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Review

The importance of regulatory RNAs in *Staphylococcus aureus*

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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.

Contents

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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 trans2013135-cription requires 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; Mulhbacher 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, hemolytic 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

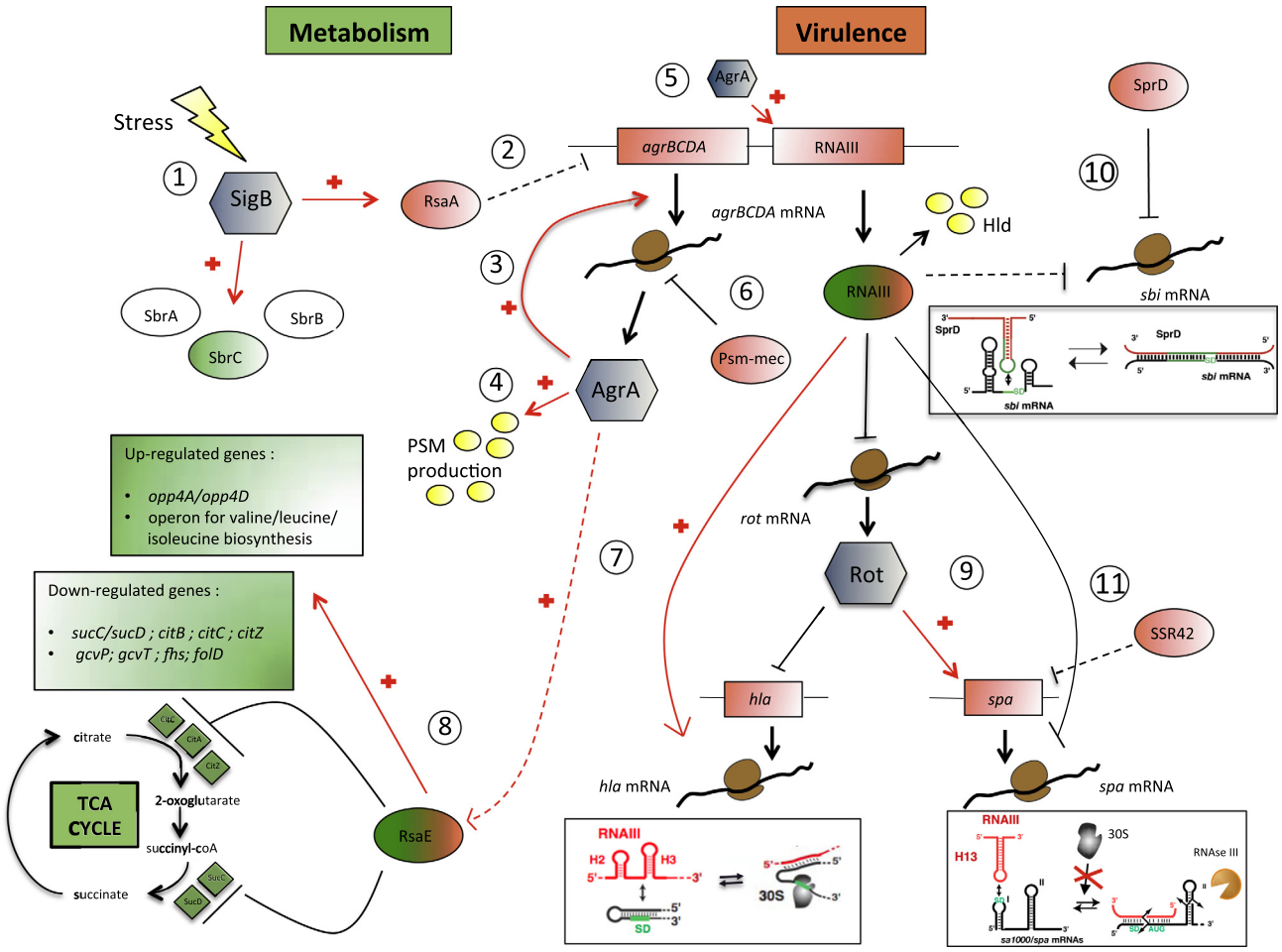

Fig. 1. RNAs and transcriptional factors link metabolism and virulence in *S. aureus*. The left panel of the figure shows molecular effectors involved mainly in *S. aureus* general metabolism (green boxes) and the right panel those involved in virulence (red boxes). Transcriptional factors are represented by grey hexagons and all regulatory RNAs by red ovals (except for RNAIII and RsaE which are also colored in green to emphasize their dual roles in central metabolism and virulence while SbrA/C are uncolored because no functions have been assigned yet). Red arrows and red crosses shows a positive effect on expression, in contrast black connections with a terminal bar denote a negative effect, dashed lines represent putative interactions: (1) Several stress factors (pH, T°C, nutrients availability) can trigger the expression of the stress-response sigma factor Sigma B, which activates the transcription of RsaA among other genes (SbrA, B, C). (2) The effect of RsaA on the *agr* operon is probably indirect. The AgrA transcriptional factor autoregulates its own transcription (3), induces PSM production (4) but also enhances RNAIII expression (5). (6) AgrA itself is subjected to a negative control by the Psm-mec RNA in methicillin resistant *S. aureus* strains. (7) It has a direct positive effect on PSMs (phenol soluble modulin) production (PSMs are small molecule with hemolytic effect) and putatively on the non-coding RsaE transcription. (8) RsaE controls negatively several genes coding for enzymes involved in the TCA cycle but also a positive effect on genes coding for membrane proteins (opp4A/D) or enzymes contributing to valine/leucine/isoleucine synthesis pathways. (9) RNAIII is a bi-functional RNA, which contains its own ORF encoding hemolysin-∂, and possessing regulatory properties: RNAIII acts at the post-transcriptional level on *hla* and *spa* mRNA