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Staphylococcus aureus Regulatory RNAs Driving Fitness upon Antibiotic Exposure

Wenfeng Liu

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Staphylococcus aureus regulatory RNAs driving fitness upon antibiotic exposure

Thèse de doctorat de l'Université Paris-Saclay
préparée à l'université Paris-Sud

École doctorale n°577: Structure et Dynamique des
Systèmes Vivants (SDSV)
Spécialité de doctorat: Sciences de la Vie et de la Santé
Microbiologie et Biologie moléculaire

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Abbreviation

2-DOS	2-deoxystreptamine
AACs	aminoglycoside acetyltransferases
ABC	ATP-binding-cassette
<i>agr</i>	accessory gene regulator
ANTs	aminoglycoside nucleotidyltransferases
APHs	aminoglycoside phosphotransferases
ArmA	aminoglycoside resistance methyltransferase A
asRNAs	antisense RNAs
aTc	anhydrotetracycline
CA	community acquired
CA-MRSA	community-associated MRSA
CLSI	Clinical and Laboratory Standards Institute
CoNS	coagulase-negative strains
CTD	C-terminal domain
daptomycin-NS	daptomycin non-susceptibility
<i>erm</i>	erythromycin ribosomal methylase
EOP	efficiency of plating
FDA	Food and Drug Administration
GMP	guanosine mono phosphate
HA	hospital-acquired
HGT	horizontal gene transfer
hVISA	heterogeneous-VISA
IVDU	intravenous drug users
LPG	lysyl-phosphatidylglycerol
LPS	lipopolysaccharide
MDR	multidrug resistant
MFS	major facilitator superfamily
MGE	mobile genetic elements
MIC	minimal inhibitory concentration
MLS	macrolides, lincosamides and streptogramins
MLS _B	macrolide, lincosamide and streptogramin B
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>

NAG	N-acetylglucosamine
NAM	N-Acetylmuramic acid
NGS	next generation sequencing
NTD	N-Terminal domain
PBPs	penicillin-binding-proteins
PC1	2,5,6-triaminopyrimidin-4-one
PG	peptidoglycan
PG	phosphatidylglycerol
PhLOPSA	phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A
PSM	phenol-soluble modulin
PTC	peptidyl transferase centre
QRDR	quinolone resistance-determining region
RNAP	RNA polymerase
RND	resistance nodulation division
RRDR	rifampicin resistance-determining regions
SCC	staphylococcal chromosome cassette
SD	Shine-Dalgarno
sRNAs	small RNAs
SSSS	staphylococcal scalded skin syndrome
TA	toxin-antitoxin
Topo IV	Topoisomerase IV
TSS	toxic shock syndrome
TSS	transcriptional start site
TSST-1	toxic shock syndrome toxin-1
UTR	untranslated region
VISA	vancomycin-intermediate <i>S. aureus</i>
VRE	vancomycin-resistant enterococci
VRSA	vancomycin-resistant <i>S. aureus</i>
VSSA	vancomycin-sensitive <i>S. aureus</i>
WHO	World Health Organization
WWTPs	wasterwater treatment plants

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Introduction

1 *Staphylococcus aureus*

1.1 General characteristics

Staphylococcus aureus (*S. aureus*), often referred as the Golden Staph, was first isolated from the pus of surgical wounds in a knee joint in 1880 in Aberdeen (Scotland) by surgeon Sir Alexander Ogston, who originally referred to the bacteria as micrococci (Ogston 1881; Ogston 1882). Under a microscope, *S. aureus* forms grape-like clusters of sphere-shaped bacteria, which prompted him to name the organism staphylococci distinguishing it from chain-forming streptococci that is also associated with surgical wound infections (Ogston 1882). In 1884, Rosenbach differentiated staphylococci isolated from humans based on the pigmentation of the colonies, and proposed the nomenclature *Staphylococcus aureus* for yellow-orange or “gold” pigmented colonies and *staphylococcus albus* (now designated as *Staphylococcus epidermidis*) for white colonies. The yellow pigmentation is produced by the staphyloxanthin, a membrane-bound carotenoid.

S. aureus is a Gram-positive bacterium with diameters of 0.5-1.5 μm (Figure 1). It is non-motile, forms no spore, and is a facultative anaerobe, which can grow through aerobic respiration or fermentation. *S. aureus* is tolerant to high concentrations of salt and shows resistance to heat (Harris *et al.* 2002). It reproduces asexually by binary fission; complete separation of the daughter cells is mediated by *S. aureus* autolysin (Varrone *et al.* 2014). The genus it belongs named *Staphylococcus* is catalase-positive and oxidase-negative, which differentiates from *Streptococcus* genus that is catalase-negative; in addition, they have different cell wall compositions (Harris *et al.* 2002).

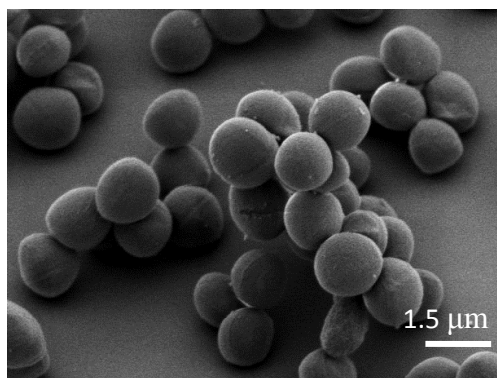


Figure 1 *Staphylococcus aureus* under electron microscope.

(Thierry Meylheuc, Claire Morvan and David Halpern, INRA, Micalis, Jouy-en-Josas)

The cell wall of *S. aureus* is a thick and tough protective coat (G D Shockman and Barren 1983). In general, *S. aureus* cell wall presents the following characteristics: i) thick peptidoglycan

layer, it makes up 50% of the cell wall mass and is capable of withstanding high internal osmotic pressure; ii) teichoic acids, a group of phosphate-containing polymers contributing to about 40% of cell wall mass (Knox and Wicken 1973). Two types of teichoic acids are present, cell wall teichoic acid covalently bound to the peptidoglycan and membrane-associated lipoteichoic acid inserted to the phospholipid bilayer of the bacteria, which serve as chelating agents and certain types of adherence; iii) surface proteins, exoproteins and peptidoglycan hydrolases (autolysins), which compose the other 10% of cell wall weight. Some of these components are involved in adhesion and are virulence determinants (Harris *et al.* 2002). Underneath the cell wall is the cytoplasm that is enclosed by the cytoplasmic membrane. Eventually, some *S. aureus* clinical strains have been shown to possess capsular polysaccharides (Fournier 1990; Thakker *et al.* 1998); it is reported that capsule production decreases phagocytosis *in vitro*, thus enhancing *S. aureus* virulence in a mouse bacteraemia model (Wilkinson and Holmes 1979; Thakker *et al.* 1998).

1.2 Pathogenicity and Infectious diseases

S. aureus is part of animal normal flora and has a remarkable capacity to adapt to different niches (van Belkum *et al.* 2009). It preferentially colonizes anterior nares (Williams 1963) but is found in extra-nasal sites including the skin, the pharynx (Ridley 1959), the gastrointestinal tract (Rimland and Roberson 1986), the women uro-genital tract (Guinan *et al.* 1982) and the axillae (Dancer and Noble 1991). Approximately 30% of human population are asymptotically and persistently colonized (Wertheim *et al.* 2005; van Belkum *et al.* 2009). However, this commensal microorganism is now globally seen as an important opportunistic pathogen related to a wide array of community-associated and hospital-acquired infections, from superficial infections to invasive and life-threatening diseases. A remarkable epidemiologic transition was observed in the recent two decades: i) a growing number of health care-associated infections, particularly endocarditis and prosthetic device infections, ii) an epidemic of community-associated skin and soft tissue infections (SSTIs) driven by strains with particular virulence factors (Tong *et al.* 2015); which both are resulting in considerable morbidity and mortality worldwide.

❖ Skin and soft tissue infections (SSTIs)

S. aureus has traditionally been the leading cause of SSTIs, with the emergence of a worldwide epidemic of community-associated MRSA (CA-MRSA) SSTIs (Tong *et al.* 2015). The skin and mucous membrane are excellent natural barriers against local tissue invasion by *S. aureus*.

However, breaches in skin barriers following trauma and surgical procedures favor the entry of *S. aureus* into subcutaneous tissues, thus creating local abscesses (Elek 1956; Elek and Conen 1957) and can lead to septicaemia if it reaches the lymphatic channels or blood. Nevertheless, SSTIs caused by *S. aureus* can occur at sites without apparent breaches including folliculitis (hair follicles), impetigo (bullous or superficial lesions) or furuncles/carbuncles (deep-seated or confluent abscesses) (David and Daum 2010). The primary defense against *S. aureus* infection is the neutrophil and macrophages responses (Tong *et al.* 2015). However, *S. aureus* can escape this immune response in a multitude of ways, such as blocking chemotaxis of leukocytes, sequestering host antibodies, hiding from detection *via* polysaccharide capsule or biofilm formation, and resisting destruction after ingestion by phagocytes (Tong *et al.* 2015).

❖ Bloodstream Infections

The dissemination of *S. aureus* in the blood is known as bacteremia, which can be categorized into three groups based on its onset: i) hospital-acquired (HA) (Klevens *et al.* 2008), ii) community acquired (CA), iii) HA with community onset (infection in an outpatient who has had recent, extensive contact with the healthcare system) (Thomer *et al.* 2016). Bacteremia is a life-threatening condition, which can result in sepsis and acute shock. Endocarditis is a typical bloodstream infection as a result of *S. aureus* long-term colonization of vasculature (Dastgheyb and Otto 2015). It is largely associated with intravenous drug users, who introduce *S. aureus* directly into the bloodstream through contaminated needles or poor sterilization of the injection site (Miro *et al.* 2005; Shrestha *et al.* 2015). Moreover, prosthetic devices including central venous catheters, surgically implanted materials and orthopedic prostheses serve as a direct conduit into the intravascular space and are risk factors for bacteremia (Jensen *et al.* 1999).

1.3 Adaptability and antibiotic resistance

S. aureus infections can be both common and serious, particularly because of the waves of antimicrobial resistance increase and changes in clinic spectrum (Chambers and DeLeo 2009; Tong *et al.* 2015). *S. aureus* is known to be highly adaptable.

The innate adaptability of *S. aureus* has led to the emergence of resistance to multiple classes of antibiotics through the acquisition of mobile genetic elements (MGE) encoding resistance determinants, or mutations in loci influencing antibiotic sensitivity (DeLeo and Chambers 2009; Jensen and Lyon 2009; Fitzgerald 2014). Methicillin-resistant *S. aureus* (MRSA) have been documented at a rapid and increasing rate since methicillin was first introduced in 1959. Hospital-associated MRSA (HA-MRSA) clones are now recognized to be the leading cause of

nosocomial infections worldwide (Carleton *et al.* 2004; Fridkin *et al.* 2005; Nickerson *et al.* 2009). The emergence of community-associated MRSA (CA-MRSA) in the past several decades has also become a point of concern, as CA-MRSA virulent strains are fast-spreading and can affect seemingly healthy individuals (Kourbatova *et al.* 2005; Giersing *et al.* 2016). The treatment of MRSA isolates requires the use of vancomycin, clindamycin, linezolid or daptomycin (Liu *et al.* 2011). The emergence of vancomycin-resistant *S. aureus* (VRSA) and vancomycin-intermediate *S. aureus* (VISA) is concerning as vancomycin is considered as the last resort treatment against MRSA. At the same time, *S. aureus* vaccine development has thus far not been proved successful, neither the use of antibodies against staphylococcal polysaccharide (Shinefield *et al.* 2002) or against secreted virulence factors (Kernodle 2011; Fowler *et al.* 2013; Thomer *et al.* 2016).

1.4 Virulence factors of *S. aureus*

The pathogenic *Staphylococcus* are commonly identified by their ability to produce coagulase and clot human and animal blood (Kloos and Musselwhite 1975). This distinguishes the coagulase positive strains including *S. aureus* from coagulase-negative strains (CoNS), such as *S. epidermidis*.

Virulence is defined as the ability of a pathogen to reduce host fitness, in other words, as the ability of an organism to establish an infection and cause disease in a host. *S. aureus* encodes a wide variety of adhesins and virulence factors that are involved in diverse virulence mechanisms, such as adhesion, colonization, biofilm formation, immune evasion, immune stimulation or cell lysis and resistance to phagocytosis (Dastgheyb and Otto 2015). *S. aureus* has three well documented global regulators of virulence: *agr* (Recsei *et al.* 1986; Morfeldt *et al.* 1988), *sar* (Cheung *et al.* 1992) and *sae* (Giraudou *et al.* 1994), which regulate the expression of surface proteins and exoproteins (*e.g.* toxins) (Harris *et al.* 2002). For instance, the phenol-soluble modulins (PSM) family of peptides, which is the most potent cytotoxin (Peschel and Otto 2013), is under the control of the *agr* regulation network that also controls many other toxins such as hemolysins (*e.g.* α -toxin) (Queck *et al.* 2008) and leukotoxins (Recsei *et al.* 1986). *S. aureus* can produce a range of extracellular toxins during the immune evasion process, including toxic shock syndrome toxin-1 (TSST-1), enterotoxins and exfoliative toxins (Harris *et al.* 2002). TSST-1 is the toxin responsible for toxic shock syndrome (TSS) that is only caused by strains carrying the TSST-1 gene (Jamart *et al.* 2005). Ingestion of enterotoxin produced by *S. aureus* in contaminated food can cause food poisoning (Argudín *et al.* 2010; Hennekinne *et*

al. 2012). The exfoliative toxins are associated with staphylococcal scalded skin syndrome (SSSS) (Mishra *et al.* 2016).

Some virulence factors are encoded by genes that are located on MGE, such as plasmids, transposons, insertion elements, pathogenicity islands (*e.g.* some enterotoxins associated with food poisoning) (Dinges *et al.* 2000) or lysogenic bacteriophages (*e.g.* Panton-Valentine leucocidin) (Narita *et al.* 2001), and factors interfere with or even clearly suppress host innate immunity such as staphylokinase (Rooijackers *et al.* 2005).

2 Overview of small regulatory RNAs in *S. aureus*

S. aureus is often exposed to a wide range of stresses in its constantly changing natural environments, such as nutrients starvation, temperature, pH, oxygen level and antibiotics. It has developed in response to these changes, a plethora of signaling pathways that sense the environment and coordinate the temporal alterations in gene expression and protein activity that favors survival and proliferation. Therefore, the comprehension and exploration of intricate regulatory networks and their dynamics that underlie fast adaptive responses and production of virulence factors is a prerequisite to find alternative strategies to combat *S. aureus* infections. Regulatory RNAs, together with two-component systems and other regulatory proteins, are implicated in these regulatory circuits.

Regulatory RNAs, often referred as small RNAs (sRNAs), are usually non-coding and short (50-500 nts) (Waters and Storz 2009). Their main function is often the posttranscriptional regulation of gene expression (Mandin and Guillier 2013). To date, numerous sRNAs have been predicted and identified in *S. aureus* by bioinformatics (Pichon and Felden 2005; Geissmann *et al.* 2009; Marchais *et al.* 2009), DNA-arrays (Anderson *et al.* 2006; Roberts *et al.* 2006; Mader *et al.* 2016), cDNA sequencing (Hüttenhofer and Vogel 2006), and RNA-seq (Bohn *et al.* 2010; Howden *et al.* 2013; Broach *et al.* 2016; Carroll *et al.* 2016) methods. sRNAs have various modes of action, they act either through base pairing with nucleic acid targets (*e.g.* mRNA), changing their stability and the translation efficiency; or through the modulation of protein activity by mimicking their substrates (Mandin and Guillier 2013).

2.1 Diversity of sRNA-mRNA interactions

sRNAs act in *cis* or in *trans* depending upon their structural relationship with their target genes, and affect genes in transcriptional or posttranscriptional levels:

- i) *cis-acting* regulatory RNAs are usually located in the 5' or 3' untranslated region (UTR) of mRNA. They regulate the transcription of adjacent genes by responding to transacting agents or environmental cues, such as temperature (thermosensors), intracellular concentration of metabolites (riboswitches), uncharged tRNAs (T-boxes) or proteins (Romby and Charpentier 2010).
- ii) *trans-acting* regulatory RNAs are often located in intergenic regions and remote from their mRNA targets. They usually exhibit partial base pairing complementarities with their targets (Waters and Storz 2009; Richards and

Vanderpool 2011; Jagodnik *et al.* 2017). They can have multiple targets (Romby and Charpentier 2010).

- iii) Antisense RNAs (asRNAs) are transcribed from the opposite strand of the target gene. Most of them act as *trans-acting* sRNA and often show high degree or complete complementarity with the targeted mRNA (Romby and Charpentier 2010). A specific example shown that asRNA regulated the expression of target *ubiG* operon in *cis* through transcriptional interference at the *ubiG* locus (Andre *et al.* 2008).

2.1.1 *Cis-acting* regulatory RNAs

The untranslated regions (UTRs) of mRNA contain important features affect the post-transcriptional and translational regulation of gene expression (Pesole *et al.* 2001; Ren *et al.* 2017).

cis-acting sRNAs are usually part of the 5'UTR or 3'UTR of an mRNA, whose expression is regulated by the sRNA (Cho and Kim 2015). The length of 5' or 3' UTR varies from a few to hundreds of nucleotides (Bouloc and Repoila 2016). They contain dedicated regulatory sites that can not only be recognized by a variety of *trans-acting* regulators (metabolites, uncharged tRNAs, proteins), but also function as direct sensors of the environmental signals (temperature, divalent ions, pH) (Breaker 2009; Narberhaus 2010; Ramesh and Winkler 2010; Smith *et al.* 2010). Some *cis-acting* RNAs are well known to alter the expression of virulence factors (Somerville and Proctor 2009; Caldelari *et al.* 2013).

