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A novel \textit{Staphylococcus aureus} cis–trans type I toxin–antitoxin module with dual effects on bacteria and host cells

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\textbf{INTRODUCTION}

\textit{Staphylococcus aureus} is a serious human bacterial pathogen that causes life-threatening nosocomial and community-acquired infections (1). The success of a staphylococcal infection relies on the coordinated expression of a large set of genes involved in virulence, including toxins and surface proteins (2). Gene expression and the adjustment of bacterial physiology to external signals are both usually dependent on regulators such as regulatory RNAs (sRNAs, also called non-coding RNAs) or transcription factors (2,3). In \textit{S. aureus}, sRNAs range in size from 50 to 500 nucleotides (nt), are usually non-coding, and control the expression of RNA targets that are involved in a wide range of biological processes which include virulence, antibiotic resistance, and central metabolism regulation (4–6). Recently, the sRNA repertoire was compiled into the Staphylococcal regulatory RNA database (SRD: http://srd.genouest.org/), which proposes a simple sRNA gene identifier (srn) for each molecule, and acts as a storehouse of useful information for the sRNA community (7). Most \textit{S. aureus} sRNAs act by base-pairing with an RNA target to modulate translation initiation and/or RNA stability. They normally fall into two categories: \textit{cis}- or \textit{trans}-encoded sRNAs (4). \textit{cis}-encoded sRNAs are transcribed from the opposite strand of another RNA (i.e. mRNA or sRNA), and they display perfect sequence complementarity with their target sequences. \textit{trans}-encoded sRNAs are transcribed apart from their targets, and display only partial complementarity with them. Among the \textit{cis}-encoded sRNAs, some studies evidenced the presence of small sense–antisense clusters, some of which can belong to toxin–antitoxin (TA) modules (7–13). TA modules are divided into six types, and are widespread in bacteria and archaea (14,15). They

\textbf{ABSTRACT}

Bacterial type I toxin–antitoxin (TA) systems are widespread, and consist of a stable toxic peptide whose expression is monitored by a labile RNA antitoxin. We characterized \textit{Staphylococcus aureus} SprA2/SprA\textsubscript{2AS} module, which shares nucleotide similarities with the SprA1/SprA\textsubscript{1AS} TA system. We demonstrated that SprA2/SprA\textsubscript{2AS} encodes a functional type I TA system, with the \textit{cis}-encoded SprA\textsubscript{2AS} antitoxin acting in \textit{trans} to prevent ribosomal loading onto SprA2 RNA. We proved that both TA systems are distinct, with no cross-regulation between the antitoxins \textit{in vitro} or \textit{in vivo}. SprA2 expresses PepA2, a toxic peptide which internally triggers bacterial death. Conversely, although PepA2 does not affect bacteria when it is present in the extracellular medium, it is highly toxic to other host cells such as polymorphonuclear neutrophils and erythrocytes. Finally, we showed that SprA\textsubscript{2AS} expression is lowered during osmotic shock and stringent response, which indicates that the system responds to specific triggers. Therefore, the SprA2/SprA\textsubscript{2AS} module is not redundant with SprA1/SprA\textsubscript{1AS}, and its PepA2 peptide exhibits an original dual mode of action against bacteria and host cells. This suggests an altruistic behavior for \textit{S. aureus} in which clones producing PepA2 \textit{in vivo} shall die as they induce cytotoxicity, thereby promoting the success of the community.

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share a common feature, which is that they all encode a stable toxin and a labile antitoxin which neutralizes its toxicity (15,16). To date, three types of TA systems (types I, II and III) have been identified in S. aureus, and their functions are slowly being elucidated (15,17–20). Among the S. aureus TA systems, two belong to type I TA modules and are expressed from pathogenicity islands, genomic islands acquired through horizontal gene transfer. These are SprA1/SprA1AS and SprG1/SprF1 (17,18,20), referred to respectively in the SRD as SprA3800/SprA3850 and SprG3840/SprG3830 (15). Type I TA modules are composed of a toxic peptide and an unstable antisense sRNA that inhibits toxin translation (21). These peptide toxins have multiple roles. These include small membrane damaging peptides inducing cell death, plasmid maintenance through post-segregational killing, global translation inhibition and commitment to persistence (15,19,21–24). Previous work in our lab revealed that in the SprA3850/SprA3850AS/SprA3850/SprA1AS TA system, SprG3840/SprG3830 expresses a single toxic peptide repressed, allowing the bacterium to grow. More recently, the SprA3850/SprA1AS system was also characterized (20). This module produces two toxic peptides from the same SprA3850/SprA1AS transcript, each causing cell death. Toxicity is counterbalanced in cis by the SprA3850/SprA1AS antisense sRNA, which has dual functions because it represses toxicity at both the transcriptional and translational levels (16). Computational analysis revealed the presence of additional putative TA modules in S. aureus which share nucleotide and genetic organization similarities with either SprA3850/SprA3850AS/SprA3850/SprA1AS or SprG3840/SprG3830/SprF1 (13,17,20), and these have been reported in the SRD (7). Here, we describe the functional identification of a novel type I TA system SprA4540/SprA2AS/SprA4550, which shares 75% sequence similarity with the SprA1/SprA1AS TA system. We provide evidence that the SprA4540/SprA2AS antisense RNA binds SprA4550/SprA2AS at the ribosomal binding site through partial complementary base-pairing to prevent translation initiation. Based on mutational analysis and cell viability experiments, we demonstrate that SprA2 encodes PepA2, a cytotoxic peptide that also intracellularly triggers S. aureus death. We show here that there is no cross-regulation between the SprA2/SprA2AS and SprA1/SprA1AS TA modules, thus demonstrating their specificity. Finally, we also reveal that antitoxin expression is reduced during an osmotic stress and in a stringent medium. These events might be the signals that trigger PepA2 expression to influence colonization, spread and/or infection.

