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Noncoding RNA

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ABSTRACT Regulatory RNAs, present in many bacterial genomes and particularly in pathogenic bacteria such as *Staphylococcus aureus*, control the expression of genes encoding virulence factors or metabolic proteins. They are extremely diverse and include noncoding RNAs (sRNA), antisense RNAs, and some 5′ or 3′ untranslated regions of messenger RNAs that act as sensors for metabolites, tRNAs, or environmental conditions (e.g., temperature, pH). In this review we focus on specific examples of sRNAs of *S. aureus* that illustrate how numerous sRNAs and associated proteins are embedded in complex networks of regulation. In addition, we discuss the CRISPR-Cas systems defined as an RNA-interference-like mechanism, which also exist in staphylococcal strains.

GENERAL INTRODUCTION

Regulatory RNAs have been identified in many bacteria and in pathogenic bacteria such as *Staphylococcus aureus*, where they play major roles in the regulation of virulence or the synthesis of metabolic proteins, besides transcriptional factors and two-component systems (1–4). Most of them are noncoding RNAs (sRNAs), but some of them express small peptides. Certain sRNAs, acting in *cis*, are situated at the 5′ untranslated regions (UTRs) of mRNAs and act as sensors of metabolites, tRNA, or environmental stimuli (e.g., temperature, pH) or are situated at the 3′ UTR. In contrast, the genes encoding sRNAs, which act *in trans*, sit on the opposite strand of the regulated mRNA or at genomic locations distant from the mRNAs they regulate. *Cis*-encoded sRNAs, also called antisense RNAs (asRNAs), are fully complementary to their targets. In contrast, *trans*-encoded sRNAs share only partial complementarity, and as a consequence, they can regulate many mRNAs. Most of them are encoded mainly in the core genome, while a few of them are localized within mobile elements, pathogenic islands, or plasmids. In this review, we will focus on the most recent mechanisms of RNA regulation discovered in *S. aureus* and how regulatory RNAs are part of sophisticated networks that allow the bacteria to adapt quickly to their environment or survive in their host.

mRNA 5′ UTRs: RIBOSWITCHES, T-BOXES, AND THERMOSENSORS

5′ UTRs of mRNAs contain riboswitches, T-boxes, or thermosensors with potential impacts for novel anti-biotherapy. Riboswitches and T-boxes are found in the 5′ UTR of some mRNAs and contain highly structured domains, which recognize metabolites such as cofactors, vitamins, amino-acids, nucleotides, second messenger cyclic di-GMP, Mg^{2+}, or nonaminoacylated tRNAs (5). Binding of these metabolites induces structural changes that modify the expression of the downstream mRNA,

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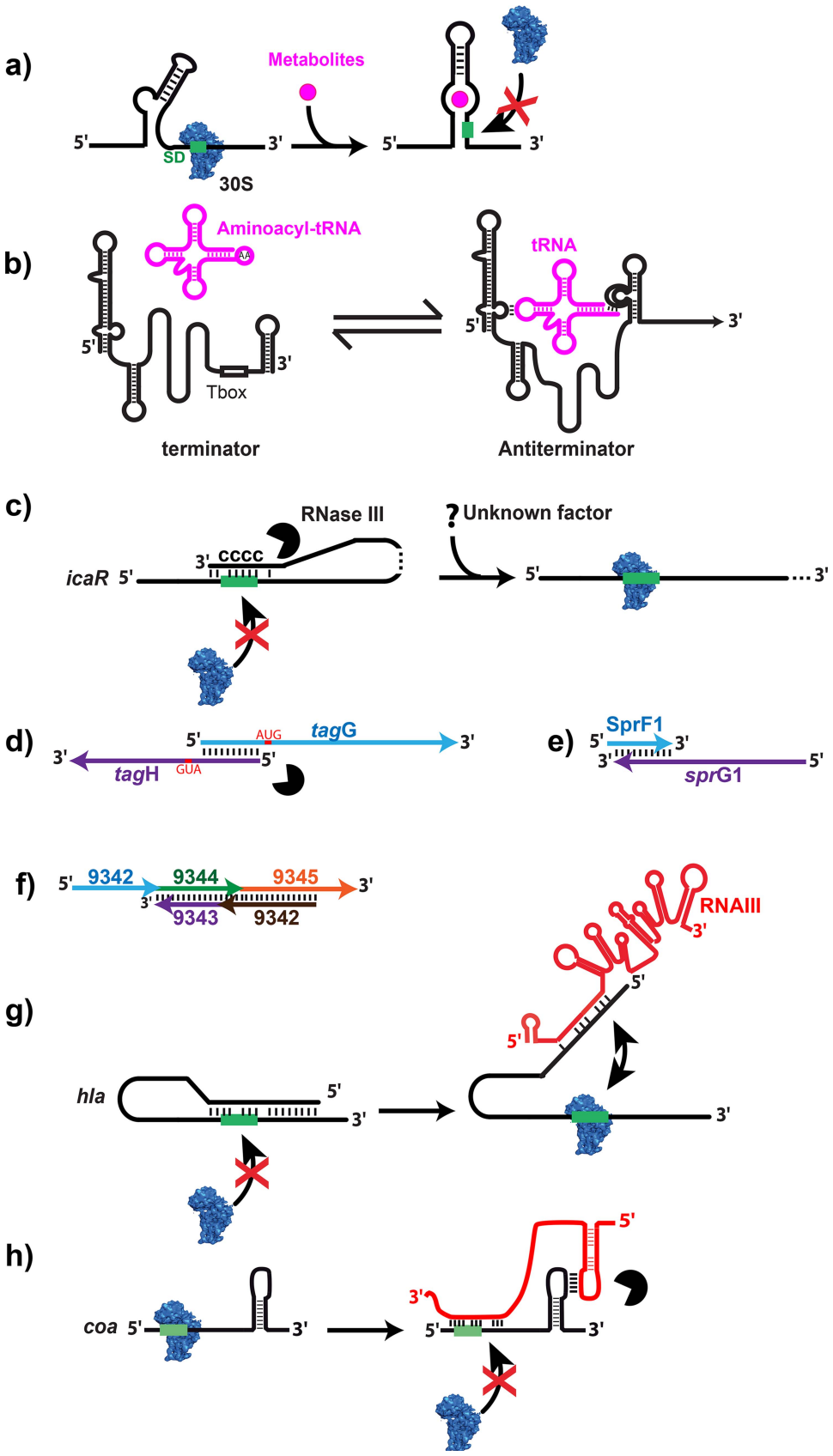
a)
Metabolites
5'
3'
SD
30S
b)
Aminoacyl-tRNA
AA
5'
3'
Tbox
terminator
tRNA
5'
3'
Antiterminator
c)
RNase III
? Unknown factor
3'
CCCC
icaR 5'
5'
3'
d)
5'
AUG
tagG
3'
3'
tagH
GUA
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e)
SprF1
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sprG1
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9342
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3'
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coa 5'
3'
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for example, by inducing premature transcription arrest, repression/activation of translation, or cleavage (Fig. 1a). A T-box senses the aminoacylation status of tRNAs and mainly controls transcription of downstream genes that encode proteins involved in biosynthesis, transport of amino acids, or aminoacylation of tRNAs (Fig. 1b). Based on sequence and structure conservation, most of the T-boxes and riboswitches were predicted in *S. aureus* genomes (4). A large proportion of riboswitches control the expression of genes involved in metabolic pathways. Because these genes are often essential for growth, they represent interesting targets for the development of alternative antimicrobial drugs in the battle against multidrug-resistant *S. aureus*. This strategy was used with the guanine and glucosamine-6-phosphate (GlcN6P) riboswitches. Mulhbacher et al. (6) identified a pyrimidine derivative that binds to the guanine riboswitch and represses the expression of *guaA*. This compound significantly attenuated *S. aureus* infections in a mouse model. In Gram-positive bacteria, the *glm*S mRNA is both a ribozyme, which catalyzes its own cleavage, and a riboswitch responding to GlcN6P. Its product encodes an essential enzyme, which converts fructose-6-phosphate into GlcN6P, a building block of bacterial peptidoglycan. Tight regulation of *glm*S mRNA is crucial to maintain a homeostatic level of GlcN6P in the cell. At high concentrations of GlcN6P, its binding to the 5′ UTR of *glm*S leads to site-specific self-cleavage, which generates a 5′ hydroxylated end molecule rapidly degraded by the RNase J1 (7, 8). A recent study led to the design, synthesis, and characterization of a GlcN6P analogue, carba-GlcN6P, which constitutively activates the *glm*S ribozyme of vancomycin-resistant *S. aureus* and destabilizes its mRNA (7). This compound was experimentally shown to induce the efficient self-cleavage of the *glm*S mRNA in a similar fashion as the natural metabolite and thus represented an important step in the development of antibiotics with a new mode of action. Very recently, a new approach called "Term-seq" revealed that several antibiotic resistance genes are under the control of riboswitches responding to antibiotics commonly used against Gram-positive pathogenic bacteria such as *Listeria* spp. and *Enterococcus faecalis* (9). These results suggest that the same phenomenon could exist in *S. aureus* and that RNA-mediated regulation could play a broader role in antibiotic resistance mechanisms than has been envisioned.

