RNA molecules with regulatory functions in pathogenic bacteria have benefited from a renewed interest these two last decades. In Staphylococcus aureus, recent genome-wide approaches have led to the discovery that almost 10-20% of genes code for RNAs with critical regulatory roles in adaptive processes. These RNAs include trans-acting RNAs, which mostly act through binding to target mRNAs, and cis-acting RNAs, which include regulatory regions of mRNAs responding to various metabolic signals. Besides recent analysis of S. aureus transcriptome has revealed an unprecedented existence of pervasive transcription generating a high number of weakly expressed antisense RNAs along the genome as well as numerous mRNAs with overlapped regions. Here, we will illustrate the diversity of trans-acting RNAs and illustrate how they are integrated into complex regulatory circuits, which link metabolism, stress response and virulence.
The importance of regulatory RNAs in *Staphylococcus aureus*

Arnaud Tomasini\(^a\), Patrice François\(^b\), Benjamin P. Howden\(^{c,d,e,f}\), Pierre Fechter\(^a\), Pascale Romby\(^a\), Isabelle Caldelari\(^a\)*

\(^a\) Architecture et Réactivité de l’ARN, Université de Strasbourg, CNRS, IBMC, Strasbourg, France  
\(^b\) Genomic Research Laboratory, Department of Medical Specialties, Geneva University Hospitals, University of Geneva, Genève, Switzerland  
\(^c\) Austin Centre for Infection Research (ACIR), Infectious Diseases Department, Austin Health, Heidelberg, Victoria, Australia  
\(^d\) Microbiology Department, Austin Health, Heidelberg, Victoria, Australia  
\(^e\) Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria, Australia  
\(^f\) Department of Microbiology, Monash University, Clayton, Victoria, Australia

**Abstract**

RNA molecules with regulatory functions in pathogenic bacteria have benefited from a renewed interest these two last decades. In *Staphylococcus aureus*, recent genome-wide approaches have led to the discovery that almost 10–20% of genes code for RNAs with critical regulatory roles in adaptive processes. These RNAs include trans-acting RNAs, which mostly act through binding to target mRNAs, and cis-acting RNAs, which include regulatory regions of mRNAs responding to various metabolic signals. Besides recent analysis of *S. aureus* transcriptome has revealed an unprecedented existence of pervasive transcription generating a high number of weakly expressed antisense RNAs along the genome as well as numerous mRNAs with overlapped regions. Here, we will illustrate the diversity of trans-acting RNAs and illustrate how they are integrated into complex regulatory circuits, which link metabolism, stress response and virulence.

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* Corresponding author.  
E-mail address: l.caldelari@ibmc-cnrs.unistra.fr (I. Caldelari).
1. Introduction

*Staphylococcus aureus* is a versatile microorganism that is able to adapt to various ecological niches. It is also present in a large proportion of the population (almost 30% of individuals) as a commensal of skin and anterior nares, and conversely, causes a large range of hospital-acquired and community infections (Chambers and DeLeo, 2009; Tristan et al., 2007). This large variety of lifestyles is due to the fact that the bacteria produces a large array of virulence factors responsible for adhesion, invasion, and dissemination in host tissues, the acquisition of nutrients, and for combating the host immune system (Novick, 2003). In addition, two component systems, transcriptional regulatory proteins, and RNAs form intricate interactions and regulatory circuits that sense and respond to a large variety of signals to coordinate gene expression in a dynamic manner (Cheung et al., 2004; Romilly et al., 2012a).

During the last decades, tremendous works have shown that besides transcriptional regulatory proteins, small and usually non-coding RNAs (sRNAs) act as important players in adaptive processes in *Escherichia coli* and many other bacteria (Caldelari et al., 2013; Papenfort and Vogel, 2010). In *S. aureus*, the first reported regulatory RNA, the so-called RNAIII, was shown to be one of the main intracellular effectors of the agr quorum-sensing system (Novick and Geisinger, 2008; Novick et al., 1993). Later on, research on the discovery and functional characterization of sRNAs in *S. aureus* has exploded leading to an impressive repertoire of various regulatory RNAs (reviewed in Felden et al., 2011; Romilly et al., 2012a). Approaches for identifications were based on various computing softwares (Geissmann et al., 2009; Marchais et al., 2009; Pichon and Felden, 2005), Affymetrix microarrays (Anderson et al., 2006; Roberts et al., 2006) and tiling arrays (Lasa et al., 2011), conventional cloning/sequencing of small sized cDNAs (Abu-Qatouseh et al., 2010), and high throughput sequencing (Beaume et al., 2010; Bohn et al., 2010; Howden et al., 2013; Lasa et al., 2011; Lioliou et al., 2012). All these studies (see Table 1 for details) revealed that the sRNA genes are located randomly in the core genome and are also present in the horizontally acquired mobile elements. Transcription of many of these sRNAs is tightly controlled and responds to growth phase and to stress conditions suggesting that they are engaged in regulatory functions (Beaume et al., 2010; Bohn et al., 2010; Geissmann et al., 2009; Howden et al., 2013; Lasa et al., 2011; Roberts et al., 2006).

