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## **Gram-positive pathogens, 3<sup>rd</sup> Edition (ASM)**

Staphylococcus section

Chapter 5: non-coding RNA

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## General introduction

Regulatory RNAs have been identified in many bacteria, and in pathogenic bacteria such as *Staphylococcus aureus*, where they play major roles in the regulation of virulence or metabolic proteins synthesis, beside transcriptional factors and two component systems (Bischoff and Romby, 2016, Tomasini *et al.*, 2014, Guillet *et al.*, 2013, Caldelari *et al.*, 2011). Most of them are non-coding RNAs (sRNAs) but some of them express small peptides. Certain sRNAs, acting in *cis*, are situated at the 5'-untranslated regions (UTR) of mRNAs, and act as sensors of metabolites, tRNA or environmental stimuli (temperature, pH, ...) or at the 3'-UTR. In contrast, the genes encoding sRNAs, which act in *trans*, sit on the opposite strand of the regulated mRNA or at genomic locations distant from the mRNAs they regulate. *Cis*-encoding sRNAs, also called antisense RNAs (asRNA), are fully complementary to their targets. In contrast, *trans*-encoding sRNAs share only partial complementarity, and as a consequence, they can regulate many mRNAs. Most of them are encoding mainly on the core genome while few of them are localised within mobile elements, pathogenic islands or plasmids. In this review, we will focus on the most recent mechanisms of RNA regulation discovered in *S. aureus* and how regulatory RNAs are part of sophisticated networks that allow the bacteria to adapt quickly to its environment or survive into its host.

## 5'-UTR of mRNAs contain riboswitches, T-boxes or thermosensors with potential impact for novel antibiotherapy

The riboswitches and T-box are found in the 5'-UTR of some mRNAs and contain highly structured domains, which recognize metabolites such as cofactors, vitamins, amino-acids, nucleotides, second messenger cyclic di-GMP,  $Mg^{2+}$  or non aminoacylated tRNAs (Quereda and Cossart, 2017). Binding of these metabolites confers structural changes that modify the expression of the downstream mRNA, for example by inducing premature transcription arrest, repression/activation of translation, or cleavage. T-box senses the aminoacylation status of tRNAs and mainly control transcription of downstream genes that encode proteins involved in the biosynthesis, transport of amino acids or aminoacylation of tRNAs. Based on sequence and structure conservation, most of the T-box and riboswitches were predicted in *S. aureus* genomes (Caldelari *et al.*, 2011). A large part of riboswitches control expression of genes involved in metabolic pathways. Because these genes are often essential for growth, they thus represent interesting targets for the development of alternative antimicrobial drugs in the battle against multi-drugs resistant *S. aureus*. This strategy was used with the guanine and the glucosamine-6-phosphate (GlcN6P) riboswitches. Mulhbacher

*et al.* (2010) have identified a pyrimidine derivative that binds to the guanine riboswitch and represses the expression of *guaA*. This compound significantly reduced the infection by *S. aureus* in a mouse model of infection. In Gram-positive bacteria, the *glmS* mRNA is both a ribozyme, which catalyses its own cleavage and a riboswitch responding to GlcN6P. Its product encodes an essential enzyme, which converts fructose-6-phosphate into GlcN6P, a building block of bacterial peptidoglycan. A tight regulation of *glmS* mRNA is crucial to maintain a homeostatic level of GlcN6P in the cell. At high concentrations of GlcN6P, its binding to the 5'-UTR of *glmS* leads to a site-specific self-cleavage, which generates a 5'-hydroxylated end molecule rapidly degraded by the RNase J1 (Collins *et al.*, 2007, Lünse *et al.*, 2011). A recent study leads to the design, synthesis, and characterization of a GlcN6P analogue, carba-GlcN6P, which constitutively activates the *glmS* ribozyme of vancomycin-resistant *S. aureus* and destabilizes its mRNA (Lünse *et al.*, 2011). This compound was experimentally shown to induce an efficient self-cleavage of the *glmS* mRNA similar to the natural metabolite and thus represented an important step in the development of antibiotics with a new mode of action. Very recently, a new approach called “Term-seq” revealed that several antibiotic resistance genes are under the control of riboswitches responding to commonly used antibiotics against Gram-positive pathogenic bacteria such as *Listeria* or *Enterococcus faecalis* (Dar *et al.*, 2016). These results suggest that the same phenomenon could exist in *S. aureus* and that RNA-mediated regulation could play a broader role than expected in antibiotic resistance mechanisms.

Not surprisingly, the presence of antibiotics can also modulate the regulatory activity of T-box. In *S. aureus*, an unusual *glyS* T-box regulates transcription antitermination of the unique glycyl-tRNA synthetase (*glyRS*) gene responsible to catalyse the aminoacylation of the five tRNA<sup>Gly</sup> isoacceptors, independently of their anticodon (GCC or UCC) and with different binding affinities (Apostolidi *et al.*, 2015). Thereafter, the T-box senses the availability of glycine not only for its incorporation into nascent polypeptide chains during translation but also for the formation of pentaglycine bridges into the peptidoglycan molecule, linking two essential pathways. Antibiotics targeting the small ribosomal subunit stabilized the T-box/tRNA complex and induced a read-through of transcription while chloramphenicol and linezolid attenuated *glyS* transcription (Stamatopoulou *et al.*, 2017). The outcome depended on the binding sites of the protein synthesis inhibitors (Stamatopoulou *et al.*, 2017). Although T-box and riboswitches can be targets for antibiotics against important human pathogens as *S. aureus*, they also presented unexpected off-target effect.

RNA thermosensors are regulatory elements often localised at the 5'-UTR of mRNA

encoding heat or cold shock proteins and virulence factors (for review Kortmann and Narberhaus, 2012). Briefly, at low temperatures, the mRNA cannot be translated since the Shine and Dalgarno (SD) sequence is trapped into a hairpin structure, which melts gradually when temperature increases. The best-studied example in Gram-positive bacteria is the thermosensor regulating the expression of the transcriptional factor *prfA*, which further activates most of the virulence genes in *Listeria monocytogenes* (Johansson *et al.*, 2002, Loh *et al.*, 2012). Such example has not been demonstrated yet in other Gram-positive bacteria including *S. aureus*.