MATERIALS AND METHODS

Bacterial strains, growth, toxin induction, viability assays, and stress conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Except where stated otherwise, cells were grown in brain heart infusion broth (BHI, Oxoid) or in tryptic soy broth (TSB, Oxoid) under agitation at 37°C. For all experiments, overnight cultures were diluted to an OD600 of 0.1, and then monitored at different time points. For co-culture assays, species were mixed to a ratio of 50:50 to reach an OD600 of 0.05. When necessary, 5 μg/ml chloramphenicol and/or erythromycin was used, while kanamycin was used at 50 μg/ml. Toxin production was induced by the addition of either 100 nM or 1 μM anhydrotetracycline (aTc). Viability assays were conducted prior aTc induction and then 2.5 h afterwards. For these assays, cell density was adjusted to an OD600 of 0.02, 10-fold serial dilutions were performed with BHI, and 10 μl of each dilution was placed on BHI agar plates supplemented with the appropriate antibiotics. For the analysis of RNA levels while undergoing different stresses, S. aureus was cultured as previously described (9). Stresses were respectively induced by the addition of 10 mM H2O2 (for oxidative stress), HCl (to lower the pH to 5.5), or NaOH (to increase the pH to 9.5), or by changing the temperature to 18 or 42°C (cold and heat shocks). In addition, 60 ml of culture was centrifuged at 4500 rpm for 8 min at room temperature, and the pellets re-suspended in TSB supplemented with 1 M NaCl (osmotic stress); in NZM medium (stringent response); or in fresh TSB as a control.

Total RNA extractions

Cells were harvested by centrifugation at 4500 rpm for 10 min at 4°C, and pellets washed with 500 μl lysis buffer (20 mM sodium acetate, 1 mM EDTA, 0.5% SDS, pH 5.5). Cell pellets were broken using acid-washed glass beads (Sigma) in the presence of phenol (pH 4) in a FastPrep FP120 cell disrupter (MP Biomedicals) for 30 s at a power setting of 6.5. Lysates were centrifuged at 16 000 g for 10 min at 4°C. Total RNAs were extracted with phenol/chloroform and precipitated overnight at −20°C with ethanol supplemented with 0.3 M sodium acetate.

Northern blot analysis, RNA stability assays and RACE mapping

Northern blots were performed as previously described (9). Briefly, 10 μg of total RNAs were loaded and separated in 8% polyacrylamide/8M urea gels. The RNAs were probed with 32P 5′-end labeled oligonucleotides (Supplementary Table S1) and detected using a Typhoon FLA 9500 scanner (GE Healthcare). RNA half-life determinations were done using 200 μg/ml rifampicin, with or without 100 nM aTc. Staphylococcus aureus Newman strain with pALC sprA2 + pCN35 sprA2AS was cultured for 2 h, and SprA2 induced by the addition of aTc. After 30 min induction, rifampicin was added to the culture and total RNAs extracted at different time intervals. RNA amounts were quantified using a Typhoon FLA 9500 scanner (GE Healthcare) and tmRNA as an internal loading control. RACE mapping (rapid amplification of cDNA ends) was performed as previously described (20) using the primers listed in Supplementary Table S1.
Table 1. Bacterial strains and plasmids used in this study

| Strains and plasmids | Characteristics | References |
|----------------------|----------------|------------|
| **S. aureus strains** | | |
| Newman | Methicillin-sensitive *S. aureus* strain | (58) |
| ΔsprA2ΔsprA2AS | Newman strain deleted for *sprA2* and *sprA2AS* and containing Km<sup>+</sup> cassette | This study |
| N516 | Erythromycin-resistant *S. aureus* strain | (59) |
| **Other strains** | | |
| XL-1 Blue | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacIΔ[F^-proAB lacF^-ΔZAM15Tn90] (Tet<sup>+</sup>) | Stratagene |
| EC101 | Kanamycin-resistant *Escherichia coli* strain | (60) |
| *Salmonella typhimurium* | *Salmonella enterica* serovar Typhimurium LT2 | (61) |
| *E. faecium* N4004 | Enterococcus faecium strain Aus004 | (62) |
| **Plasmids** | | |
| pALC | pALC2073 with the tetracycline-inducible *Pxy* promoter with Amp<sup>+</sup> in *E. coli* and Chlor<sup>+</sup> | (63) |
| pALC_sprA1 | pALC2073 with *sprA1* cloned under *Pxy* promoter | This study |
| pALC_sprA2 | pALC2073 with *sprA2* cloned under *Pxy* promoter | This study |
| pALC_sprA2AS_TT4A | pALC2073 with *sprA2AS* cloned under *Pxy* promoter | This study |
| pALC_sprA2G52C | pALC2073 with *sprA2G52C* cloned under *Pxy* promoter | This study |
| pCN35c | Modified high-copy-number shuttle with Amp<sup>+</sup> in *E. coli* and Chlor<sup>+</sup> instead of *Erm<sup>+</sup>* in *S. aureus* | (29) |
| pCN35c_sprA2Flag | pCN35c with *sprA2* under its own promoter and fused with flag at the 5′ end of its own promoter | This study |
| pCN35c_sprA2AS | pCN35c with *sprA2AS* under its own promoter | This study |
| pCN35c_sprA1AS | pCN35c with *sprA1AS* under its own promoter | This study |
| pCN35c_sprA2Flag | pCN35c with *sprA2* Flag containing *sprA2* under its own promoter | This study |
| pCN41 | Vector carrying *blaZ*, reporter gene and conferring Amp<sup>+</sup> in *E. coli* and *Erm<sup>+</sup>* in *S. aureus* | (29) |
| pCN41_PsprA2AS | pCN41c with *blaZ* under the control of *PsprA2AS* | This study |
| pCN35 | High-copy-number shuttle with Amp<sup>+</sup> in *E. coli* and *Erm<sup>+</sup>* in *S. aureus* | (29) |
| pCN35_sprA1AS | pCN35 with *sprA1AS* under its own promoter | This study |
| pCN35_sprA2AS | pCN35 with *sprA2AS* under its own promoter | This study |