by base-pairings. The effect on target mRNAs translation is either positive (*hla*) or negative (*spa*, *rot*). Rot inhibits transcription of *hla* and *spa* genes. (10) SprD negative regulation of *sbi* mRNA translation occurs via the ncRNA SprD, but hypothetically also via RNAIII. (11) The long ncRNA SSR42 plays potentially an indirect role on surface proteins like protein A. All the references are given in the text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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 (Lioliou 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 σ^B-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 strains of *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 RsaOI) were upregulated. RsaOI 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). RsaOI was proposed to adopt a particular structure involving two long distant interactions (Marchais et al., 2010). Intriguingly, *rsaOI* 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.

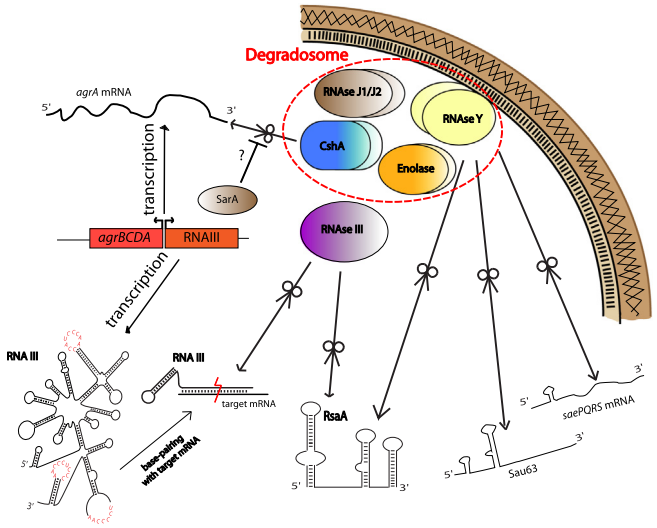

Fig. 2. Interplay between RNAs and RNA-binding proteins in *Staphylococcus aureus*. The major complex of RNA degradation, the so-called degradosome (circled in red), comprises the membrane-anchored RNAse Y, the two non-essential RNAse J1/J2, the RNA helicase CshA and the central metabolism-related enolase. PNPase 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 *sau63* (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 *agrA* 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 RNAII, 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 *agrA* mRNA itself is negatively regulated by different factors (see Figs. 1 and 2 and text below). The *agr* 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 RNAIII *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, RNAIII 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 RNAIII 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 5′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 G-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, RNAIII 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 adhesin 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 RNAIII 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 RNAIII, 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 RNAIII 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, PIϕ (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

Table 1
Summary of experimentally validated sRNAs in *S. aureus*.[a]