As in many bacteria, sRNAs in *S. aureus* are heterogeneous in size, structure and function. They can be very long (>500 nts) and some of them encode peptides. A large number of regulatory RNAs act on independently expressed targets (mRNA or proteins). Moreover, genome-wide analysis unexpectedly revealed the expression of a large number of short and long antisense RNAs (asRNAs), as well as mRNAs that present overlaps with the entire length or with some parts of their 5’ or 3’-untranslated regions (Lasa et al., 2011). The biological consequences of such antisense transcripts require further experimental investigation, but some examples have recently indicated regulatory roles (Howden et al., 2013; Lasa et al., 2011; Lioliou et al., 2012; Sayed et al., 2011). Another prominent class of regulatory RNA elements, the so-called riboswitches, are transcribed as part of their target mRNAs (Breaker, 2011; Dambach and Winkler, 2009). They are mostly located in the 5’ UTRs of some mRNAs and exhibit a structured receptor domain specifically recognized by a small metabolite. The binding induces a conformational change of the downstream mRNA that provokes premature transcription arrest, translation repression/activation, or cleavage. In general, riboswitches regulate the associated downstream genes, which are involved in the uptake or metabolism of the sensed metabolite (Breaker, 2011; Serganov, 2010). In *S. aureus*, riboswitches control almost 2% of the genes associated with metabolism and the most common respond to the intracellular concentrations of S-adenosylmethionine, thiamine pyrophosphate, flavin mononucleotide, lysine, glycine, guanine, 7-aminomethyl-7-deazaguanine, and glucosamine-6-phosphate (reviewed in Caldelari et al., 2011; Felden et al., 2011). Riboswitches are considered as promising targets for the design of novel antibacterial compounds that can overcome the current mechanisms of resistance, because they regulate essential genes and their structures reveal specific pockets for binding small compounds (Breaker, 2009; Mulhacher et al., 2010b).

Conservation of most sRNAs is restricted to the genus *Staphylococcus*, except for the cis-acting regulatory sequences and housekeeping RNAs. Although many of them are encoded within the *S. aureus* species, their expression appears to be strain-specific (Bohn et al., 2010; Geissmann et al., 2009; Pichon and Felden, 2005). Therefore the sRNA expression profiles might contribute to the diversity of phenotypes. With the new development of genome-wide approaches, the functional analysis of *S. aureus* sRNAs is expected to undergo a paradigm shift because it is now possible to monitor the expression profiles of, in principle, all RNAs and to gain knowledge on the impact of sRNAs on metabolism, adaptability to environment or host, resistance pathways or pathogenicity mechanisms. In this review, we will focus on trans-acting sRNAs in *S. aureus* for which the function has been solved. We will illustrate by specific examples that numerous sRNAs and associated proteins are found embedded in complex regulatory circuits, which links metabolism, stress response and virulence.

2. *S. aureus* sRNA at the crossroads of bacterial metabolism, stress response, and virulence

*S. aureus* is exposed to a number of environmental stress conditions during the infection process. It has been well documented that the transcription and synthesis of the virulence determinants are influenced by environmental and nutritional stresses, and by the energy metabolism (Somerville and Proctor, 2009). Interestingly, the synthesis of trans-encoded sRNA is induced upon stresses such as temperature change, acid or alkali, nutrient limitation, oxidative stress or antibiotic treatment that can be encountered during the host infection (Anderson et al., 2006; Beaume et al., 2010; Bohn et al., 2010; Geissmann et al., 2009). Other sRNAs are presumably part of the growth phase specific transcriptional responses controlling metabolism and possibly virulence.

2.1. sRNA and small-colony variant phenotype

Abu-Qatouseh et al. (2010) constructed cDNA libraries from samples collected at different growth stages for two isogenic derivatives of a clinical strain displaying the normal and the variant small-colony variant (SCV) phenotypes, respectively. SCVs are slow-growing and persistent bacteria with an increased ability to form biofilm and high resistance to aminoglycoside antibiotics. Although many alterations in metabolism can be associated with slow-growth, a limited effect on deficient electron transport has been described for the *S. aureus* SCV phenotype (Proctor et al., 2006). Additional features concern decreased pigmentation, hemo-lytic and coagulase activity, and respiration that can be linked to electron transport deficiency. A total of 142 sRNAs were identified and expression of 18 candidates was validated by Northern blot. These sRNAs originated from intergenic regions (IGRs) or were antisense or partially overlapping with ORFs. The expression of the newly identified sRNAs was growth phase regulated and most interestingly, for a subset of them comprising RNAIII, was phenotype-specific. Certain sRNAs were exclusively expressed in the
wild-type (WT) strain like RNAIII or were up-regulated in the SCV like RsaA and the pH responsive riboswitch element yybP-ykoY. Six deregulated candidate sRNAs encoded from pathogenicity islands or inserted phage sequences were also identified. An SCV-specific sRNA Sau-66, not expressed in the WT strain, may have a role in controlling inorganic ion transport through antisense control of SA0671. Whether the deregulation of sRNAs is a consequence or is responsible for the development of the SCV phenotype in S. aureus has to be clarified.

2.2. sRNA and the TCA cycle

The tricarboxylic acid (TCA) cycle is one of the pathways leading to the production of biosynthetic intermediates required for the synthesis of macromolecules. When environmental conditions change and nutrients become growth limiting, S. aureus increases TCA cycle activity to catabolize nonpreferred carbon sources, i.e., acetate. Numerous studies have shown that the TCA cycle has an impact on the production of virulence factors through the inhibition of biosynthetic enzyme activity but also by regulating the transcription of target genes (Somerville and Proctor, 2009). Interestingly, two studies have shown that RsaE, a small non-coding RNA, is involved in the regulation of several metabolic pathways (Bohn et al., 2010; Geissmann et al., 2009). RsaE is conserved in Staphylococcus, Macrococcus and in Bacillus (Geissmann et al., 2009), all derive from a common Gram-positive ancestor. Using combined transcriptome/proteome analysis, it was demonstrated that RsaE regulates enzymes involved in amino acid and peptide transport, cofactor synthesis, lipid, purine and carbohydrate metabolisms, and the TCA cycle (Fig. 1). Consistently, overproduction of RsaE induced a growth defect that is partially alleviated by acetate (Bohn et al., 2010). For three targets, oppB (amino acid and peptide transporter), sucD (succinyl-CoA synthetase of the TCA cycle) and SA0873 (unknown function) mRNAs, direct repression was proposed to occur by the formation of base-pairings between a conserved and unpaired C-rich motif of RsaE with the SD sequence.
of these mRNAs to inhibit translation (Geissmann et al., 2009). More recently, new RsaE target candidates have been identified using bioinformatics combined with high throughput sequencing approaches (Lioliu et al., 2012, 2013). In many clinical strains, RsaE is expressed at the exponential phase and repressed at the stationary phase of growth (Bohn et al., 2010; Song et al., 2012). Hence, RsaE would facilitate the transition of energy metabolism, purine biosynthesis and amino acid transport in response to nutrient availability, and would contribute to the cell reprogramming during the growth phase. However, in RN6390, a σE-deficient strain, the expression of RsaE was enhanced at the stationary phase of growth in a manner dependent on the agr system (Geissmann et al., 2009). Due to the interplay between metabolism and virulence factor production (Somerville and Proctor, 2009), RsaE is expected to play an indirect role in virulence (Fig. 1).