### 3'-UTRs of mRNAs act in *cis* or are reservoirs of sRNAs

Transcriptome analysis of the human pathogen *S. aureus* revealed that at least one-third of mRNAs carry long 3'-UTRs, and thus might display multiple regulatory functions (Lasa *et al.*, 2012). Some of them have direct action on the expression of their own mRNA (Lasa, *et al.*, 2012, Ruiz de los Mozos *et al.*, 2013). Long 3'-UTRs (>100 nucleotides) can end at an intrinsic Rho-independent terminator of transcription (TT), can be generated from a specific RNase cleavage, or from a terminating-read-through of the RNA polymerase. Remarkably, several 3'-UTRs contained riboswitches as the result of a metabolite-sensing regulation (Ruiz de los Mozos *et al.*, 2013). Indeed, the TT of the riboswitch in an OFF conformation also serves as the TT of the gene encoded upstream of the riboswitch.

A singular example of a new post-transcriptional regulatory mechanism was shown for the *icaR* mRNA, which possesses an unusual long 3'-UTR (390 bp). In this case, the expression of the mRNA is modulated through a long-distant interaction between 3'-UTR and 5'-UTR (Ruiz de los Mozos *et al.*, 2013). Because *icaR* codes for a transcriptional repressor of the *icaADBC* operon encoding enzymes involved in the synthesis of PIA-PNAG, the main polysaccharides of the biofilm matrix, the regulation has direct impact on biofilm formation. Base-pairing interactions between the long 3'-UTR of *icaR* mRNA and the SD sequence of the same mRNA hindered efficient translation initiation (Ruiz de los Mozos *et al.*, 2013). This long range RNA duplex generated a specific site for the double-strand endoribonuclease III (RNase III) for cleavage. As a consequence, PIA-PNAG synthesis increased. Yet the mechanism allowing IcaR translation is unknown to prevent the action of the 3'-UTR as a *cis*-acting antisense RNA (figure 1).

It is also postulated that 3'-UTR length provides other types of transcript-specific regulation. Indeed in *Salmonella* sp., the 3'-UTRs are also reservoirs for sRNAs, which originated either by transcription from an internal promoter or by processing. In both cases,

the sRNA generated from the 3'-UTR regulated trans-encoded mRNA targets. For instance, the sRNA CpxQ is cleaved by RNase E from the 3'-UTR of *cpxP* and repressed the translation of mRNAs encoding a family of envelope proteins while DapZ is transcribed from an internal promoter within the *dapB* gene and inhibited translation of the major ABC transporters, DppA and OppA (Chao *et al.*, 2012, Chao and Vogel, 2016, for review see Miyakoshi *et al.*, 2015). Recent works showed that such 3'-UTR-derived sRNA also existed in *S. aureus* although their functions remained to be addressed (Desgranges *et al.*, unpublished data).

### AsRNAs in pervasive transcription and acting as anti-toxins

AsRNAs are transcribed from the opposite strand of the mRNAs they regulate, so that they display perfect complementarities with their target. Short asRNAs are often encoded on mobile elements such as plasmids, transposons, and phage-like elements. Such elements can potentially be transferred horizontally to other bacterial species or be duplicated (Fozo *et al.*, 2008). In *S. aureus*, they were first described to control plasmid conjugation and replication (Novick *et al.*, 1989). The size of asRNAs can vary from 10 to thousands of nucleotides, because these RNAs can overlap with part of a gene (3' or 5' ends), the entire gene or a group of genes (figure 1). This phenomenon is called pervasive transcription. Initially pervasive transcription was considered as a non-functional transcriptional noise. However, considering the large number of asRNAs expressed from the entire genome and in several bacterial species, these RNAs might play an important role in the regulation of gene expression. Genome-wide analysis of *S. aureus* highlighted that the expression of a significant proportion (75%) of antisense transcripts to annotated open reading frames are synthesized from the complementary strand and that these sense/antisense duplexes are digested by RNase III generating short fragments all along the genome (Lasa *et al.*, 2011). Another study using RIP-Seq approach confirmed the involvement of RNase III in the regulation of sense/antisense transcripts and overlapping UTRs (Lioliou *et al.*, 2012). The situation might be even more complex as recent results suggested that the termination factor Rho play a major role to prevent pervasive transcription in *B. subtilis*, but also in *S. aureus* (Bidnenko *et al.*, 2017, Mäder *et al.*, 2016). Although the biological outcome of pervasive transcription is not clearly understood, some of the produced asRNAs are functional and control several biological processes (Lasa *et al.*, 2012).

Type I toxin-antitoxin (TA) systems are particular cases of short asRNAs, in which the antitoxin is an asRNA regulating the translation or the fate of the toxin encoding mRNA,

whereas in the type III system, the anti-toxin sequesters the toxin (review by Goeders *et al.*, 2016; Coray *et al.*, 2017). In *S. aureus*, several type I TA module systems have been described (review of Brielle *et al.*, 2016). One of them, called SprF1/SprG1, expresses SprF1 as the asRNA and *sprG1* mRNA, which encodes two short secreted peptides with haemolytic and antibacterial activity (Pinel-Marie *et al.*, 2014). The anti-toxin SprF1 binds to the 3'-end of *sprG1* mRNA that leads to mRNA degradation and inhibition of peptide synthesis to protect cells against lethality (Pinel-Marie *et al.*, 2014). The SprA1/asSprA1 pair is another intriguing and unconventional system. The asSprA1 is transcribed from the opposite strand of the *sprA1* mRNA producing a cytolytic peptide. Their 3'ends overlapped by 35 nucleotides but experimental data indicated that the functional domain of asSprA1 is outside the complementary sequence with *sprA1* mRNA. Both RNAs were expressed concomitantly and asSprA1 5' base-paired with the ribosome-binding site (RBS) of *sprA1* impairing its translation (Sayed *et al.*, 2012). Thus asSprA1 acts as a *trans* regulator with its complementary target, suggesting that it can potentially interact with other RNA targets. Finally, a cluster of five genes encoding sRNAs specific to the *S. aureus* Newman strain contained a putative TA system (Bronsard *et al.*, 2017) (figure 1). Three genes were transcribed from the positive strand, two from the negative strand. Moreover, one small open reading frame was detected within one of the gene from the minus strand and coded for a secreted peptide with similarity to the RelE toxin (Neubauer *et al.*, 2009). Whether these two overlapping genes corresponded to a novel TA system remained to be addressed. Interestingly this locus was expressed in a growth-phase dependent manner, in nutrient starvation and oxidative stress. Type I TA systems have been involved in many functions (membrane depolarization, plasmid maintenance) mainly in *Escherichia coli* among which persistence (for review Berghoff and Wagner, 2017), but not in *S. aureus* until now (Brielle *et al.*, 2016).