Not surprisingly, the presence of antibiotics can also modulate the regulatory activity of T-boxes. In *S. aureus*, an unusual *gly*S T-box regulates transcription antitermination (Fig. 1b) of the unique glycyl-tRNA synthetase (glyRS) gene responsible for catalyzing the aminoacylation of the five $tRNA^{Gly}$ isoacceptors, independently of their anticodon (GCC or UCC) and with different binding affinities (10). Thereafter, the T-box senses the availability of glycine not only for its incorporation into nascent polypeptide chains during translation but also for the formation of pentaglycine bridges into the peptidoglycan molecule, linking two essential pathways. Antibiotics targeting the small ribosomal subunit stabilized the T-box/tRNA complex and induced a readthrough of transcription, while chloramphenicol and linezolid attenuated *gly*S transcription (11). The outcome depended on the binding sites of the protein syn-

FIGURE 1 Several mechanisms of RNA regulation in *S. aureus*. **(a)** Schematic drawing of the flavin mononucleotide riboswitch. The 5′ UTR adopts a particular structure recognized by the flavin mononucleotide, which in turn leads to the stabilization of a stem-loop structure sequestrating the SD sequence to inhibit translation. 30S is for the small ribosomal subunit. **(b)** An example of a T-box motif as found in the 5′ UTR of many mRNAs encoding aminoacyl-tRNA synthetases. Nonaminoacylated tRNA binds to the leader region at two sites and stabilizes an antiterminator structure, allowing transcription of the downstream gene. The drawing is adapted from reference 4. **(c)** The 3′ UTR of the biofilm repressor IcaR possesses a cytosine-rich motif, which binds to the SD sequence and hinders ribosomes from its binding site on the mRNA (see text for details). **(d)** Overlapping 5′ UTRs of *tag*G and *tag*H mRNAs are processed by the endoribonuclease III (Rnase III). Shorter 5′ ends might facilitate ribosome recruitment. **(e)** The antitoxin RNA SprF1 interacts at the 3′ end of the toxin encoded by sprG1 and triggers its degradation. **(f)** A cluster of five sRNAs was sequenced in the *S. aureus* Newman strain that encodes a putative toxin-antitoxin system (see text for details). **(g,h)** sRNAs act by an antisense mechanism. Binding of the 5′ UTR of RNAIII to the 5′ UTR of *hla* mRNA liberates its SD and activated translation (g), whereas the 3′ domain of RNAIII acts as a repressor domain, which contains C-rich motifs for base-pairing with the SD sequence of mRNA as *coa* mRNA depicted in the figure (h). Green bar, SD sequence; black circle, RNase III (for references and more details, see text).

thesis inhibitors (11). Although T-boxes can be direct targets for antibiotics against *S. aureus*, it was also reported that a high concentration of the antibiotic tigecycline might induce possible off-target inhibition of the RNA polymerase (11).

