positive charges, and this is probably responsible for its higher cytolytic effect against erythrocytes. In L. lactis, E. coli, and B. brevis, increasing the positive charges of signal peptide sequences improves secretion efficiency (Ravn et al., 2003; Izard et al., 1995; Takimura et al., 1997). Depending on environmental conditions, S. aureus may prefer to use the second initiation codon, increasing shorter peptide synthesis when a subset of cells is lyzed. This is either an altruistic community behavior or is a way to eradicate bacterial rivals. Growth conditions that mimic the effect of the artificial induction are currently unknown. If produced in sufficient amounts, the membrane peptides investigate a programmed cell death, which could promote the secretion of the peptides able to lyse host cells and competing bacteria and the release of virulence factors to facilitate spread of infections. Their toxicity accounts for their obligatory repression by SprF1 during growth. In S. aureus, the phenol-soluble modulins (PSMs) are
Figure 6. Secreted SprG1 Peptides Inactivate Host Cells and Competing Bacteria Differently: Schematic Diagram of SprG1 Regulation by SprF1 and Roles

(A) Immunoblotting with anti-FLAG to detect the SprG1-encoded flagged peptides during growth of strain N315 pCN35 U SprG1FLAG in the culture supernatants. The anti-SarA is the control for the total fraction, with anti-Protein A and anti-Eap as controls for the extracellular fractions.

(B) Hemolytic activity of increasing concentrations of the long (white) and short (black) synthetic peptides. The delta-hemolysin produced by RNAIII (gray) is a hemolytic peptide used as a positive control. The results are expressed as a percentage of lysis compared with the 100% lysis of the positive control (1% Triton) and are the mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

(C) The Minimal Inhibitory Concentration (MIC) of the two peptides encoded by SprG1 onto Gram-positive and Gram-negative bacteria. Nisin and cecropin were used as positive controls for the Gram-positive and -negative bacteria, respectively.

(D) Membrane extract antibacterial activity in liquid cultures after being purified from strains sprG1/sprF1 (gray), isogenic pALC-U SprG1 (pink), and pALC-U SprG1-STOP1,14 (blue). The growth of the S. aureus N315 strain is measured at 600 nm in the absence (5% isopropanol control, black) or presence of 230 μg/ml of membrane extracts. Nisin (100 μg/ml, green) is used as a positive control. The error bars indicate variations between three replicates.

(E) Working model summarizing the locations, outcomes, and functions of the two SprG1-expressed peptides and their negative regulation (red bar) by cis-acting SprF1 (blue). The black arrows represent the gene pair; the short and long peptides expressed by SprG1 (black) are the small and large ovals, respectively. The size of the gray arrows is proportional to the amount of peptides produced, as well as to their functional implications in host cells and bacterial lyses.
proinflammatory peptides also hemolytic and secreted, as is Δ-hemolysin (PSM gamma), another peptide that interacts with membranes (Ott, 2010; Cheung et al., 2012). PSMs contribute to S. aureus virulence (Wang et al., 2007) and biofilm maturation (Perezaxamy et al., 2012). The peptides encoded by SprG1 may also influence these biological events.

Type I TA systems are commonly found in multiple copies, consistent with the expression of three to four sprF and sprG copies in numerous S. aureus strains. Because there is significant sequence conservation between these copies (Figure S6), there might be interference and crosstalk between them. This will be addressed in future studies by deleting each copy and addressing its role in toxicity. Interestingly, a comparison of “wild” and laboratory strains of E. coli suggested that the number of copies would correspond to the ecological niche occupied by the bacterial host (Fozo, 2012). The biological roles of type I TA systems are unclear, but the systems aid in adaptive stress responses, recovery from starvation, and immunity against predators (Blower et al., 2011), and they can be temperature sensitive (Jahn et al., 2012). Upon oxidative and acidic stress in S. aureus, PepA1 type I toxin expression is induced, maybe to ease bacterial escape from phagolysosomes (Sayed et al., 2012b). Persister cells are dormant bacteria tolerant to antibiotics that show increased levels of TA transcripts (Dörr et al., 2010). TheSprG1/SprF1 pair might be involved in metabolic dormancy and persister formation. There are differences between other type I TA modules and the SprG1/SprF1 pair, including the fact that two toxic peptides are produced by the pair. Based on their sequences, the additional copies of sprG expressed in strain N315 may encode shorter peptides that are 25, 26, or 18 amino acids long for sprG2, sprG3, and sprG4, respectively (Figure S6). These additional copies may have acquired divergent regulatory functions. Global regulation of virulence factor expression by type I TA systems in pathogenic bacteria is an exciting concept, but it remains to be experimentally proved.