2.3. SigmaB-dependent sRNAs

The stress-dependent activation of Sigma B has been the subject of intensive works in order to understand the influence of environmental conditions on virulence factor biosynthesis. Sigma B was found activated under general stress conditions, upon growth phase transitions and during morphological changes of S. aureus (reviewed in Van Schaik and Abee, 2005). Sigma B is also involved in the regulatory networks controlling the expression of virulence determinants, modulates antibiotic resistance and biofilm formation. Hence the Sigma B regulon includes many different cellular processes. Sigma B regulates the expression of genes by binding to a consensus promoter sequence upstream of the target genes. However, it was also found that many of these Sigma-B-dependent genes are not preceded by a consensus promoter sequence, implicating a role for other downstream regulators. This is why several teams searched for sRNAs that might be part of the Sigma B regulon (Geissmann et al., 2009; Nielsen et al., 2011) (Fig. 1). Among them, rsaA gene is under the control of a typical Sigma B-dependent promoter (Geissmann et al., 2009). Recent work suggests that RsaA regulates the translation of a master regulatory protein to regulate capsule and biofilm formation (Romilly et al., unpublished data). Prediction of Sigma B-promoters within intergenic regions of S. aureus revealed three other sRNAs, which were induced by KOH treatment. Two of them might be bifunctional RNAs because they potentially encode small and highly basic peptides, which are conserved among staphylococci (Beaume et al., 2010; Nielsen et al., 2011). The third encodes a genuine asRNA, which interacts in vitro with the 3′-end of the downstream gene on the opposite strand (Nielsen et al., 2011). Although antisense regulation has not yet been demonstrated in vivo, these data suggested that SbrC would regulate the synthesis of an ABC transporter specific for the uptake of manganese that is important for effective host colonization by S. aureus (Nielsen et al., 2011). Global approaches such as chromatin immunoprecipitation followed by HTS would be useful to reveal the whole set of sRNAs that are part of the Sigma B regulon.

2.4. Expression of sRNAs under antibiotic exposure

Antibiotic treatment can be regarded as one of the most selective pressures on S. aureus, including within the host environment. As a major public health problem, some S. aureus clones have become resistant to most antimicrobial agents including the commonly used penicillin-related antibiotics. Multi-drug resistant S. aureus have been now reported with increasing frequency worldwide. Strains that are resistant to methicillin (methicillin-resistant S. aureus; MRSA) are often also resistant to lincosamides, macrolides, aminoglycosides, fluoroquinolones, or combinations of these antibiotics. More recently, intermediate resistance to vancomycin – a glycopeptide antibiotic that is one of the last line agents active against MRSA – has been reported globally. Community-associated MRSA (CA-MRSA) strains seem also to be particularly virulent (Chambers and Deleo, 2009). Recently, the transcriptional response of sequence type 239 (ST239) multidrug resistant S. aureus to antibiotic exposure was investigated using Illumina HTS, with a particular focus to decipher the potential role of sRNAs in antimicrobial response (Howden et al., 2013). In this study, four antibiotics were used (vancomycin, linezolid, ceftobiprole and tigecycline). A large number of putative sRNAs were identified in this strain (409 in total), and 42% of them (171) were antisense to mRNAs. Seventy-nine of the sRNAs were encoded from the S. aureus accessory genome, indicating that 330 core genome encoded putative sRNAs are conserved in all S. aureus included in the analysis (19 strains). Upon drug exposure, the most significant changes were differential regulation of asRNAs, predominantly targeted against genes encoding proteins involved in translation and ribosomal function, such as subunits of RNA polymerase, translation elongation factors and ribosomal proteins. Linezolid, an oxazolidinone antibiotic that acts by binding to 23S rRNA, produced the most profound transcriptional responses. These results suggested a contribution, through control of protein synthesis and of asRNAs to the bacterial response to antimicrobials. In addition, one putative sRNAs (sRNA260) and one experimentally validated sRNA RNA131 (also called RsaOII) were upregulated. RsaOII is a small non-coding RNA conserved in all staphylococcal strains and its synthesis accumulates at the stationary phase of growth (Marchais et al., 2009). RsaOII was proposed to adopt a particular structure involving two long distant interactions (Marchais et al., 2010). Intriguingly, rsaOII gene is located in the vicinity of the vra operon that is upregulated in some vancomycin intermediate S. aureus strains (Howden et al., 2013). Ongoing work is underway to definitely link sRNA expression to drug resistance.