Western blots

Recombinant plasmids (Table 1) were transformed in *S. aureus* as previously described (25). *S. aureus* was cultured in BHI broth supplemented with the appropriate antibiotics at 37°C and under agitation until reaching an appropriate OD<sub>600nm</sub> (see Results). Cells were harvested by centrifugation, and cellular protein extracts were prepared with protease inhibitors as previously described (9). Proteins from the supernatants were precipitated with trichloroacetic acid (TCA), then washed with acetone as previously described (9). Samples were separated on 16% Tricine-SDS-PAGE gels and transferred onto Hybond P PVDF membranes (Amersham). After overnight blocking at 4°C under gentle agitation, the membranes were incubated with horseradish peroxidase-conjugated (HRP) anti-FLAG antibodies (Sigma). The membranes were revealed using an ECL Prime western blotting detection kit (Amersham) and scanned with an ImageQuant LAS 4000 (GE).

Peptide synthesis, hemolytic and cytotoxic assays

PepA2 and PepA1 peptides were synthesized by automated solid-phase synthesis with the kind help of Dr BaudyFloch’s CoInt group (ISCR-UMR 6226, University of Rennes 1), and characterized as described previously (26). High-performance liquid chromatography (HPLC) analysis was done to ensure that the purity of the final products was above 95%, and their expected molar mass was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).

Cytotoxic assays were conducted using human blood provided by the Etablissement Français du Sang from at least three independent samples (EFS Rennes). For hemolytic assays, blood was washed and then resuspended at 5% (v/v) in phosphate-buffered saline (PBS). The peptide solutions were added at 2 μM concentrations to V-bottom 96-well plates after which 75 μl of 5% blood was added. The negative control was performed using PBS instead of peptides, while 100% hemolysis was assessed using 1% Triton X-100. Plates were incubated for 2 h at 37°C, then centrifuged at 1400 rpm for 15 min at room temperature. Finally, 100 μl supernatant was transferred into a flat-bottom 96-well plate and the OD<sub>414nm</sub> measured to assess the presence of released haemoglobin.

Polymorphonuclear neutrophils (PMNs) were isolated using the EasySep<sup>™</sup> Direct Human Neutrophil Isolation kit (Stemcell) per the manufacturer’s recommendations. Neutrophils were resuspended in RPMI medium supplemented with 2% fetal vac serum (FVS), then 20 000 neutrophils were distributed in each of the 96 wells of a tissue culture plate. Cells were incubated for 2 h at 37°C and 5% CO<sub>2</sub> with increasing concentrations of PepA2. Neutrophils were harvested by centrifugation at 350 g for 8 min at 4°C, washed with RPMI 2% FVS, then stained with 50 μg/ml propidium iodide (PI) (Sigma-Aldrich) for 15 min in the dark at room temperature. After washing, cell viability was determined and analysed on a BD Biosciences LSR II cytometer using FACSDiva<sup>™</sup> software. Since PI is not permeant to live cells, the percentage of unstained PMNs corresponds to the viable cell count.

In vitro transcription and translation assays

*In vitro* transcription was conducted from PCR-amplified fragments cloned into pUC19 under the control of the T7 promoter, and containing a HaeIII restriction site at the sequence’s 3′ end. Plasmids were linearized with HaeIII and subjected to *in vitro* transcription using the MEGAscript T7 kit (Thermo Fisher) according to the manufacturer’s rec-
Electrophoretic mobility shift assays and toeprints

RNAs were probed with oligonucleotides labelled at the 5’ ends with \( \gamma^{32} \text{P} \). For electrophoretic mobility shift assays, RNAs were denatured in buffer (80 mM HEPES pH 7.5, 330 mM KCl, 4 mM MgCl\(_2\)) for 2 min at 80°C, chilled on ice, then refolded for 20 min at 20°C. Binding was conducted in 10 \( \mu \text{L} \) for 30 min at 30°C. 0.1 pmol of labelled RNA was incubated with increasing amounts of unlabelled RNA. The specificity of the complex was assessed by adding an excess of unlabelled RNA (for concentrations used, see figures 6 and 8). Samples were supplemented with glycerol to a final concentration of 10%, then loaded onto native 8% polyacrylamide gels containing 5% glycerol. Electrophoresis was performed in 0.5 \( \times \) Tris–borate–EDTA buffer at 4°C and 100 V. The results were analysed on a PhosphorImager and \( K_d \) values determined accordingly.

For toeprinting assays, annealing mixtures containing 0.25 pmol unlabelled SprA2 and 1.5 pmol labelled primer in a buffer (20 mM Tris-acetate pH 7.5, 60 mM NH\(_4\)Cl, 1 mM DTT) were incubated for 1 min at 90°C, and then quickly chilled on ice for 1 min. Renaturation was done in 10 mM MgCl\(_2\) at 20°C for 15 min. The influence of SprA2AS was assayed by adding 0.25 pmol to the annealing mixtures, then incubating for 5 min at 37°C. Purified Escherichia coli 70S ribosomes were diluted in the reaction buffer in the presence of 1 mM MgCl\(_2\) then activated for 15 min at 37°C.