2.1.1.1 5'UTRs as a source of regulatory RNAs

- i) *Cis-acting* sRNAs sensing metabolites used as antibiotic putative target

A widespread *cis-acting* RNA element in 5'UTR of mRNAs is the riboswitches which are metabolite-sensing and feedback regulate the associated genes (Breaker 2011). Riboswitches comprise two functional domains: an aptamer and an expression platform (Nudler and Mironov 2004; Coppins *et al.* 2007; Dambach and Winkler 2009; Henkin 2009). The aptamer or the sensor domain is a conserved and structured receptor that specifically recognizes by a defined ligand; the expression platform undergoes significant structural changes, and then typically switches off the expression of the downstream ORF as a result of ligand binding (Figure 2), but some turn it on (Serganov 2010). This regulation happens at the transcriptional or

posttranscriptional levels depending on the loading positions of the riboswitch on the mRNA, resulting in repressing or activating of gene expression (Breaker 2011).

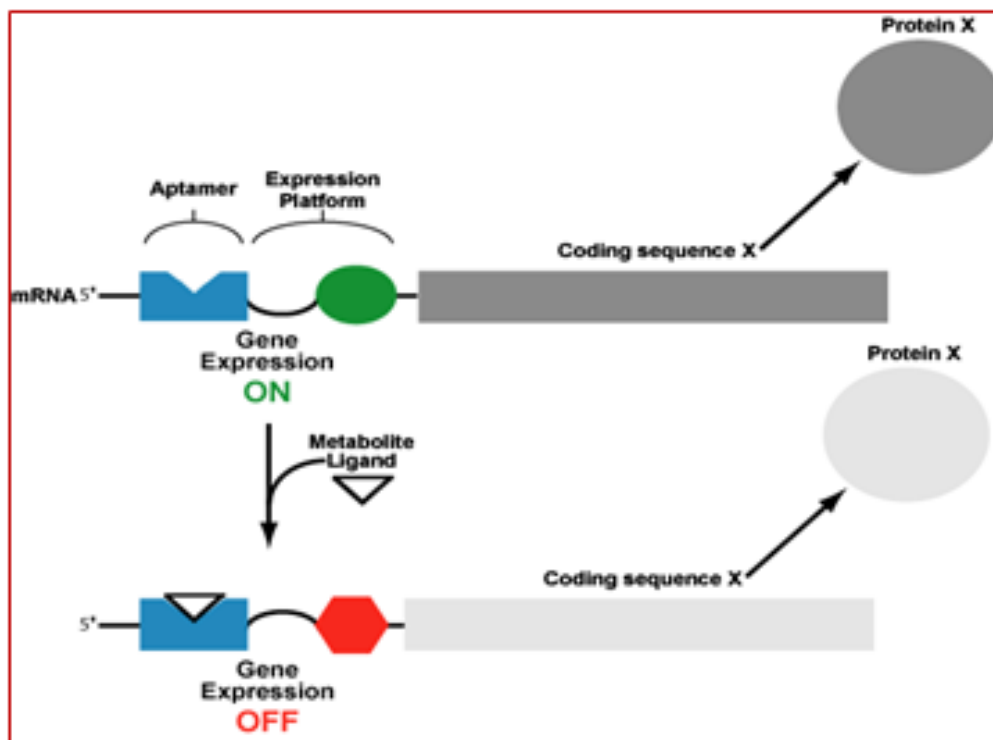


Figure 2 Common mechanism of riboswitches. (Edwards and Batey 2010)

Riboswitches are attractive targets for the development of novel antibacterial compounds, which possibly offer an alternative solution for the growing multiple drug resistant nosocomial pathogens (Breaker 2009; Mulhbacher *et al.* 2010). As an example, PC1 (2,5,6-triaminopyrimidin-4-one), a pyrimidine derivative compound binds guanine riboswitches and constitutively switches off the essential *guaA* gene, which encodes guanosine mono phosphate (GMP) synthetase (a purine nucleotide). PC1 thus has been shown to have a bactericidal activity against *S. aureus* and to reduce infection in mice model (Mulhbacher *et al.* 2010). Importantly, PC1 has a narrow spectrum activity as it targets exclusively bacteria containing the purine riboswitch, which should reduce selective pressure for resistance on non-targeted bacteria (Caldelari *et al.* 2013); in contrast, this is also the major limitation to validate PC1 clinically given that it does not target all bacteria containing guanine riboswitches, but only those in which *guaA* is under the control of a riboswitch. Furthermore, there was no apparent cytotoxicity for mammals (mice) since riboswitches are generally absent in the eukaryotic host (Mulhbacher *et al.* 2010).

ii) *Cis-acting* sRNAs sensing antibiotics

Antibiotic-induced stabilization of transcripts of *erm* family genes in *S. aureus* is a well characterized example for 5' untranslated regulatory region. It is a translational attenuation mechanism controlled by site-specific ribosome stalling, and used for inducible expression of antibiotic resistance genes *erm* (methyltransferase) (Gryczan *et al.* 1980; Horinouchi and Weisblum 1980). The *erm* family specifies rRNA methylases that confer resistance to macrolide, lincosamide and streptogramin B (MLS_B) antibiotics by reducing the affinity between these antibiotics and ribosomes (Sandler and Weisblum 1989). Briefly, *ermC* mRNA is transcribed constitutively but translated in an extremely low level due to the masking of the ribosome-binding site and initiation codon within mRNA secondary structure; in the presence of sub-inhibitory concentrations of an inducer (erythromycin or similar lincosamides) (Weisblum *et al.* 1971), the antibiotic binds to ribosomes causing ribosome stalling. Stalling during translation of the leader peptide triggers the conformational change that releases *ermC* RBS and activates the expression of the methylase gene (Vazquez-Laslop *et al.* 2008). A similar mechanism was proposed for *S. aureus ermA* gene which has a more complex structure and encodes two peptides in contrast to one of *ermC* (Murphy 1985).

2.1.1.2 3'UTRs as a source of regulatory RNAs

Bacterial 5' UTRs are more spotlighted compared to 3'UTRs. However, bacterial 3'UTRs especially long 3'UTRs i) have recently emerged as a new class of post-transcriptional regulatory elements (Ren *et al.* 2017), ii) are considered as a rich reservoir of small regulatory RNAs either by processing of the long 3'UTR or by *de novo* transcription from an internal promoter (Kawano *et al.* 2005; Chao *et al.* 2012), iii) were found to regulate RNA decay, iv) can be targeted by regulatory sRNAs, and v) interact with 5' UTRs to regulate translation initiation (Ruiz de los Mozos *et al.* 2013).

It is demonstrated that at least one third of *S. aureus* transcripts carry 3'UTRs longer than 100 nt, which provides significant potential for transcript-specific regulation (Ruiz de los Mozos *et al.* 2013). Recently, it has been reported that the long 3'UTR of *icaR*, which contains a UCCCCUG motif, is complementary to the Shine-Dalgarno (SD) region in the 5'UTR of *icaR*. 5' and 3' UTRs when associated, form a double-stranded RNA substrate for RNase III cleavage, promote mRNA decay and simultaneously inhibit ribosome loading and the formation of a translational complex, affecting *icaR* mRNA stability and translation (Ruiz de los Mozos *et al.* 2013; Ren *et al.* 2017). *icaR* encodes the transcriptional repressor of the main exopolysaccharidic compound of *S. aureus* biofilm matrix (Arciola *et al.* 2012), deletion or

substitution of the motif (UCCCCUG) within the 3'UTR is sufficient to destroy the interaction with the 5'UTR and cause the accumulation of IcaR and the inhibition of biofilm formation (Ruiz de los Mozos *et al.* 2013).

2.1.2 *Trans-acting* regulatory RNAs

trans-acting sRNA typically base pairs with multiple mRNAs, this capacity which results from limited contacts with their target mRNAs in discontinuous patches rather than extended stretches of perfect complementarity for asRNA (Gottesman 2005; Waters and Storz 2009; Jagodnik *et al.* 2017). To date, the potential base-pairing region between *trans-acting* sRNA and mRNA is mainly centralized at the ribosome-binding site and encompass 10-25 nt. Subsequently, the sRNA-mRNA duplex is frequently subject to degradation by RNase III in *S. aureus*. However, sRNAs can also activate the expression of target mRNA by disrupting the modification of secondary structure that covers the ribosome-binding site. Theoretically, the interaction between *trans-acting* sRNA and mRNA could repress or promote translation. This class of sRNAs acts in concert with transcriptional regulatory proteins or two-component systems to regulate cellular metabolism (*e.g.*, quorum sensing system), and are critical in adaptive strategies during environmental changes (Felden *et al.* 2011).

The most intensively studied *trans-acting* sRNA in *S. aureus* is RNAIII, which is the main intracellular effector of the *agr* (accessory gene regulator) system used for quorum sensing (Novick and Geisinger 2008). Quorum sensing often regulates virulence gene expression (Bassler and Losick 2006). This system is composed of two transcription units, RNAII and RNAIII. RNAII encodes the quorum-sensing cassette (the membrane protease AgrB and the secreted autoinducer peptide AIP) and the two-component system (the sensor kinase AgrC and the response regulator AgrA). When cell density increase, AIP concentration increases proportionally. When AIP reaches a certain threshold, it activates the membrane kinase AgrC and the response regulator AgrA through phosphorylation. Subsequently, AgrA induces the transcription of RNAII and RNAIII (the promoter P2 and P3, respectively) (Novick and Jiang 2003) (Figure 3). RNAIII was the first regulatory RNA shown to regulate multiple targets involved in virulence. It is known to activate the translation of *hla* gene, which encodes alpha-hemolysin within RNAIII region, by a competitive binding of the large 5'UTR of RNAIII that prevents the formation of an intramolecular inhibitory secondary structure in the 5'UTR of *hla* mRNA and releases the RBS of *hla* mRNA (Morfeldt *et al.* 1995; Boisset *et al.* 2007; Pitman and Cho 2015). RNAIII targets mRNAs encoding surface virulence factor genes such as *rot*

(repressor of toxins, pleiotropic transcriptional factor), *spa* (surface adhesion protein A), *sbi* (immunoglobulin-binding protein) (Chabelskaya *et al.* 2014), *lytM* (peptidoglycan hydrolase) and *coa* (staphylocoagulase) (Brantl and Bruckner 2014). Interestingly, RNAIII is also involved in the metabolism of peptidoglycan, which may contribute to the cell wall integrity at high cell density (Boisset *et al.* 2007; Lioliou *et al.* 2012; Mu *et al.* 2012). In summary, RNAIII is a multi-functional sRNA, which primarily functions at the post-transcriptional level by regulating the translation and degradation of multiple target mRNAs, among which, many virulence factors.

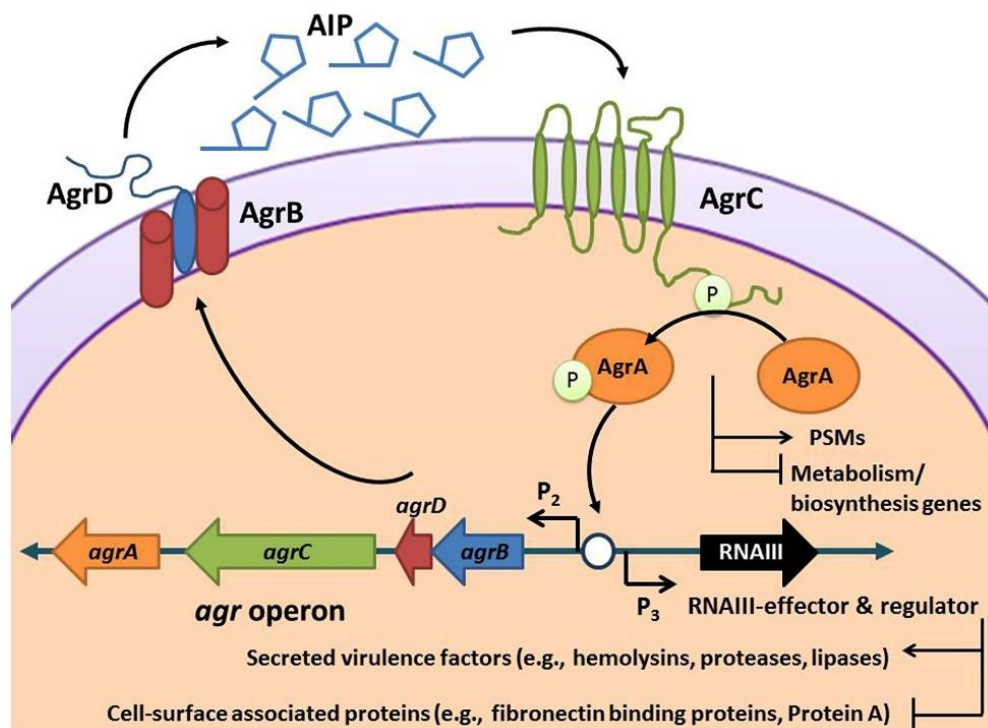


Figure 3 Schematic of the *S. aureus* agr system (Quave and Horswill 2014).

2.1.3 *S. aureus* type I toxin-antitoxin systems

Antisense RNAs (asRNAs) are RNAs encoded on the opposite DNA strand of the genomic locus of their mRNA target. Plenty of asRNAs are expressed from pathogenicity islands and mobile elements such as plasmids and transposons. Among these asRNAs, type I toxin-antitoxin (TA) systems are striking due to their significant biological functions. Type I TA modules consist of a gene encoding a stable toxin (a small protein with bacteriostatic or bactericidal properties), and a gene encoding a cognate antitoxin, a sRNA, counteracting the effect of the toxin by acting as a direct inhibitor or by controlling the production of toxin. The development of type I TA systems in *S. aureus* was strongly promoted by the first investigation of the enterococcal plasmid pAD1 in Gram-positive bacterium (Weaver *et al.* 1996), and by the

identification of numerous sRNAs (Pichon and Felden 2005; Beaume *et al.* 2010; Bohn *et al.* 2010). Recently, two particular mechanisms of antisense regulation have been reported in *S. aureus*, SprA1/SprA1_{AS} and SprF1/SprG1 (Sayed *et al.* 2011; Pinel-Marie *et al.* 2014).

i) SprA1/SprA1_{AS}

The *sprA* genes are present in *S. aureus* genomes in two to five copies depending on the strains. SprA1_{AS} is encoded together with the toxin gene SprA1 in a pathogenicity island in strains such as N315, Newman, NCTC8325 and USA300. It encodes the cytolytic peptide, which lyses human erythrocyte and has an antimicrobial activity against Gram-negative and Gram-positive bacteria (including *S. aureus*). PepA1 disrupts cell membrane integrity by inserting within membranes and inducing apoptosis-like death in bacteria; it might also be implicated in bacteria persistence in acidic environment and under oxidative stress (Sayed *et al.* 2012). *sprA1* mRNA has a compact secondary structure made of two RNA pseudoknots flanked by stable stem loops; it encodes a peptide whose Shine-Dalgarno sequence and translational start site are sequestered within the 5' stem-loop and the first RNA pseudoknot, thus disfavoring peptide translation (Sayed *et al.* 2011). SprA1_{AS} represses the production of SprA1 *in vivo*, by forming a helix with SprA1 at its internal RNA pseudoknot and occluding translation initiation signals. Despite such a structural lock, the ribosomes of *S. aureus* can still load onto *sprA1* mRNA *in vivo* to produce a 30 amino acid toxic peptide, named PepA1, which inhibits *S. aureus* growth when the attenuation function of SprA1_{AS} acting is off (Sayed *et al.* 2012). In addition, structural evidence showed that the functional domain of SprA1_{AS} is outside its overlapping region with SprA1 (Sayed *et al.* 2011). Furthermore, the SprA1_{AS} is constitutively and concomitantly expressed with SprA1 during bacterial growth preventing SprA1 translation and toxicity against *S. aureus* cells (Sayed *et al.* 2011).

ii) SprF1/SprG1

SprF and SprG present multiple copies from the pathogenicity islands and core genome of *S. aureus* that were originally detected by computer searches and transcriptomic analysis (Pichon and Felden 2005). SprF1/SprG1 pair might belong to type I TA system according to sequence comparisons (Fozo *et al.* 2010). Notably, the toxin gene *sprG1* encodes two peptides from one single internal open reading frame with two different AUG initiation codons, SprG1-long (44 amino acid peptide) and SprG1-short (31 amino acid peptide); the latest is the most abundant form. Inducible expression of SprG1 inhibits *S. aureus* growth probably due to peptides accumulation at the membrane presumably forming pores altering membrane integrity and thus

causing cell death (Pinel-Marie *et al.* 2014). The antitoxin gene *sprF1* negatively regulates SprG1 by forming duplexes, which might be subject to ribonuclease III leading to RNA degradation. The two peptides are extracellular toxins that can lyse host cells, which is mainly contributed by the longer peptide; they are antibacterial against Gram-positive and Gram-negative bacteria, with the most effective activity against *S. aureus* obtain with the shorter peptide (Pinel-Marie *et al.* 2014). SprG1/SprF1 is an unconventional type I TA system in *S. aureus* secreting two hemolytic and antibacterial peptides from the dual-coding toxin RNA SprG1, negatively modulated by the antisense RNA SprF1 (Pinel-Marie *et al.* 2014).

2.2 sRNA-protein interactions

The regulatory RNAs often work by base pairing with target mRNAs, but some work in conjunction with specific target proteins. These sRNA-protein interactions can be grouped into two general categories: i) sRNAs interacting with proteins and regulating their activities; ii) sRNAs providing the specificity and the primary activity to the RNA-protein partnership. Yet there is not a lot information on RNA-binding proteins (RNA chaperone, enzymes, RNA helicase, post-transcriptional regulators...) associated with sRNA regulation in *S. aureus*.

2.2.1 sRNA with protein sequestration activity

Some regulatory RNAs with enzymatic activity act by sequestering proteins, and thereby regulate their activities. This ability is for instance characterized in *E. coli* on the 6S RNA, which binds to the housekeeping form of RNA polymerase (σ^{70} -RNAP), changing its promoter preference (Willkomm and Hartmann 2005; Wassarman 2007). This sequestration of σ^{70} holoenzyme by 6S RNA down-regulates σ^{70} -dependent transcription, therefore, facilitating transcription from σ^S -dependent promoters in stationary phase. 6S RNA has a well-conserved secondary structure, which mimics open promoters binding the σ^{70} -RNA polymerase during transcription initiation, thus keep the transcriptional factors away from the DNA promoters, which suggests 6S RNA could act as a direct competitor (Figure 4). This competition, which mainly occurs in stationary phase when 6S is abundant (Trotochaud and Wassarman 2005), indicates that 6S RNA is a critical factor in bacterial adaptation to stationary phase (Wassarman 2007). Interestingly, the 6S RNA serve as a template for the transcription of a 14-20 nucleotide RNA product (pRNA) during outgrowth from stationary phase (Wassarman and Saecker 2006; Gildehaus *et al.* 2007). pRNA production is a way to release σ^{70} -RNAP from 6S in response to NTP concentration. It remains unclear whether the pRNA have another function (Wassarman 2007; Waters and Storz 2009).