### Transcriptional factors and sRNAs build complex regulatory networks

*Trans*-acting sRNAs regulate mRNAs by imperfect base-pairings, which signifies that one sRNA can modulate several targets and one target can be controlled by several sRNAs. In *S. aureus*, the annealing region between sRNA and mRNAs are often longer than in *E. coli* and mostly targets the ribosome binding site (RBS) of mRNAs affecting translation. In several cases, a second distinct site of interaction occurs in the coding region. Unlike Gram-negative bacteria, in which Hfq and ProQ proteins participate to the sRNA regulation by stabilizing and facilitating their pairings with mRNA targets (see reviews Updegrave *et al.*, 2016; Attaiech *et al.*, 2017), no RNA chaperones have been yet identified in *S. aureus*.

Indeed, *proQ* is not encoded and the role of Hfq is still unclear. Recent work has shown that the rim domain of Hfq has an amino-acid composition (low in arginine) incompatible with RNA annealing activity compared to *E. coli* Hfq (Zheng *et al.*, 2016). The sRNAs belong to intricate networks of regulation and their synthesis is often dependent of transcription factors or of two components systems, in addition sRNAs can also control transcription factors at the post-transcriptional level (figure 2). Examples will be described below.

AgrA, the responsive regulator of the *agr* quorum sensing system, activates the transcription of RNAIII. This bifunctional RNAIII codes for  $\delta$ -hemolysin and regulates the expression of virulence genes at the post-transcriptional level (see below). It interacts with various mRNAs either to activate or to repress translation. The RBS of *hla* mRNA encoding  $\alpha$ -hemolysin is embedded into a hairpin, which prevents the ribosome to bind and start translation. The 5'-UTR of RNAIII possesses complementary sequences to the leader region of *hla*. Interactions between both RNAs would recruit ribosomes to initiate Hla translation. In its 3'-UTR, RNAIII carries conserved UCCC motifs that are used as the seed sequences to bind with the RBS of the target mRNAs coding for the Protein A, the coagulase, the Sbi protein, the transcription factor Rot, the repressor of exotoxins, and the endopeptidase LytM (for review, Bronesky *et al.*, 2016). Moreover, AgrA repressed the sRNA ArtR, which inhibits translation of the SarA homolog SarT (Xue *et al.*, 2014) and activated RsaE (see below, Geissmann *et al.*, 2009) (figure 2).

The staphylococcal accessory regulator SarA is synthesised from three distinct promoters (P1, P2, P3) and binds DNA or RNA (Bischoff and Romby, 2016). As a transcription factor, it regulates many genes involved in virulence, autolysis, biofilm formation, stress response, antibiotic resistance, or metabolism, but also two sRNAs, SprC and *Srn\_9340* located on the same pathogenicity island (Mauro *et al.*, 2016). SprC prevented ribosomes to bind the SD of the *atl* mRNA coding for an autolysin, then reducing internalization by macrophages and attenuating virulence (Le Pabic *et al.*, 2015). SarA repressed both *sprC* and *srn\_9340* transcription and required an ATTTTAT sequence in its binding site (Mauro *et al.*, 2016). However, while SarA level remains relatively constant along the bacterial growth, the expression of SprC fluctuated, which suggest that additional factors might control its synthesis and that a mechanism of derepression should co-exist under specific conditions. These are very first examples of two sRNAs regulated by the same transcription factor.

In the following, we will describe an sRNA, whose transcription is controlled by three independent transcription factors. The sRNA RsaE possesses two consensus sequence motifs

UCCC as found in RNAlII, which interact with the RBS of several mRNAs involved in central metabolism to repress their translation (Geissmann *et al.*, 2009, Bohn *et al.*, 2010). RsaE is highly conserved between *Staphylococcaceae* and *Bacillaceae* families. Not only the sequence of RsaE is conserved between *B. subtilis* and *S. aureus* species, but also its regulation and functions. Recent studies have shown that its transcription is activated by the two component systems SrrAB (staphylococcal respiratory response) in response to NO and a binding site for the redox sensing repressor Rex has been predicted (Geissmann *et al.*, 2009, Durand *et al.*, 2015, 2017). In *B. subtilis*, the Rex-repression of RsaE (also called RoxS) has been proposed to readjust the cellular balance of NAD<sup>+</sup>/NADH upon various signals (Durand *et al.*, 2017).

The alternative sigma B factor ( $\sigma^B$ ) together with the RNA polymerase guides transcription of genes mainly in the stationary phase of growth and under stress conditions. Its regulon comprises more than 200 genes including several virulence factors, transcription factors and sRNAs (figure 2, Bischoff *et al.*, 2004). Among the sRNAs induced by  $\sigma^B$  are SbrA, B, C activated by KOH (Nielsen *et al.*, 2011) and RsaA (Geissman *et al.*, 2009). The stability of RsaA depended on RNase III and the endoribonuclease RNase Y, which is part of the degradosome in *S. aureus* (Lioliou *et al.*, 2012, Marincola *et al.*, 2012, Roux *et al.*, 2011). RsaA acts as an acute virulence attenuator in *S. aureus* (see below) by inhibiting translation of the MgrA transcription factor (figure 2, Romilly *et al.*, 2014) and indirectly by activating the synthesis of several surface proteins (Tomasini *et al.*, 2017). RsaA possesses two UCCC motifs, which in the case of *mgrA* mRNA bind to two distant regions, involving an imperfect duplex masking the SD sequence of the mRNA and a loop-loop interaction occurring downstream in the coding region. These two distant binding sites are required for efficient repression and RNase III-dependent degradation of the repressed mRNA (Romilly *et al.*, 2014). Finally, the sRNA Teg49 is generated from the  $\sigma^B$ -dependent P3 promoter of *sarA* and is most probably processed by RNase III and RNase Y with the help of the helicase CshA (Beaume *et al.*, 2010, Kim *et al.*, 2014, Manna *et al.*, 2017). Transcriptomic analyses revealed that beside genes involved in virulence and autolysis, Teg49 might post-transcriptionally affected the SaeRS and LytRS two-component systems, yet the exact mechanism is not known (Manna *et al.*, 2017). On top of that, another sRNA Teg48, whose role is not known, was processed from the P1 promoter of *sarA* (figure 2). Even if the maturation process and the function of Teg48 and Teg49 are not clearly established, it describes a putative novel reservoir of sRNA from long 5'-UTR of a mRNA encoding a master regulator of virulence in *S. aureus*.