RNA thermosensors are regulatory elements often localized at the 5′ UTR of mRNAs encoding heat or cold shock proteins and virulence factors (for review see 12). Briefly, at low temperatures, the mRNA cannot be translated, since the Shine and Dalgarno (SD) sequence is trapped in a hairpin structure, which melts gradually when the temperature increases. The best-studied example in Gram-positive bacteria is the thermosensor regulating the expression of the transcriptional factor *prf*A, which activates the expression of most of the virulence genes in *Listeria monocytogenes* at high temperatures (13, 14). No such example has yet been demonstrated for other Gram-positive bacteria, including *S. aureus*.

3′ UTRs OF mRNAs ACT IN *CIS* OR ARE RESERVOIRS OF sRNAs

Transcriptome analysis of the human pathogen *S. aureus* revealed that at least one-third of mRNAs carry long 3′ UTRs and thus might display multiple regulatory functions (15). Some of them have direct action on the expression of their own mRNA (15, 16). Long 3′ UTRs (>100 nucleotides) can end at an intrinsic Rho-independent terminator of transcription (TT) and can be generated from a specific RNase cleavage or from a termination read through of the RNA polymerase. Remarkably, several 3′ UTRs contain riboswitches functioning in metabolite-sensing regulation (16). Indeed, the TT of the riboswitch in an OFF conformation also serves as the TT of the gene encoded upstream of the riboswitch.

A singular example of a newly identified posttranscriptional regulatory mechanism was shown for the *icaR* mRNA (Fig. 1c), which possesses an unusually long 3′ UTR (390 bp). In this case, the expression of the mRNA is modulated through a long-distance interaction between 3′ UTR and 5′ UTR (16). Because *icaR* codes for a transcriptional repressor of the *icaADBC* operon, encoding enzymes involved in the synthesis of PIA-PNAG, the main polysaccharides of the biofilm matrix, this regulation has a direct impact on biofilm formation. Base-pairing interactions between the long 3′ UTR of *icaR* mRNA and the SD sequence of the same mRNA hindered efficient translation initiation (16). This long-range RNA duplex generated a specific site for the double-stranded endoribonuclease III (RNase III) for cleavage. As a consequence of this cleavage, PIA-PNAG synthesis increased. However, the mechanism allowing IcaR translation is not known to prevent the action of the 3′ UTR as a *cis*-acting antisense RNA (Fig. 1c).

It is also postulated that the 3′ UTR length provides other types of transcript-specific regulation. Indeed, in *Salmonella* spp., the 3′ UTRs are also reservoirs for sRNAs, which originate either by transcription from an internal promoter or by processing. In both cases, the sRNA generated from the 3′ UTR regulates trans-encoded mRNA targets. For instance, the sRNA CpxQ is generated by RNase E cleavage of the 3′ UTR of *cpxP* and represses the translation of mRNAs encoding a family of envelope proteins, whereas the sRNA DapZ is transcribed from an internal promoter within the *dapB* gene and inhibits translation of the major ABC transporters, DppA and OppA (17, 18; for review see 19). Recent work has shown that such 3′ UTR-derived sRNAs also exist in *S. aureus*, although their functions remain to be addressed (E. Desgranges, S. Marzi, P. Romby and I. Caldelari, unpublished data).

asRNAs IN PERVASIVE TRANSCRIPTION AND ACTING AS ANTITOXINS

asRNAs are transcribed from the opposite strand of the mRNAs they regulate, so that they display perfect complementarities with their targets. Short asRNAs are often encoded on mobile elements such as plasmids, transposons, and phage-like elements. Such elements can potentially be transferred horizontally to other bacterial species or be duplicated (20). In *S. aureus*, they were first described to control plasmid conjugation and replication (21). The size of asRNAs can vary from 10 to thousands of nucleotides, because these RNAs can overlap with part of a gene (3′ or 5′ ends), the entire gene, or a group of genes (Fig. 1d to f). This phenomenon is called pervasive transcription. Initially, pervasive transcription was considered a nonfunctional transcriptional noise. However, considering the large number of asRNAs expressed from the entire genome and in several bacterial species, these RNAs might play an important role in the regulation of gene expression. Genome-wide analysis of *S. aureus* highlighted that the expression of a significant proportion (75%) of antisense transcripts to annotated open reading frames are synthesized from the complementary strand and that these sense/antisense duplexes are digested by RNase III, generating short fragments all along the genome (22). Another study using the RIP-Seq approach confirmed the involvement of RNase III in the