Fig. 2. Interplay between RNAs and RNA-binding proteins in Staphylococcus aureus. The major complex of RNA degradation, the so-called degradosome (circled in red), comprises the membrane-anchored R하세요 A, the two non-essential RNases J1/J2, the RNA helicase CshA and the central metabolism-related enolase. PNFase and phosphofructokinase are also part of the complex but not shown for figure simplicity (see Roux et al., 2011 for further details). RNase Y is involved in the degradation of several mRNAs (saePQRS mRNAs shown for example) and ncRNAs RsaA and sun63 (Marincola et al., 2012). The double-stranded RNA specific RNAse III is not associated with the degradosome but is nonetheless one of the most important enzyme involved in RNA degradation. The agrBCDA mRNA degradation is mediated by CshA, the RNA helicase component of the degradosome (Oun et al., 2013). It has also been shown that the transcriptional factor SarA has a positive effect on agr mRNA stability but whether it is indirect by inhibiting degradation by CshA is yet to be elucidated (Morrison et al., 2012a).
3. sRNAs acting directly on virulence gene expression

Regulatory RNAs dedicated to the regulation of virulence genes are thought to be acquired more recently in the evolution of the bacteria, and could be considered as key adaptors to the host (Queck et al., 2008). In this paragraph, we will highlight recent studies to illustrate the direct role of sRNAs in the virulence of S. aureus, their integration in pre-existing regulatory circuits and the diversity of their expression according to the strain and/or infection, that may contribute to the adaptation of the bacteria to the variability of the host responses.

3.1. Two intertwined regulatory networks quorum sensing and virulence factor control

Quorum sensing is widely used by pathogens to regulate virulence gene expression (Bassler and Losick, 2006). In S. aureus, quorum sensing is mainly accomplished by the agr system. This system is composed of two divergent transcripts, RNAII encoding a quorum sensing cassette and a two-component system, and RNAIII encoding hemolysin delta. The quorum sensing cassette produces the autoinducer peptide, AIP, which upon a threshold concentration, activates the membrane kinase AgrC and the response regulator AgrA through a phosphorylation mechanism. AgrA induces the expression of RNAI, and RNAIII. While most studies were dedicated to decipher the function of RNAIII, the main intracellular effector of gene virulence (Novick et al., 1993; Novick, 2003), a recent study on a methicillin-resistant S. aureus strain revealed that the agr system is composed of two independent regulatory circuits, with two principal effectors, AgrA and RNAIII (Queck et al., 2008). While RNAIII coordinates the expression of virulence factors, AgrA up-regulates Phenol-soluble-modulin (PSM) cytolysin genes by direct binding to promoters and down-regulates genes involved in carbohydrate and amino acid metabolism by an unknown mechanism (Queck et al., 2008). Thus the AgrA-dependant regulatory circuit may be reminiscent of the ancient function of the quorum sensing system, in a non-pathogenic life style of S. aureus, where agr enables cells to adapt to low nutrition conditions in high cell density population. Otherwise, not only the agr operon is controlled by transcriptional factors like SarA, MgrA, but agr mRNA itself is negatively regulated by different factors (see Figs. 1 and 2 and text below). Theagr system is confirmed as a pivotal element in S. aureus virulence allowing the bacteria to integrate a multitude of signals.

3.2. S. aureus RNAIII regulates the temporal expression of virulence determinants

The bifunctional RNAIII encodes a PSM, delta hemolysin. If both delta hemolysin and RNAIII play roles in virulence, they do not act synergistically. Interestingly, hld expression is delayed by 1 h after the transcription of RNAIII (Novick, 2003). This is not in line with the accessibility of the ribosome binding site (RBS) sequence of hld since it was shown that ribosomes bind to RNAII in vitro (Bénito et al., 2000). Thus, it is possible that a trans-acting factor could be involved either to repress hld translation during the first hour, or to activate its translation. As a regulator, RNAII promotes the switch between the expression of surface proteins and the synthesis of excreted toxins in a time-dependent manner (Novick, 2003). The regulation mechanisms by RNAII have been extensively documented in the last years (reviewed in Caldelari et al., 2011; Romby and Charpentier, 2010; Romilly et al., 2012a). It controls gene expression at the post-transcriptional level via an antisense mechanism. The 3′UTR of hld binds to the leader region of hla mRNA to facilitate ribosome recruitment while the large 3′UTR is acting primarily as a repressor domain. The 3′UTR contains three redundant hairpin structures with a conserved C-rich sequence located in the apical loops. This motif is often used to bind the C-rich sequences of the ribosome binding sites of mRNAs. The duplexes formed between the RNAIII and the targeted mRNAs prevent ribosome binding and recruit the double-strand specific RNase III, which initiates the rapid degradation of both the mRNAs and the RNAIII (Boisset et al., 2007; Chevalier et al., 2010; Huntzinger et al., 2005). These mRNAs encode virulence factors expressed at the surface of the cell (protein A, coagulase, SA1000), and the transcriptional repressor of toxins, Rot. Through the inhibition of Rot, RNAII indirectly activates the transcription of exotoxins and represses the transcription of adhesins such as protein A (Boisset et al., 2007; Geisinger et al., 2006). Thus, RNAIII acts directly at the post-transcriptional level by targeting mRNAs, and indirectly at the transcriptional level by repressing the translation of Rot, through the formation of two coherent feed-forward regulatory loops as exemplified in Fig. 1. Such networks ensure a tight regulation of gene expression regulation (Shimoni et al., 2007, 2009). They may also explain the delay between repression of adhesins synthesis, which occurs directly through RNA–RNA pairing, and the induction of exoprotein synthesis, which requires first the inhibition of rot translation (Novick, 2003). Interestingly, RNAIII also represses several hydrolases and amidases involved in the metabolism of peptidoglycan and hence it may contribute to the cell wall integrity at high cell density (Boisset et al., 2007; Chunhua et al., 2012; Dunman et al., 2001) (Lioliou et al., personal communication). Regulation of peptidoglycan metabolism, and not virulence only, might be the common function of RNAIII in all staphylococcal species including Staphylococcus epidermidis.