Study	Strain used	sRNA discovery methodology (number of in silico predicted sRNAs)	Experimentally validated sRNAs[e]	Experimental validation method	Target and mechanism	Comment
Roberts et al. (2006)	UAMS-1	Gene chip analysis	SSR42	NB, RT-PCR	Spa, hla, hglC, lukF Unknown	
Pichon and Felden (2005) and Sayed et al. (2011)	Mu50 (clonal complex 5)	Bioinformatic predictions (xx)	SprA SprA2, SprA3, SprB, SprC, SprD, SprE, SprF, SprG, SprFG2, SprFG3 4.5S RNA, 6S RNA, RNAIII, tmRNA, RNase P	 NB[b] NB[b] NB[b]	ABC transporter (SA2216), possible antisense sRNA Unknown, SprA2 encodes a cytolytic peptide Housekeeping ncRNAs	
Geissmann et al. (2009)	RN6390, Col, Newman, HG001	Bioinformatic predictions and experimental validation	RsaE RsaA, RsaB, RsaC, RsaD, RsaF, RsaG, RsaH, RsaI, RsaJ, RsaK	NB, PE, RACE NB, PE, RACE	Masking of ribosomal binding site for oppB, sucD, SA0873 Unknown	Genetic manipulation demonstrated a role for RsaE in controlling metabolic pathways
Marchais et al. (2009)	N315 (clonal complex 5)	Bioinformatic (NAPP) (189) and Northern analysis.	RsaOA, RsaOB, RsaOC, RsaOD, RsaOE, RsaOF, RsaOG[b]	NB	Unknown	
Nielsen et al. (2011)	N315 (clonal complex 5)	Bioinformatic search for intergenic σ^B consensus sites and experimental validation	SbrA (RsaOO), SbrB, SbrC	NB	σ^B regulated. SbrC interacts with SA0587 (mntC)	*sbrA* and *sbrB* potential CDS
Abu-Qatouseh et al. (2010)	–	Cloning and sequencing of cDNAs	Ssr-72, Ssr-80, Ssr-87 Sau-02, Sau-13, Sau-19, Sau-24, Sau-27, Sau-30, Sau-31, Sau-41, Sau-50, Sau-53, Sau-59, Sau-63, Sau-64, Sau-66, Sau-5949, Sau-5971, Sau-6053, Sau-6072	NB[c] NB	Unknown Unknown. Sau-66, putative posttranslational control of antisense gene SA0671	142 candidate sRNA identified
Bohn et al. (2010)	N315 (clonal complex 5)	454 pyrosequencing followed by experimental validation	RsaON (RsaE)[d] RsaOH, RsaOI, RsaOL, RsaOM, RsaOO, RsaOP, RsaOQ, RsaOR, RsaOT, RsaOU, RsaOV, RsaOW, RsaOX	NB NB	Binds *opp3A* mRNA ribosome-binding site. Overexpression of RsaE reduces central metabolic pathways and increases amino acid pool	30 sRNAs identified, 14 new
Beaume et al. (2010)	N315 (clonal complex 5)	Illumina sequencing	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, Teg2pl	RT-PCR	Unknown	195 sRNAs predicted by HTS

[a] Validated using Northern blot, RNA extremity mapping, or RT-qPCR.
[b] Also experimentally validated using Northern blot in the study by Abu-Qatouseh et al. (2010).
[c] Originally described by Anderson et al. (2006).
[d] 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).
[e] A number of different names have been assigned to many of these sRNAs. In this table the initial name from the relevant study has been used. A full list of alternate names can be found in the supplementary table from Felden et al. (2011). In the nearest future, it would be appropriate to adopt a common way to annotate the sRNA genes.

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 α (PSMα), a cytolytic toxin. In addition, the psm-mec RNA 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.

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 tiling 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; Mikulecky 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 prominently in Gram-positive bacteria such as *S. aur-*