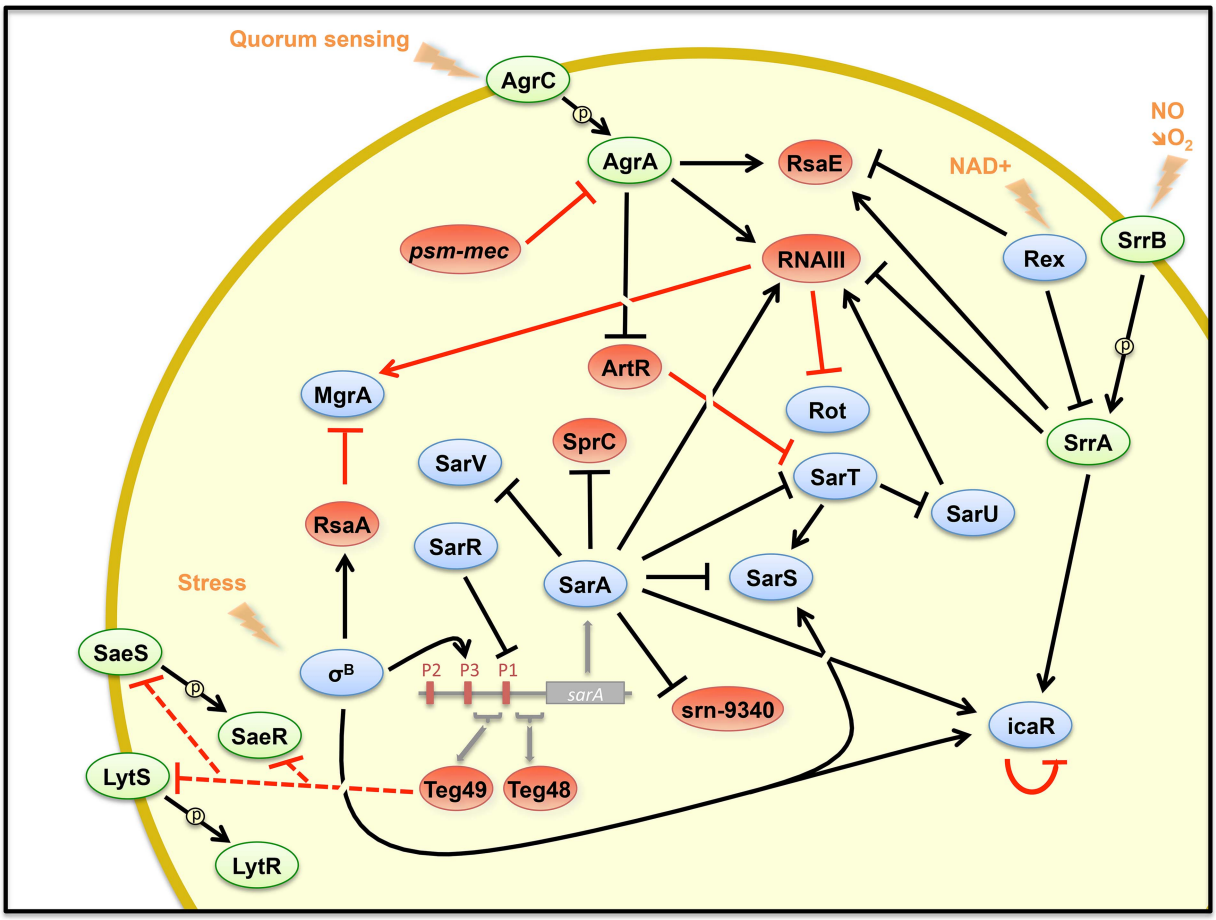

FIGURE 2 Examples of the complex network between sRNAs and transcriptional factors in *S. aureus* in response to stress. Arrows show activation and bars show repression. Blue, transcriptional regulators; green, two-component systems; red, regulatory sRNAs. Red lines corresponded to posttranscriptional regulation, and black lines, to transcriptional regulation. Dotted lines are for the target mRNAs that were not experimentally validated. Only sRNA-dependent mRNA targets encoding transcriptional factors are depicted in the figure.

regulation of sense/antisense transcripts and overlapping UTRs (23). The situation might be even more complex, because recent results suggested that the termination factor Rho plays a major role in preventing pervasive transcription in *Bacillus subtilis*, but also in *S. aureus* (24, 25). Although the biological outcome of pervasive transcription is not clearly understood, some of the asRNAs produced are functional and control several biological processes (15).

Type I toxin-antitoxin (TA) systems are particular cases of short asRNAs, in which the antitoxin is an asRNA regulating the translation or the fate of the toxin-encoding mRNA, whereas in the type III system, the antitoxin sequesters the toxin (reviewed in 26, 27). In *S. aureus*, several type I TA module systems have been described (reviewed in 28). One of them, called SprF1/SprG1, expresses SprF1 as the asRNA and *spr*G1 mRNA (Fig. 1e), which encodes two short secreted peptides with hemolytic and antibacterial activity (29). The antitoxin SprF1 binds to the 3′ end of *spr*G1 mRNA, which leads to mRNA degradation and inhibition of peptide synthesis to protect cells against lethality (29). The SprA1/asSprA1 pair is another intriguing and unconventional system. The asSprA1 is transcribed from the opposite strand of the *spr*A1 mRNA, producing a cytolytic peptide. Their 3′ ends overlapped by 35 nucleotides, but experimental data indicated that the functional domain of asSprA1 is outside the complementary sequence with *spr*A1 mRNA. Both RNAs were expressed concomitantly, and asSprA1 5′ base-paired with the ribosome-binding site (RBS) of *spr*A1, impairing its translation (30). Thus, asSprA1 acts as a *trans* regulator with its

complementary target, suggesting that it can potentially interact with other RNA targets. Finally, a cluster of five genes encoding sRNAs specific to the *S. aureus* Newman strain contained a putative TA system (31) (Fig. 1f). Three genes were transcribed from the positive strand and two from the negative strand. Moreover, one small open reading frame was detected within one of the genes from the minus strand and coded for a secreted peptide with similarity to the RelE toxin (32). Whether these two overlapping genes corresponded to a novel TA system remains to be addressed. Interestingly, this locus was expressed in a growth-phase-dependent manner, in nutriment starvation, and in oxidative stress. Type I TA systems have been involved in many functions (e.g., membrane depolarization, plasmid maintenance), mainly in *Escherichia coli*, including the persistence phenomenon (for review see 33), but not in *S. aureus* until now (28).

TRANSCRIPTIONAL FACTORS AND sRNAs BUILD COMPLEX REGULATORY NETWORKS

The sRNAs belong to intricate networks of regulation, and their synthesis is often dependent on transcription factors or on two-component systems. In addition, sRNAs can also control transcription factors at the posttranscriptional level (Fig. 2). Typical examples will be described below. In addition, *trans*-acting sRNAs regulate mRNAs by imperfect base-pairings, which signifies that one sRNA can modulate several targets and one target can be controlled by several sRNAs. In *S. aureus*, the annealing region between sRNA and mRNAs are often longer than in *E. coli* and mostly targets the RBS of mRNAs affecting translation. In several cases, a second distinct site of interaction occurs in the coding region. Unlike Gram-negative bacteria, in which Hfq and ProQ proteins participate in sRNA regulation by stabilizing and facilitating their pairings with mRNA targets (see reviews in 34, 35), no RNA chaperones have yet been identified in *S. aureus*. Indeed, there is no identifiable *proQ* homolog, and the role of Hfq is still unclear. Recent work has shown that the rim domain of Hfq has an amino acid composition (low in arginine) incompatible with RNA annealing activity compared to *E. coli* Hfq (36).