For each sample, 4 pmol purified 70S ribosomes were added, followed by 5 min incubation at 37°C and adjustment of the MgCl\(_2\) concentration to 10 mM. After 10 min at 37°C, 10 pmol tRNA\(^{\text{Met}}\) was added and the samples were incubated for 5 min at 37°C. The cDNAs were synthesized using 4 units AMV RT (New England Biolabs) for 15 min at 43°C. The reaction was stopped by adding 10 \( \mu \text{L} \) loading buffer II (Ambion). The samples were incubated for 5 min at 95°C, then placed on ice. The cDNAs were loaded and separated onto 8% urea–PAGE gels. The toeprint position on SprA2 was determined by DNA sequencing, and the results were analysed on a PhosphorImager.

Reporter gene experiments

Overnight cultures were adjusted to an OD\(_{600 \text{ nm}}\) of 0.1. For each time point, cells were centrifuged and resuspended in 1× PBS to obtain an equal cell density throughout the assay. Cell lysis was performed using 0.7 mg/ml lysozyme, 0.2 U/\( \mu \text{L} \) benzonase and 0.1 mM MgCl\(_2\) at 37°C for 20 min. After adding 0.25 mg/ml nitrocefin (a ß-lactamase substrate), ß-lactamase activity was quantified on a BioTek instrument every 10 min for 40 min at 492 nm. This activity was normalized against the protein quantities determined by a Bradford assay.
Figure 1. The putative type I toxin–antitoxin module Srn\_4550/sprA2/Srn\_4540/sprA2AS is expressed in *Staphylococcus aureus* Newman. (A) Schematic of the genetic organization of the srn\_4550/sprA2/srn\_4540/sprA2AS locus. (B) Srn\_4550/sprA2/Srn\_4540/sprA2AS expression profile during growth in BHI. Growth was monitored by measuring the optical density at 600 nm (OD\_600nm) every hour (upper chart). Expression was monitored by northern blotting, with tmRNA as an internal loading control (lower chart), with the relative amount of Srn\_4540/SprA2AS RNA indicated under each band. (C) In silico best prediction of the molecular interaction between the two components made with intaRNA program. The putative ribosome-binding site is green and the start codon is red. The coordinates appearing on panels A and C were experimentally determined by RACE mapping.

Figure 2. *sprA2/sprA2AS* encodes and expresses a functional toxin–antitoxin system. (A) Comparative growth curves after induction with 1 µM anhydrotetracycline (aTc) on *Staphylococcus aureus* Newman replicating empty plasmids pALC and pCN35 (squares); pALC\_sprA2 and pCN35 (circles); and pALC\_sprA2 and pCN35\_sprA2AS (triangles). Growth was conducted in a Biotek instrument for 2.5 h, then expression was induced by aTc. Data points shown represent the growth after this addition, and are the mean of three independent biological replicates. (B) Assessment of cell viability by plating 10-fold serial dilutions, prior induction (left panel) or 2.5 h post-induction (right panel). All cultures (broth and agar) contain chloramphenicol and erythromycin concentrations appropriate for plasmid maintenance.
although complete suppression of toxicity could not be achieved. Similar experiments were performed with the sprA/sprAAS system (Supplementary Figure S2), known to have antibacterial activity. Here, SprA1 overexpression led to cell death which was fully relieved by SprA1AS overexpression, suggesting that SprA2 RNA expression leads to stronger toxicity than does SprA1. To verify whether cell toxicity was due to the expression of a peptide rather than the SprA2 RNA itself, we tagged PepA2 with a FLAG sequence at its N-terminus. As described previously, the addition of a FLAG sequence usually decreases peptide toxicity, allowing for its expression to be monitored (17). Then, to avoid any potential repression of FLAG-PepA2 expression by endogenous SprA2AS, we deleted the entire putative type I TA locus in the Newman strain. We expressed SprA2-FLAG under its own promoter and in the presence or absence of SprA2AS in Newman ΔsprA2ΔsprA2AS (Figure 3A and Supplementary Figure S3). Western blotting indicated that a peptide was expressed from sprA2, especially at OD600 nm values of 1 and 2, and that the presence of SprA2AS lowered peptide expression. Additionally, we collected the growth supernatant and found that a fraction of the FLAG-PepA2 was released in the extracellular medium, mostly at OD600 nm of 6 and in the absence of SprA2AS (Figure 3A). To prevent PepA2 expression, we made point mutations in sprA2, as shown in Figure 3B. Each mutated version was cloned into pALC and expression was induced with aTc (Figure 3C). Mutating the start codon (SprA2A53T,T54A) to prevent translation initiation did not inhibit growth. Since this absence of growth inhibition could be due to a substantial structural modification in SprA2 RNA rather than to a lack of PepA2 expression, we generated a point mutation just before the start codon that allows translation (SprA2G5SC in Figure 3B). This construct led to a sudden growth arrest similar to that observed previously, confirming that toxicity is dependent on PepA2 expression and that it is not due to the RNA. Altogether, our results indicate that sprA2/sprA2AS encodes a functional type I TA system, with sprA2 triggering S. aureus death.