RNAIII is thus a multi-functional RNA and this is probably not an exception in S. aureus (Kaito et al., 2011; Fig. 1). Although it is clear that RNAIII regulates gene expression at the post-transcriptional level, the whole set of direct targets has not yet been fully elucidated. Strong evidences suggested that RNase III acts as a co-factor of RNAII to induce rapid degradation of repressed target mRNAs. However, no protein such as Hfq has been found to be involved in the formation of RNAIII–mRNA complexes, or in RNAIII stability. Do the two functions of RNAII, synthesis and regulation, interfere with each other? Recent work suggested that part of the coding sequence of hld could bind to map mRNA encoding the major histocompatibility complex class II analogous protein to activate translation (Liu et al., 2011). In addition, part of the ribosome binding site of hld is also probably engaged in base pairings with hla mRNA. Therefore activation of translation of target mRNAs and hld translation are most probably two exclusive functions of RNAIII. Finally, although all sequenced S. aureus strains contain the RNAII locus, the yield of RNAIII strongly varies. The regulators involved in the transcriptional control or its stability are not fully understood although it is well known that many transcriptional regulators converged to the agr system to modulate the virulence factor production (Figs. 1 and 2). This might confer the advantage to the bacteria to respond to multiple environmental cues in addition to cell density (Priest et al., 2012).

3.3. SprD mediates a crosstalk between pathogenicity island and the core genome to regulate virulence

SprD is the second sRNA emerging as an important player in the regulation of virulence. It is a 142-nts long sRNA expressed from a pathogenic island, Pla (Chabelskaya et al., 2010; Pichon and Felden, 2005) and interacts through base pairings with the sbi mRNA, which encodes an immune evasion molecule protecting the bacteria against host immune responses. Interestingly, sbi is encoded on the core genome, suggesting that SprD mediates connections between the pathogenic island and the core genome. SprD contains
four hairpin structures and the third one interacts with the ribosome binding site of the sbi mRNA to form a long duplex that prevent translation initiation in vitro (Fig. 1). As for RNAIII, SprD does not require trans-acting factors like Hfq, but sbi mRNA degradation is not promoted by SprD expression. Interestingly enough, the yield of sbi mRNA strongly decreased at the stationary phase of growth independently of SprD (Chabelskaya et al., 2010). This data suggested that a second regulatory event might take place. Finally, SprD contributes to S. aureus diseases in a mouse model of infection, but this effect was not linked to the repression of Sbi production, indicating that SprD might regulate the expression of other proteins important for infection.

3.4. A pathogenicity-island sRNA attenuates MRSA virulence

In the USA, community acquired methicillin resistant S. aureus (CA-MRSA) has been shown to be more virulent than hospital associated MRSA. Recent work discovered that this behavior is at least partly linked to the existence of a transcription product of the psm-mec gene which is located in the SCCmec of the hospital acquired MRSA (HA-MRSA) but not in the equivalent SCCmec locus of CA-MRSA (Kaito et al., 2011, 2013). Psm-mec is a bifunctional RNA, which encodes a phenol-soluble modulin (PSMα), a cytolytic toxin. In addition, the psm-mec mRNA inhibits translation of agrA mRNA by direct base-pairings, with the coding sequence of the mRNA (Kaito et al., 2013). It remains to be determined how psm-mec RNA is able to repress the translation of agrA mRNA. It is more likely that duplex formation might recruit a ribonuclease to initiate mRNA degradation. Deletion of the psm-mec RNA from HA-MRSA strains increased the expression of AgrA which results in an increase of toxin production and of PSMα and enhanced virulence in mice (Kaito et al., 2013). Interestingly 25% of 325 clinical MRSA isolated in Japan contained a mutation in the psm-mec promoter and 9% did not carry psm-mec leading to high level of PSM in the supernatant. Identification of the psm-mec mutation could be a novel method to predict virulence properties of MRSA strains. This study shows that another bi-functional RNA is able to affect virulence through AgrA inhibition, and illustrates how the acquisition of novel genetic elements and of sRNA alters the behavior of the bacteria.