## crRNA, tracrRNA and the CRISPR Cas adaptive immunity systems in *S. aureus*

Phages are the most abundant forms of life on earth and the natural killers of bacteria as in most cases their lytic life cycle ends with the death of the bacterial cell. Outnumbering their microbial hosts, phages impose a selective pressure to the diversification of microbial defense systems (Breitbart and Rohwer, 2005). These include various innate phage-resistance mechanisms such as restriction/modification enzymes, receptor masking, blocking DNA injection, abortive infection (Labrie *et al.*, 2010; Westra *et al.*, 2012), and the adaptive defense mechanism based on clustered, regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (*cas*) genes (Barrangou *et al.*, 2007; Makarova *et al.*, 2006). The latter RNA-interference-like mechanism relies on small non coding RNAs, crRNA and in some cases tracrRNA, through which prokaryotic hosts (bacteria and archaea) can acquire heritable resistance to genetic parasites like phages, but also plasmids and transposons (for reviews see (Westra *et al.*, 2012; Wiedenheft *et al.*, 2012)). To date, CRISPR-Cas systems have been found in about 50% of bacterial genomes and 95% of archaeal genomes (Grissa *et al.*, 2007; Makarova *et al.*, 2015). The CRISPR and the *cas* locus are often located next to each other in the genomes, sometimes organized into operons, but a significant number of genomes have also isolated *cas* loci and/or CRISPRs (Haft *et al.*, 2005).

Despite a large diversity of the CRISPR-cas systems, they share common features. Briefly, the CRISPR loci are characterized by an array of short and palindromic repetitive sequences interspaced by sequences called “spacers” that are coming from plasmid and viruses. These spacers are first integrated into the host genome to provide immunity to the host (acquisition step, i.e., (Horvath and Barrangou, 2010; Nunez *et al.*, 2014)) and during a second event of infection they are transcribed and used as guides to inactivate the viral genome. This two-step pathway involves a variety of Cas proteins, leading to several major types of CRISPR-cas systems (reviewed in (Makarova *et al.*, 2015; Marraffini, 2015)). However, the acquisition step involves two highly conserved Cas1 and Cas2 proteins (Makarova *et al.*, 2015; Yosef *et al.*, 2012). The Cas1-Cas2 integrase is a heterohexameric complex of four Cas1 and two Cas2 which preferentially incorporates foreign DNA at the first CRISPR repeat, participates in the discrimination against self-DNA and in the minimization of off-targeting insertions (Levy *et al.*, 2015; Modell *et al.*, 2017; Wright *et al.*, 2017). Both proteins have been found in several *S. aureus* strains (Grissa *et al.*, 2007). During the second step, activation of transcription from a promoter located in an AT-rich leader sequence preceding the first CRISPR repeat (Jansen *et al.*, 2002; Pougach *et al.*, 2010) leads to

expression of the whole array into precursor CRISPR transcripts (pre-crRNA). The pre-crRNAs are then processed into mature crRNAs consisting of partial repeat(s) and a single spacer sequence, each complementary to a unique invader sequence (Haurwitz *et al.*, 2010). Different endonucleases participate into the maturation step, which might vary in different bacteria. Type I and III system performs the function by a multisubunit Cas protein complex and are characterized by Cas6 processing (Haurwitz *et al.*, 2010). Type II uses another sRNA, tracrRNA, to direct RNase III-dependent maturation of the pre-crRNA in the presence of Cas9, the hallmark protein of the type II system (Deltcheva *et al.*, 2011). Cas9 endonuclease remains associated to the dual-tracrRNA:crRNA structure which, during the interference phase, guides the cleavage of site-specific cognate target DNA (Jinek *et al.*, 2012). Type I and II CRISPR-Cas systems target DNA (Brouns *et al.*, 2008; Zhang and Ye, 2017), whereas type III systems provide immunity against DNA and RNA (Strutt *et al.*, 2018).

With the completion of genome sequences of several *S. aureus* strains, it is now clear that CRISPR-Cas system is not prevalent and only few strains have been reported to harbor this system. Indeed, a CRISPR Finder analysis (Grissa *et al.*, 2007) of the 115 sequenced *S. aureus* genomes present on the CRISPRdb (<http://crispr.i2bc.paris-saclay.fr>) showed that the majority of CRISPR-like loci contain only few spacers (1 or 2), a few have between 3 and 10 spacers, while only one genome (from methicillin resistant *S. aureus* 08BA02176, isolated from a patient) has 15 spacers. The last example has been analyzed using CRISPRone (<http://omics.informatics.indiana.edu/CRISPRone>) (Zhang and Ye, 2017) showing the presence of elements belonging to the Type III-A CRISPR system (including Csm2, Cas1, Cas2 and Cas6; **Figure 3A**). Other isolates share similar organization of the CRISPR-Cas systems (MSHR1132, JS395, CIG290 and 21252) and more than 30 CRISPRs are predicted to be present in the available *S. aureus* genomes (draft or complete) (Holt *et al.*, 2011; Zhang and Ye, 2017). Nevertheless, multi-locus sequencing typing (MLST) performed on one of these isolate (MSHR1132) has shown that it belongs to a divergent clonal complex which lacks the genes for production of staphyloxanthin, and which appears to be closely related to *S. epidermidis* and *S. lugdunensis* (Holt *et al.*, 2011). It has been thus hypothesized that CRISPR/cas was present in a common ancestor of *S. epidermidis*, *S. lugdunensis*, and *S. aureus* and was later lost in most conventional *S. aureus* strains. Noteworthy, numerous repeat-spacer-like structures resemble CRISPR elements but lack spacer diversity, and have been classified as false-CRISPR (Zhang and Ye, 2017). *Staphylococcus aureus* repeat (STAR)-like elements (GC-rich direct repeats) also belong to this class of RNAs for which the functions remained to be addressed (Cramton *et al.*, 2000; Purves *et al.*, 2012). More