The 6S RNA is conserved among bacterial species with two homologs in *B. subtilis* and one in *S. aureus*. In *B. subtilis*, two abundant 6S RNAs are expressed differentially through growth (*bsrA* accumulating during stationary phase and *bsrB* constantly growth in all growth phase), emphasizing the critical role of 6S RNA in bacterial adaptation (Trotochaud and Wassarman 2005); in *S. aureus*, the 6S RNA is constitutively expressed under various conditions but the associated regulations are unknown (Mader *et al.* 2016).

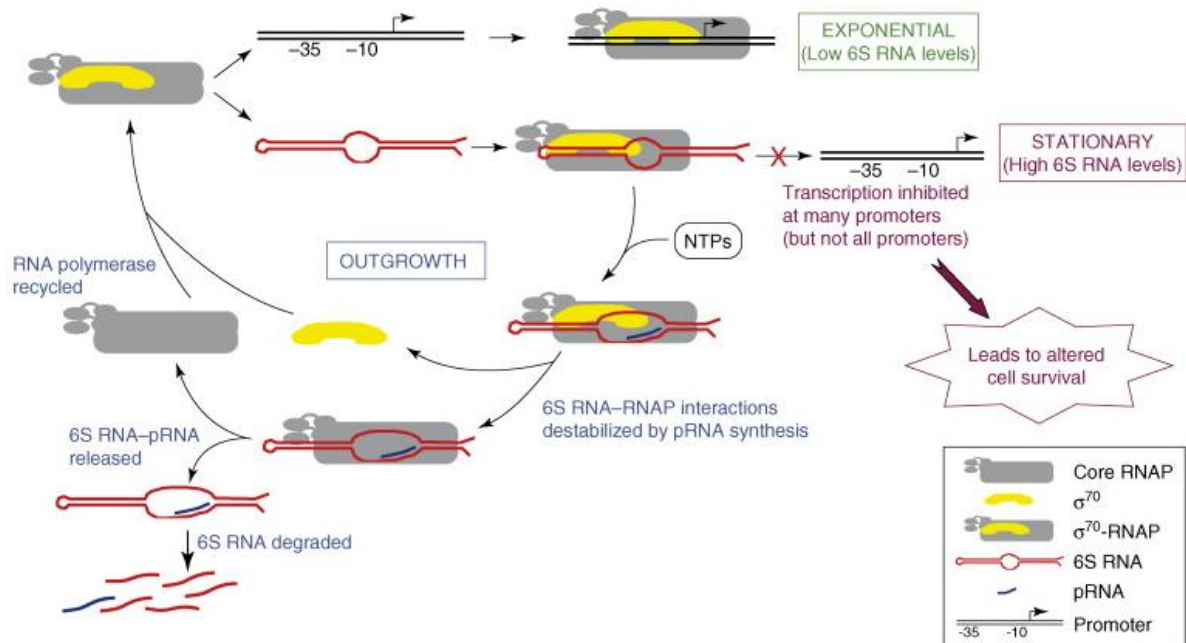


Figure 4 Model for dynamic interactions between 6S RNA, promoter DNA and RNA polymerase during different phases of growth (Wassarman 2007).

2.2.2 Mysterious functions of Hfq protein in *S. aureus*

Hfq is an RNA chaperone whose function has been highly studied in Gram-negative bacteria. It plays a crucial role in most *trans-acting* sRNAs-dependent regulations by stabilizing sRNAs against degradation, helping them anneal to their mRNA targets, modifying mRNA structure for better accessibility, optimizing sRNA/mRNA duplex formation, and recruiting important nucleases such as RNase E (Brennan and Link 2007; Vogel and Luisi 2011). It has been shown that Hfq is required for the fitness and virulence of an increasing number of bacterial pathogens (Chao and Vogel 2010).

The function of Hfq remains unclear in most Gram-positive bacteria, some pathogens including streptococci, lack a recognizable *hfq* gene, whereas some others, such as staphylococci, encode Hfq but possibly weakly or not expressed in some strains (Roberts *et al.* 2006; Liu *et al.* 2010). An *hfq* deletion mutant has no significant phenotype in stress response, virulence factor production, antibiotics resistance and metabolism (Bohn *et al.* 2007). Although *S. aureus* Hfq

binds to sRNA *in vitro*, the protein does not stimulate *trans-acting* sRNA-dependent regulation for target mRNAs *in vivo* and is dispensable for sRNA-mRNA interaction (Huntzinger *et al.* 2005; Pichon and Felden 2005; Bohn *et al.* 2007; Boisset *et al.* 2007). In addition, it was reported that an *hfq*-deficient mutant of *E. coli* cannot be fully complemented by *S. aureus* Hfq. This phenomenon may result from the lack of the C-terminal extension of Hfq in *S. aureus* that is present in the *E. coli* Hfq. The C-terminal extension of *E. coli* Hfq constitutes an RNA interaction surface with specificity for mRNAs, which suggests an RNA binding defect for *S. aureus* Hfq (Vecerek *et al.* 2008). In summary, the function of *S. aureus* Hfq has not been determined clearly yet (Bouloc and Repoila 2016). Whether other RNA chaperones or proteins are required in sRNA-mRNA interactions remains to be determined.

2.2.3 Ribonuclease III as a possible co-factor of RNAs

Bacterial Ribonuclease III, often referred to as RNase III or RNase C, belongs to the Class I RNase III family of enzymes consisting of a catalytic domain and a double-strand RNA (dsRNA) binding domain (Blaszczyk *et al.* 2001). RNase III is a highly conserved bacterial Mg²⁺-dependent double-strand-specific endoribonuclease that was initially discovered in *E. coli* extracts. It cleaves dsRNA generating short RNA duplexes ended by 3'-overhang 2 nt. It recognizes various topologies rather than specific sequences, and cleaves a variety of structures such as imperfect duplexes, helices interrupted by bulged residues, kissing loops and stacked helices (Li and Nicholson 1996; Franch *et al.* 1999; Calin-Jageman and Nicholson 2003; Chevalier *et al.* 2008).

RNase III is the most documented RNase in *S. aureus*. Its function was mainly determined by the characterization of virulence genes regulated by the *agr* system (Novick *et al.* 1993; Huntzinger *et al.* 2005; Boisset *et al.* 2007; Liu *et al.* 2011). This enzyme acts as a co-factor of the quorum-sensing dependent RNAIII; It facilitates the degradation of dsRNA formed by the base-pairing of RNAIII with its mRNA targets (Romilly *et al.* 2012). Due to its dsRNA specificity, RNase III is a key factor involved in various cell processes including i) rRNA maturation by stem-loops cleavage inside the primary rRNAs (Deutscher 2009), ii) mRNA turnover, *e.g.* RNase III feedback autoregulation by self-cleavage (Bardwell *et al.* 1989), iii) processing/cleavage of mRNAs and sRNA-mRNA duplexes (Durand *et al.* 2012; Lioliou *et al.* 2012; Bonnin and Bouloc 2015). Furthermore, RNase III has also been clearly identified as a major partner in antisense regulation in two independent studies (Lasa *et al.* 2011; Lioliou *et al.* 2012).

Accordingly, RNase III is often associated with antisense regulation in *S. aureus* (Boisset *et al.* 2007), however, the role of RNase III in antisense regulation is probably more widespread than previously expected (Romilly *et al.* 2012). A recent study concentrating on the role of RNase III at a genome scale was performed in *S. aureus* using a comparative transcriptomic analysis of wild-type and RNase III deficient (Δrnc) strains to analyze short RNA fractions (Lasa *et al.* 2011). It has revealed that a large collection of 22 nt short RNA transcripts that covers more than 75% of all mRNAs throughout *S. aureus* chromosome are generated by the digestion of RNase III endoribonuclease; removal of RNase III activity significantly reduces the amount of short RNA transcripts and is accompanied by the accumulation of discrete antisense transcripts (Lasa *et al.* 2011). This suggests that genome-wide pervasive antisense transcriptions are hidden owing to RNase III processing of sense/antisense transcripts. Interestingly, this posttranscriptional process of RNase III appears to be restricted to Gram-positive bacteria (Lasa *et al.* 2011). The second study shows that RNase III processes overlapping 5' UTRs of divergently transcribed genes and generates functional mRNAs but with shorter 5'UTRs, thus, RNase III is associated with RNA quality control of pervasive transcription (Lioliou *et al.* 2012).

2.2.4 Transcriptional regulator affects RNA stability, the example of SarA regulator in *S. aureus*

Currently, there is little information about other RNA-binding proteins that might be involved in sRNA regulation; however, new RNA binding proteins keep emerging by chance. The pleiotropic transcriptional regulatory protein SarA in *S. aureus* was unexpectedly characterized as an RNA-binding protein, which modulates mRNA turnover by stabilizing few transcripts related to virulence such as *spa* and *agrA* in late exponential or stationary phase of growth, suggesting that binding of SarA protein might protect mRNAs from degradation (Roberts *et al.* 2006; Morrison *et al.* 2012; Tomasini *et al.* 2014). The *sarA* locus encodes a DNA binding protein and consists of three overlapping transcripts designated as *sarA*, *sarC*, and *sarB* driven by three distinct promoters, P1, P3 and P2, each of which shares a termination site (Bayer *et al.* 1996). SarA is constitutively produced throughout *S. aureus* growth phases, however, the individual *sar* transcripts are expressed in a growth phase-dependent fashion, *sarA* and *sarB* are primarily transcribed during exponential phase whereas *sarC* is predominantly expressed during stationary phase (Manna *et al.* 1998; Blevins *et al.* 1999; Morrison *et al.* 2012). SarA protein functions as a repressor or an activator by binding to conserved AT-rich DNA motifs (ATTTTAT) in the promoter regions of target genes (Chien *et al.* 1999; Sterba *et al.* 2003).

3 Regulatory RNAs functions in *S. aureus*

Regulatory RNAs are a heterogeneous group of molecules, which act by various mechanisms to modulate and fine tune gene expression involved in physiology and pathogenesis. The diverse biological roles of sRNAs encompass: i) the regulation of metabolism including carbohydrate metabolism, metabolite transport and synthesis/degradation; ii) adaptation to environmental stresses and varying culture conditions, such as toxicity, biofilm formation and virulence. All these processes are under the control of multiple transcriptional regulatory proteins, the alternative σ^B and the regulatory RNA (Novick and Jiang 2003).

3.1 The role of σ^B -dependent sRNAs in stress response

Sigma factors are dissociable subunits of RNA polymerase (RNAP). They bind RNAP, forming RNAP holoenzyme, and this complex can recognize promoters and 'melt' DNA, thus initiating transcription. *S. aureus* encodes four sigma factors: σ^A , the housekeeping sigma factor that modulates transcription in exponentially growing cells; the stress responsive σ^B (Wu *et al.* 1996); σ^H , which may be required for natural competence (Morikawa *et al.* 2003); σ^S , which is cryptic but could be implicated in stress and virulence responses (Shaw *et al.* 2008). The alternative sigma factor, σ^B , which is the best studied among the three, functions as a central regulator of the stress response in *S. aureus*. It is activated under general stress conditions, such as growth phase transitions and morphological changes of *S. aureus* (Ferreira *et al.* 2004; van Schaik and Abee 2005). σ^B plays an important role in regulatory networks controlling the expression of virulence determinants, the modulation of antibiotic resistance and cellular differentiation processes such as biofilm formation. Hence, σ^B is a crucial regulator that may influence a wide range of cellular processes. It regulates gene expression usually by recognizing a consensus promoter sequence upstream of target genes (Tomasini *et al.* 2014), exceptionally, some genes lacking this consensus sequence are partially regulated by σ^B , along with other regulators (Geissmann *et al.* 2009; Nielsen *et al.* 2011).

Among the σ^B regulated sRNAs, RsaA gene is under the control of a typical σ^B -dependent promoter (Geissmann *et al.* 2009). The σ^B -dependent RsaA RNA represses the synthesis of the global transcriptional regulator MgrA by forming an imperfect duplex with the Shine and Dalgarno sequence and a loop-loop interaction within the coding region of the target mRNA, consequently, RsaA causes an enhanced production of biofilm and a decreased synthesis of capsule formation (Geissmann *et al.* 2009). Furthermore, RsaA functions as a virulence suppressor of acute infections through mice animal models (Geissmann *et al.* 2009).

3.2 sRNAs acting on virulence gene expression

A large number of virulence factors are known to contribute to the pathogenesis of *S. aureus* whose expression is subject of temporal control and affected by RNAIII (*c.f.* Section 2.1.2).

Apart from RNAIII, several sRNAs, such as *sprA-G*, which were identified in *S. aureus* pathogenicity islands (SaPIs), are also known to be involved in the regulation of virulence (Pichon and Felden 2005; Chabelskaya *et al.* 2010; Le Pabic *et al.* 2015). sRNAs expressed from SaPIs were horizontally acquired from mobile genetic elements. Interestingly, these sRNAs can regulate target genes located on bacterial chromosome or present complex regulations, such as the type I toxin-antitoxin *SprA1/SprA1_{AS}* and *SprF1/SprG1* (Sayed *et al.* 2011; Pinel-Marie *et al.* 2014). A well-characterized example of *spr* genes is *SprD*, which mediates a crosstalk between pathogenicity island and the core genome to regulate virulence (Chabelskaya *et al.* 2010). *SprD* was shown to repress translation initiation of *sbi* mRNA, a gene located on the core genome which encodes an immune-evasion molecule preventing bacteria from host immune responses. The accessible *SprD* central region interacts with *sbi* mRNA translational start site to form a long duplex of 40 base pairs interrupted by bulged nucleotides, which is sufficient to prevent translation initiation *in vivo* and *in vitro* (Chabelskaya *et al.* 2010). The yield of *sbi* mRNA strongly decreased during stationary phase independently of *SprD* expression, suggesting that an additional regulatory event is involved (Chabelskaya *et al.* 2010). Furthermore, *SprD* contributes significantly to virulence in a mouse model of infection, but with no relationship with *Sbi* production, demonstrating that other proteins important for pathogenesis may be under the regulation of *SprD* (Chabelskaya *et al.* 2010).

Another bi-functional sRNA *psm-mec* that encodes a cytolytic toxin *PSM α* (phenol-soluble modulins α), is capable of inhibiting the translation of *agrA* mRNA by base pairing directly with its coding sequence, thus affecting virulence (Kaito *et al.* 2013). *psm-mec* gene is located in the SCCmec (staphylococcal chromosome cassette), which confers resistance to methicillin and other antibiotics to MRSA strains (Otto 2010).

3.3 Regulatory RNAs involved in metabolic regulation

Regulatory RNAs are crucial regulators involved in a wide variety of physiological functions. Their regulatory pathways allow bacteria to fine-tune metabolism during cell growth, to sense population density, to modulate and modify cell-surface properties and to regulate stress adaptation or virulence. Fine-tuning functions are reflected by the lack of severe phenotypes upon deletion or overexpression of sRNAs (Brantl and Bruckner 2014). The first staphylococcal

sRNA involved in metabolic regulation in *S. aureus* is RsaE. RsaE is highly conserved in *Staphylococcus*, *Micrococcus* and *Bacillus* (Geissmann *et al.* 2009). It is a global regulator associated with various metabolic pathways including amino acid synthesis, peptide transport, cofactor synthesis, carbohydrate and folate metabolism, arginine catabolism and the TCA cycle (Geissmann *et al.* 2009; Bohn *et al.* 2010; Rochat *et al.* 2018). A conserved and unpaired UCCC sequence motif within RsaE interacts with target mRNAs at ribosome binding site to prevent the formation of translation initiation complex (Geissmann *et al.* 2009; Rochat *et al.* 2018).

4 Overview of antibiotics

The term “antibiotic” has a broad definition, it describes the activity of any compound or chemical that can destroy or inhibit the growth of microorganisms and is used in the treatment of external or internal infections. While some antibiotics were traditionally produced by microorganisms, most are now manufactured synthetically.

For decades, antibiotics have been used widely in the fight against infectious diseases caused by bacteria (Odonkor and Addo 2011). Since penicillin has been discovered in the late 1920s by Alexander Fleming, and the sulpha drugs have been introduced in the 1930s by Domagk, large numbers of new antimicrobials have been developed, especially between the 1940s and the 1960s. ‘The era of antibiotics’ led to optimism till the early 1970s. Infectious diseases could be controlled and prevented and mankind was confident that modern medicine would prevail against infectious diseases (Yoneyama and Katsumata 2006; Bockstael and Aerschot 2009; Davies and Davies 2010).

However, since the 70s, very few new antimicrobial agents have been discovered, and modifications for already existing antibiotics are the only way to combat resistant bacteria. This has resulted in a standstill between the growing of antibiotic resistance and the research of new types of drugs. Vancomycin, which was first introduced in 1956, is considered as the ‘last resort’ treatment of life-threatening Gram-positive bacterial infections when they are unresponsive to other antibiotics. Since the reports of vancomycin intermediate resistance and vancomycin resistance came out, evolution of vancomycin resistance is a growing issue. Fortunately, alternatives to vancomycin have been developed in the past two decades for the treatment of multidrug resistant (MDR) Gram-positive bacterial infections, such as linezolid and daptomycin (Duplessis and Crum-Cianflone 2011). Linezolid was approved for clinical use in 2000, daptomycin was discovered in the late 1980s and got the approval for commercial using in 2003, and both molecules are highly efficacious. In the last decade, a novel drug, ceftaroline, has shown a high activity against MDR Gram-positive bacteria, Gram-negative bacteria and many anaerobic species (Sader *et al.* 2005; Ge *et al.* 2008; Steed and Rybak 2010; Duplessis and Crum-Cianflone 2011).