### Table 1
Summary of experimentally validated sRNAs in S. aureus.

<table>
<thead>
<tr>
<th>Study</th>
<th>Strain used</th>
<th>sRNA discovery methodology (number of in silico predicted sRNAs)</th>
<th>Experimentally validated sRNAs#</th>
<th>Experimental validation method</th>
<th>Target and mechanism</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roberts et al. (2006)</td>
<td>UAMS-1 (clonal complex 5)</td>
<td>Gene chip analysis</td>
<td>SsrA2</td>
<td>NB, RT-PCR</td>
<td>Spa, hla, hgcL, lukF</td>
<td>Unknown</td>
</tr>
<tr>
<td>Pichon and Felden (2005) and Sayed et al. (2011)</td>
<td>Mu50 (clonal complex 5)</td>
<td>Bioinformatic predictions (xx)</td>
<td>SprA, SprA2, SprA3, SprB, SprC, SprD, SprF, SprG, SprFG2, SprFG3</td>
<td>NB#</td>
<td>ABC transporter (SA2216), possible antisense sRNA</td>
<td>Unknown, SprA2 encodes a cytolytic peptide</td>
</tr>
<tr>
<td>Geissmann et al. (2009)</td>
<td>RN6390, Col, Newman, HG001</td>
<td>Bioinformatic predictions and experimental validation</td>
<td>RsAE, RsA, RsB, RsC, RsA, RsD, RsA, RsC, RsA, RsH, Rsald, Rsao, Rsak</td>
<td>NB, PE, RACE</td>
<td>Masking of ribosomal binding site for oppB, sucD, SA0873</td>
<td>Unknown, SprA encodes a ribonuclease</td>
</tr>
<tr>
<td>Marchais et al. (2009)</td>
<td>N315 (clonal complex 5)</td>
<td>Bioinformatic (NAPP) (189) and Northern analysis</td>
<td>RsOA, RsDB, RsOC, RsOD, RsOE, RsOF, RsAg, RsOG</td>
<td>NB#</td>
<td>Unknown</td>
<td>Genetic manipulation demonstrated a role for RsAE in controlling metabolic pathways</td>
</tr>
<tr>
<td>Nielsen et al. (2011)</td>
<td>N315 (clonal complex 5)</td>
<td>Bioinformatic search for intergenic conserved sites and experimental validation</td>
<td>Sbra (SaOO), SbraA, SbraB, SbraC</td>
<td>NB#</td>
<td>c^2 regulated. SbraC interacts with SA0587 (mntC)</td>
<td>sbra and sbrB potential CDS</td>
</tr>
<tr>
<td>Abu-Qataseh et al. (2010)</td>
<td>-</td>
<td>Cloning and sequencing of cDNAs</td>
<td>Ssr-72, Ssr-80, Ssr-87</td>
<td>NB#</td>
<td>Unknown</td>
<td>142 candidate sRNA identified</td>
</tr>
<tr>
<td>Bohn et al. (2010)</td>
<td>N315 (clonal complex 5)</td>
<td>454 pyrosequencing followed by experimental validation</td>
<td>RsaON (RsaE)</td>
<td>NB#</td>
<td>Unknown</td>
<td>30 sRNAs identified, 14 new</td>
</tr>
<tr>
<td>Beaume et al. (2010)</td>
<td>N315 (clonal complex 5)</td>
<td>Illumina sequencing</td>
<td>Teg1, Teg4, Teg17, Teg18, Teg19b, Teg21, Teg24, Teg26, Teg28, Teg35, Teg38, Teg42, Teg45, Teg47, Teg55, Teg56, Teg57, Teg60, Teg61, Teg69, Teg70, Teg72, Teg73, Teg76, Teg91, Teg2ipl</td>
<td>RT-PCR</td>
<td>NB</td>
<td>195 sRNAs predicted by HTS</td>
</tr>
</tbody>
</table>

# Validated using Northern blot, RNA extremity mapping, or RT-qPCR.
# Also experimentally validated using Northern blot in the study by Abu-Qataseh et al. (2010).
# Originally described by Anderson et al. (2006).
# Previously described and validated. NB, Northern blot; PE, primer extension; RACE, random amplification of cDNA ends; RT-PCR, real-time PCR. Several long and stable RNAs (SSR) expressed under particular conditions of growth have been assigned by microarrays Roberts et al. (2006).
3.5. Another long sRNA contributes to pathogenesis

A subset of RNA molecules, called small stable RNAs (SSRs), has been previously identified by microarrays (Anderson et al., 2006; Roberts et al., 2006). These SSRs are either transcribed or stabilized in response to growth phase, stringent, heat and cold shocks, alkaline and acidic conditions. Among these RNAs, stability of SSR42 is greatly enhanced under stationary phase of growth (Anderson et al., 2006; Morrison et al., 2012b). This 891-long RNA affects erythrocyte lysis, resistance to opsonisation killing, and pathogenesis in a murine model of skin and soft tissue infection (Morrison et al., 2012b). The RNA upregulates genes involved in capsule biosynthesis, and downregulates 80 genes including several cell surface virulence factors. While no direct binding was demonstrated between SSR42 and mRNA encoding virulence determinants, the effect is predicted to be indirect by modulating the expression of a transcriptional regulator, and more experiments would need to be performed to decipher the molecular mechanisms of regulation. The genomic localization of SSR42 does not exclude that the RNA might regulate the upstream and divergent gene through an antisense mechanism, because the two genes are separated only by few nucleotides and recent highthroughput sequencing analysis suggested that the 3′ end of SSR42 overlaps with the end of the coding sequence of the upstream gene (Lioliou et al., 2012). This long RNA was co-immunoprecipitated with the endoribonuclease III (RNaseIII) together with an asRNA at the exponential phase of growth in RN6390 (Lioliou et al., 2012). While SSR42 is unstable at the exponential phase of growth (Morrison et al., 2012b), it is tempting to propose that antisense regulation mediated through RNase III would regulate either the stability of SSR42 or of the flanking genes in a growth phase dependent manner. Recent tilting arrays analysis discovered that S. aureus genome expressed long antisense RNAs that are rapidly degraded by RNase III (Lasa et al., 2011). Such asRNA regulation is expected to play important regulatory functions (reviewed in Lasa et al., 2012).

3.6. Expression of sRNAs in S. aureus during infection

Most sRNAs have been identified in a few strains, and their expression profiles during infection in human have been studied for only a subset of sRNAs including RNAIII. The vast majority of clinical isolates from acute infections express RNAIII (Traber et al., 2008), but the level of RNAIII expression varies among isolates (Jelsbak et al., 2010). However, agr-mutant strains, which most probably arose during infection, were isolated from patients (Traber et al., 2008). Interestingly some of the agr-mutant strains have been associated with persistent bacteremia and dormant states, notably for methicillin-resistant S. aureus infection (Fowler et al., 2004; Sakoulas et al., 2002). These observations are also supported by the fact that the SCV phenotype is deficient in RNAIII synthesis (Proctor et al., 2006). Thus, the appearance of agr mutations might reflect an adaptation of the pathogen within the infected host. Recently Song et al. (2012) compared the expression profile of 5 sRNAs (RNAIII, RsaA, RsaE, RsaG, RsaH) of 60 isolates from acute and chronic infections, and from nasal colonization. The expression levels of the sRNAs were measured in the clinical samples and compared with those of the corresponding strains grown in rich culture medium. They showed that all studied sRNAs are expressed in human, and that their expression is strain specific, with consequent variation among strains. Particularly, their levels were highly variable in the abscessed patients, more homogeneous in the cystic fibrosis patients, and highly uniform in the nasal carriers samples. The comparison of the sRNA expression in infection versus colonization specimens suggests that the sRNA expression levels observed in infection would be a consequence of the great variability of the host response, while colonization is apparently a more uniform scenario.