recently, a type II-C CRISPR-Cas system was found in a methicillin-resistant *S. aureus* strain isolated from an Irish patient (Figure 3B). This CRISPR element is located on a pseudo SCC-mec composite island, which has been most probably horizontally acquired from *S. epidermidis* strain (Kinnevey *et al.*, 2013). The peculiarity of this system is that it contains a shorter version of Cas9 protein. The crystal structure of a complex containing SaCas9, the sgRNA (an artificial fusion product of a crRNA and a tracrRNA (Jinek *et al.*, 2012)) and its target DNA provided a model to understand how crRNA and tracrRNA guide Cas9 on the target DNA and prepare it for double stranded DNA cleavages (Nishimasu *et al.*, 2015). In this structure, the DNA duplex of the protospacer adjacent region (PAM) contains the signals recognized by the Cas9 PAM-interacting (PI) domain, which discriminates the invader DNA against self-DNA and facilitates the target DNA unwinding leading to the formation of heteroduplex. The beginning of the RNA–DNA heteroduplex (“seed” region) adopts a distorted structure critical for Cas9-catalyzed DNA cleavage. This conformation resulted from the RNA-DNA helix structure, the interactions with protein residues and the RNA helix formed by the repeat/anti-repeat regions mimicking the tracrRNA-crRNA interactions (Figure 3C). The discovery of the smaller Cas9 protein led to recent improvements in genome editing (Ran *et al.*, 2015). The seminal works of Jennifer Doudna and Emmanuelle Charpentier on the use of CRISPR-Cas systems for genome editing have inspired many studies showing the incredible potency of the system (Cong *et al.*, 2013; Jinek *et al.*, 2013; Mali *et al.*, 2013). Using Cas9 from *Streptococcus pyogenes* (SpCas9) (Sakuma *et al.*, 2014) directed by the sequence of a single-guide RNA (sgRNA), thousands of publications have reported its application for site-specific genome modifications, gene knockouts or replacements, gene expression control and functional genome screenings in over 40 different species. Interestingly, SaCas9 has been successfully used for eukaryotic genome editing since its smaller size makes it easier to be delivered *via* adeno-associated virus vectors to somatic tissues (Ran *et al.*, 2015).

The fact that CRISPR-Cas has been mapped on mobile elements in *S. aureus* MRSA confirms the importance of horizontal genes transfer from other co-colonizing bacteria in the acquisition of novel functions and in the evolution of *S. aureus* strains. It remains to be analyzed what could be their advantages and why these CRISPR elements are rare in *S. aureus*. Interestingly, transcription of the CRISPR-Cas genes can be highly regulated and induced upon infection (Agari *et al.*, 2010; Quax *et al.*, 2013; Young *et al.*, 2012) by membrane stress (Perez-Rodriguez *et al.*, 2011) and, in Gram-negative bacteria, by quorum sensing (QS) signaling (Hoyland-Kroghsbo *et al.*, 2017; Patterson *et al.*, 2016). Recent work

has developed genetic engineering tools in order to apply CRISPR/Cas9 system as an antimicrobial strategy against *S. aureus* (Park *et al.*, 2017). Clearly, the CRISPR/Cas9 system offers an alternative therapeutic to the conventional antibiotics.

### Role of sRNAs in physiopathology

The pathogenesis related to *S. aureus* can take different forms depending on the infected tissue and the invading bacterial strain. This is often accompanied by the expression of various virulence factors involved in the colonization and the alteration of the tissue but also by the capacity to escape from the host immune response. Among key regulators of virulence, several sRNAs have been shown to modulate the synthesis of virulence factors in a dynamic manner, and some of them contribute to specific aspects of bacterial virulence in animal models of infection (Bischoff and Romby, 2016).

The most studied RNA in *S. aureus* is one of the main intracellular effectors of the quorum sensing system *agr*. As described above, RNAIII regulates expression of virulence factors known to be associated with infectious diseases. For instance, it represses the synthesis of protein A, which triggers inflammatory signalling pathways and contributes to evasion of the immune response. Conversely, RNAIII induces the synthesis of a battery of toxins, which contribute to the degradation of tissues and subversion of host defenses, such as the pore-forming toxins, and peptides with proinflammatory and lytic activities. A recent modelisation of the quorum sensing system and of its regulators has illustrated the importance of the *agr* system in promoting dissemination of the bacteria from biofilms or dense population (Audretsch *et al.*, 2013; Nitzan *et al.*, 2015). Nevertheless, despite the fact that many clinical isolates from acute infections express RNAIII, its steady state level varies considerably among them (Jelsbak *et al.*, 2010; Song *et al.*, 2012). In particular, higher level of RNAIII has been observed in the increased virulence community acquired MRSA strains as compared to other *S. aureus* lineages (Montgomery *et al.*, 2010; 2008). Interestingly, a recent study showed that the level of RNAIII is lower in strains isolated from patients with sepsis than those from commensal carrier patients (Bordeau *et al.*, 2016). Perhaps more surprisingly, heterogeneity has been reported in patients where *agr*-positive and *agr*-negative strains co-existed. This has been proposed as one of the criteria, which might modulate the outcome of the infections (Painter *et al.*, 2014; Pollitt *et al.*, 2014).