Unfortunately, resistance has been seen to nearly all antibiotics developed, since antimicrobial resistance was first recognized soon after the deployment of sulfonamides and penicillins (Figure 5). The extensive use of antibiotics has raised serious public health problem due to MDR bacterial pathogens (Yoneyama and Katsumata 2006). The infections caused by bacterial pathogens are remarkably resilient and have developed several ways to resist antibiotics and

other antimicrobial drugs; what's more, the inappropriate and irrational use of antimicrobials stimulated the increasing of antibiotic resistance. While a number of bacterial species contribute to this emerging issue, the most striking example, and probably the most costly in terms of morbidity and mortality, concern *S. aureus*, it remains one of the key challenges for clinicians and scientists in this regard (Howden *et al.* 2014).

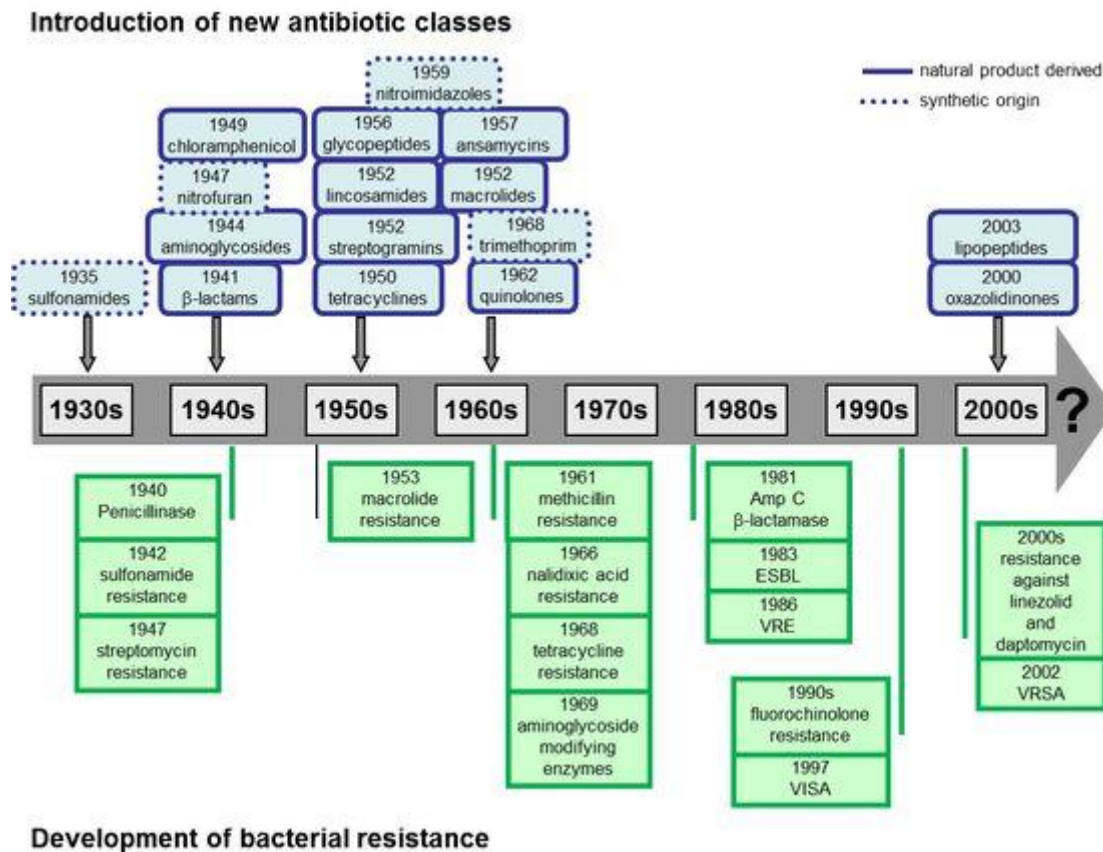


Figure 5 Development of antibiotic resistance: key events based on timeline. (Brötz-Oesterhelt and Sass 2010)

4.1 Antibiotics pollution

4.1.1 Sources of environmental pollution

Currently, MDR bacteria are a major threat to public health. The basic and general question is, from where do these resistant and MDR bacteria emerge? Environmental contamination with antibiotics and the propagation of antibiotic resistance elements are probably the main contributors (Berglund 2015). The main sources of environmental pollution by antibiotics are: i) Pharmaceutical plants, that release antibiotic residues; ii) Hospitals and long-term health care facilities waste effluent; iii) large-scale animal farms, aquaculture and agriculture, using antibiotics as prophylactic treatment or growth factors (Cabello 2006) and spreading manure on crop land.

When human and animals are given antibiotics, part of the antibiotics is excreted unaltered in feces and urine. Waste from domestic animals rich in nutrients is often used as fertilizer on crop fields directly (or indirectly together with sewage water from toilets end up in manure storage tanks, lagoons or compost toilets which can be also used as fertilizer or as a substrate for methane production in biogas plants), leading to contamination of soil with both antibiotic residues and resistant bacteria. Antibiotic residues end up in wastewater (Berkner *et al.* 2014). It has been reported that the treated wastewater by wasterwater treatment plants (WWTPs) still contains higher proportions of various resistant bacterial populations which is corresponding to the respective proportions contained in surface water (Goni *et al.* 1999; Iwane *et al.* 2001; Guardabassi *et al.* 2002; Huang *et al.* 2012; Guardabassi and Dalsgaard 2018). Then residues eventually find their ways into rivers, lakes and dams where the water is used for agriculture irrigation, animal drinking and aquatic farming, therefore, causing contamination of natural environment (Figure 6).

Antibiotics can be degraded in natural ecosystems by processes including photodegradation, chemical degradation and biodegradation at different rates depending on temperature (Dolliver and Gupta 2008), moisture, chemical composition of the environment (Stoob *et al.* 2007) and the microbiota that can contribute to biodegradation.

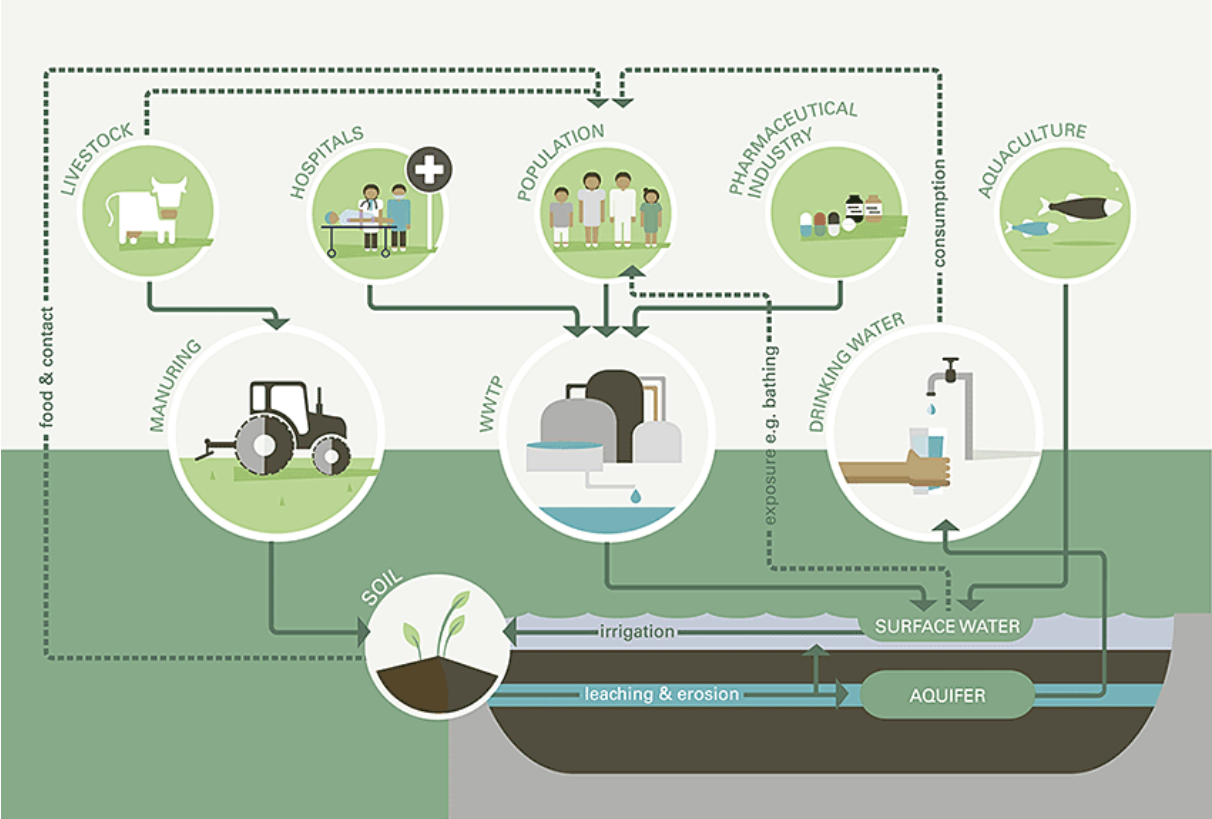


Figure 6 How antibiotics spread emerging as environmental contaminants. (Czekalski *et al.* 2012)

4.1.2 The environmental “resistome” and “mobilome”

Soil, one of the largest and most diverse microbial habitats on earth, is considered as a vast reservoir of antibiotic resistance genes that are available for exchange with clinical pathogens (D'Costa *et al.* 2006; Aminov and Mackie 2007; D'Costa *et al.* 2011). The role of soil in global exchanges of antibiotic resistance genes is not only due to the direct contact with antibiotics, but also to the presence of actinomycete and streptomyces genus whose species account for the majority of all naturally-produced antibiotics (glycopeptides and lipopeptides, lincosamides and rifamycins, respectively), (Forsberg *et al.* 2012; Ainsa 2018). Antibiotic producing organisms harbor resistance determinants for self-immunity that are often clustered in antibiotic biosynthetic operons (Cundliffe *et al.* 2001; Hubbard and Walsh 2003; D'Costa *et al.* 2006).

Wastewater has long been implicated as a significant environmental reservoir of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) found in human pathogens. In light of some studies, WWTPs provide favorable conditions for the proliferation of ARB, which may in turn stimulate the transfer of resistance genes to non-resistant bacteria (Bouki *et al.* 2013). Several studies support the probability of gene transfer enhanced by the environmental conditions in WWTPs (Mach and Grimes 1982; Poté *et al.* 2003; Davies 2012). Consequently, all resistance genes that are relevant to antibiotic environmental pollution can be regarded as a big gene “pool”, named environmental “resistome”, which can potentially transfer to pathogenic bacteria (Wright 2007). Microorganisms in the environmental “resistome” collectively carry and share enormous numbers of resistance genes. These genes can hop from one bacterial species to another through horizontal gene transfer (HGT) platforms (*e.g.*, plasmids, transposons, integrons and prophages) in the presence of antibiotics pressure. This magnitude and diversity of the mobile gene pool is broadly defined as the “mobilome”. The mobilome can spread among water and soil bacterial communities, and modify the local environmental microbiota *via* changes in its composition or activity, thus possibly affecting human and environmental health by enabling pathogens to develop new forms of resistance to antibiotic treatments, eventually making them “superbugs” that are immune to all current antibiotics (Barkay and Smets 2005). Moreover, as a result of the wide dissemination of genes frequently present in human pathogens in places without high antibiotic load, reveals that the probability for their maintenance in natural ecosystems can be high once resistant elements are present in HGT platforms (Pallecchi *et al.* 2008; Martinez 2009).

4.1.3 Environmental sub-lethal/sub-inhibitory concentrations

Lethal concentrations of antibiotics, which are superior to the minimal inhibitory concentration (MIC), either bactericidal and/or bacteriostatic are sufficient to kill or inhibit bacterial proliferation, respectively. It has been widely accepted that the selection of resistance mainly occurs at high and therapeutic levels of antibiotics (Martinez 2009; Martinez 2009; Bernier and Surette 2013), however, antibiotics that are present in natural environment are generally fall well below lethal concentration used in antibiotic therapy due to anthropogenic pollution, therefore, antibiotic resistance genes are likely involved in response mechanisms to non-lethal concentrations too. Bacteria can grow in sub-lethal concentrations of antibiotics, while they are under selection of specific physiological and genetic responses that are triggered (Goh *et al.* 2002; Tsui *et al.* 2004; Davies *et al.* 2006; Yim *et al.* 2006; Fajardo and Martinez 2008).

Sub-lethal levels of antibiotics act as stress inducers that often induce the SOS stress response which is implicated in various antibiotic resistance mechanisms (Bernier and Surette 2013). The induction of the SOS response can also be essential for bacterial survival in stressful environments (*e.g.* nutrient starvation). The associated genetic responses can directly or indirectly modify antibiotic resistance through the increase of the mutation rates or the increase of horizontal transfer possibilities and prophage induction (Bernier and Surette 2013). It has been shown, for instance, that sub-inhibitory concentration of fluoroquinolones induces the SOS response and increase the mutation frequency and prophage mobilization in *S. aureus* promoting bacterial genetic diversity (Cirz *et al.* 2007; Mesak *et al.* 2008).

Not surprisingly, not only fluoroquinolones, but also β -lactams (Miller *et al.* 2004; Maiques *et al.* 2006; Cortes *et al.* 2008), aminoglycosides (Henderson-Begg *et al.* 2006), rifamycins (Cirz *et al.* 2005), trimethoprim (Lewin and Amyes 1991), tetracycline (Baharoglu and Mazel 2011) and chloramphenicol (Cortes *et al.* 2008), all have significant transcriptional effects on the SOS response of various bacteria. The increased number of mutations, horizontal resistance genes transfer and phage release induced by one antibiotic can increase resistance to other classes of antibiotics across bacteria species. This phenomenon is thus tightly connected with “resistome” and “mobilome”, and sub-lethal concentration of antibiotics can lead to resistance at higher concentrations.

Apart from the specific transcriptional responses, sub-lethal levels of antibiotics can also result in the small colony variant phenotype causing intermediate antibiotic resistance in staphylococcal (Matar, Suzan 2004).

4.1.4 Fitness costs

It is generally accepted that most antibiotic resistance mechanisms impair bacterial fitness and confer fitness costs, which is typically observed as a reduced bacterial growth rate (Andersson and Hughes 2010; Martinez 2011). Without antibiotic selection, resistant bacteria will be outcompeted by the susceptible ones (Martinez 2011). The magnitude of fitness cost is the main biological parameter that affects the rate of development of resistance, the stability of the resistance and the rate of reversing resistance when antibiotic selection is reduced (Andersson and Hughes 2010). The fitness cost of antibiotic resistance is variable. Five groups have been categorized based on the different extent of fitness costs:

- i) Cost-free: it has been shown that some resistance mechanisms have no cost for bacteria (Tubulekas and Hughes 1993; Balsalobre and de la Campa 2008). As a result, wild-type bacteria cannot out-compete their resistant counterparts.
- ii) Inducible cost: the resistance cost can be reduced through regulation of the resistance mechanism. For instance, the VanA-type resistance phenotype is induced by glycopeptides (*c.f.* Section 5.1.2.2.1), VanA-type resistance is very costly for MRSA in presence of inducers, whereas maintaining the genes has minimal biological cost in absence of glycopeptides (Arthur *et al.* 1992; Andersson and Hughes 2010).
- iii) Correlated cost: cost compensation and resistance can be positively correlated, it is reported that the acquisition of an additional fluoroquinolone resistance mutation can not only increase antibiotic resistance but also remarkably increase bacterial fitness (Marcusson *et al.* 2009; Andersson and Hughes 2010).
- iv) Environmental condition dependent cost: bacterial fitness is strongly dependent on the environment where bacteria survive. For example, rifampicin-resistant mutants with amino acid substitutions in RpoB, RNA polymerase subunit β , have a reduced fitness in exponential growth and a notable growth advantage in the environment of the ageing colonies in *E. coli* and *Salmonella enterica* (Wrande *et al.* 2008).
- v) Possible epistatic effect: epistasis refers to a situation in which the fitness effect of a mutation depends on its genetic background (Melnik *et al.* 2015). For instance, a fluoroquinolone-resistant *Campylobacter jejuni* isolate with a single point mutation in *gyrA* gene presents an enhanced fitness compared to the related susceptible counterpart in the absence of antibiotic selection pressure in chicken infection model, however, the same mutation is costly in a different genetic variant of *C. jejuni* (Luo *et al.* 2005).

Notably, fitness costs can be counterbalanced by secondary mutations that keep the resistance but reduce the metabolic burden of the primary mutation. Compensatory mutations are the result

of a special form of epistasis; as they confer advantage in presence of selective pressure, they are easy to be selected (Bjorkman *et al.* 2000; Paulander *et al.* 2007). Compensatory evolution can stabilize and maintain resistant bacterial populations in the absence of antibiotics selection. Fitness costs are not sufficient for eliminating resistant bacteria from the natural environment; in contrast, resistant bacteria can invade different ecosystems. Fortunately, we could take advantages of fitness costs to reduce the probability of resistance development by choosing high fitness cost targets for the development of new antibiotics (Andersson and Hughes 2010).

5 Classifications of antibiotics

Antibiotics can be classified in several ways, the most common and useful classification scheme is based on the different interaction targets. Currently, a large number of antibiotics are used clinically, but the variety of targets that they inhibit is limited. To understand how antibiotics work and how bacteria become resistance to them, a brief description of the targets of the main classes of antibiotics is required. The main classes of antibiotics inhibit four classical targets, which are the focus of this thesis (Figure 7): (i) bacterial envelope biosynthesis including cell wall and cell membrane, (ii) protein biosynthesis, (iii) RNA biosynthesis, and (iv) DNA biosynthesis (Yoneyama and Katsumata 2006; Kohanski *et al.* 2010).