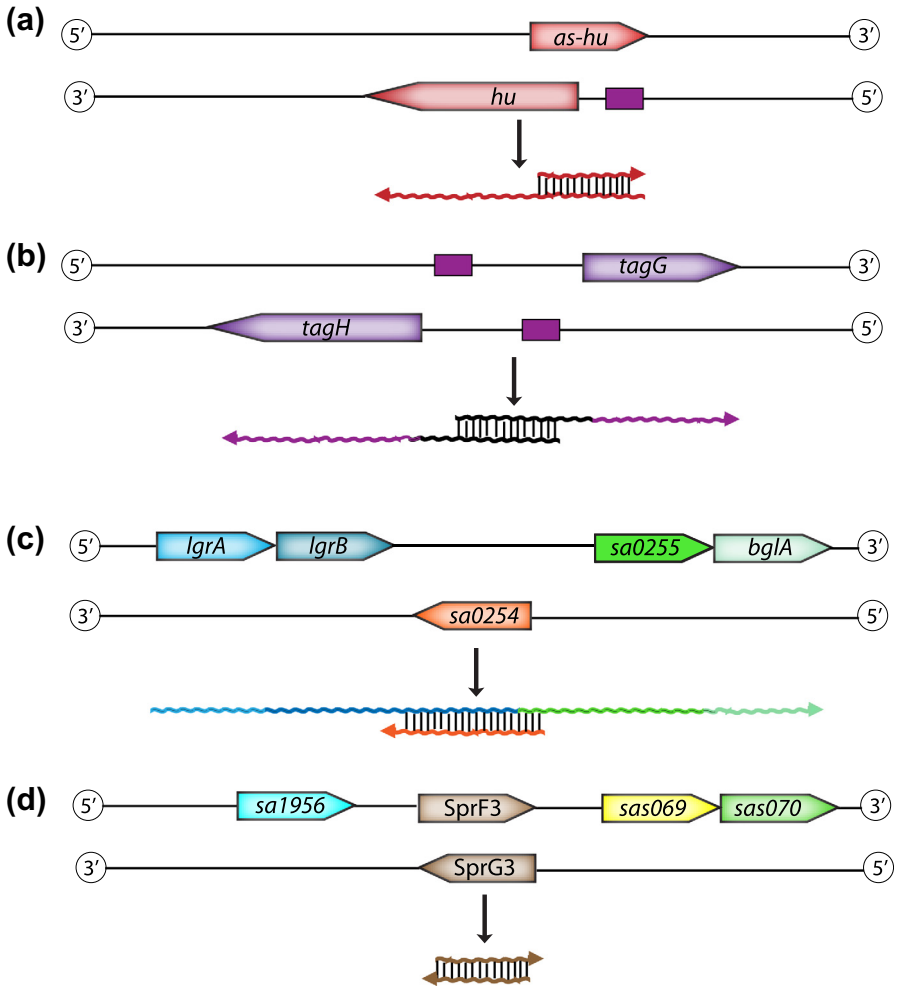

Fig. 3. Four mechanisms to generate overlapping transcription in *S. aureus*. Purple boxes represent the transcriptional start sequences and genes annotations are according to the *S. aureus* N315 genome. (a) Bone fide antisense RNA (asRNA): *hu* mRNA may be rapidly degraded by the dual action of the *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 *lgrABsa0255bglA* 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.)

eus (Lasa et al., 2011). 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. Alongside its major role in the processing of virulence genes, the enzyme is involved in the turnover of two sRNAs RsaA and Sau63 (Fig. 2). Indeed, half-life of both sRNAs was increased more than 5 times in the *rny* mutant compared to the wild type strain. Interestingly, we could also show that RNase III cleaves RsaA at two sites in the bulged loop of its 5′ hairpin structure and that RsaA is more stable in the *rnc* mutant strain compared to the wild-type strain (Lioliou et al., 2012) suggesting that RNase III contributes to RsaA maturation. Nevertheless, we cannot exclude that like many ncRNAs, RsaA regulates expression of its target by an antisense mechanism, and RNase III would be a partner to induce degradation of the duplex between RsaA and mRNAs.

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

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 exposition 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-metabolizable agonists were designed to constitutively switch off the expression of the essential *guaA* 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.

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References

Abu-Qatouseh, L.F., Chinni, S.V., Seggewiß, J., Proctor, R.A., Brosius, J., Rozhdestvensky, T.S., Peters, G., von Eiff, C., Becker, K., 2010. Identification of differentially expressed small non-protein-coding RNAs in *Staphylococcus aureus* displaying both the normal and the small-colony variant phenotype. J. Mol. Med. 88, 565–575.

Anderson, K.L., Roberts, C., Disz, T., Vonstein, V., Hwang, K., Overbeek, R., Olson, P.D., Projan, S.J., Dunman, P.M., 2006. Characterization of the *Staphylococcus aureus* heat shock, cold shock, stringent, and SOS responses and their effects on log-phase mrna turnover. J. Bacteriol. 188, 6739–6756.

Bassler, B.L., Losick, R., 2006. Bacterially speaking. Cell 125, 237–246.

Beaume, M., Hernandez, D., Farinelli, L., Deluen, C., Linder, P., Gaspin, C., Romby, P., Schrenzel, J., Francois, P., 2010. Cartography of methicillin-resistant *S. aureus* transcripts: detection, orientation and temporal expression during growth phase and stress conditions. PLoS One 5, e10725.

Benito, Y., Kolb, F.A., Romby, P., Lina, G., Etienne, J., Vandenesch, F., 2000. Probing the structure of RNAIII, the *Staphylococcus aureus* agr regulatory RNA, and identification of the RNA domain involved in repression of protein A expression. RNA 6, 668–679.

Bohn, C., Rigoulay, C., Bouloc, P., 2007. No detectable effect of RNA-binding protein Hfq absence in *Staphylococcus aureus*. BMC Microbiol. 7, 10.

Bohn, C., Rigoulay, C., Chabelskaya, S., Sharma, C.M., Marchais, A., Skorski, P., Borezée-Durant, E., Barbet, R., Jacquet, E., Jacq, A., Gautheret, D., Felden, B., Vogel, J., Bouloc, P., 2010. Experimental discovery of small RNAs in *Staphylococcus aureus* reveals a riboregulator of central metabolism. Nucleic Acids Res. 38, 6620–6636.

Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M., Chevalier, C., Helfer, A.C., Benito, Y., Jacquier, A., Gaspin, C., Vandenesch, F., Romby, P., 2007. *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. Genes Dev. 21, 1353–1366.

Breaker, R.R., 2009. Riboswitches: from ancient gene-control systems to modern drug targets. Future Microbiol. 4, 771–773.

Breaker, R.R., 2011. Prospects for riboswitch discovery and analysis. Mol. Cell 43, 867–879.

Brennan, R.G., Link, T.M., 2007. Hfq structure, function and ligand binding. Curr. Opin. Microbiol. 10, 125–133.

Caldelari, I., Chao, Y., Romby, P., Vogel, J., 2013. RNA-mediated regulation in pathogenic bacteria. Cold Spring Harb. Perspect. Med. 3(9).

Caldelari, I., Fechter, P., Lioliou, E., Romilly, C., Chevalier, C., Gaspin, C., Romby, P., 2011. A current overview of regulatory RNA in *Staphylococcus aureus*. In: Marchfelder, A., Hess, W. (Eds.), Regulatory RNAs in Prokaryotes. Wiley Verlag, Wien and New York.

Chabelskaya, S., Gaillot, O., Felden, B., 2010. A *Staphylococcus aureus* small RNA is required for bacterial virulence and regulates the expression of an immune-evasion molecule. PLoS Pathog. 6, e1000927.
Chambers, H.F., DeLeo, F.R., 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat. Rev. Microbiol. 7, 629–641.
Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H., Xiong, Y.-Q., 2004. Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. FEMS Immunol. Med. Microbiol. 40, 1–9.
Chevalier, C., Boisset, S., Romilly, C., Masquida, B., Fechter, P., Geissmann, T., Vandenesch, F., Romby, P., 2010. *Staphylococcus aureus* RNAIII binds to two distant regions of coa mRNA to arrest translation and promote mRNA degradation. PLoS Pathog. 6, e1000809.
Chunhua, M., Yu, L., Yaping, G., Jie, D., Qiang, L., Xiaorong, T., Guang, Y., 2012. The expression of LytM is down-regulated by RNAIII in *Staphylococcus aureus*. J. Basic Microbiol. 52, 636–641. http://dx.doi.org/10.1002/jobm.201100426.
Dambach, M.D., Winkler, W.C., 2009. Expanding roles for metabolite-sensing regulatory RNAs. Curr. Opin. Microbiol. 12, 161–169.
Dunman, P.M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., Brown, E.L., Zagursky, R.J., Shlaes, D., Projan, S.J., 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the agr and/or sarA loci. J. Bacteriol. 183, 7341–7353.
Felden, B., Vandenesch, F., Bouloc, P., Romby, P., 2011. The *Staphylococcus aureus* RNome and its commitment to virulence. PLoS Pathog. 7, e1002006.
Fowler Jr., V.G., Sakoulas, G., McIntyre, L.M., Meka, V.G., Arbeit, R.D., Cabell, C.H., Stryjewski, M.E., Eliopoulos, G.M., Reller, L.B., Corey, G.R., Jones, T., Lucindo, N., Yeaman, M.R., Bayer, A.S., 2004. Persistent bacteremia due to methicillin-resistant *Staphylococcus aureus* infection is associated with agr dysfunction and low-level in vitro resistance to thrombin-induced platelet microbicidal protein. J. Infect. Dis. 190, 1140–1149.
Geisinger, E., Adhikari, R.P., Jin, R., Ross, H.F., Novick, R.P., 2006. Inhibition of rot translation by RNAIII, a key feature of agr function. Mol. Microbiol. 61, 1038–1048.
Geissmann, T., Chevalier, C., Cros, M.-J., Boisset, S., Fechter, P., Noirot, C., Schrenzel, J., François, P., Vandenesch, F., Gaspin, C., Romby, P., 2009. A search for small noncoding RNAs in *Staphylococcus aureus* reveals a conserved sequence motif for regulation. Nucleic Acids Res. 37, 7239–7257.
Horstmann, N., Orans, J., Valentin-Hansen, P., Shelburne III, S.A., Brennan, R.G., 2012. Structural mechanism of *Staphylococcus aureus* Hfq binding to an RNA A-tract. Nucleic Acids Res. 40, 11023–11035.
Howden, B.P., Beaume, M., Harrison, P.F., Hernandez, D., Schrenzel, J., Seemann, T., Francois, P., Stinear, T.P., 2013. Analysis of the small RNA transcriptional response in multidrug-resistant *Staphylococcus aureus* after antimicrobial exposure. Antimicrob. Agents Chemother. 57, 3864–3874.
Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., Etienne, J., Ehresmann, B., Ehresmann, C., Jacquier, A., Vandenesch, F., Romby, P., 2005. *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate spa gene expression. EMBO J. 24, 824–835.