*S. aureus* is also frequently exposed to other microbes during colonization and infection, providing opportunities to acquire mobile genetic elements that contribute to the evolution of the genome. Some of these genomic islands play key roles in pathogenesis

through the complementation of new virulence factors (pathogenicity islands) or through the synthesis of novel regulators modulating the expression of genes of the core genome. As an example, SprD is an important small regulatory sRNA (142-nts long nucleotides) expressed from a pathogenic island, which promoted significantly *S. aureus* diseases in a mouse sepsis model of infection (Chabelskaya *et al.*, 2010; Pichon and Felden, 2005). SprD interacted through base-pairings with the *sbi* mRNA, which encodes an immune evasion molecule protecting the bacteria against the host immune responses (Haupt *et al.*, 2008; Zhang *et al.*, 1998). However, the phenotype of the  $\Delta sprD$  mutant strain was not linked to the SprD-dependent regulation of *sbi* since the  $\Delta sbi$  mutant strain behaved as the wild type strain in the mouse sepsis model (Chabelskaya *et al.*, 2010). Therefore, these data strongly suggested that SprD might regulate the expression of other proteins important for infection.

SSR42 (for small stable RNAs) is a 891-nts long sRNA whose stability is greatly enhanced under stationary phase of growth (Anderson *et al.*, 2006; Morrison *et al.*, 2012). It regulated the expression of approximately 80 mRNAs in two genetically different *S. aureus* strain backgrounds. While it increased the expression of capsule Cap5a, SSR42 down-regulated the expression of protein A,  $\alpha$  and  $\gamma$  hemolysin, and Panton Valentin Leukocidin (PVL) (Morrison *et al.*, 2012). Because no direct binding was evidenced between SSR42 and mRNAs encoding virulence determinants, the effect was predicted to be indirect through the modulation of the expression of a transcriptional regulator. Phenotypically, the deletion of SSR42 gene affected erythrocyte lysis, resistance to opsonisation killing, and pathogenesis in a murine model of skin and soft tissue infections (Morrison *et al.*, 2012). More recently, SSR42 was selected as an important gene for the intracellular virulence after screening of a transposon mutant library pool. After internalization in epithelial cells, the  $\Delta SSR42$  mutant strain was significantly enriched in the intracellular fraction most likely due to an attenuated cytotoxicity (Das *et al.*, 2016).

In contrast to RNAIII, SprD and SSR42, which contributed to enhance virulence of *S. aureus*, other regulatory RNAs behave as attenuators of virulence. This is for instance the case of another encoded pathogenicity island sRNA, the so-called SprC (Pichon & Felden, 2005). Indeed, the virulence of the isogenic strain lacking SprC was significantly and reproducibly improved in a mouse systemic model. Furthermore, SprC reduces *S. aureus* phagocytosis by human monocytes and macrophages, and resistance to oxidative stress. This phenotype appears to be due, at least in part to the formation of base pairings between SprC and *atl* mRNA which encodes the staphylococcal autolysin (ATL) (Le Pabic *et al.*, 2015). Another example is the Psm-mec RNA, which is a bifunctional RNA located into the

SCCmec mobile genetic element. It encodes a phenol-soluble modulins (PSMmec) cytolytic toxin and acted as a translational repressor through direct binding with *agrA* mRNA (Kaito *et al.*, 2013). AgrA activated the transcription of all *psm* genes such as the PSM $\alpha$ . Therefore, the deletion of the *psm-mec* RNA increased the expression of AgrA which resulted in an increase of toxin and PSM $\alpha$  production and enhanced virulence in mice (Kaito *et al.*, 2013). Interestingly, community acquired methicillin resistant *S. aureus* (CA-MRSA) that do not carry the *psm-mec* gene has been shown to be more virulent than the hospital associated MRSA that harbors the gene (Kaito *et al.*, 2013; 2011). Finally, a mutant strain that did not express the non coding RNA RsaA attenuated the severity of acute systemic infection in a mouse model (Romilly *et al.*, 2014). This deletion is linked to the deregulation of MgrA, a master regulator of capsule synthesis and clumping (Crosby *et al.*, 2016). This phenotype in pathogenesis is most probably linked to the high sensitivity of the mutant strain to opsonophagocytosis by host PMNs. Because the expression of these three sRNAs is detrimental for bacterial spreading into colonized organisms, one may suggest that during evolution they have favour commensalism with the host.

In order to evaluate the impact of the sRNA expression in the context of the host infection, two studies have explored the possible relationships between infection severity and RNA expression levels. In the first study, the expression levels of five sRNAs (RNAIII, RsaA, RsaE, RsaG and RsaH) were analyzed in samples from acute cutaneous infection, cystic fibrosis sputum or nasal colonization. The expression profiles did not correlate with the type of infection but the authors have noticed that the expression of these five RNAs were more homogenous in the nasal colonization isolates than in those responsible for infection (Song *et al.*, 2012). More recently, the expression levels of RNAIII and SprD were measured in 40 strains cultivated from patients with sepsis or septic shock and compared to 21 strains isolated from asymptomatic colonized carriers. It appeared that strains from septic shock had significantly lower levels of RNAIII and to a lesser extent for SprD (Bordeau *et al.*, 2016). It is important to note that this analysis was performed on clinical isolates cultured *in vitro* and does not necessarily reflect the expression of these RNAs during infection within the host. In fact, studies to assess the role and importance of RNAs in the establishment or evolution of infection are difficult to achieve. The great variability of *S. aureus* strains, the difficulty to obtain highly controlled cohorts of patients, the reliability of sampling protocols, sample processing and RNA expression analysis are all obstacles to overcome. Moreover, biological variables may influence the analysis since the relationships between host immune system and microbe seems to be particularly individualized and can influence the disease outcome

(Krismer *et al.*, 2017; Brown *et al.*, 2014). Furthermore, interspecies interactions between bacterial pathogens and the commensal microbiota, as well as limited nutrients play major roles in promoting or in preventing *S. aureus* colonization (Krismer *et al.*, 2017). Interestingly, it was shown that the *agr* system is repressed by high concentrations of haemoglobin in the nasal fluids leading to the expression of several cell surface proteins and favouring nasal colonization (Pynnonen *et al.*, 2011). Similar data were observed when *S. aureus* was co-cultivated with the nasal strain of *Corynebacterium striatum* (Ramsey *et al.*, 2016). A recent study also demonstrated that the commensal *S. epidermidis* can influence the expression of one ncRNA of *S. aureus* (Hermansen *et al.*, 2018).