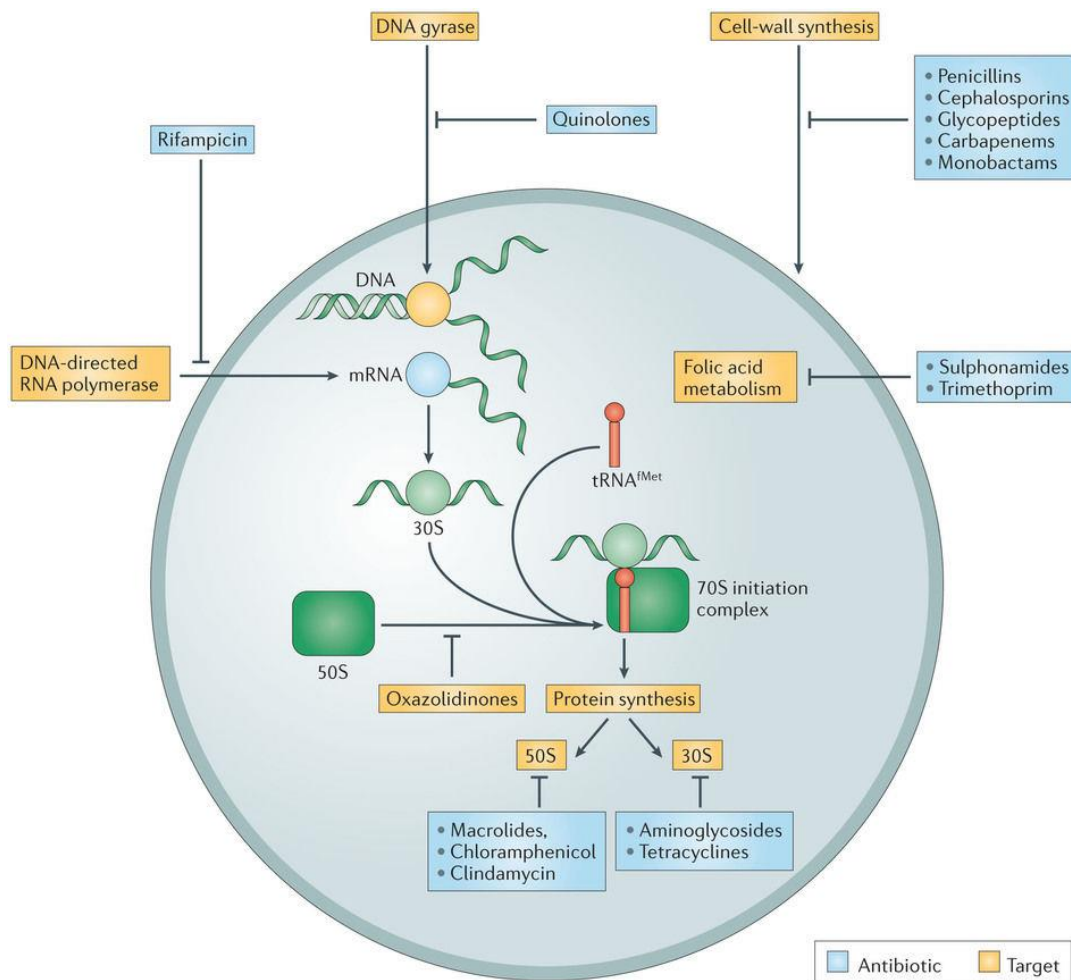


Figure 7 Major targets of antibiotics (Lewis 2013).

5.1 Inhibition of bacterial envelope biosynthesis

The cell wall is the principal stress-bearing and shape-maintaining element in bacteria, and its structural integrity is of critical importance to cell viability. In both Gram-positive and Gram-negative bacteria, the scaffold of the cell wall consists of the cross-linked polymer

peptidoglycan (PG) (Scheffers and Pinho 2005). Peptidoglycan is made of a polysaccharide backbone consisting of alternating *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) residues in equal amounts (Nikolaidis *et al.* 2014). Since bacterial cell wall is required for survival, several classes of antibiotics (notably the penicillins, cephalosporins and glycopeptides) stop bacterial infections by interfering with cell wall synthesis. Gram-positive bacteria, focusing on *S. aureus*, surround itself with a thick cell wall that is a major target of antibiotics (*c.f.* Section 1.1) (Romaniuk and Cegelski 2015).

5.1.1 β -lactam antibiotics

Penicillins and cephalosporins are the major antibiotics that inhibit bacterial cell wall synthesis. They are called beta-lactams because of the unusual 4-member ring that is common to all their members (Figure 8). The β -lactams include some of the most effective, widely used, and well-tolerated agents available for the treatment of microbial infections, such as cloxacillin, flucloxacillin and cefazolin (Katzung & Trevor's Pharmacology). However, β -lactam antibiotics have faced obsolescence with the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) (Blazquez *et al.* 2014). Due to various mechanisms of acquired β -lactam resistance, several resistance phenotypes have been described so far in *S. aureus*: i) production of β -lactamases (*e.g.* penicillinases, cephalosporinases *etc.*), ii) alteration of penicillin-binding proteins, and iii) acquisition of low-drug-affinity penicillin-binding proteins (Nikolaidis *et al.* 2014). The ability to survive in the presence of β -lactam antibiotics remains the main problem in the therapy.

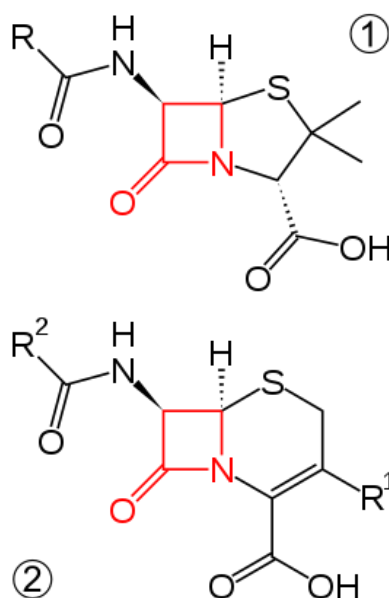


Figure 8 Core structure of penicillins (top) and cephalosporins (bottom); β -lactam ring in red. (Wikipedia, β -lactam antibiotic)

5.1.1.1 Penicillins

Cloxacillin and Flucloxacillin are narrow-spectrum β -lactam antibiotics of the penicillin class. They are used to treat infections caused by susceptible Gram-positive bacteria. Unlike other penicillins, cloxacillin and flucloxacillin have activity against β -lactamase-producing organisms such as *S. aureus* as they are β -lactamase stable (Sutherland *et al.* 1970). However, both of them are ineffective against MRSA.

5.1.1.2 Cephalosporins

Cephalosporins are one of the major classes of β -lactam antibiotics. They are frequently defined by their generations, first to fifth, to suggest a general spectrum of activity of each generation. For example, within this classification, cefazolin is a first-generation cephalosporin, it has activity against Gram-positive cocci, but has limited activity against Gram-negative pathogens.

5.1.1.3 Mechanism of action

An important discovery was the existence of penicillin-binding-proteins (PBPs), which are transpeptidase enzymes involved in the final stage of peptidoglycan construction (Lowy 2003). There are four native PBPs in *S. aureus*, PBP1, PBP2, PBP3, and PBP4. High molecular weight PBPs (PBP1, PBP2 and PBP3) have two protein domains, one involved in transpeptidation (cross-linking), some of the other involved in transglycosylation (extending the glycan chain) (Georgopapadakou and Liu 1980; Park and Matsuhashi 1984; Henze and Berger-Bachi 1995). The role of low molecular weight PBP4 in *S. aureus* is a carboxypeptidase and is needed for the secondary cross-linking of peptidoglycan (Henze and Berger-Bachi 1995). The β -lactams, which resemble the terminal D-alanyl-D-alanine extremity of the stem peptide, inhibit the transpeptidation domain of PBPs and carboxypeptidase activity of low molecular weight PBPs, thus interfering with the crosslinking reaction (Nguyen-Distèche *et al.* 1982; Navratna *et al.* 2009; Bugg *et al.* 2011). Without cross-linking of the peptidoglycan, the cell wall becomes mechanically weak, some of the cytoplasmic contents are released and the cell lyse rapidly (Stapleton and Taylor 2002).

5.1.1.4 Mechanism of resistance

The issue of antimicrobial resistance to β -lactam antibiotics has been thoroughly researched over the years. Bacteria fight back β -lactam by the acquisition of a plasmid that contains *blaZ*, a gene encoding a β -lactamase enzyme. All β -lactam related drugs have a β -lactam ring at the core of their structure. The β -lactamase enzymes (also known as penicillinases) hydrolyze the peptide bond in the β -lactam ring, opening the ring and thus preventing the binding to PBPs. β -

lactamases are widespread enzymes in bacteria, and are produced by many species (Odonkor and Addo 2011).

Modified β -lactam drugs, such as ampicillin, methicillin and oxacillin were then developed. They have a modified structure to protect the β -lactam ring from attack by β -lactamases. Unfortunately, as soon as methicillin was used clinically, methicillin-resistant *S. aureus* (MRSA) strains were isolated (Chambers 1997; Stapleton and Taylor 2002). In this case, resistance is not due to β -lactamase production but due to the expression of an additional penicillin-binding protein, named PBP2a, which has a much lower affinity for methicillin and most other β -lactam drugs and a higher rate of drug release compared with the intrinsic set of PBPs (PBP1 to 4). (Tschierske *et al.* 1997; Ma *et al.* 2002; Stapleton and Taylor 2002).

PBP2a is a peptidoglycan transpeptidase that, in cooperation with the transglycosylase domain of PBP2 of *S. aureus*, can catalyze cell wall biosynthesis in the presence of β -lactam antibiotics, thus enabling survival and growth of the bacteria (Kim *et al.* 2012). PBP2a is encoded by *mecA* gene, which is acquired through horizontal transfer of a mobile genetic element (MGE) that is designated as staphylococcal cassette chromosome *mec* (SCC*mec*) (Ma *et al.* 2002; Deurenberg *et al.* 2007). These genetic elements contain two required components: the *mec* gene complex, and the *ccr* (cassette chromosome recombinase) gene complex (which contains site-specific recombinase genes) (Lowy 2003). The SCC*mec* elements are highly diverse, and have been classified into different types based on: i) the type of *ccr* gene complex, (ii) the class of the *mec* gene complex, and iii) the chromosome background (Okuma *et al.* 2002; Lowy 2003; Hanssen and Ericson Sollid 2006; 2009). These elements are responsible for integration and excision of SCC*mec* and β -lactam resistance phenotypes (2009).

mecA is regulated by the transcriptional DNA binding repressor MecI and the β -lactam sensor/signal transducer membrane protein MecR1, both of which are co-transcribed divergently. MecR1 is present in the cytoplasmic membrane; it detects the presence of β -lactams by means of an extracellular penicillin-binding domain and transmits the signal *via* an intracellular zinc metallo-protease signalling domain. The promoters of these genes are situated between *mecA* and *mecR1*, and an operator region that encompasses the -10 sequence of *mecA* and the -35 sequence of *mecR1* (Sharma *et al.* 1998; Stapleton and Taylor 2002). In absence of β -lactam antibiotics, MecI represses the transcription of both *mecA* and *mecR1*–*mecI*. When β -lactam antibiotics are present, extracellular penicillin-binding domain of MecR1 is activated, then MecRI is cleaved autocatalytically, and the intracellular zinc metallo-protease domain becomes active. The metallo-protease cleaves MecI that binds to the operator region of *mecA*, which allows the transcription of *mecA* and the subsequent production of PBP2a (Stapleton and

Taylor 2002; Deurenberg *et al.* 2007; Blazquez *et al.* 2014) (Figure 9). This system is homologous to the *blaI-blaR1-blaZ* signal transduction system that triggers synthesis of β -lactamase (*blaZ*) in both MRSA and methicillin-susceptible *S. aureus* (Clarke and Dyke 2001; Stapleton and Taylor 2002; Arede, Botelho *et al.* 2013).

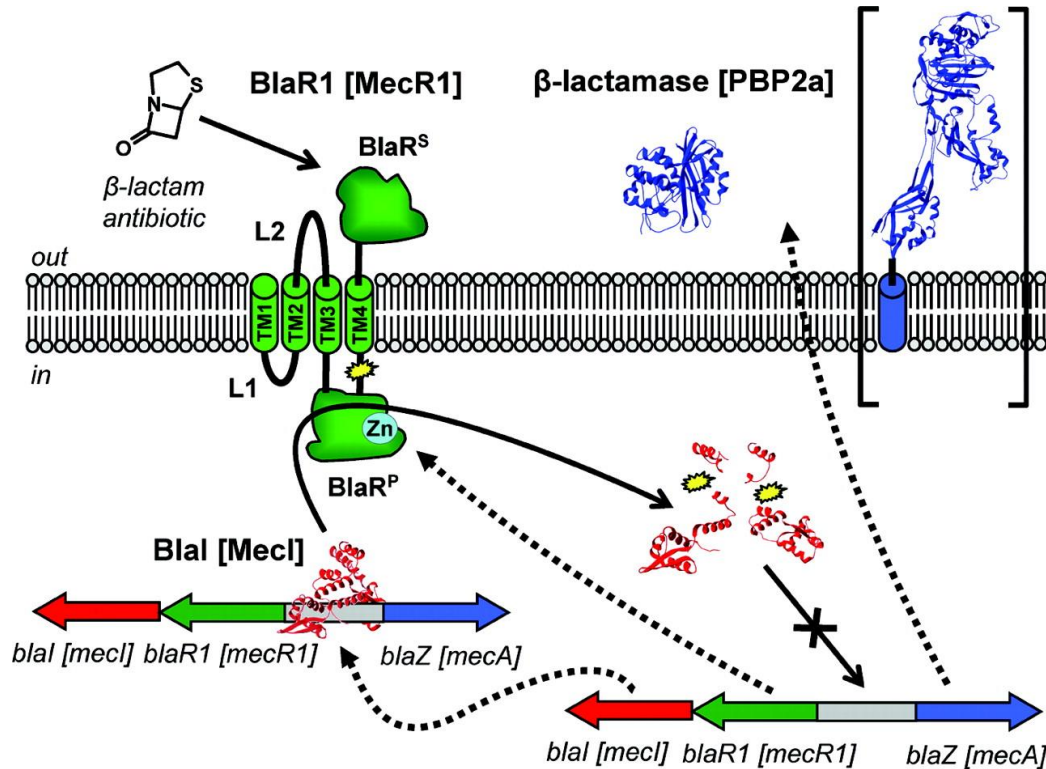


Figure 9 Regulation systems controlling the expression of β -lactamase and PBP2a (Wilke *et al.* 2004; Arêde *et al.* 2012).

5.1.2 Glycopeptides

Glycopeptides are glycosylated non-ribosomal peptides produced by a diverse group of soil actinomycetes. Glycopeptides, particularly vancomycin, are considered as the last resort for the treatment of life-threatening infections caused by multidrug-resistant Gram-positive human pathogens, such as *S. aureus* (MRSA) and *Enterococcus* spp (Binda *et al.* 2014; Mirza 2017). Vancomycin is a type of glycopeptides, which was first isolated by Edmund Kornfeld in 1953, and was approved for clinical use by U.S Food and Drug Administration (FDA) in 1958 (Howden *et al.* 2010; Xu *et al.* 2014; Mirza 2017).

5.1.2.1 Mechanism of action

Glycopeptides target Gram-positive bacteria by inhibiting proper synthesis of the cell wall. The principle component of the cell wall is the heavily cross-linked peptidoglycan, which is made up of glycan chains NAG (*N*-acetylglucosamine) and NAM (*N*-acetylmuramic acid) cross-linked to one another by glycine bridges and stem peptides (UDP-Mur-NAc-L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala). Glycopeptides bind to the acyl-D-alanyl-D-alanine (D-Ala-D-Ala)

terminus of the growing peptidoglycan precursors with high affinity and prevents the cross-linking by inhibiting the action of transglycosylases and transpeptidases (Levine 2006; Arthur 2010; Binda *et al.* 2014; McGuinness *et al.* 2017; Mirza 2017).

5.1.2.2 Mechanism of resistance in *S. aureus*

Starting from the early 1980s, a dramatic increase of glycopeptides (vancomycin) use was observed because of the advent of pseudomembranous enterocolitis coupled with the spread of MRSA. This led to the emergence of two types of glycopeptides resistant *S. aureus*, vancomycin intermediate-resistant *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) (Levine 2006; Perichon and Courvalin 2009).

5.1.2.2.1 Vancomycin-resistant *S. aureus* (VRSA)

Vancomycin-resistant *S. aureus* (MIC $\geq 16\mu\text{g/ml}$) is conferred by the *vanA* operon encoded on transposon *Tn1546*, which is originally a part of vancomycin-resistant enterococci (VRE) conjugative plasmid, that could reprogram cell wall biosynthesis, thus evade the action of the antibiotics (Arthur and Quintiliani 2001; Perichon and Courvalin 2009; Zhu *et al.* 2013; McGuinness *et al.* 2017). Acquisition of vancomycin resistance results from two genetic events. Firstly, the plasmid was transferred from the *Enterococcus* donor to the *S. aureus* recipient by conjugation; secondly, *Tn1546* transposed from the incoming plasmid to a resident replicon (plasmid or chromosome) in the recipient. The acquired plasmid behaves as a suicide gene delivery vector, and the incoming DNA is rescued by illegitimate recombination (Figure 10) (Perichon and Courvalin 2009).

vanA operon-mediated vancomycin resistance is modulated by two key events: i) synthesis of peptidoglycan precursors ending in D-Ala-D-lactate (D-Ala-D-Lac) and/or D-Ala-D-serine (D-Ala-D-Ser), which cannot bind vancomycin (Figure 11) (Mainardi *et al.* 2008), ii) hydrolysis of the normal D-Ala-D-Ala-terminating precursors, which can bind vancomycin (Bugg *et al.* 1991; Perichon and Courvalin 2000; Perichon and Courvalin 2009; McGuinness *et al.* 2017).