In conclusion, the SprG1/SprF1 pair expressed by numerous S. aureus clinical isolates is an unconventional type I TA system producing two membrane toxins with different cytolytic activities and that are controlled by a dual-acting RNA antitoxin. In bacterial pathogens, the programmed induction of toxin expression from TA pairs could lead to promising antibacterial strategies (Williams and Hergenrother, 2012) although in this case chemical optimization will be needed to remove human cell toxicity. Detecting the native peptides will provide direct evidence of their expression. The next challenges are to identify under what conditions they are expressed, and what are their functions might be during staphylococcal growth, colonization, and infection.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Genetic Manipulations**

Strains, plasmids, and primers are listed in Tables S2–S4. Strains were grown at 37°C in BH buffer (Oxoid). When necessary, chloramphenicol and erythromycin were added at 10 μg/ml. In pCN35 ΔsprF1, the SprF1 sequence of 187 nt upstream and 63 nt downstream was amplified from N315 genomic DNA as a 385 bp fragment, with flanking BamHI/EcoRI sites, and inserted into pCN35 (Charpentier et al., 2004). For the flagged SprG1 constructs, amplification from N315 DNA was done of an SprG1-229 sequence consisting of 149 nt upstream and 4 nt downstream. A 3XFLAG in-frame sequence of the SprG1-encoded peptides followed by a termination codon (Zehouf et al. 2004) was added, resulting in a 658 bp fragment with flanking BamHI/EcoRI sites. This was then inserted into pCN35. For the flagged SprG1 constructs expressing SprF1, the SprG1 sequence corresponds to a 715 bp fragment of 149 nt upstream and 61 nt downstream. In these constructs, the RNAs were expressed from their endogenous promoters. To generate an anthracycline (aTo)-inducible construct for SprG1, a fragment containing the SprG1 sequence with +1 and 4 nt downstream was amplified from N315 DNA as a 500 bp fragment with flanking KpnI/EcoRI sites then inserted into pALC (Bateman et al., 2001). To inactive the sprF1/SprG1 gene pair, DNA fragments of 1,050 bp upstream and 915 bp downstream of the sprF1/SprG1 gene were amplified by PCR from N315 DNA and cloned into BamHI/PstI sites of temperature-sensitive plasmid pBT2 (Brückner, 1997). The resulting plasmid pBT2ΔsprF1/sprG1 was transformed into S. aureus strain RN4220 and then into S. aureus N315. Mutants were enriched by growth at 42°C. Cells from stationary-phase cultures were plated onto Tripti-case Soy Agar (TSA) plates and incubated at 37°C. Colonies were imprinted onto plates supplemented with 10 μg/ml chloramphenicol. Chloramphenicol-sensitive colonies were tested by PCR for the deletion of sprF1/sprG1 and then confirmed by northern blot (Figure S7).

**RNA Extractions, Northern Blots, and Half-Life Determinations**

RNA extractions were performed as previously described (Chabetskaya et al., 2010). The DNA probes for RNA detection are listed in Table S4. Total RNAs (10 μg) were separated on denaturing PAGE and transferred onto Zeta-Probe GT membranes (Bio-Rad). Specific 3′-end digoxigenin-labeled probes were hybridized with DIG Easy Hyb solution overnight at 37°C, washed, revealed by an antidigoxigenin antibody-AP (Roche), exposed, and then viewed with the ImageQuant LAS4000 imager (GE Healthcare). Quantifications were performed with the ImageQuant and normalized to 5S rRNA or tmRNA. For the RNA half-life measurements, S. aureus was cultured overnight, diluted 1:100, then grown for an additional 5 hr at 37°C to the E phase, and then incubated with 200 μg x ml⁻¹ of rifampicin for 1–120 min.