AgrA, the response regulator of the *agr* quorum sensing system, activates the transcription of RNAIII. The bifunctional RNAIII codes for δ-hemolysin and regulates the expression of virulence genes at the posttranscriptional level (see below). It interacts with various mRNAs either to activate or to repress translation (Fig. 1g,h). The RBS of *hla* mRNA encoding α-hemolysin is embedded in a hairpin, which prevents ribosome binding and blocks the start of translation. The 5′ UTR of RNAIII possesses complementary sequences to the leader region of *hla*. Interaction between these RNAs enables the recruitment of ribosomes to initiate Hla translation. In its 3′ UTR, RNAIII carries conserved UCCC motifs that are used as the seed sequences to bind with the RBS of the target mRNAs coding for protein A, coagulase, Sbi protein, the transcription factor Rot (a repressor of exotoxins), and the endopeptidase LytM (for review see 37). Moreover, AgrA represses the sRNA ArtR, which inhibits translation of the SarA homolog SarT (38), and activates RsaE (see 39) (Fig. 2).

The staphylococcal accessory regulator SarA is synthesized from three distinct promoters (P1, P2, P3) and binds DNA or RNA (1). As a transcription factor, it regulates many genes involved in virulence, autolysis, biofilm formation, stress response, antibiotic resistance, and metabolism, but also two sRNAs, SprC and Srn_9340, located on the same pathogenicity island (40). SprC prevents ribosome binding to the SD of the *atl* mRNA coding for an autolysin. Deletion of the *spr*C gene causes enhanced phagocytosis of *S. aureus* by monocytes and macrophages, and this effect was found to be partly due to the deregulation of *atl* expression (41). SarA represses both *spr*C and *srn_9340* transcription and requires an ATTTTAT sequence in its binding site (40). However, while the SarA level remains relatively constant during bacterial growth, the expression of SprC fluctuates, which suggests that additional factors might control its synthesis and that a mechanism of derepression should coexist under specific conditions. These are the first examples of two sRNAs regulated by the same transcription factor.

In the following, we will describe an sRNA whose transcription is controlled by three independent transcription factors. The sRNA RsaE possesses two consensus sequence motifs UCCC as found in RNAIII, which interact with the RBS of several mRNAs involved in central metabolism to repress their translation (39, 42). RsaE is highly conserved between the *Staphylococcaceae* and *Bacillaceae* families. Not only is the sequence of RsaE conserved between *B. subtilis* and *S. aureus* species, but so are its regulation and functions. Recent studies have shown that its transcription is activated by AgrA (see above), the two-component system SrrAB (staphylococcal respiratory response) in response to NO, and a binding site for the redox sensing repressor Rex has been predicted (39, 43, 44). In *B. subtilis*, the Rex-repression of RsaE (also called RoxS) has been