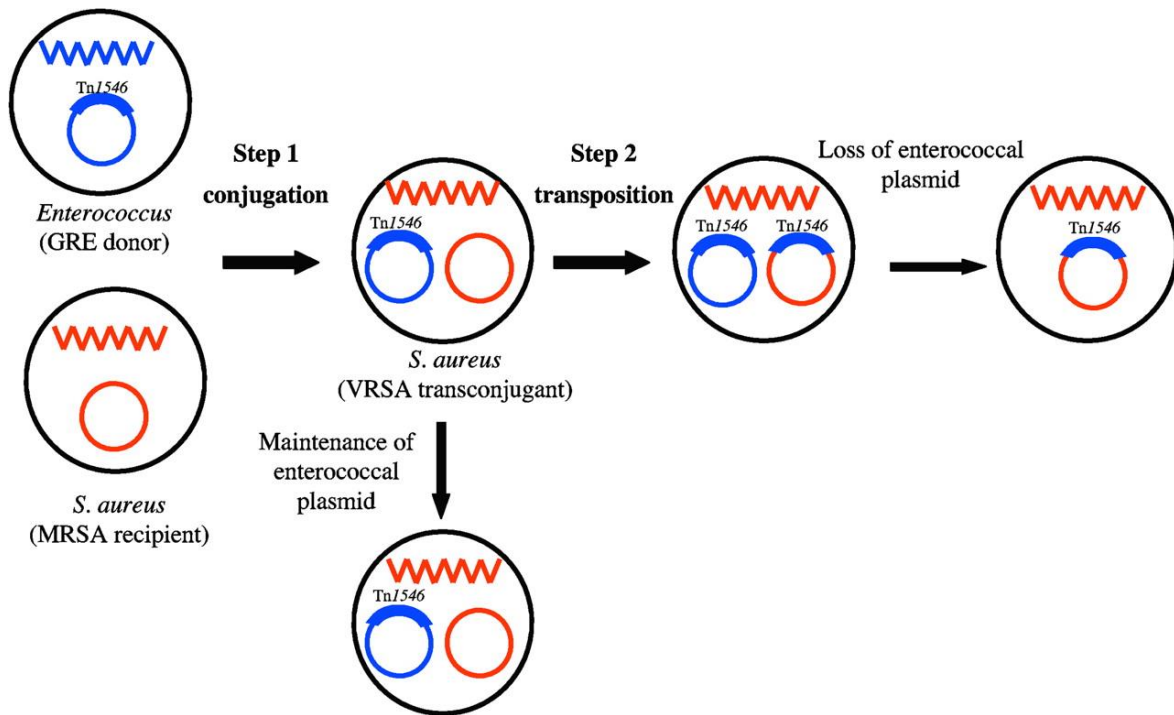


Figure 10 Schematic representation of Tn1546 transfer from *Enterococcus* to *S. aureus* (Perichon and Courvalin 2009).

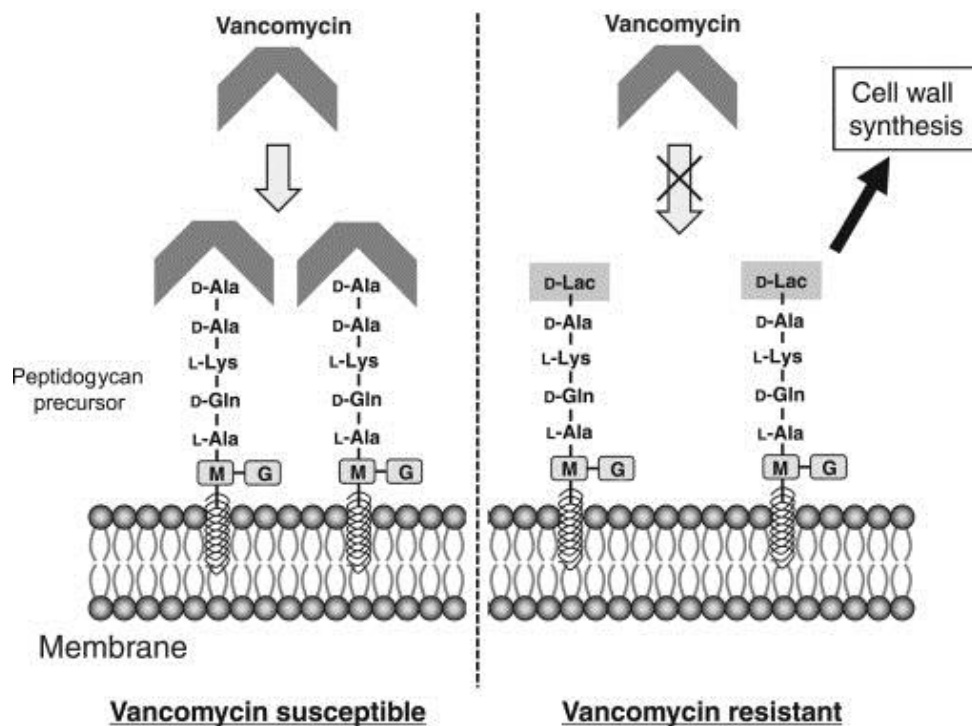


Figure 11 Vancomycin resistance mechanism in *S. aureus*. (Kawada-Matsuo and Komatsuzawa 2012)

Vancomycin-resistant *Staphylococcus aureus* (VRSA) are still exceedingly rare. The first VRSA case was reported in 2002. To date, there are only 14 reported cases of VRSA in United States (Zhu *et al.* 2013; Mirza 2017), several reports in India (Tiwari and Sen 2006; Banerjee and Anupurba 2012) and several cases in Iran (Aligholi *et al.* 2008; Azimian *et al.* 2012; Dezfulian *et al.* 2012; Fasihi *et al.* 2017; Fasihi *et al.* 2017). Although it is of serious concern for the infected patients, it seems that, due to several biological constraints (such as restriction modification system of *S. aureus*), dissemination of VRSA has so far been limited (Perichon and Courvalin 2009). However, the potential spread of such clinical isolates should not be underestimated because of the minimal biological cost in absence of induction (Perichon and Courvalin 2009).

5.1.2.2.2 Vancomycin-intermediate *S. aureus* (VISA)

VISA strains demonstrated moderate reductions in susceptibility to vancomycin, and are distinct from vancomycin-resistant *S. aureus* (VRSA) (Perichon and Courvalin 2009; Howden *et al.* 2014). The vancomycin-intermediate *S. aureus* (VISA) Mu50 strain, which was isolated clinically in Japan, was first reported in 1997 (Hiramatsu *et al.* 1997). Meanwhile, a new model of vancomycin resistance (hVISA) was defined (Hiramatsu *et al.* 1997). Heterogeneous-VISA (hVISA) refers to a strain of *S. aureus* that is susceptible to vancomycin by the standard broth microdilution reference method (vancomycin MIC $\leq 2\mu\text{g/ml}$), however a test using a higher inoculum or prolonged incubation leads to the detection of resistant subpopulations with a higher MIC at a greater rate than one in every 10^5 - 10^6 , for which the vancomycin MIC is in the intermediate range, currently defined as 4-8 $\mu\text{g/ml}$ by the Clinical and Laboratory Standards Institute (CLSI) (Hiramatsu 2001; Tenover 2010; Mirza 2017).

The VISA and hVISA status seems to be a successful ecological achievement of *S. aureus* survival against vancomycin pressure (Hiramatsu 2001; Mirza 2017). Although vancomycin suppresses the majority of bacterial population, the rest survives and grows in the presence of high level concentration of vancomycin. These subpopulations produce thickened cell walls to survive under vancomycin pressure. Once the vancomycin pressure is alleviated, the hVISA cells return to the VSSA status. The scheme of stepwise acquisition of vancomycin-intermediate resistance is VSSA \rightarrow hVISA \rightarrow VISA (Hiramatsu 2001).

Changes in cell wall volume and composition are key features that have been repeatedly described in hVISA and VISA strains. One of the most common phenotypic features is the thickened cell wall with reduced peptidoglycan cross-linking. Reduced cross-linking of peptidoglycan results in an increasing of free D-Ala-D-Ala residues (binding sites for

vancomycin) (Peleg *et al.* 2009). It is supposed that vancomycin binds to these free D-Ala-D-Ala residues in the outer layers of the thickened cell wall and is unable to reach its site of action at the cell membrane (Howden 2005). The trapped vancomycin molecules within the cell wall clog the peptidoglycan meshwork and form a physical barrier towards further incoming vancomycin molecules. Thus, collaboration of the clogging and cell wall thickening leads to vancomycin resistance (Howden 2005; Cui *et al.* 2006). In addition to thickened cell wall, there are other phenotypic changes that are likely relevant to the cell wall architecture, such as reduced cell wall turnover, reduced autolytic activity, and activated cell wall synthesis (Deresinski 2013; Howden *et al.* 2014).

The molecular mechanisms of vancomycin resistance in hVISA and VISA are less well understood than the one of VRSA. So far, no specific genetic determinants of hVISA and VISA have been demonstrated, however, several genes and mutations are known to contribute to the evolution of VISA. For instance, some mutations within genes encoding two-component regulatory systems, such as *vraSR* (vancomycin resistance-associated sensor/regulator), *graRS* (glycopeptide resistance-associated sensor/regulator), *walKR* and *rpoB* (RNA polymerase gene) have been linked to vancomycin resistance (Meehl *et al.* 2007; Deresinski 2013; McEvoy *et al.* 2013; Howden *et al.* 2014). Furthermore, some genes whose expression has been found altered in VISA strains, include *atl* (autolysin), *mprF* (phosphatidylglycerol lysyltransferase), *sceD* (transglycosylase), *sarA*, *sigB*, *tcaA*, *mgrA* and *ccpA* (Samanta and Elasri 2014; Hu *et al.* 2016; Mirza 2017). Taken together, these studies provide some new insights towards the understanding of hVISA and VISA resistance mechanism.

The prevalence of hVISA/VISA is greater than that of VRSA, but the spread of these strains appears limited at present (Howe *et al.* 2004; Kos *et al.* 2012). The failure of these strains to spread is perhaps linked to the transient nature of the hVISA phenotype, as the organism can revert rapidly to VSSA in the absence of selective pressure imparted by glycopeptide antibiotics (McGuinness *et al.* 2017).

5.1.2.3 β -lactam antibiotics and vancomycin (“seesaw effect”)

The high prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) has caused an increase in the utilization of glycopeptides such as vancomycin, resulting in the emergence of vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA) (Appelbaum 2006; Werth *et al.* 2013). Isolates with reduced susceptibility to vancomycin have been reported much more frequently, and associated with vancomycin treatment failures (Roch *et al.* 2014). Interestingly, among these VISA and hVISA isolates, the susceptibility of β -lactams increases

accompanied by a paradoxical decrease of glycopeptides susceptibility, a process known as the “seesaw effect”. This phenomenon has been demonstrated both *in vitro* and *in vivo*. For instance, an *in vitro* pharmacokinetic/pharmacodynamic model simulating *in vivo* antibiotic exposure pointed that vancomycin in combination with cefazolin improved antibacterial activity against MRSA and hVISA isolates compared to vancomycin alone (Hagihara *et al.* 2012; Dilworth *et al.* 2014). This observation is consistent with the previous *in vivo* combination study, which illustrated that vancomycin and a β -lactam combination was more effective in an *in vivo* rabbit model of infective endocarditis caused by VRSA (Fox *et al.* 2006). The explanation of the β -lactams susceptibility increase in VRSA is probably due to the production of cell walls lacking the terminal D-Ala–D-Ala residues, thus resulting in the inability for cells to do the PBP2a cross-link (Severin *et al.* 2004). It has also been suggested that PBP4 modulates the reduction of secondary cell wall cross-linking, and release additional free peptidoglycan precursors and thickening the cell wall which is the characterized phenotype of VISA. PBP2a has low affinity for β -lactams, in this situation, PBP2 is thought to work as a major factor in cell wall assembly, reverts the susceptibility to β -lactams and results in the “seesaw effect” phenomenon. This hypothesis is supported partially by the observation that VISA strains exposed to β -lactams targeting PBP2 become more susceptible to vancomycin (van Hal and Paterson 2011; Ortwine *et al.* 2013). Overall, the use of combination antimicrobial therapy is a common occurrence and represents a potential treatment option for infections caused by VISA and hVISA.

5.1.3 Lipopeptides

Lipopeptides are versatile molecules produced by a variety of bacterial and fungal genera, whose functions include i) antimicrobial activity, ii) bacterial motility and swarming (Raaijmakers *et al.* 2006; Raaijmakers *et al.* 2010). They are a remarkable class of self-assembling molecule that is able to form peptide-functionalized supramolecular nanostructures. Lipopeptides are amphiphilic molecules that are composed of a fatty acid tail linked to a short oligopeptide, which is cyclized to form a lactone ring between two amino acids in the peptide chain (Raaijmakers *et al.* 2010; Hamley 2015).

Daptomycin, a fermentation product produced by the Gram positive bacterium *Streptomyces roseosporus*, is a cyclic antimicrobial lipopeptide with bactericidal activity; it is approved for clinical use against serious infections caused by Gram-positive organisms, such as *Staphylococcus aureus* and *Enterococcus faecalis*, including strains that are resistant to β -

lactam antibiotics and vancomycin (Steenbergen *et al.* 2005; Baltz 2009; Zhang *et al.* 2014; Hamley 2015; Ma *et al.* 2017).

5.1.3.1 Mechanism of action

The unique structure of daptomycin consists of a cyclic peptide moiety with 10 amino acids, from which the N-terminal 3 amino acids protrude; the N-terminus carries a decanoyl fatty acyl side chain (Zhang *et al.* 2014) (Figure 12). This distinctive structure contains several non-standard amino acids, including three D-amino acids, ornithine, 3-methyl-glutamic acid, and kynurinine (Humphries *et al.* 2013).

Daptomycin works at the bacterial cytoplasmic membrane. Various molecular targets and action modes have been proposed, including the inhibition of peptidoglycan (Mengin-Lecreulx *et al.* 1990) or lipoteichoic acid synthesis (Canepari *et al.* 1990). However, the proposal of permeabilization and depolarization of the bacterial cell membrane is reported consistently in studies from different laboratories (Silverman *et al.* 2003; Rubinchik *et al.* 2011; Taylor and Palmer 2016). The activity of daptomycin is strictly dependent on the presence of physiological levels of Ca^{2+} , which induce conformational changes in daptomycin (Straus and Hancock 2006; Ho *et al.* 2008; Humphries *et al.* 2013), these changes also facilitate daptomycin membrane insertion and oligomerization (Muraih *et al.* 2011).

The proposed mechanism involves the insertion of daptomycin lipophilic tail into bacterial cell membrane in a phosphatidylglycerol-dependent fashion, where it aggregates and forms oligomers, generates an ion conduction channel, leads to ion leakage, depolarization of the cell, and thus the disruption of the functional integrity of the cell (Steenbergen *et al.* 2005; Pogliano *et al.* 2012).

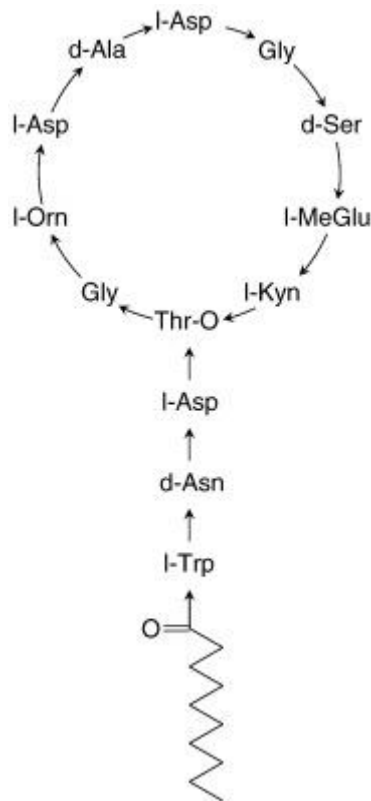


Figure 12 Schematic representation of daptomycin (Zhang *et al.* 2014).

5.1.3.2 Mechanism of non-susceptibility in *S. aureus*

Although bacterial resistance against daptomycin is still relatively rare, clinical cases of daptomycin non-susceptibility emerging during therapy have been documented with important pathogens such as *S. aureus* as well as *Enterococcus* and *Streptococcus* species (Bayer *et al.* 2013; Tran *et al.* 2015; Taylor and Palmer 2016).

S. aureus non-susceptibility to daptomycin is multifactorial, the pathway of which appears to be isolate specific, and involved in both cell membrane and cell wall homeostasis *via* adaptations in metabolic function and stress response regulatory pathways (Bayer *et al.* 2013; Humphries *et al.* 2013; Tran *et al.* 2015). Daptomycin non-susceptible strains often exhibit a progressive accumulation of single nucleotide polymorphisms in *mprF* (a lysyl-phosphatidylglycerol synthetase), *ycyFG* (sensor histidine kinase), *rpoB* and *rpoC* (RNA polymerase subunits), each giving about a twofold increases in the minimal inhibitory concentration (MIC), the combinations giving higher MICs (Friedman *et al.* 2006; Julian *et al.* 2007; Murthy *et al.* 2008; Baltz 2009).

mprF encodes a bifunctional membrane protein, that catalyzes the lysinylation of PG (phosphatidylglycerol) to form the positively charged lysyl-phosphatidylglycerol (LPG) in the

inner phospholipid layer. Subsequently, *mprF* translocates LPG to the outer layer *via* the flippase domain (Ernst and Peschel 2011; Humphries *et al.* 2013). Membranes rich in PG and LPG are less acidic than those are lacking. The two distinct functions of *mprF* result in a partial neutralization of the normally anionic bacterial cell surface, and thus reduces the binding of Ca²⁺ bound daptomycin (Baltz 2009; Ernst and Peschel 2011). The *mprF*-defective mutants have higher LPG/PG ratios in the outer membrane and bind less daptomycin than the wild-type strain (Jones *et al.* 2008; Baltz 2009). The susceptibility to daptomycin increases about fourfold with the deletion of *mprF* in *S. aureus* (Jones *et al.* 2008). This result is consistent with studies showing that Ca²⁺ bound daptomycin acts as a cationic peptide (Scott *et al.* 2007; Ho *et al.* 2008; Jung *et al.* 2008). Interestingly, *mprF* is not a target of daptomycin, but its expression level modulates the entry of daptomycin into bacterial membranes, affects daptomycin oligomerization and cell wall depolarization and permeabilization (Baltz 2009).