**RACE End Mapping**

Primers used are shown in Table S4. Total RNAs (5 μg) from S. aureus N315 were circularized using T4 RNA Ligase (Promega). After precipitation, the reaction was reverse transcribed by the M-MLV RT (Promega) using primer R1. Two PCR reactions were performed with Taq polymerase (Invitrogen) using R2-F1 and R2-F2 primers, respectively. The PCR products were cloned in pGEM vector (Promega), transformed into E. coli DH5α, and sequenced with M13 reverse vector primer using MegaBACE DNA sequencers (Amersham Pharmacia Biotech).

**In Vitro Transcription, RNA Labeling, and Translation Assays**

All the RNAs used in this study were transcribed from PCR-amplified templates using N315 genomic DNA and forward primers containing a T7 promoter sequence (Table S4). PCR-generated DNA was used as a template for transcription using a MEGAscript T7 kit (Ambion). RNA was gel-purified, eluted, and ethanol precipitated. 5′ end labeling of RNA was performed as previously described (Antal et al., 2005). In vitro translation of [35S]-methionine was performed (Sayed et al., 2012b) using an E. coli S30 Extract System for Linear Templates (Promega), following the manufacturer’s instructions.

**Gel-Shift Assays and Tooprints**

Gel retardation assays were performed as previously described (Antal et al., 2005). For the labeled gel shift, 0.4 pmol of labeled SprF1 was incubated with various concentrations (0.1–8 pmol) of unlabeled SprG1. For the unlabeled gel shift, 1 pmol of SprG1 WT, SprG1-5′ORF, or SprG1-3′ was incubated with 0.5 or 1 pmol of SprF1. RNA mixes were incubated in binding buffer (80 mM K-HEPES [pH 7.5], 4 mM MgCl₂, 330 mM KCl) for 20 min at 30°C before native gel separation. Tooprint assays were performed (Chabetskaya et al., 2010) using 0.25 pmol of SprG1 and 1 pmol of labeled “SprG1-Toe” primer.

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Protein Extractions, Cell Fractionations, and Western Blots
Protein extracts and cell fractionations were performed as previously described (Sayed et al., 2012a, 2012b). Bradford assays were performed on the samples and equal amounts of intracellular, extracellular, cytosolic, and membrane proteins were used for the western blots and antibacterial assays. For the western blots, the samples were separated on 16% Tricine SDS-PAGE and transferred onto Hybond-P PVDF membrane (Amersham). After blocking, membranes were incubated with one of the following primary antibodies: monoclonal mouse anti-FLAG horseradish peroxidase (HRP) (Sigma); polyclonal rabbit anti-M. luteus ATPase (Downer et al., 2002); rabbit anti-S. aureus SarA (performed in the laboratory); mouse monoclonal anti-S. aureus Protein A (Sigma); or sheep polyclonal anti-S. aureus Eap (Hagger et al., 2005). After incubation with HRP-conjugated secondary antibodies, the membranes were revealed using the ECL Plus Western Blotting Detection kit (Amersham) and scanned with the LAS4000 imager. For the antibacterial assays, the membrane extracts were dissolved in lysis buffer with 0.05% Triton X-100, EDTA-free cocktail protease inhibitor, and 25% isopropanol, and concentrated between 3 to 10 kDa with Centricon and Amicon Centrifugal Filters (Millipore), following the manufacturer’s instructions.

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.012.

AUTHOR CONTRIBUTIONS
M.L.P.-M. and R.B. prepared samples. M.L.P.-M. and B.F. designed the experiments, analyzed the data, and wrote the manuscript.

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Statistical Analysis
Data are expressed as mean ± SD of at least three to four independent experiments. Significant differences were evaluated using the t test. Standard deviations: *p < 0.05; **p < 0.01; and ***p < 0.001 were considered to be statistically significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.012.

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