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

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## Figures legends

**Figure 1:** Several mechanisms of RNA regulation in *Staphylococcus aureus*. **A.** Schematic drawing of the flavin mononucleotide (FMN) riboswitch. The 5' UTR adopts a particular structure recognized by FMN which in turn leads to the stabilization of a stem-loop structure sequestering the Shine and Dalgarno sequence (SD) to inhibit translation. 30S is for the small ribosomal subunit. **B.** The 3'-UTR of the biofilm repressor IcaR possesses a cytosine-rich motif, which binds to the Shine-Dalgarno (SD) and hinders ribosomes from its binding site on the mRNA (see text for details). **C.** Overlapping 5'-UTRs of *tagG* and *tagH* mRNAs are processed by the endoribonuclease III (RNase III). Shorter 5'ends might facilitate ribosome recruitment. **D.** The anti-toxin RNA SprF1 interacted at the 3'end of the toxin encoding by *sprG1* and triggered its degradation. **E.** A cluster of five sRNAs was sequenced in the *S. aureus* Newman strain that encodes a putative toxin-antitoxin system (see text for details). **F** and **G.** sRNAs act by an antisense mechanism. Binding of the 5'-UTR of RNAIII to the 5'-UTR of *hla* mRNA liberated its SD and activated translation (F) whereas the 3' domain of RNAIII acted as a repressor domain, which contains C-rich motifs for base-pairing with the SD of mRNA as *coa* mRNA depicted in the figure (G). Green bars represent SD sequence, black circles are for RNase III (for references and more details, see text).

**Figure 2:** Examples of complex network between sRNAs and transcriptional factors in *Staphylococcus aureus* in response to stress. Arrows are for activation, bars for repression. In blue, are the transcriptional regulators, in green the two-component systems and in red the regulatory sRNAs. Red lines corresponded to post-transcriptional regulation and black lines to transcriptional regulation. Dotted lines are for the target mRNAs that were not experimentally validated. For clarity, only sRNA-dependent mRNA targets encoding transcriptional factors were depicted in the figure.

**Figure 3. A.** Genomic organization of the loci for the Type III-A CRISPR system of the *Staphylococcus aureus* 08BA02176 strain. Type III is the typical *S. aureus* CRISPR organization. The scheme has been obtained using CRISPRone (Zhang and Ye, 2017) and the GenBank deposited 08BA02176 genome sequence (RefSeq accession number GCF\_000296595.1). **B.** Genomic organization of the loci for the Type II-C CRISPR system of the *Staphylococcus aureus* M06/0171 strain. The CRISPR-Cas genes have been found on a staphylococcal cassette chromosome *mec* (SCC*mec*) inserted into the 3' end of the

chromosomally located *orfX* gene. The scheme has been obtained using CRISPRone (Zhang and Ye, 2017) and the GenBank deposited SCCmec sequence (GenBank accession number HE980450.1). C. Cartoon (RNA and DNA) and surface (Cas9) representations of the SaCas9–sgRNA–target DNA complex (pdb file 5AXW (Nishimasu *et al.*, 2015)). The SaCas9 sgRNA consists of the crRNA guide region (crGUIDE represented in pale yellow color) forming a heteroduplex with the target DNA strand (tDNA in magenta) and the repeat/anti-repeat helix (in blue the repeat crRNA derived strand, in green the anti-repeat tracrRNA derived strand). The PAM containing DNA duplex is red. Cas9 domains are colored as follow: WED domain is cyan, REC domain is pale orange, NUC domain is grey. Molecular graphics images have been prepared using PyMol (Schrodinger, 2015).