5.1.3.3 Relationship between vancomycin exposure and daptomycin non-susceptibility

Daptomycin non-susceptibility (daptomycin-NS) appears to be linked in some *S. aureus* isolates to increased vancomycin MICs (Humphries *et al.* 2013). Daptomycin-NS phenotype is observed mainly in vancomycin-intermediate *S. aureus* (VISA) isolates and a minority of *S. aureus* isolates with vancomycin heteroresistance (hVISA) (Cui *et al.* 2006; Julian *et al.* 2007; Mwangi *et al.* 2007; Kelley *et al.* 2011). The VISA phenotype is likely associated with a thickened cell wall, which acts as a physical barrier and affect daptomycin penetration to cell membrane (Cui *et al.* 2006). It is important to note that vancomycin-resistant *S. aureus* (VRSA) isolates are susceptible to daptomycin (Patel *et al.* 2006; Saravolatz *et al.* 2012), because VRSA phenotype is mediated by *vanA*, which does not affect daptomycin susceptibility (Patel *et al.* 2006; Humphries *et al.* 2013). In addition, mutations frequently observed in daptomycin-NS *S. aureus* (*vraSR*, *walKR*, *rpoB* and *rpoC*) also emerged during vancomycin therapy (Bayer *et al.* 2013). This result suggests that vancomycin treatment might be a major dangerous factor for subsequent use of daptomycin. In summary, it appears that prior vancomycin exposures may well provide a microbiologic foundation for development of subsequent daptomycin-NS (Bayer *et al.* 2013).

5.1.3.4 Relationship between β -lactam antibiotics and daptomycin non-susceptibility

The phenotype “seesaw effect” was first observed in VISA and VRSA isolates, for which the vancomycin MIC is inversely related to that of β -lactams (Mishra *et al.* 2009; Yang *et al.* 2010; Rose *et al.* 2012). A similar effect is seen in some daptomycin-NS isolates with the presence of

subinhibitory concentrations of β -lactam antibiotics (Yang *et al.* 2010). Several studies showed that daptomycin and β -lactam antibiotics are highly synergistic against both daptomycin-susceptible and daptomycin-non-susceptibility MRSA (Rand and Houck 2004; Snyderman *et al.* 2005; Mehta *et al.* 2012). For example, daptomycin combination with oxacillin shows a synergistic effect *in vitro* against vancomycin and daptomycin none susceptible MRSA using clinical bloodstream and endocarditis isolates (Yang *et al.* 2010; Dhand *et al.* 2011). Mechanistically, co-incubation of daptomycin with a β -lactam induces a significant reduction of the net positive charge of cell surface, it reverts the increased repulsion provoked by daptomycin alone and results in the restoration of daptomycin susceptibility by binding to the cell surface (Figure 13) (Dhand *et al.* 2011; Mehta *et al.* 2012). However, the precise mechanism of the “seesaw effect” and the synergy between daptomycin and β -lactams remains to be explained at the cellular level. Recently, a study observed a significant diversity among different β -lactams and their relative efficacies in combination with daptomycin. It has been reported that the synergy is more pronounced with β -lactams known to preferentially bind PBP1, whereas β -lactam antibiotics with preferential binding to PBP2, PBP3, or PBP4 showed significantly less synergy (Berti *et al.* 2013; Berti *et al.* 2015). More research is needed to further characterize the effects of these different β -lactams on the process of “seesaw effect” in *S. aureus* with reduced daptomycin activity.

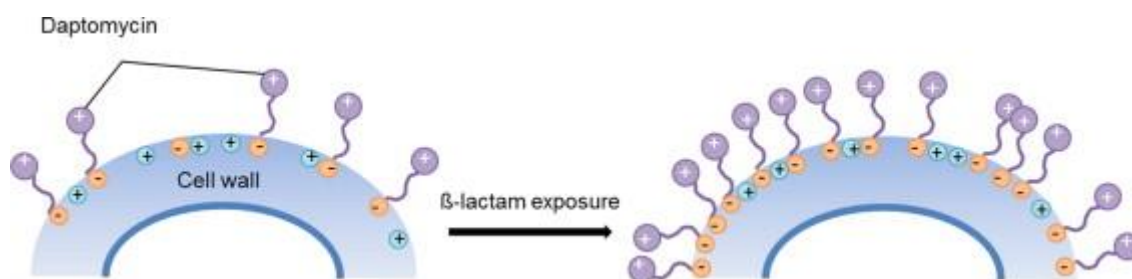


Figure 13 Proposed mechanism for daptomycin and β -lactam synergy (Ortwine *et al.* 2013).

5.2 Inhibition of protein biosynthesis

Protein synthesis inhibitors are substances that can stop or slow cell growth or proliferation by the disruption of various stages of bacterial translation without affecting the host. This broad definition can be used to describe many antibiotics. The majority of antibiotics that block bacterial translation *via* interfering with i) the formation of 30S initiation complex, ii) the elongation process generating the newly synthesized polypeptide chain (including aminoacyl tRNA entry, conformational proofreading, peptidyl transfer and ribosomal translocation), *etc.*

5.2.1 30S ribosomal subunit inhibitors

There are many antibiotics available inhibiting bacterial translation by binding to the 30S ribosomal subunit (Lambert 2012). It functions as targets for antimicrobial drugs, such as aminoglycosides.

Aminoglycoside antibiotics were the first drugs discovered by systematic screening of natural product sources for antibacterial activity (Hermann 2007). Aminoglycosides demonstrate highly potent concentration-dependent killing action and are broad-spectrum bactericidal antibiotics, with many desirable properties for the treatment of Gram-negative aerobes and some anaerobic bacilli and act synergistically with β -lactams against certain Gram-positive organisms. *S. aureus* including MRSA, is among the responsive Gram-positive susceptible groups (Abou-Zeid *et al.* 1978).

Aminoglycosides share a central 2-deoxystreptamine (2-DOS) scaffold connected to amino sugar substituents at the 4,5 or 4,6 positions (Figure 14) (Busscher *et al.* 2005). The 4,6-disubstituted 2-DOS derivatives, which is the largest group of aminoglycosides, includes several antibiotics, such as kanamycin, tobramycin, gentamicin and amikacin. Neomycin and paromomycin are part of the 4,5-disubstituted 2-DOS derivatives. Gentamicin, tobramycin and amikacin are the most frequently used antibiotics in clinics, especially gentamicin (Saleh *et al.* 2016).

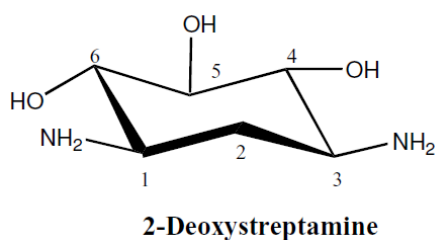


Figure 14 Backbone structures of aminoglycosides (Kumar *et al.* 2008).

Gentamicin was isolated from the bacteria *Micromonospora purpurea* in 1963. It is the most commonly and clinically used aminoglycoside because of its rapid bactericidal activity, its low level of resistance in most community and hospital-associated Gram-negative pathogens, and its low cost. However, gentamicin bears severe adverse effects, which occur with all aminoglycosides: nephrotoxicity and ototoxicity. They are thought to be dose related with higher doses or prolonged therapy causing greater chance of toxicity, unlike nephrotoxicity, ototoxicity is irreversible (Edson and Terrell 1999).

5.2.1.1 Mechanism of action

Aminoglycosides are multifunctional hydrophilic sugars that possess at least two amino and hydroxyl functions (Chittapragada *et al.* 2009). They are natural polycationic molecules with high affinity for nucleic acids, *e.g.* certain RNAs, especially the rRNAs of prokaryote (Chittapragada *et al.* 2009).

Aminoglycosides causes leakage out of the outer membrane of Gram-negative organisms in a self-promoted uptake pathway. Aminoglycosides competitively displace divalent cations which cross-bridge adjacent lipopolysaccharide (LPS) molecules, thus disrupt the stability and permeabilize the outer membrane, facilitate the uptake of other molecules of the permeabilizing polycations (Hancock *et al.* 1991).

Aminoglycosides are the best characterized class of antibiotics that bind directly to ribosomal RNA (Yoshizawa *et al.* 1998). They bind to the asymmetric interior loop of 16S rRNA of the 30S ribosomal subunit that contains three unpaired adenine residues formed by the universally conserved nucleotides A1492 and A1493, and the prokaryotic specific nucleotide A1408, imparting their specificity (Recht *et al.* 1999; Hermann 2007; Tsai *et al.* 2013), which interferes with the decoding A site. In the absence of tRNA or drug, A1492 and A1493 are stacked within an asymmetric internal loop at the base of Helix 44 (h44) (Figure 15) (Tsai *et al.* 2013). Upon mRNA decoding, the flexible adenine sensors (A1492 and A1493) of the decoding site contact directly with mRNA-tRNA codon-anticodon hybrid. 4,5 or 4,6 disubstituted aminoglycosides bind at the decoding site, displacing A1492 and A1493 and mimicking the tRNA conformational effect. This binding stabilizes the tRNA-mRNA interaction in A site by decreasing tRNA dissociation rates (Karimi and Ehrenberg 1994). Thus, it perturbs the elongation of the nascent polypeptide chain by impairing the proofreading process that ensures translational fidelity (misreading and/or premature termination) (Figure 16) (Melancon *et al.* 1992; Yoshizawa *et al.* 1998). Over time, the accumulation of aberrant proteins that are truncated or incorrectly folded leads to oxidative stress and bacterial cell death (Hermann 2007; Kohanski *et al.* 2007; Kohanski *et al.* 2008).

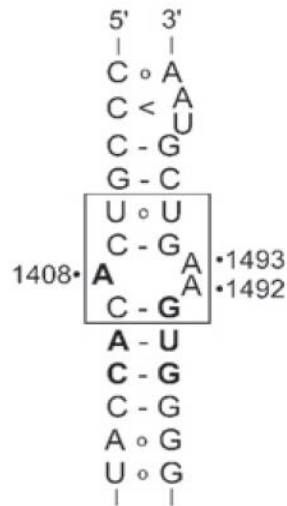


Figure 15 Secondary structure of the decoding site in 16S ribosomal RNA in *E. coli*. The aminoglycoside binding site is marked by a box; methylated residues are marked by red circle (Hermann 2007).

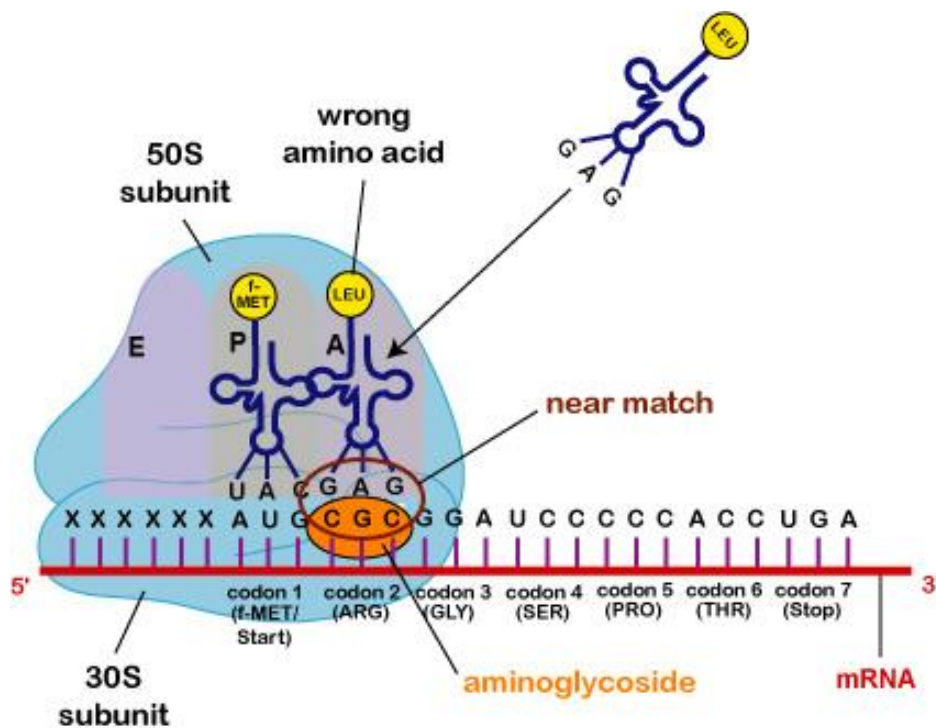


Figure 16 Aminoglycosides interfere with translation by causing a misreading of the codons along the mRNA. (from Gary E. Kaiser)

5.2.1.2 Mechanism of resistance

Three distinct mechanisms have been proposed for bacterial resistance to aminoglycosides: i) the decrease of intracellular drug concentration, ii) target site alterations, and iii) enzymatic modification of aminoglycosides (Vakulenko and Mobashery 2003).

- i) Intracellular drug concentration decreasing

Intracellular aminoglycoside levels can be reduced in target cells by a decreased drug uptake and accumulation, likely due to membrane impermeabilization (low level/absence of transmembrane potential), however, the underlying cause of molecular mechanisms remains unknown (Mingeot-Leclercq *et al.* 1999). It is highly significant in the clinic since it is a common characteristic of all aminoglycosides and leads to intermediate susceptibility or intrinsic resistance. Moreover, alteration of the respiratory chain in anaerobic bacteria and energy-dependent multidrug efflux systems in Gram-negative bacteria are involved in moderate resistance to aminoglycosides (MacArthur *et al.* 1984; Edgar and Bibi 1997; Karlowsky *et al.* 1997; Magnet and Blanchard 2005; Lambert 2012). In *S. aureus*, chromosomal mutations influencing transmembrane electrical potential have also been shown to provide aminoglycoside resistance (Miller *et al.* 1980). Such mutations lower growth rate, give rise to small colony variants and allow bacteria to survive during aminoglycoside therapy (Vakulenko and Mobashery 2003).

ii) Target alterations

Target modifications that cause aminoglycoside resistance include mutational changes in ribosomal proteins or 16S rRNA, and enzymatic methylation of the rRNA.

Target mutation is a rare mechanism of aminoglycoside resistance due to the multiple copies of rRNA operon existing in most bacteria (seven in *E. coli*, for example), except for few species of *Mycobacterium* (Hermann 2007). Streptomycin treatment of *Mycobacterium tuberculosis* infection revealed clinical resistance cases that involved ribosomal mutations (Cohn *et al.* 1997). The single ribosomal operon of this pathogen allows the production of a homogeneous population of aminoglycoside-resistant ribosomes after a single base change (Magnet and Blanchard 2005; Hermann 2007).

Methylation of 16S rRNA has been more recently reported as a mechanism of resistance against aminoglycosides among Gram-negative human pathogens. This methylation confers high-level resistance to all clinically administered aminoglycosides. It was first described in aminoglycoside-producing organisms (Doi and Arakawa 2007). Two sites within the A site decoding region of 16S rRNA are concerned with methylation: methylated residues G1405 and A1408 (Figure 15). The most prevalent type of resistance methyltransferases, ArmA (aminoglycoside resistance methyltransferase A), confers high-level resistance to 4,6 disubstituted aminoglycosides (kanamycin and gentamicin groups) but not to neomycin that is a 4,5 substituted 2-DOS and apramycin by methylation of the N7 position of guanine 1405

(Kotra *et al.* 2000; Galimand *et al.* 2003; Doi and Arakawa 2007; Lioy *et al.* 2014). The second type of methyltransferases is composed of a unique member, NpmA, which methylates the *N1* position of A1408 and confers high-level resistance to both 4,6 and 4,5 disubstituted 2-DOS (Wachino *et al.* 2007; Lambert 2012; Lioy *et al.* 2014).

iii) Enzymatic modification

Enzymatic modification of the amino or hydroxyl groups plays by far the most important role in aminoglycoside resistance in clinical isolates of Gram-negative and Gram-positive bacteria. Three families of enzymes that perform cofactor-dependent drug modification in the bacterial cytoplasm have been recognized; these are aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases (AACs), and aminoglycoside nucleotidyltransferases (ANTs) (Wright *et al.* 1998; Vakulenko and Mobashery 2003).

The sites modified by these enzymes for different classes of aminoglycosides, are further divided into subclasses with different regiospecificities for aminoglycoside modifications: there are four nucleotidyltransferases [ANT(6), ANT(4'), ANT(3''), and ANT(2'')], seven phosphotransferases [APH(3'), APH(2''), APH(3''), APH(6), APH(9), APH(4), and APH(7'')], and four acetyltransferases [AAC(2'), AAC(6'), AAC(1), and AAC(3)] (Kotra *et al.* 2000). There is also a bifunctional enzyme, AAC(6')-APH(2''), that can acetylate and phosphorylate its substrates sequentially (Kotra *et al.* 2000). It is important to note that the distribution of the various modifying enzymes is specific to either Gram-positive or Gram-negative bacteria (Lambert 2012). The most common modifying enzymes in Gram-positive bacteria are summarized in Table 1 (Lambert 2012).

The level of resistance produced in various microorganisms and individual strain is significantly different and depends on diverse factors, including the amount of enzyme produced, its catalytic efficiency, and the type of aminoglycoside *etc.* (Vakulenko and Mobashery 2003). In general, only phosphotransferases produce high levels of resistance.

Enzymatic modification is the major and primary mechanism of aminoglycosides resistance. In theory, the antibacterial activity of various aminoglycosides could be restored if the resistance mechanisms are to be inhibited. It is a successful concept for overcoming resistance in other antibiotics classes, like β -lactams. However, little progress has been made in the development of clinically useful inhibitors of aminoglycoside-modifying enzymes. The difficulty of finding universal inhibitors for the large number of different enzymes is compounded by the fact that

the biochemical mechanism of many aminoglycoside-modifying enzymes is not known in detail (Vakulenko and Mobashery 2003; Hermann 2007; Herzog *et al.* 2016; Zarate *et al.* 2018).

Table 1 The main aminoglycoside-modifying enzymes in Gram-Positive bacteria (Lambert 2012).