**PepA2 kills Staphylococcus aureus intracellularly and acts as an extracellular cytotoxin against human erythrocytes and polymorphonuclear neutrophils**

We pursued our investigations on cell toxicity, conducting co-cultures with both Gram-positive and Gram-negative bacteria. The bacteria were mixed at equal optical densities, and growth was followed in a Biotek instrument. After 2.5 h growth, cultures were subjected to 1 μM aTc to promote SprA2 expression (Figure 4). For all co-cultures tested, we could discriminate the species based on the use of selective or elective media (Supplementary Figure S4). When mixed with erythromycin-resistant S. aureus N315, S. aureus Newman growth stopped upon SprA2 induction, but N315 continued growing, which indicates that the SprA2 toxin acts intracellularly and cannot kill S. aureus from the outside (Figure 4A and Supplementary Figure S4). Conversely, no significant growth differences were observed in cultures not challenged with aTc (Supplementary Figure S5). We next tested the effects of the toxin on the growth of kanamycin-resistant Escherichia coli EC101 (Figure 4B), Salmonella typhimurium (Figure 4C), and Enterococcus faecium Aus004 (Figure 4D). In all of the tests, PepA2 overexpression arrested the growth of S. aureus Newman pALC_sprA2AS, but not of the other species. To confirm that extracellular PepA2 was inefficient for bacterial killing, we chemically synthesized and purified PepA2. Synthetic PepA2 was added to bacterial cultures at various concentrations to determine the minimum inhibitory concentration (MIC). PepA2 did not substantially inhibit the growths of S. aureus, E. coli or S. typhimurium, and only weakly inhibited E. faecium (Supplementary Figure S6). Furthermore, SprA2 overexpression in E. coli inhibited growth, indicating that PepA2 can also target Gram-negative bacteria from the inside, although not from the outside (Supplementary Figure S7). We further characterized synthetic PepA2’s toxic activity by adding it extracellularly to human erythrocytes and to polymorphonuclear neutrophils (PMNs) from different blood donors (Figure 5). PepA2 was highly hemolytic, with 50% hemolysis (H50) reached with ~0.4 μM PepA2 (Figure 5A). On the other hand, chemical synthesis of PepA1 and its subsequent use for hemolysis revealed that more than 30 μM of this peptide was needed to obtain the same effect on human red blood cells (Figure 5B). We next tested the cytotoxic effect of PepA2 against PMNs (Figure 5C). PepA2 was also cytotoxic in this case, with an IC50 around 6 μM. Taken together, our data indicate that PepA2 induces bacterial cell death internally and, when it is released in the medium it is cytotoxic against human host cells, but not efficiently antibacterial.

**The non-overlapping 5' end of SprA2AS regulates toxin production and prevents PepA2 translation initiation**

To investigate the regulation between the toxin and anti-toxin RNA in the sprA2/sprA2AS type I TA system, we began with gel shift assays. We mixed labelled SprA2 with increasing amounts of SprA2AS and found that the RNAs interact to form a complex (Figure 6A). This indicated a direct RNA-RNA interaction, without the need of a third party. The apparent binding constant between the two is ~13 nM, and the complex formation specificity was assessed by adding a 50-fold excess of various unlabelled RNAs (Figure 6A, last three lanes). While an excess of unlabelled SprA2 displaced the complex, the addition of unrelated Srr3610_SprC sRNA (30) or a fragment of autoysin mRNA did modify the binding of SprA2AS onto SprA2 RNA, implying specificity. We then went on to examine whether the 5’ non-overlapping region of SprA2AS was responsible for the interaction between the two RNAs (Figure 6B and C). For this, we transcribed 5’ and 3’ moieties of SprA2 and SprA2AS (Supplementary Figure S8). As shown in Figure 6B, only the 5’ half of SprA2AS formed a specific complex with SprA2. Similarly, unlabelled SprA2AS only slowed the migration of 5’ SprA2 (Figure 6C). These results indicate that the 5’ non-overlapping domains of both RNAs interact together in vitro, and demonstrate that although they are transcribed from the same locus (Figure 1A and C), SprA2AS regulates SprA2 expression in trans.