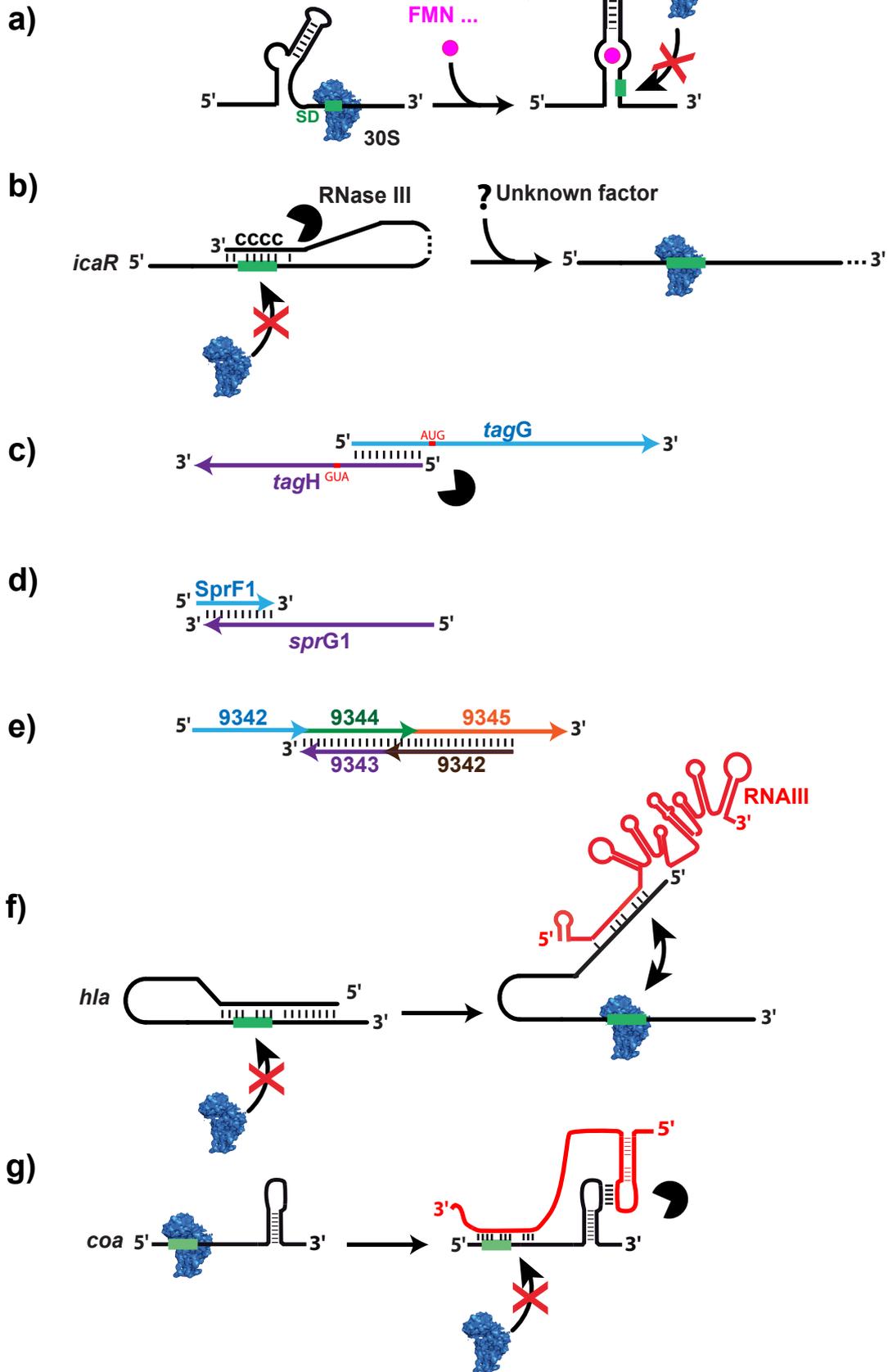
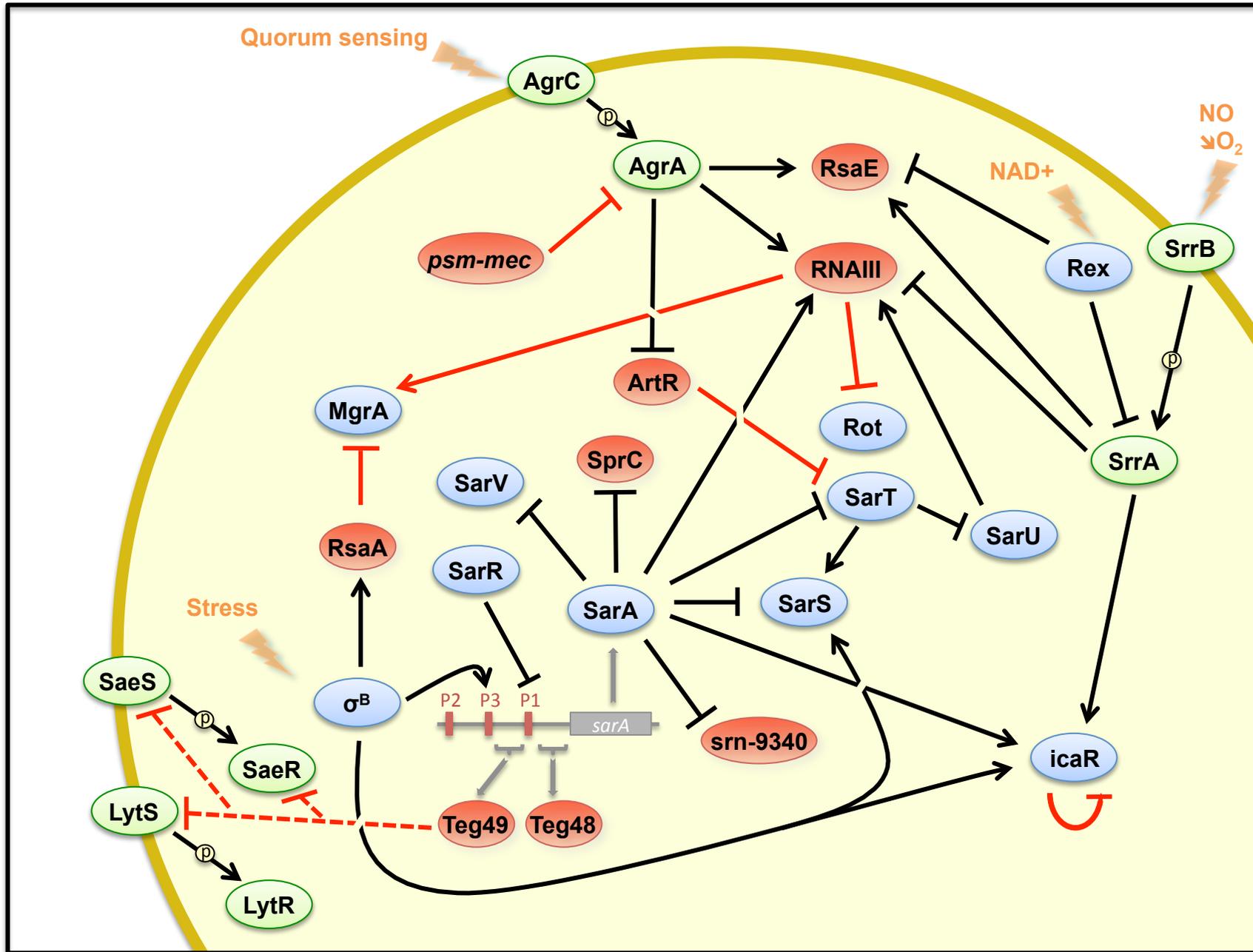
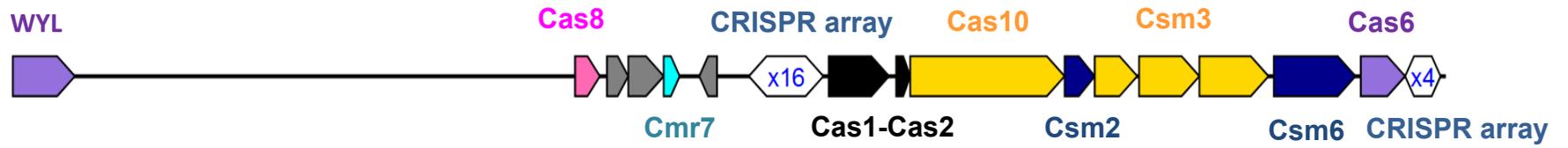
**Figure 1**

Figure 2



a) *S. aureus* type III-A CRISPR-Cas system (08BA02176 strain)



b) *S. aureus* type II-C CRISPR-Cas system (M06/0171 strain)



c) *S. aureus* Cas9-sgRNA-DNA structure from type II CRISPR-Cas system