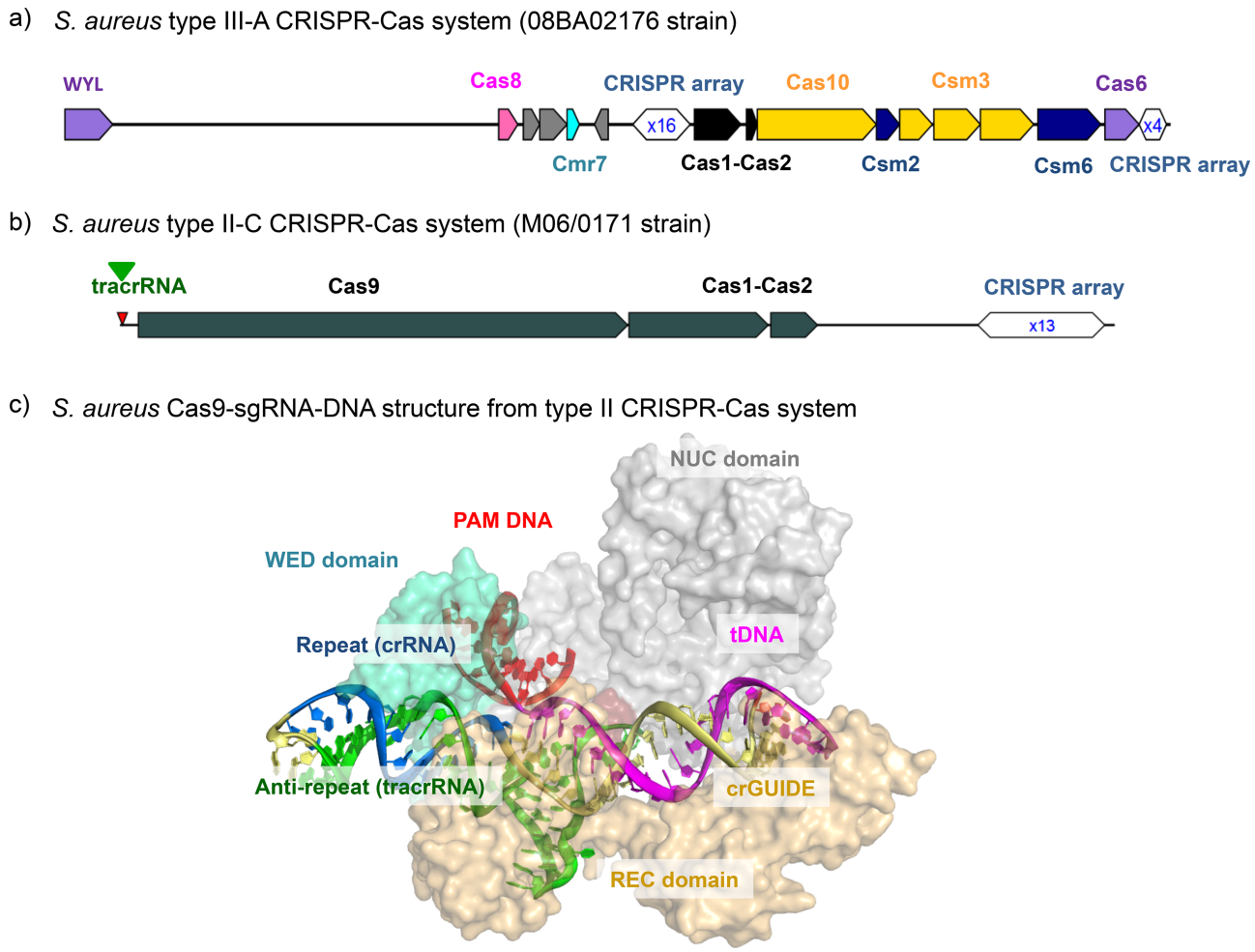

FIGURE 3 **(a)** Genomic organization of the loci for the type III-A CRISPR system of *S. aureus* strain 08BA02176. Type III is the typical *S. aureus* CRISPR organization. The scheme was obtained using CRISPRone (72), and the genome sequence was deposited in GenBank (accession number 08BA02176; RefSeq accession number GCF_000296595.1). **(b)** Genomic organization of the loci for the type II-C CRISPR system of *S. aureus* strain M06/0171. The CRISPR-Cas genes were found on an SCC*mec* inserted into the 3′ end of the chromosomally located *orfX* gene. The scheme was obtained using CRISPRone (72), and the SCCmec sequence was deposited in GenBank (GenBank accession number HE980450.1). **(c)** Cartoon (RNA and DNA) and surface (Cas9) representations of the SaCas9-sgRNA-target DNA complex (pdb file 5AXW) (80). The SaCas9 sgRNA consists of the crRNA guide region (crGUIDE represented in pale yellow) forming a heteroduplex with the target DNA strand (tDNA in magenta) and the repeat/antirepeat helix (blue, the repeat crRNA-derived strand, green, the antirepeat trascrRNA-derived strand). The protospacer adjacent region-containing DNA duplex is red. Cas9 domains are colored as follows: cyan, WED domain; pale orange, REC domain; gray, NUC domain. Molecular graphics images were prepared using PyMol.

proposed to readjust the cellular balance of NAD^+/NADH on various signals (44).

The alternative sigma B factor (σ^B) together with RNA polymerase guides transcription of genes mainly in the stationary phase of growth and under stress conditions. Its regulon comprises more than 200 genes, including several virulence factors, transcription factors, and sRNAs (Fig. 2). Among the sRNAs induced by σ^B are SbrA, B, and C activated by KOH (45), and RsaA (39). The stability of RsaA depends on RNase III and the endoribonuclease RNase Y (23, 46). RsaA acts as an acute virulence attenuator in *S. aureus* (see below) by

inhibiting translation of the MgrA transcription factor (Fig. 2) (47), which in turn causes the activation of the synthesis of several surface proteins (48). RsaA possesses two UCCC motifs, which in the case of *mgrA* mRNA, bind to two distant regions, involving an imperfect duplex masking the SD sequence of the mRNA and a loop-loop interaction occurring downstream in the coding region. These two distant binding sites are required for efficient repression and RNase III-dependent degradation of the repressed mRNA (47). Finally, the sRNA Teg49 is transcribed from the σ^B- dependent P3 promoter of *sar*A and is probably processed by RNase III and RNase Y with the help of the helicase CshA (49–51). Transcriptomic analyses revealed that besides genes involved in virulence and autolysis, Teg49 might posttranscriptionally affect the SaeRS and LytRS two-component systems, yet the exact mechanism is not known (51). In addition, another sRNA, Teg48, whose role is not known, is transcribed from the P1 promoter of *sar*A (Fig. 2). Even if the maturation process and the function of Teg48 and Teg49 are not clearly established, the long 5′ UTR of *sar*A mRNA encoding a master regulator of virulence in *S. aureus* represents a putative reservoir for novel sRNAs.

crRNA, tracrRNA, AND THE CRISPR-Cas ADAPTIVE IMMUNITY SYSTEMS IN *S. AUREUS*

Phages are the most abundant forms of life on earth and the natural killers of bacteria because in most cases their lytic life cycle ends with the death of the bacterial cell. Outnumbering their microbial hosts, phages impose selective pressure for the diversification of microbial defense systems. These include various innate phage-resistance mechanisms such as restriction/modification enzymes, receptor masking, blocking DNA injection, abortive infection (52, 53), and the adaptive defense mechanism based on clustered, regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (*cas*) genes (54, 55). The latter RNA-interference-like mechanism relies on small noncoding RNAs, CRISPR RNA (crRNA), and in some cases trans-activating crRNA (tracrRNA), through which prokaryotic hosts (bacteria and archaea) can acquire heritable resistance to genetic parasites such as phages, but also plasmids and transposons (for reviews see 53, 56). To date, CRISPR-Cas systems have been found in about 50% of bacterial genomes and 95% of archaeal genomes (57, 58). The CRISPR and the *cas* loci are often located next to each other in the genomes, sometimes organized into operons, but a significant number of genomes have also isolated *cas* loci and/or CRISPRs (59).

Despite the large diversity of the CRISPR-cas systems, they share common features. Briefly, the CRISPR loci are characterized by an array of short and palindromic repetitive sequences interspaced by sequences called "spacers" that are derived from plasmid and viral DNAs (in some cases, also RNAs). During the initial infection with a virus or plasmid, these spacers are first integrated into the CRISPR array in the host genome to provide the host with immunity (acquisition step [60, 61]). During a second infection event they are transcribed and used as guides to inactivate the viral or plasmid genome. This two-step pathway involves a variety of Cas proteins, leading to several major types of CRISPR-cas systems (reviewed in 58, 62). However, the acquisition step involves two highly conserved Cas1 and Cas2 proteins (58, 63). The Cas1-Cas2 integrase is a heterohexameric complex of four Cas1s and two Cas2s which preferentially incorporates foreign DNA at the first CRISPR repeat and participates in the discrimination against self-DNA and in the minimization of off-targeting insertions (64–66). Both proteins have been found in several *S. aureus* strains (57). During the second step, activation of transcription from a promoter located in an AT-rich leader sequence preceding the first CRISPR repeat (67, 68) leads to expression of the whole array into precursor CRISPR transcripts (pre-crRNA). The pre-crRNAs are then processed into mature crRNAs consisting of partial repeat(s) and a single spacer sequence, each complementary to a unique invader sequence (69). Different endonucleases participate in the maturation step, which might vary in different bacteria. Type I and III systems perform the function by a multisubunit Cas protein complex and are characterized by Cas6 processing (69). Type II uses another sRNA, tracrRNA, to direct RNase III-dependent maturation of the pre-crRNA in the presence of Cas9, the hallmark protein of the type II system (70). Cas9 endonuclease remains associated with the dual-tracrRNA:crRNA structure, which during the interference phase, guides the cleavage of site-specific cognate target DNA (71). Type I and II CRISPR-Cas systems target DNA (72, 73), whereas type III systems provide immunity against DNA and RNA (74).