As presented in Figure 1C, this trans-regulation is predicted to mask the SprA2 ribosomal binding site (RBS). We therefore conducted toeprinting assays to see whether
SprA2AS would compete with ribosome binding onto SprA2. We began by looking at SprA2 in the presence of increasing amounts of purified *E. coli* ribosomes (Figure 7A). The assays showed that SprA2 recruits ribosomes to form translation initiation complexes in the presence of the initiator tRNA^fMet_. From 1 pmol of 70S ribosomes, the toeprint was detected approximately 14 nucleotides downstream from the predicted initiation codon (Figure 7A, lanes 9 and 10). We then tested whether the antitoxin SprA2AS could prevent ribosomal loading onto SprA2 (Figure 7B), as the antitoxin and/or ribosomes were mixed with SprA2. A strong pause was detected starting at position A72 during the reverse transcription of SprA2 in the presence of its RNA antitoxin (Figure 7B, lane 6), suggesting that the association between the two RNAs induces a structural modification in SprA2 which prevents primer extension. The addition of both SprA2AS and ribosomes resulted in an absence of ribosomal loading, whereas the pause due to the antitoxin’s presence was still detected (Figure 7B, lane 8 and right panel). This indicated that SprA2AS prevents ribosomal loading onto SprA2 and therefore PepA2 translation. To support this, we performed *in vitro* translation of SprA2 in the presence of either the full-length RNA antitoxin or its 5’ or 3’ truncated versions (Figure 7C). Whereas SprA2 translation did occur *in vitro* (lane 1), it was inhibited by both SprA2AS and by the truncated version containing the main binding domain (lanes 2 and 3). Conversely, the 3’ part of the antitoxin did not significantly inhibit translation (lane 4). Also, the addition of non-cognate antitoxin SprA1AS did not prevent translation *in vitro* (lane 5), suggesting that members of the SprA TA systems are specific to each other. SprA2AS thus regulates the expression of SprA2, at least at the translational level, and its non-overlapping 5’ domain binds SprA2 at the RBS to prevent ribosomal loading and PepA2 expression.
Figure 4. The SprA2 toxin does not kill other bacteria during co-cultures. (A) The effects of sprA2 expression on the growth of *Staphylococcus aureus* N315. Shown are: *S. aureus* N315 (blue diamonds); *S. aureus* Newman pALC (red squares); Newman pALC sprA2 (green triangles); Newman pALC + N315 (purple crosses); and Newman pALC sprA2 + N315 (blue crosses). (B) The effects of sprA2 expression on *Escherichia coli* growth. Shown are: *E. coli* EC101 (blue diamonds); *S. aureus* Newman pALC (red squares); Newman pALC sprA2 (green triangles); Newman pALC + *E. coli* (purple crosses); and Newman pALC sprA2 + *E. coli* (blue crosses). (C) The effects of SprA2 expression on *Salmonella typhimurium* growth. Shown are: *S. typhimurium* (blue diamonds); *S. aureus* Newman pALC (red squares); Newman pALC sprA2 (green triangles); Newman pALC + *S. typhimurium* (purple crosses); and Newman pALC sprA2 + *S. typhimurium* (blue crosses). (D) The effects of SprA2 expression on *Enterococcus faecium* growth. Shown are: *E. faecium* Aus004 (blue diamonds); *S. aureus* Newman pALC (red squares); Newman pALC sprA2 (green triangles); Newman pALC + *E. faecium* (purple crosses); and Newman pALC sprA2 + *E. faecium* (blue crosses). Growth was conducted in a Biotek instrument and 1 µM aTc was added after 2.5 h. The data presented are the mean of three independent experiments performed in triplicate. To improve readability, the standard deviations (which never exceeded 0.057) were omitted.

Figure 5. SprA2 is cytotoxic against human erythrocytes and polymorphonuclear neutrophils (PMNs) from blood donors. (A) Hemolytic activity of synthetic SprA2-encoded peptides on human erythrocytes (RBCs). (B) Hemolytic activity of synthetic SprA1-encoded peptides on human RBCs. (C) Cytotoxicity of synthetic SprA2-encoded peptides on PMN was estimated by flow cytometry using propidium iodide staining. The data are the mean of at least three independent biological experiments performed in triplicate. *P < 0.05; **P < 0.001.
The SprA2/SprA2AS and SprA1/SprA1AS pairs act independently, with no cross-regulations between the antitoxins to rescue cell toxicity

These results revealed a regulation mechanism similar to that observed in the SprA1/SprA1AS TA system (17). However, although both TA systems have nucleotide sequence similarities (Supplementary Figure S1), in vitro translation assays indicated that SprA1AS cannot inhibit SprA2 translation. Therefore, we wondered whether the TA systems act independently, or if there is a cross-reactivity. First, we performed gel shift assays to demonstrate whether such cross interactions can occur in vitro (Figure 8A). When increasing amounts of unlabeled SprA2AS RNA were added to labeled SprA2 or SprA1 RNA, RNA–RNA interactions were observed only between SprA2 and SprA2AS (Figure 8A, left panel). Conversely, unlabeled SprA1AS RNA only slowed the migration of labeled SprA1 (Figure 8A, right panel), indicating that both TA systems are specific to themselves in vitro. To support this, we tested the potential of each antitoxin to interact with their non-cognate toxins during bacterial culture. Genes coding the toxins were cloned into the aTc-inducible pALC vector, while the antitoxins were cloned into pCN35 under their own promoters (Figure 8B). Cell death due to SprA2 overexpression was not relieved by the presence of the SprA1AS antitoxin (Figure 8B; crosses in left panel), consistent with the in vitro results. Similarly, SprA1 toxicity was relieved only when its SprA1AS cognate antitoxin was expressed from pCN35 (Figure 8B, right panel). Total RNA extractions performed under these growth conditions confirmed that all of the TA module components were expressed, and that overexpression of SprA2 or SprA1 was indeed effective (Figure 8C). While the antitoxin RNA levels did increase when their cognate toxins were induced, the RNA levels of the other antitoxins went down (Figure 8C, conditions 2 to 4). Therefore, we monitored the transcript levels of another type I TA module (SprG1/SprF1) and of two regulatory RNAs, Srn_9340 and Srn_3610_SprC (Supplementary Figure S9). SprF1 and these regulatory RNAs have half-lives of 10, 5 and 20 min, respectively (25, 30), and they all showed reduced transcript levels upon SprA1/2 induction. Conversely, the RNA levels of SprG1, which has a half-life of ~2 h (20), did not decrease after SprA1/2 induction. This suggests that the decreased levels of RNAs with short or moderate half-lives are probably due to cell death, which favours rapid RNA degradation.