4. RNA-binding proteins

4.1. Hfq protein, a mysterious protein in S. aureus

There is currently very limited information on staphylococcal RNA-binding proteins that might be involved in sRNA regulation. Hfq is present in most of the staphylococcal genomes, but differentially expressed (Liu et al., 2010). Its role in vivo is still controversial, while an hfq deletion mutant has no significant phenotype (Bohn et al., 2007; Romilly et al., 2012a). Several sRNAs, such as RNAIII or RsAE, and SprD, repress their target mRNAs either by forming long irregular duplexes, or by forming bi-partite binding sites involving distant and unpaired regions of both RNAs. In all cases, fast binding is initiated by G–C base pairs present in loop regions (Fig. 1). Hence, long complementarities or bipartite binding sites might compensate the need of Hfq. The dispensability or requirement of Hfq might have resulted from numerous specific features of Hfq, sRNAs and their RNA duplexes, which still have to be clearly addressed. Although the crystal structures of S. aureus Hfq associated with short RNA sequences were the first to be solved (Brennan and Link, 2007), its function still remains mysterious. The protein adopts an homohexamer structure organized into a doughnut with a proximal and distal faces recognizing unpaired uridine-rich and adenine-rich sequences, respectively (e.g., Brennan and Link, 2007; Horstmann et al., 2012; Link et al., 2009; Mukeley et al., 2004; Someya et al., 2012; Soper et al., 2011). In enterobacteriaceae, the proximal and distal faces of the Hfq hexamer specifically bind sRNA and mRNA targets. Interestingly, a recent study discovered, that a motif rich in arginines (RRER) located on the outer rim of the hexamer, are required for the chaperone activity of Hfq (Panja et al., 2013). Mutations in the arginine patch of E. coli Hfq affected sRNA regulation and prevented annealing of the two RNAs eventhough binding was not affected suggesting that the RRER motif constitutes the active site of the chaperone activity. This arginine patch is replaced by a KANQ motif in S. aureus Hfq (Panja et al., 2013). It remains to be analyzed whether this sequence difference explains why Hfq is not required for many sRNAs in S. aureus. These different RNA binding sites might explain why S. aureus Hfq cannot complement Salmonella typhimurium or E. coli hfq mutant strains (Rochat et al., 2012; Vecerek et al., 2008).

4.2. Ribonucleases as possible co-factors of sRNAs

The role of several ribonucleases has been recently well documented in S. aureus virulence. RNase III is responsible to degrade double-stranded RNA formed by base-pairing of RNAIII with its mRNA targets such as spa, coa and rot (Fig. 1). The enzyme recognizes various topologies, rather than specific sequences and cleaves at sites such as imperfect duplexes, helices interrupted by bulged residues and kissing loops between RNAIII and coa or rot mRNAs by examples (Romilly et al., 2012b). Moreover, in two different studies, RNase III has also been clearly identified as a major partner in antisense regulation, beside its known role in mRNA and sRNA turnover and rRNA or tRNA maturation (Lasa et al., 2011; Lioliou et al., 2012). At least four key types of antisense transcription have been defined in S. aureus (see Fig. 3): (a) bona-fide short and long antisense RNAs, (b) overlapping 5′ or 3′-untranslated region of divergent or convergent mRNAs, (c) overlapping operons and (d) type I toxin-antitoxin modules (Lasa et al., 2012). The biological significance of such overlapping transcription is still unclear, but the role of RNase III in antisense regulation seems to happen most frequently in Gram-positive bacteria such as S. aur-
Indeed, RNase III is responsible to cleave sense–antisense RNAs duplexes leading to accumulation of 20 nucleotides fragments from all over the chromosome. At least 75% of mRNAs were subject to this processing, which reflects the importance of the phenomenon that might function as a general purpose global control on bacterial gene expression.

Regarding the membrane-bound endoribonuclease RNase Y, it cleaves unpaired regions of RNA and was shown to be part of the degradosome-like complex together with RNases J1/J2, PNPase, enolase, phosphofructokinase, and a DEAD box RNA helicase in S. aureus (see below, and Roux et al., 2011)(Fig. 2). RNase Y was shown to affect production of virulence factors either through expression of the accessory gene regulatory locus, agr or independently (Kaito et al., 2005; Nagata et al., 2008). Recently Marincola et al. (2012) performed a transcriptomic analysis to decipher the effect of RNase Y on global gene regulation. At least 75% of mRNAs were subject to this processing, which reflects the importance of the phenomenon that might function as a general purpose global control on bacterial gene expression.

The CshA DEAD-box helicase was reported to interact with proteins from the degradosome (Roux et al., 2011). It has the typical RNA-dependent ATPase activity of DEAD-box proteins, and plays a role in the steady-state level and stability of agrA mRNA, which indirectly affects the level of RNAIII and the virulence of the bacterium (Oun et al., 2013)(Fig. 2).