Jelsbak, L., Hemmingsen, L., Donat, S., Ohlsen, K., Boye, K., Westh, H., Ingmer, H., Frees, D., 2010. Growth phase-dependent regulation of the global virulence regulator Rot in clinical isolates of *Staphylococcus aureus*. Int. J. Med. Microbiol. 300, 229–236.
Jia, X., Zhang, J., Sun, W., He, W., Jiang, H., Chen, D., Murchie, A.I.H., 2013. Riboswitch regulation of aminoglycoside resistance acetyl and adenyl transferases. Cell 153, 1419–1420.
Kaito, C., Kurokawa, K., Matsumoto, Y., Terao, Y., Kawabata, S., Hamada, S., Sekimizu, K., 2005. Silkworm pathogenic bacteria infection model for identification of novel virulence genes. Mol. Microbiol. 56, 934–944.
Kaito, C., Saito, Y., Nagano, G., Ikuo, M., Omae, Y., Hanada, Y., Han, X., Kuwahara-Arai, K., Hishinuma, T., Baba, T., Ito, T., Hiramatsu, K., Sekimizu, K., 2011. Transcription and translation products of the cytolysin gene psm-mec on the mobile genetic element SCCmec regulate *Staphylococcus aureus* virulence. PLoS Pathog. 7, e1001267.
Kaito, C., Saito, Y., Ikuo, M., Omae, Y., Mao, H., Nagano, G., Fujiyuki, T., Numata, S., Han, X., Obata, K., Hasegawa, S., Yamaguchi, H., Inokuchi, K., Ito, T., Hiramatsu, K., Sekimizu, K., 2013. Mobile genetic element SCCmec-encoded psm-mec RNA suppresses translation of agrA and attenuates MRSA virulence. PLoS Pathog. 9, e1003269.
Lasa, I., Toledo-Arana, A., Dobin, A., Villanueva, M., de Mozos, I.R., Vergara-Irigaray, M., Segura, V., Fagegaltier, D., Penadés, J.R., Valle, J., Solano, C., Gingeras, T.R., 2011. Genome-wide antisense transcription drives mRNA processing in bacteria. PNAS 108, 20172–20177.
Lasa, I., Toledo-Arana, A., Gingeras, T.R., 2012. An effort to make sense of antisense transcription in bacteria. RNA Biol. 9, 1039–1044.
Link, T.M., Valentin-Hansen, P., Brennan, R.G., 2009. Structure of *Escherichia coli* Hfq bound to polyriboadenylate RNA. Proc. Natl. Acad. Sci. USA 106, 19292–19297.
Lioliou, E., Sharma, C.M., Altuvia, Y., Caldelari, I., Romilly, C., Helfer, A.-C., Margalit, H., Romby, P., 2013. In vivo mapping of RNA–RNA interactions in *Staphylococcus aureus* using the endoribonuclease III. Methods 63 (2), 135–143.
Lioliou, E., Sharma, C.M., Caldelari, I., Helfer, A.-C., Fechter, P., Vandenesch, F., Vogel, J., Romby, P., 2012. Global regulatory functions of the *Staphylococcus aureus* endoribonuclease III in gene expression. PLoS Genet. 8, e1002782.
Liu, Y., Wu, N., Dong, J., Gao, Y., Zhang, X., Mu, C., Shao, N., Yang, G., 2010. Hfq is a global regulator that controls the pathogenicity of *Staphylococcus aureus*. PLoS One 5, e13069.
Liu, Y., Mu, C., Ying, X., Li, W., Wu, N., Dong, J., Gao, Y., Shao, N., Fan, M., Yang, G., 2011. RNAIII activates map expression by forming an RNA–RNA complex in *Staphylococcus aureus*. FEBS Lett. 585, 899–905.
Marchais, A., Bohn, C., Bouloc, P., Gautheret, D., 2010. RsaOG, a new staphylococcal family of highly transcribed non-coding RNA. RNA Biol. 7, 116–119.
Marchais, A., Naville, M., Bohn, C., Bouloc, P., Gautheret, D., 2009. Single-pass classification of all noncoding sequences in a bacterial genome using phylogenetic profiles. Genome Res. 19, 1084–1092.
Marincola, G., Schäfer, T., Behler, J., Bernhardt, J., Ohlsen, K., Goerke, C., Wolz, C., 2012. RNase Y of *Staphylococcus aureus* and its role in the activation of virulence genes. Mol. Microbiol. 85, 817–832.
Mikulecky, P.J., Kaw, M.K., Brescia, C.C., Takach, J.C., Sledjeski, D.D., Feig, A.L., 2004. *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, rpoS and poly(A) RNAs. Nat. Struct. Mol. Biol. 11, 1206–1214.
Morrison, J.M., Anderson, K.L., Beenken, K.E., Smeltzer, M.S., Dunman, P.M., 2012a. The staphylococcal accessory regulator, SarA, is an RNA-binding protein that modulates the mRNA turnover properties of late-exponential and stationary phase *Staphylococcus aureus* cells. Front. Cell. Infect. Microbiol. 2, 26.
Morrison, J.M., Miller, E.W., Benson, M.A., Alonzo 3rd, F., Yoong, P., Torres, V.J., Hinrichs, S.H., Dunman, P.M., 2012b. Characterization of SSR42, a novel virulence factor regulatory RNA that contributes to the pathogenesis of a *Staphylococcus aureus* USA300 representative. J. Bacteriol. 194, 2924–2938.