SprA2 overexpression increases the stability of SprA2AS RNA

Increased SprA2AS antitoxin RNA in response to the overexpression of its cognate toxin suggested an effect at the levels or transcription and/or stability. To measure antitoxin expression, we cloned the sprA2AS promoter into pCN41, allowing transcriptional fusion with blaZ, which encodes β-lactamase. *S. aureus* Newman pCN41_PsprA2AS was transformed with either pALC or pALC_SprA2 (Figure 9). A 1.3-fold increase in β-lactamase activity was measured when aTc was added to promote SprA2 expression. Similarly, when aTc was added to the strain co-transformed with pALC, β-lactamase activity increased ~1.4-fold, indicating that the inducer itself weakly activates PsprA2AS in the absence of SprA2 overexpression. Conversely, no significant variations were detected between strains overexpressing SprA2 and the control, indicating that SprA2 does not directly or indirectly regulate the transcription of its antitoxin. We therefore decided to look at SprA2AS RNA stability with or without SprA2 induction. To do this we used Newman pALC_SprA2 + pCN35_sprA2AS (see Figure
Figure 7. SprA2AS prevents SprA2 translation by using its non-overlapping RNA domain. (A) Toeprinting assays of 0.25 pmol SprA2 in the presence of increasing amounts of purified E. coli 70S ribosomes revealed toeprints from position U71. The A, C, G and U lanes represent the SprA2 sequencing lanes. (B) Toeprinting assays of 0.25 pmol SprA2 in the presence of SprA2AS and/or purified E. coli 70S ribosomes. The toeprint initially observed from position U71 decreased in the presence of the antitoxin. However, a strong pause appears at position A64 upon SprA2AS addition, suggesting a conformational change in SprA2. In the right panel, a shorter exposure is provided to show the effect of SprA2AS on the toeprint. (C) In vitro translation assay of 20 pmol SprA2 in the presence of 30 pmol of SprA2AS, 5′ SprA2AS RNA, 3′ SprA2AS, or SprA1AS (lanes 1–5, respectively). SprA2 encodes PepA2, a peptide weighing ∼4 kDa and inhibited transcription with rifampicin (Figure 9B). While the half-life of SprA2AS was around 5 min in the presence of endogenous SprA2, SprA2 induction increased its antitoxin’s stability by ∼9-fold, with a half-life of around 45 min. SprA2 expression thus increases SprA2AS stability, but not its transcriptional activity.

Antitoxin expression levels are reduced under osmotic shock and stringent conditions

The triggers involved in the expression and regulation of this newly described type I TA system are unknown. To explore this question, we applied different stresses, some mimicking the changes encountered by the bacteria during infection. SprA2 and SprA2AS RNA levels were monitored by northern blots after 30 and 60 min stress, then compared with those produced without stress (Figure 10). The Mann-Whitney U test was used to locate statistically significant differences, with biologically relevant differences being anything over the defined cut-off value of 2. The SprA2 RNA levels were weakly affected by all conditions applied (Figure 10A), but although we measured variations, they were always lower than a two-fold change (i.e. a 1.7-fold decrease in 1 M NaCl after undergoing stress for 1 h). Conversely, after 30 or 60 min of stringent (NZM) or osmotic (NaCl) stress, SprA2AS RNA levels significantly decreased (Figure 10B). In 1 M NaCl, antitoxin RNA was decreased more than two-fold, whereas it was lowered by around four in NZM medium. While the RNA levels were affected in these two cases, only a slight decrease (under the cut-off) in SprA2 transcript levels was measured in 1 M NaCl (Figure 10A). Other variations in antitoxin levels were lower than two-fold increases or decreases (Figure 10B). Altogether, these data indicate that sprA2 and sprA2AS are differentially expressed in response to stressors, with antitoxin RNA levels reduced under stringent conditions and during osmotic shock. In addition, this reduction was not accompanied by a strong change in SprA2 transcript levels. Overall, this suggests that stringent conditions and elevated osmotic pressure could be triggers for promoting PepA2 toxin production.

DISCUSSION

Toxin-antitoxin modules are widespread in bacteria and archaea (14,15). They are known to participate in various functions, including irreversible membrane damage, plasmid maintenance through post-segregational killing, cell death or stasis, global translation inhibition, and a recently discovered role in persistence (15,19,22,24). They are divided into six different types, with three described in S. aureus (15). To date, two type-I systems have been characterized in S. aureus: the SprA1/SprA1AS and SprG1/SprF1 systems identified and expressed from pathogenicity islands (17,20).

In bacterial genomes, type I TA systems are commonly found in multiple copies. In this work, we describe the SprA2/SprA2AS module, which has more than 70% nucleotide sequence identity with the SprA1/SprA1AS TA system (7,13,17). We report here the second example of a type I TA system in which a cis-encoded antitoxin acts in trans with its cognate toxin to prevent ribosomal loading and thus the translation of a toxic peptide. We showed that the 5′ part of the antitoxin’s RNA is sufficient to bind SprA2 RNA in vitro, while its 3′ moiety, sharing extensive complementary due to the overlapping regions, has only a minor effect on regulation. Indeed, no inhibition of SprA2 translation was
detected with 30 pmol of 3′ SprA2AS, whereas strong inhibition was already observed using the same amount of 5′ SprA2AS (Figure 7C). Moreover, SprA2AS binds its target with high affinity: its apparent $K_d$ of 13 nM is similar to the 15 nM observed for the SprA1/SprA1AS TA system, and is slightly higher than the >1 nM value for SprG1/SprF1 (17,20). Our in-depth characterization of the mechanism involved indicates that SprA2 overexpression (and therefore PepA2 expression) has no effect on the activity of the PsprA2 promoter, but it increases the stability of the antitoxin by nine, presumably due to its titration by SprA2 and an enhanced stability of the complex.