With the completion of the genome sequences of several *S. aureus* strains, it appears that the CRISPR-Cas systems are not highly prevalent, and only a few of them have been experimentally demonstrated. A CRISPR Finder analysis (57) of the 115 sequenced *S. aureus* genomes present in the CRISPRdb (http://crispr.i2bc

.paris-saclay.fr) showed that the majority of CRISPR-like loci contain only a few spacers (1 or 2), a few have between 3 and 10 spacers, and only 1 genome (from methicillin-resistant *S. aureus* [MRSA] 08BA 02176, isolated from a patient) has 15 spacers. Using CRISPRone (http://omics.informatics.indiana.edu/CRISPRone), several genes belonging to the type III-A CRISPR system (including Csm2, Cas1, Cas2, and Cas6) (Fig. 3a) have been recently predicted (72) in different isolates (MSHR1132, JS395, CIG290, and 21252). Multilocus sequence typing performed on one of these isolates (MSHR1132) has shown that it belongs to a divergent clonal complex, which appears to be closely related to *Staphylococcus epidermidis* and *Staphylococcus lugdunensis* (75), and which has been renamed *Staphylococcus argenteus* (76). It has thus been hypothesized that CRISPR/cas was present in a common ancestor of *S. epidermidis*, *S. lugdunensis*, and *S. aureus* and was later lost in most conventional *S. aureus* strains. It is noteworthy that numerous repeat-spacer-like structures resemble CRISPR elements but lack spacer diversity and have been classified as false-CRISPR (72). *S. aureus* repeat-like elements (GC-rich direct repeats) belong to this class of RNAs, for which the functions remain to be addressed (77, 78).

More recently, a type II-C CRISPR-Cas system was found in an MRSA strain isolated from an Irish patient (Fig. 3b). This CRISPR element is located on a pseudo staphylococcal cassette chromosome *mec* (SCC*mec*) composite island, which was probably horizontally acquired from an *S. epidermidis* strain (79). The peculiarity of this system (SaCas9) is that it contains a shorter version of Cas9 protein. The crystal structure of a complex containing SaCas9, the sgRNA (single-guide RNA; an artificial fusion product of a crRNA and a tracrRNA [71]) and its target DNA provided a model to understand how crRNA and tracrRNA guide Cas9 on the target DNA and prepare it for double-stranded DNA cleavages (80). In this structure, the DNA duplex of the protospacer-adjacent region contains the signals recognized by the Cas9 protospacer-adjacent region-interacting (PI) domain, which discriminates the invader DNA against self-DNA and facilitates unwinding of the target DNA, leading to the formation of a heteroduplex. The beginning of the RNA-DNA heteroduplex ("seed" region) adopts a distorted structure critical for Cas9-catalyzed DNA cleavage. This conformation results from the RNA-DNA helix structure, the interactions with protein residues, and the RNA helix formed by the repeat/antirepeat regions mimicking the tracrRNA-crRNA interactions (Fig. 3c). The discovery of the smaller Cas9 protein led to recent improvements in genome editing (81). The seminal works of Jennifer Doudna and Emmanuelle Charpentier on the use of CRISPR-Cas systems for genome editing have inspired many studies showing the incredible potency of the system (82–84). Thousands of publications have reported the use of *Streptococcus pyogenes* Cas9 (85) directed by the sequence of a sgRNA for site-specific genome modifications, gene knockouts or replacements, gene expression control, and functional genome screenings in over 40 species. Interestingly, SaCas9 has been successfully used for eukaryotic genome editing since its smaller size makes it easier to be delivered via adeno-associated virus vectors to somatic tissues (81).

The fact that CRISPR-Cas has been mapped on mobile elements in MRSA confirms the importance of horizontal gene transfer from other cocolonizing bacteria in the acquisition of novel functions and in the evolution of *S. aureus* strains. Interestingly, transcription of the CRISPR-Cas genes can be highly regulated and induced upon infection (86–88) by membrane stress (89) and, in Gram-negative bacteria, by quorum sensing signaling (90, 91). Recent work has developed genetic engineering tools to apply the CRISPR/Cas9 system as an antimicrobial strategy against *S. aureus* (92). Clearly, the CRISPR/Cas9 system offers an alternative therapy to conventional antibiotics.

ROLE OF sRNAs IN PHYSIOPATHOLOGY

S. aureus pathogenesis can take different forms depending on the infected tissue and the invading bacterial strain. This is often accompanied by the expression of various virulence factors involved in the colonization and the alteration of the tissue but also by the capacity to escape from the host immune response. Among key regulators of virulence, several sRNAs have been shown to modulate the synthesis of virulence factors in a dynamic manner, and some of them contribute to specific aspects of bacterial virulence in animal models of infection (1).

The most studied RNA in *S. aureus*, *agr*-RNAIII, is the main intracellular effector of the quorum sensing system *agr*. As described above, RNAIII regulates expression of virulence factors known to be associated with infectious diseases. For instance, it represses the synthesis of protein A, which triggers inflammatory signaling pathways and contributes to evasion of the immune response. Conversely, RNAIII induces the synthesis of a battery of toxins, which contribute to the degradation of tissues and subversion of host defenses, such as the pore-

forming toxins, and peptides with proinflammatory and lytic activities. Recent modeling of the quorum sensing system and of its regulators has illustrated the importance of the *agr* system in promoting dissemination of the bacteria from biofilms or dense populations (93, 94). Nevertheless, despite the fact that many clinical isolates from acute infections express RNAIII, its steady state level varies considerably among them (95, 96). In particular, a higher level of RNAIII has been observed in the community-acquired MRSA strains with increased virulence compared to other *S. aureus* lineages (97, 98). Interestingly, a recent study showed that the level of RNAIII is lower in strains isolated from patients with sepsis than in those from commensal carrier patients (99). Perhaps more surprisingly, heterogeneity has been reported in patients in which *agr*-positive and *agr*-negative strains coexisted. This has been proposed as a factor that might modulate the outcome of the infections (100, 101).