Mulhbacher, J., Brouillette, E., Allard, M., Fortier, L.-C., Malouin, F., Lafontaine, D.A., 2010a. Novel riboswitch ligand analogs as selective inhibitors of guanine-related metabolic pathways. PLoS Pathog. 6, e1000865.
Mulhbacher, J., St-Pierre, P., Lafontaine, D.A., 2010b. Therapeutic applications of ribozymes and riboswitches. Curr. Opin. Pharmacol. 10, 551–556.
Nagata, M., Kaito, C., Sekimizu, K., 2008. Phosphodiesterase activity of CvfA is required for virulence in *Staphylococcus aureus*. J. Biol. Chem. 283, 2176–2184.
Nielsen, J.S., Christiansen, M.H.G., Bonde, M., Gottschalk, S., Frees, D., Thomsen, L.E., Kallipolitis, B.H., 2011. Searching for small σB-regulated genes in *Staphylococcus aureus*. Arch. Microbiol. 193, 23–34.
Novick, R.P., 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol. Microbiol. 48, 1429–1449.
Novick, R.P., Geisinger, E., 2008. Quorum sensing in *Staphylococci*. Annu. Rev. Genet. 42, 541–564.
Novick, R.P., Ross, H.F., Projan, S.J., Kornblum, J., Kreiswirth, B., Moghazeh, S., 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. 12, 3967–3975.
Oun, S., Redder, P., Didier, J.-P., François, P., Corvaglia, A.-R., Buttazzoni, E., Giraud, C., Girard, M., Schrenzel, J., Linder, P., 2013. The CshA DEAD-box RNA helicase is important for quorum sensing control in *Staphylococcus aureus*. RNA Biol. 10, 157–165.
Panja, S., Schu, D.J., Woodson, S.A., 2013. Conserved arginines on the rim of Hfq catalyze base pair formation and exchange. Nucleic Acids Res. 41 (15), 7536–7546.
Papenfort, K., Vogel, J., 2010. Regulatory RNA in bacterial pathogens. Cell Host Microbe 8, 116–127.
Pichon, C., Felden, B., 2005. Small RNA genes expressed from *Staphylococcus aureus* genomic and pathogenicity islands with specific expression among pathogenic strains. PNAS 102, 14249–14254.
Priest, N.K., Rudkin, J.K., Feil, E.J., van den Elsen, J.M.H., Cheung, A., Peacock, S.J., Laabei, M., Lucks, D.A., Recker, M., Massey, R.C., 2012. From genotype to phenotype: can systems biology be used to predict *Staphylococcus aureus* virulence? Nat. Rev. Microbiol. 10, 791–797.
Proctor, R.A., von Eiff, C., Kahl, B.C., Becker, K., McNamara, P., Herrmann, M., Peters, G., 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat. Rev. Microbiol. 4, 295–305.
Queck, S.Y., Jameson-Lee, M., Villaruz, A.E., Bach, T.-H.L., Khan, B.A., Sturdevant, D.E., Ricklefs, S.M., Li, M., Otto, M., 2008. RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*. Mol. Cell 32, 150–158.
Roberts, C., Anderson, K.L., Murphy, E., Projan, S.J., Mounts, W., Hurlburt, B., Smeltzer, M., Overbeek, R., Disz, T., Dunman, P.M., 2006. Characterizing the effect of the *Staphylococcus aureus* virulence factor regulator, SarA, on log-phase mRNA half-lives. J. Bacteriol. 188, 2593–2603.
Rochat, T., Bouloc, P., Yang, Q., Bossi, L., Figueroa-Bossi, N., 2012. Lack of interchangeability of Hfq-like proteins. Biochimie 94, 1554–1559.
Romby, P., Charpentier, E., 2010. An overview of RNAs with regulatory functions in gram-positive bacteria. Cell. Mol. Life Sci. 67, 217–237.
Romilly, C., Caldelari, I., Parmentier, D., Lioliou, E., Romby, P., Fechter, P., 2012a. Current knowledge on regulatory RNAs and their machineries in *Staphylococcus aureus*. RNA Biol. 9, 402–413.
Romilly, C., Chevalier, C., Marzi, S., Masquida, B., Geissmann, T., Vandenesch, F., Westhof, E., Romby, P., 2012b. Loop–loop interactions involved in antisense regulation are processed by the endoribonuclease III in *Staphylococcus aureus*. RNA Biol. 9, 1461–1472.
Roux, C.M., DeMuth, J.P., Dunman, P.M., 2011. Characterization of components of the *Staphylococcus aureus* mRNA degradosome holoenzyme-like complex. J. Bacteriol. 193, 5520–5526.
Sakoulas, G., Eliopoulos, G.M., Moellering Jr., R.C., Wennersten, C., Venkataraman, L., Novick, R.P., Gold, H.S., 2002. Accessory gene regulator (agr) locus in geographically diverse *Staphylococcus aureus* isolates with reduced susceptibility to vancomycin. Antimicrob. Agents Chemother. 46, 1492–1502.