In the present work, we investigated whether cross-regulation occurs between SprA1/SprA1AS and SprA2/SprA2AS type I TA modules. Instances of crosstalk are largely under documented (31,32). To our knowledge, so far crosstalk has only been reported in other bacteria for one type II TA system (33) and also elegantly between a type I and a type II TA system (34) and between a type II and type V TA system (35). Here, our in vivo and in vitro analyses showed that when it comes to toxicity regulation, the antitoxins involved are specific to their cognate modules. Moreover, we showed that the SprA2AS antitoxin responds to an osmotic shock and to nutrient starvation, whereas previous studies conducted on the SprA1/SprA1AS TA system revealed that PepA1 expression increased upon oxidative and acidic stresses due to a decrease in antitoxin levels (18). Both studies indicate that the key factor for allowing or repressing PepA1 or PepA2 expression is the corresponding antitoxin RNA level. PepA2 shares 50% amino acid identity with PepA1 (Supplementary Figure S1). Both of the PepA1 and PepA2...
N-terminal domains are rich in hydrophobic aliphatic amino acids that confer hydrophobicity and hemolytic potencies (26,36,37). Such hydrophobic content is also encountered in SprG1, another staphylococcal type I toxin (20) or also in the phenol-soluble modulins (38). Many type I TA systems form pores in bacterial membranes, inducing bacterial death (16,18,38,39). However, in our study, PepA1 and PepA2 exhibit different features. While PepA1 has both antimicrobial and hemolytic activity (17,18,26), our data indicates that PepA2 is mostly cytotoxic, rather than acting very strongly against either Gram-positive and Gram-negative bacteria. PepA2 is about 10 times more effective on human erythrocytes than PepA1 (Figure 5).

Our results suggest that PepA2 has a serious potential to damage eukaryotic membranes compared to bacterial membranes, and may belong to Staphylococcus aureus virulence arsenal, along with other leukocidins such as the phenol-soluble modulins (40).

Overall, because of the specificity of each antitoxin to a single RNA and their differences in toxin targets, effectiveness, and responses to various environmental stimuli, we raise the question of their redundancies. Redundancy within TA systems is usually attributed to the fact that deletion of a single TA system was insufficient to confer a significant phenotype (41). TA systems were originally described in post-segregational killing mechanism and to mediate programmed cell death (42), therefore being efficient killers. It was then demonstrated that they could induce stasis (42), and several recent studies have called attention to their role in commitment to persistence through fluctuations in the alarmone (p)ppGpp involved in the stringent response (41). Therefore, TA systems (including type I) have a broad spectrum of action (14,16), and are sometimes difficult to link to a phenotype (43). Here, our data do not pinpoint a specific role for the SprA2/SprA2AS module in bacterial stasis or persistence. Contrary to (42), overexpression of SprA2 led to a sudden growth arrest followed by decreased absorbance measures. This suggests cell death, a conclusion supported after spreading cells on agar plates in the absence of the inducer. Expression levels measured under different stress conditions allowed us to identify a drop in SprA2AS RNA levels in the case of hyperosmotic conditions and during nutrient starvation, which promotes a stringent response. A functional link between SprA2/SprA2AS and (p)ppGpp has not yet been investigated. However, our data does suggest that each TA system has dedicated effectors and roles and must therefore participate in the adaptive response to environmental changes (44–46), possibly including those encountered when in contact with the host during infection or colonization. Indeed, there is growing evidence in recent publications for TA systems that are not linked to persistier cell formation (45). In S. aureus, the discovery and characterization of type I TA systems is limited to a few studies (8,17,20), all of which involve cell death. In other bacterial and clinical pathogens, type I TA modules have various mechanisms of action, functions, and triggers (14,16,24,47). These include plasmid maintenance, regulation of the SOS response, abortive phage infection, and biofilm formation (48–52). Conversely, more is known about staphylococcal type II TA systems (43). A recent article demonstrated that persistier levels were unaffected when all known type II TA systems were knocked out (53). In that study, the authors showed that persistier production is critically dependent on the drop in intracellular ATP, and this might be the case for all Gram-positive bacteria. Additionally, studies on the S. aureus type II system MazEF pinpointed a possible regulatory relationship with staphylococcal pathogenicity (54). Similarly, the PemKSA TA system’s participation in staphylococcal virulence regulation could be linked to an altered translation of a large set of genes that promote virulence factor expression (55). Finally, the physiological role of SavRS, a novel type II S. aureus TA system, was recently described (56). The deletion of this system increased hemolytic activity and pathogenicity in a mouse subcutaneous abscess model. Additionally, the authors showed that the TA system could repress virulence gene expression by direct binding. Ongoing efforts
to characterize the different SprFs/SprGs type I TA modules support the hypothesis that each TA system has specific roles. SprG1 is an efficient killer (20), whereas some of the newly characterized SprG toxins induce bacterial stat- Elevated expression of SprA2 and SprA2AS was caused by stress applied, then RNA extracted to monitor (A) SprA2 and (B) SprA2AS transcript levels. Stress was induced either by centrifuging bacteria prior to resuspension in appropriate medium, or by directly adding the stressor to the growth medium. Pellets were resuspended in fresh TSB (TSB1), TSB supplemented with 1 M NaCl (mimicking osmotic stress), or in NZM medium (emulating a stringent response). Other stresses were induced by adding H2O2, HCl or NaOH, or by changing the temperature to 18 or 42 °C. The TSB lane corresponds to cells maintained in TSB under normal conditions. The relative expression levels were calculated after quantification of northern blot bands using TSB1 or TSB2 as calibrators. For all experiments, tmRNA was used as an internal loading control. The data presented are the mean of three independent experiments. Statistical analysis was conducted using the one-tailed Mann–Whitney test; differences were considered statistically significant when \( P < 0.05 \).

### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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