### 4.3. A transcriptional regulator affects RNA stability

Very recently, the pleiotropic transcriptional regulatory protein SarA was unexpectedly identified as an RNA binding protein. It altered mRNA turnover by stabilizing few transcripts associated to virulence, like clpP, spa or agrA in the late exponential or stationary phase of growth, suggesting that binding of the protein would protect mRNAs from degradation (Morrison et al., 2012a; Roberts et al., 2006). One cannot exclude that the effect on agrA mRNA might be indirect by inhibiting its degradation by CshA (see above and Fig. 2). Moreover, using in vitro and in vivo approaches, SarA

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**Fig. 3.** Four mechanisms to generate overlapping transcription in S. aureus. Purple boxes represent the transcriptional start sequences and genes annotations are according to the S. aureus N315 genome. (a) Bone fide antisense RNA (asRNA): hu mRNA may be rapidly degraded by the dual action of the hu asRNA and RNaseIII (Lioliou et al., 2012). (b) Overlapping 5' untranslated region: RNase III cleaves the overlapping 5' UTR of the tagG/tagH resulting in the formation of a tagH mRNA with a shorten leader (Lioliou et al., 2012). (c) Overlapping operon: sa0254 is located in the middle of the operon lgrA sa0255 bglA and is transcribed in opposite direction to the rest of the operon (Lasa et al., 2011). (d) Type I toxin–antitoxin: SprF3/SprG3 may belong to the group I toxin-antitoxin systems, with SprG being the putative toxin. Whether SprG3 encodes a peptide is currently unknown (Pichon and Felden, 2005). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
protein was found to bind spa mRNA in a cooperative manner. It was also found to destabilize mRNAs or bind to transcripts that mapped to intergenic regions, which might encode sRNAs. Thus, SarA modulates the production of proteins at the mRNA level via both transcriptional and post-transcriptional pathways. The use of UV cross-linking and immunoprecipitation assay (CLIP) would be appropriate to identify direct interaction sites between SarA and RNAs in vivo (Wang et al., 2009).

5. Concluding remarks

A rather good knowledge on the sRNA repertoire in specific S. aureus strains is now available but significant work is still required to gain a full appreciation of the sRNA functions, of their regulatory networks and their interactions with proteins. The few examples here demonstrate already how sRNAs modulate the expression of key transcriptional factors and how several sRNAs converged to form extensive regulatory networks to regulate virulence factors (Fig. 1). One of the future tasks will be to understand how the different networks are interconnected, and how they influence each other to develop variations in the pattern of virulence gene expression. The use of high-throughput sequencing approaches have revealed hundreds of unexpected transcripts in both sense and antisense orientations, and numerous short transcripts derived from long 5' or 3'UTRs, or internal regions of mRNAs have been detected. Whether they behave as independent regulatory RNAs requires experimental validation. Other sRNAs encode peptides that may play a role in virulence or in growth control. Continued mechanistic and functional studies of individual sRNAs and their machineries will certainly unravel unexpected functions and regulatory mechanisms. For instance, the asRNA SprA1-AS, which regulates the expression of a cytolytic peptide SprA1, binds to the target RNA in a very unusual way (Sayed et al., 2011). Unexpectedly, the interacting region does not involve the 3' end of SprA1-AS, which is fully complementary to the 3' end of the target SprA1 RNA but instead, the active region of the asRNA is in its 5' part that is partially complementary to the ribosome binding site of SprA1. These findings demonstrate that the structures of both RNAs were determinant for the regulatory mechanism.

We illustrate here how S. aureus sRNAs interconnect metabolism, stress adaptation, cell-to-cell communication and virulence, and how they are able to mediate crosstalks between pathogenicity islands and the core genome. Other avenues can be further explored such as modulation of the bacterial chromosome structure, control of mobility of pathogenicity islands, and host–pathogen interaction. One critical point is the fact that it is difficult to generalize the impact of sRNA regulation in S. aureus due to great genetic and phenotypic variability between the strains. In addition, the expression of sRNA in S. aureus grown in rich medium culture does not reflect the in vivo conditions. Hence, a major challenge is to understand the contribution of S. aureus sRNAs in the human context and to gain knowledge about the sRNA roles and their co-factors during human colonization and infection (Westermann et al., 2012).

S. aureus resistance to antimicrobial drugs has become a major health problem. Mechanisms of bacterial defense against antibiotics include multiple strategies such as mutation of the drug target, inactivation or destruction of the agent, inhibition of cell division during exposure to certain antibiotics and repression of transport systems to avoid the antimicrobial entry. Whether sRNAs are involved in the control of S. aureus adaptability to antimicrobial agents is still poorly understood. More recently, an aminoglycoside-binding riboswitch that is widely distributed among antibiotic-resistant bacterial pathogens as S. aureus, has been discovered. This riboswitch is present in the leader regions of mRNAs and activates the production of enzymes that confer resistance to aminoglycoside antibiotics when these are present in the environment (Jia et al., 2013). The authors proposed a model of an antibiotic-sensing riboswitch, whose structure will change upon drug binding unmasking the RBS and leading to mRNA translation. This simple mechanism allows the cells to rapidly respond to the antibiotic killing effect and might be exploited by other RNA-binding antibiotics. Conversely, recent promising works have shown that metabolite-binding riboswitches can be exploited to avoid multiple drug resistance. For instance, in S. aureus, several non-metallobinding agonists were designed to constitutively switch off the expression of the essential gusA gene encoding GMP synthase, which is under the control of a guanine-sensing riboswitch. These compounds inhibit growth and reduce S. aureus infection in mice (Mulhbacher et al., 2010a). Hence, manipulating individual sRNA regulatory pathways opens new avenues to explore novel ways to combat S. aureus diseases. There are still many exciting discoveries and unanswered questions to be solved on S. aureus sRNAs.

Acknowledgements

We are thankful to all other members of the team for helpful comments on the manuscript. We are grateful to François Vandenesch and to Thomas Geissmann (University of Lyon, INSERM) for helpful discussions and support. This work was supported by the Centre National de la Recherche Scientifique (CNRS, PR), the Agence Nationale de la Recherche and the “Laboratoires d’excellence” (LABEX) NetRNA Grant ANR-10-LABX-36 (PR) in the frame of «Programme d’Investissements d’Avenir», and the National Health and Medical Research Council (NHMRC), Australia (BPH).

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