Sayed, N., Jousselin, A., Felden, B., 2011. A cis-antisense RNA acts in trans in *Staphylococcus aureus* to control translation of a human cytolytic peptide. Nat. Struct. Mol. Biol. 19, 105–112.

Serganov, A., 2010. Determination of riboswitch structures: light at the end of the tunnel? RNA Biol. 7, 98–103.

Shimoni, Y., Altuvia, S., Margalit, H., Biham, O., 2009. Stochastic analysis of the SOS response in *Escherichia coli*. PLoS One 4, e5363.

Shimoni, Y., Friedlander, G., Hetzroni, G., Niv, G., Altuvia, S., Biham, O., Margalit, H., 2007. Regulation of gene expression by small non-coding RNAs: a quantitative view. Mol. Syst. Biol. 3, 138.

Somerville, G.A., Proctor, R.A., 2009. At the crossroads of bacterial metabolism and virulence factor synthesis in *Staphylococci*. Microbiol. Mol. Biol. Rev. 73, 233–248.

Someya, T., Baba, S., Fujimoto, M., Kawai, G., Kumasaka, T., Nakamura, K., 2012. Crystal structure of Hfq from *Bacillus subtilis* in complex with SELEX-derived RNA aptamer: insight into RNA-binding properties of bacterial Hfq. Nucleic Acids Res. 40, 1856–1867.

Song, J., Lays, C., Vandenesch, F., Benito, Y., Bes, M., Chu, Y., Lina, G., Romby, P., Geissmann, T., Boisset, S., 2012. The expression of small regulatory RNAs in clinical samples reflects the different life styles of *Staphylococcus aureus* in colonization vs. infection. PLoS One 7, e37294.

Soper, T.J., Doxzen, K., Woodson, S.A., 2011. Major role for mRNA binding and restructuring in sRNA recruitment by Hfq. RNA 17, 1544–1550.

Traber, K.E., Lee, E., Benson, S., Corrigan, R., Cantera, M., Shopsin, B., Novick, R.P., 2008. Agr function in clinical *Staphylococcus aureus* isolates. Microbiology 154, 2265–2274.

Tristan, A., Ferry, T., Durand, G., Dauwalder, O., Bes, M., Lina, G., Vandenesch, F., Etienne, J., 2007. Virulence determinants in community and hospital meticillin-resistant *Staphylococcus aureus*. J. Hosp. Infect. 65 (Suppl. 2), 105–109.

Van Schaik, W., Abee, T., 2005. The role of sigmaB in the stress response of Gram-positive bacteria – targets for food preservation and safety. Curr. Opin. Biotechnol. 16, 218–224.

Vecerek, B., Rajkowitsch, L., Sonnleitner, E., Schroeder, R., Bläsi, U., 2008. The C-terminal domain of *Escherichia coli* Hfq is required for regulation. Nucleic Acids Res. 36, 133–143.

Wang, Z., Tollervey, J., Briese, M., Turner, D., Ule, J., 2009. CLIP: construction of cDNA libraries for high-throughput sequencing from RNAs cross-linked to proteins in vivo. Methods 48, 287–293.

Westermann, A.J., Gorski, S.A., Vogel, J., 2012. Dual RNA-seq of pathogen and host. Nat. Rev. Microbiol. 10, 618–630.