S. aureus is also frequently exposed to other microbes during colonization and infection, providing opportunities to acquire mobile genetic elements that contribute to the evolution of the genome. Some of these genomic islands play key roles in pathogenesis through their possession of new virulence factors (pathogenicity islands) or through the synthesis of novel regulators modulating the expression of genes of the core genome. As an example, SprD is an important small regulatory sRNA (142 nucleotides) expressed from a pathogenicity island, which significantly promoted *S. aureus* diseases in a mouse sepsis model of infection (102, 103). SprD interacts through base-pairings with the *sbi* mRNA, which encodes an immune evasion molecule protecting the bacteria from the host immune responses (104, 105). However, the phenotype of the Δ*sprD* mutant strain was not linked to the SprD-dependent regulation of *sbi* since the Δ*sbi* mutant strain behaved like the wild-type strain in the mouse sepsis model (102). Therefore, these data strongly suggest that SprD might regulate the expression of other proteins important for infection.

SSR42 (for small stable RNAs) is an 891-nucleotide-long sRNA whose stability is greatly enhanced in the stationary phase of growth (106, 107). It regulated the expression of approximately 80 mRNAs in 2 genetically different *S. aureus* strain backgrounds. While it increased the expression of capsule Cap5a, SSR42 downregulated the expression of protein A, α and γ hemolysin, and Panton-Valentin leukocidin (107). Because no direct binding was evidenced between SSR42 and mRNAs encoding virulence determinants, the effect was predicted to be indirect through the modulation of the expression of a transcriptional regulator. Phenotypically, the deletion of the SSR42 gene affected erythrocyte lysis, resistance to opsonization killing, and pathogenesis in a murine model of skin and soft tissue infections (107). More recently, SSR42 was identified as an important effector of intracellular virulence by screening of a transposon mutant library pool. After internalization in epithelial cells, the ΔSSR42 mutant strain was significantly enriched in the intracellular fraction, most likely due to an attenuated cytotoxicity (108).

In contrast to RNAIII, SprD, and SSR42, which contribute to enhanced virulence of *S. aureus*, other regulatory RNAs behave as attenuators of virulence. This is, for instance, the case of another encoded pathogenicity island sRNA, the so-called SprC (103). Indeed, the virulence of the isogenic strain lacking SprC was significantly and reproducibly enhanced in a mouse systemic model. Furthermore, SprC reduces *S. aureus* susceptibility to phagocytosis by human monocytes and macrophages (41). Another example is the *psm-mec* RNA, which is a bifunctional RNA located in the SCC*mec* mobile genetic element. It encodes a phenol-soluble modulin (PSMmec) cytolytic toxin and acts as a translational repressor through direct binding with *agr*A mRNA (109). The transcription of all *psm* genes is positively regulated by AgrA. This activation is linked to a specific binding of the phosphorylated form of AgrA to the promoter sequences upstream of the *psm* genes, except that this binding was not yet demonstrated for the *psm-mec* gene (reviewed in 110). The deletion of the *psm-mec* RNA increased the expression of AgrA, which resulted in an increase of toxin and PSMα production and enhanced virulence in mice (109). Interestingly, community-acquired MRSA that does not carry the *psm-mec* gene has been shown to be more virulent than the hospital-associated MRSA that harbors the gene (109, 111). Finally, a mutant strain that did not express the sRNA, RsaA, was attenuated in the severity of acute systemic infection in a mouse model (47). This deletion is linked to the deregulation of MgrA, a master regulator of capsule synthesis and clumping (112). This phenotype in pathogenesis is probably linked to the high sensitivity of the mutant strain to opsonophagocytosis by host polymorphonuclear leukocytes. Because the expression of these three sRNAs is detrimental for bacterial spreading within colonized host organisms, one may suggest that during evolution they have favored commensalism with the host.

To evaluate the impact of sRNA expression in the context of host infection, two studies explored the possible relationships between infection severity and RNA

expression levels. In the first study, the expression levels of five sRNAs (RNAIII, RsaA, RsaE, RsaG, and RsaH) were analyzed in samples from acute cutaneous infection, cystic fibrosis sputum, or nasal colonization. The expression profiles did not correlate with the type of infection, but the authors noticed that the expression of these five RNAs was more homogeneous in the nasal colonization isolates than in those responsible for infection (96). More recently, the expression levels of RNAIII and SprD were measured in 40 strains cultivated from patients with sepsis or septic shock and compared to 21 strains isolated from asymptomatic colonized carriers. It appeared that strains from septic shock had significantly lower levels of RNAIII and to a lesser extent for SprD (99). It is important to note that this analysis was performed on clinical isolates cultured *in vitro* and does not necessarily reflect the expression of these RNAs during infection within the host. In fact, it is very difficult to obtain reliable data from *in vivo* studies to assess the role and importance of RNAs in the establishment or evolution of infection. The great variability of *S. aureus* strains, the difficulty in obtaining highly controlled cohorts of patients, and the reliability of sampling protocols, sample processing, and RNA expression analysis are all obstacles to be overcome. Moreover, biological variables may influence the analysis since the relationships between host immune systems and microbes seem to be particularly individualized and can influence the disease outcome (113, 114). Furthermore, interspecies interactions between bacterial pathogens and the commensal microbiota, as well as limited nutrients, play major roles in promoting or preventing *S. aureus* colonization (113). Interestingly, it was shown that the *agr* system is repressed by high concentrations of hemoglobin in the nasal fluids, leading to the expression of several cell surface proteins and favoring nasal colonization (115). Similar data were obtained when *S. aureus* was cocultivated with the nasal strain of *Corynebacterium striatum* (116). A recent study also demonstrated that the commensal *S. epidermidis* can influence the expression of one ncRNA of *S. aureus* (117).

Clearly, we are just beginning to better appreciate the roles of regulatory sRNAs during colonization and in the pathophysiology of *S. aureus* infections.

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