	APH(3')-III	ANT(4')-I	APH(2'')-AAC(6')
Kanamycin B	+	+	+
Gentamicin	-	-	+
Tobramycin	-	+	+
Amikacin	+	+	+
Apramycin	-	-	-
Fortimicin	-	-	-

AAC: aminoglycoside *N*-acetyltransferase
 ANT: aminoglycoside *O*-nucleotidyltransferase
 APH: aminoglycoside *O*-phosphotransferase

5.2.1.3 Synergism of aminoglycosides with β -lactams in *S. aureus*

Aminoglycosides are often combined with a β -lactam in the treatment of *S. aureus* infection. The first human data of synergistic effect of a combination therapy was published in 1976, it was reported that methicillin together with gentamicin therapy enhanced the bactericidal activity in the treatment of a patient with an endocarditis caused by a methicillin-sensitive *S. aureus* (Murray *et al.* 1976).

Afterwards, several comparative trials and randomized controlled trials were performed to evaluate the impact of the synergistic effect of a β -lactam antibiotic combined with an aminoglycoside (Watanakunakorn and Baird 1977; Abrams *et al.* 1979; Rajashekaraiyah *et al.* 1980; Korzeniowski and Sande 1982; Frimodt-Moller *et al.* 1987). Among these, only one study demonstrated that the combined therapy lead to a more rapid clinical response (defervescence, normalization of leukocyte count and shortened time of bacteremia); but did not alter morbidity or mortality in either experimental group (Korzeniowski and Sande 1982; Harder and Ensom 2007).

Interestingly, two subsequent non-comparative trials showed that a short-course combination regimen (2 weeks) was particularly successful in the therapy of right-sided endocarditis in intravenous drug users (IVDU) (Chambers *et al.* 1988; Torres-Tortosa *et al.* 1994).

In point of fact, these trials are not comparable, and even if combination therapy is deemed effective, the benefit of synergistic effect with aminoglycosides and β -lactams is still unpredictable.

5.2.2 50S ribosomal subunit inhibitors

The 50S is the larger subunit of the 70S ribosome. It consists of 5S and 23S rRNA and dozens of ribosomal proteins.

5.2.2.1 Macrolides and related compound

Macrolides, lincosamides and streptogramins (MLS) are compounds that are structurally distinct but functionally similar. They exert their antibacterial effects by binding to the 50S subunit of bacterial ribosome, inhibiting protein synthesis by preventing transpeptidation and translocation reactions. These molecules have a similar spectrum of activity limited to Gram-positive cocci (mainly staphylococci and streptococci), mycoplasmas and campylobacters (Leclercq 2002; Tenson *et al.* 2003; Lambert 2012).

The macrolides are a class of natural products that are classified on the basis of the number of atoms in the ring of the macrocyclic lactone, which usually contain 14-, 15-, or 16-membered lactone ring attached with deoxy sugars (desosamine and cladinose) (Tenson *et al.* 2003). Erythromycin of the 14-membered ring family, is probably the best known macrolide (Alvarez-Elcoro and Enzler 1999).

Lincosamides make up an important class of antibiotics that originates from *Streptomyces lincolnensis* in a soil sample. It constitutes a relatively small group of antibiotics with a chemical structure that is devoid of the lactone ring and consisting of amino acid and sugar moieties. Lincosamides include lincomycin and the semisynthetic derivatives, clindamycin and pirlimycin (Spizek and Rezanka 2017). They are bacteriostatic antibiotics used against Gram-positive organisms, selected Gram-negative anaerobes and protozoans; however, they may be bactericidal at high concentration (Spizek and Rezanka 2017).

Clindamycin is obtained *via* the replacement of the 7-(*R*) hydroxyl group of lincomycin by a 7(*S*)-chloro-7-substituent (Figure 17) (Meyers *et al.* 1969; Birkenmeyer and Kagan 1970). It consists of a pyrrolidinyl group linked to a galactose sugar by a peptide bond (Schlunzen *et al.* 2001). Clindamycin is the most clinically relevant lincosamide and it is highly active against Gram-positive pathogens, including MRSA (Morar *et al.* 2009).

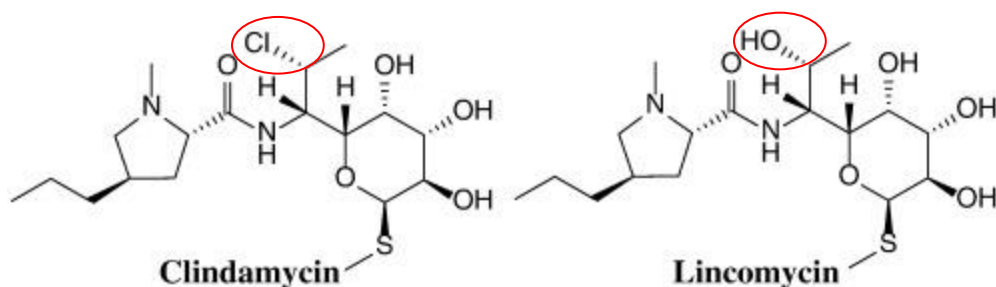


Figure 17 Chemical structure of clindamycin and lincomycin (He *et al.* 2017).

5.2.2.1.1 Mechanism of action

Briefly, the elongation process of translation can be explained as the entire nascent polypeptide chain is transferred from the A site to the incoming amino acid of the P site, with the help of a peptide bond which is catalyzed by the peptidyl-transferase, during each elongation cycle. As the nascent polypeptide chain is being elongated, it passes through a tunnel in the large ribosomal subunit. Antibiotics that inhibit peptide bond formation and nascent chain progression can be categorized into distinct classes based on the precise binding sites (the peptidyl transferase center and/or in the ribosomal tunnel): i) within the A site of the PTC (peptidyl transferase center), ii) exclusively at the P site, iii) cover both the A site and P site, or iv) within the ribosomal tunnel adjacent to the PTC (Wilson 2009).

Clindamycin inhibits protein synthesis and acts specifically through the binding of the 23S rRNA; the binding site is within the A site of the peptidyl transferase center (PTC) of 50S subunit (Schlunzen *et al.* 2001; Tu *et al.* 2005; Dunkle *et al.* 2010), where peptide-bond formation occurs during elongation (Wilson 2014). It inhibits peptide-bond formation by perturbing or preventing the correct positioning of the aminoacylated ends of tRNAs in the PTC (Wilson 2014), thus it blocks the extension of the peptide chain and leads to the dissociation of peptidyl-tRNA from ribosomes (Figure 18). Such “drop-off” events occur just after the initiation of protein synthesis, when the nascent polypeptide chain is short (Tenson *et al.* 2003; Spizek and Rezanka 2017).

Interestingly, the binding site of clindamycin is slightly different from the binding sites of chloramphenicol, oxazolidinone (linezolid) and some macrolide antibiotics. Specifically, the prolyl-moiety of clindamycin overlaps the aminoacyl-moiety of an A site tRNA and the binding site of chloramphenicol, whereas the sugar moiety of clindamycin extends into the ribosomal tunnel and overlaps the binding position of the macrolide antibiotics, such as erythromycin (Schlunzen *et al.* 2001; Tu *et al.* 2005; Wilson 2009). The crystal structure of linezolid bound to bacterial ribosome (Wilson *et al.* 2008) reveals that linezolid binds to the A site of the PTC,

in a position overlapping the binding sites of chloramphenicol as well as the aminoacyl moiety of an A site bound tRNA (Wilson 2009).

Consistently, this is a good agreement that lincosamides compete with chloramphenicols, oxazolidinones and some macrolide antibiotics for ribosome binding site (Lin *et al.* 1997; Skripkin *et al.* 2008).

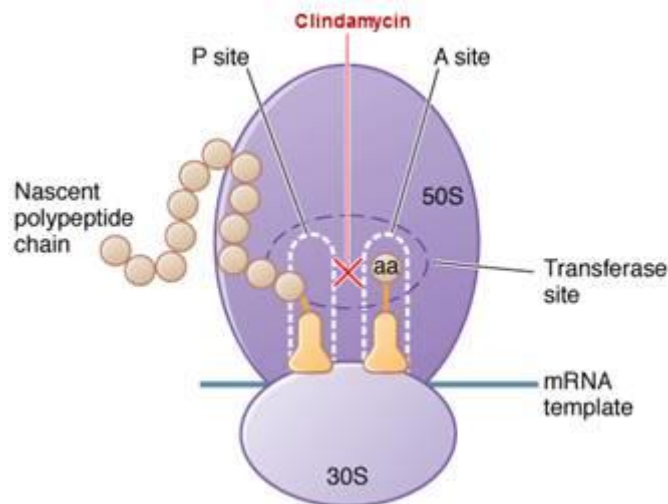


Figure 18 Clindamycin mechanism of action (Wilson 2014)

5.2.2.1.2 Mechanism of resistance in *Staphylococcus* spp.

Bacteria resist MLS_B (macrolides, lincosamides and streptogramin B) antibiotics through three ways: i) target site modification (ribosomal binding site modification by methylation or mutation in the 23S rRNA) (Courvalin *et al.* 1985), ii) enzymatic modification of antibiotics (drug inactivation), a way more prevalent in pathogenic Gram-positive cocci (Dutta and Devriese 1982; Leclercq *et al.* 1985), and iii) active efflux of the antibiotics. So far, modification of the ribosomal target confers broad-spectrum resistance to macrolides and lincosamides, whereas inactivation and efflux affect only some of these molecules.

i) Ribosomal methylation

The main type of resistance to clindamycin is the so-called MLS_B resistance which renders sensitive microorganism cross-resistant to macrolides, lincosamides and streptogramin B (Weisblum 1995). It is a dimethylation of adenine A2058, at the N6 position which is located in the region of the peptidyl transferase loop in domain V of the 23S rRNA in the 50S ribosomal subunit, by specific ribosome methylation modification enzymes (Leclercq and Courvalin 1991). The family of genes that is responsible for this methylation is named *erm* (erythromycin

ribosomal methylase). Four major classes are detected in pathogenic microorganisms: *ermA*, *ermB*, *ermC*, and *ermF* (Weisblum 1995; Roberts *et al.* 1999).

The *ermA* and *ermC* determinants are predominant in staphylococci (Lina *et al.* 1999; Roberts *et al.* 1999). The *ermA* genes, which are borne by transposons related to *Tn554*, are mainly spread in methicillin-resistant strains (Tillotson *et al.* 1989); whereas *ermC* genes that are carried by plasmids, are mostly responsible for erythromycin resistance in methicillin-susceptible strains (Lina *et al.* 1999; Gherardi *et al.* 2009). The expression of *erm* genes is manifested as either constitutive or inducible. In constitutive resistance, active methylase mRNA is produced in the absence of an inducer (Misic *et al.* 2017). In contrast, in inducible resistance, the bacteria produce inactive methylase mRNA, bacteria are sensitive to MLS_B antibiotics as long as no sufficient inducer is present (Leclercq 2002; Hess and Gallert 2014). The induction is related to the presence of an attenuator upstream of the respective *erm* gene that determines which drug is able to act as inducer. The nature of the attenuator may serve to distinguish *erm* genes in different bacterial species (Weisblum 1995). The presence of an inducer leads to mRNA rearrangements, which allows to the translation of the methylase (Leclercq 2002; Schwendener and Perreten 2012).

ii) Drug inactivation

Organisms employing the antibiotic modification strategy inactivate lincosamides *via* an adenylation catalyzed by nucleotidyl transferases encoded by *lnu* genes (formerly *lin*): *lnuA* and *lnuB*. LinA and its variants (LnuC and LnuD) show amino acid sequence identity with the aminoglycoside antibiotic nucleotidyltransferase ANT(2'')-Ia (Petinaki *et al.* 2008). LinA is found in staphylococci and is capable of adenylation at either 3'- or 4'-OH of the methylthio lincosamide sugar of lincosamides (Brisson-Noel *et al.* 1988; Morar *et al.* 2009; Sundlov and Gulick 2009). LinB and LnuF, have some sequence similarity with the β -subunit of the DNA polymerase (Pol β) (Morar *et al.* 2009). LinB is found in enterococci and modifies the 3'-OH of methylthio lincosamide only (Bozdogan *et al.* 1999).

iii) Active efflux of antibiotics

In Gram-positive organisms, the acquisition of macrolide resistance by active efflux is caused by 2 classes of pumps: i) the ATP-binding-cassette (ABC) transporter superfamily ii) the major facilitator superfamily (MFS) (Leclercq 2002).

The active efflux of antibiotics is an important mechanism of resistance in staphylococci along with target site modification and enzymatic inactivation. The plasmid borne genes, *msrA/B*, first detected in *S. epidermidis*, belong to the ATP-dependent efflux pumps (ABC). They confer resistance to 14-membered and 15-membered macrolides and streptogramin type B in *Staphylococcus* spp (MS_B resistance phenotype), but not lincosamides (Ross *et al.* 1990; Duran *et al.* 2012). An active efflux ABC transporter-like transmembrane protein is encoded by *lsa* genes (*lsaA* and/or *lsaC*) that functions to export antimicrobials belonging to the MLS family (Dina *et al.* 2003), which are responsible for intrinsic resistance to lincosamides and streptogramin type A in *Enterococcus faecalis* (Dina *et al.* 2003; Hollenbeck and Rice 2012; Ogrodzki *et al.* 2017). Recently, a novel gene *lsaE*, which shows a high degree of similarity to *lsaA*, has been detected in staphylococci (Wendlandt *et al.* 2013). These resistance genes transfer from enterococci to *S. aureus* had been observed previously with other plasmid or transposon borne genes, for example the *vanA* gene cluster (Noble *et al.* 1992).

5.2.2.2 Oxazolidinones

The oxazolidinones is a class of new synthetic antibiotics containing 2-oxazolidone in their structure that inhibit protein synthesis at an exceedingly early stage. They are primarily effective against Gram-positive bacteria.

Linezolid has been the first oxazolidinone available and displays impressive antibacterial activity against many important multidrug-resistant human pathogens. Given its potent activity, linezolid has been reserved for the treatment of documented serious infections, including MRSA, VISA and hVISA (Fung *et al.* 2001).

5.2.2.2.1 Mechanism of action

It appears that linezolid inhibits protein synthesis preceding translation initiation by binding to the 50S subunit within domain V of the 23S rRNA peptidyl transferase center that is near the interface with the 30S subunit (Shinabarger *et al.* 1997; Swaney *et al.* 1998; Kloss *et al.* 1999; Fung *et al.* 2001), thereby preventing the formation of the initiation complex composed of the 30S subunit, fMet-tRNA, mRNA, GTP and initiation factors (Eustice *et al.* 1988; Swaney *et al.* 1998; Diekema and Jones 2000; Fung *et al.* 2001; Bozdogan and Appelbaum 2004). Consequently, mRNA translation is blocked due to inhibition of the 70S formation.

This mode of action is fairly distinct from other protein synthesis inhibitors such as aminoglycosides, macrolides and lincosamides, they either induce misreading of mRNAs or inhibit polypeptide elongation. The key benefit of linezolid unique mechanism of action is that

cross-resistance with other currently available antimicrobial agents that act by inhibiting protein synthesis would be unlikely.

Meanwhile, other studies demonstrated that oxazolidinones may behave as competitive inhibitors of initiator tRNA by binding to the ribosomal peptidyltransferase P site and the adjacent A site (the binding site for incoming aminoacyl-tRNA), thus inhibiting the formation of the first peptide bond (Patel *et al.* 2001; Bobkova *et al.* 2003; Bozdogan and Appelbaum 2004). Oxazolidinones are thus also implicated in elongation inhibition.

More recently, the consensus has been that oxazolidinones bind to the 50S A site pocket near the catalytic center, overlapping the aminoacyl moiety of an A site bound tRNA, suggesting that the inhibition involves a competition with the incoming A site substrates (Leach *et al.* 2007; Ippolito *et al.* 2008; Wilson *et al.* 2008; Wilson 2009). This binding site overlaps partially with that of many other PTC inhibitors, such as chloramphenicol and clindamycin (Wilson 2014).

5.2.2.2.2 Mechanism of resistance in staphylococci

Although some of the first reports on linezolid claimed that there would be no/rare cross-resistance to linezolid in view of its unique mechanism of action (Long and Vester 2012), further researches proved that the mechanisms of resistance are similar to those of antibiotics targeting PTC (Colca *et al.* 2003; Leach *et al.* 2007).

Oxazolidinones resistance in staphylococci has been encountered clinically as well as *in vitro*, but it is still an extremely uncommon phenomenon. The main resistance mechanism described for oxazolidinone is target modification and has been characterized into 3 categories: i) mutations in the domain V of 23S rRNA, ii) acquisition of the ribosomal methyltransferase gene *cmr*, iii) mutations in *rplC* and *rplD* genes that encode 50S ribosomal proteins L3 and L4 respectively (Stefani *et al.* 2010).

i) Resistance caused by 23S rRNA mutations

Mutation of the 23S rRNA was discovered in many microorganisms. In staphylococci, G2576U is the most frequently reported mutation in linezolid-resistant clinical isolates (Roberts *et al.* 2006; Hong *et al.* 2007; Kelly *et al.* 2008; Yoshida *et al.* 2009; Wong *et al.* 2010; Endimiani *et al.* 2011). Interestingly, some of the reports about G2576U mutation in clinical isolates correlated the dose and the duration of linezolid exposure to the level of linezolid resistance (Besier *et al.* 2008; Hill *et al.* 2010; Endimiani *et al.* 2011; Ikeda-Dantsuji *et al.* 2011),

emphasizing the importance of judicious use of linezolid in clinical settings (Long and Vester 2012).

The U2504A, U2500A and G2447U 23S rRNA mutations have been reported in linezolid resistant clinical staphylococcal isolates (Meka *et al.* 2004; Liakopoulos *et al.* 2009; Wong *et al.* 2010). Particularly, U2504A plays an important role in resistance to PTC antibiotics due to its specific location of the binding pockets of phenicols, lincosamides and oxazolidinones (Stefani *et al.* 2010).

ii) Resistance caused by 23S rRNA alteration

The main type of 23S rRNA modification that provides acquired antibiotic resistance is methylation. A multi-resistance gene, *cfr*, which encodes an rRNA methyltransferase confers the only known transferable form of linezolid resistance so far (Kehrenberg *et al.* 2005). *cfr* is related to the RlmN methyltransferases, it adds an additional methyl group at the C-2 position of 23S rRNA nucleotide A2503 (Toh *et al.* 2008; Giessing *et al.* 2009; Long and Vester 2012). Since A2503 is located in the PTC, a proximity of the overlapping non-identical binding sites of multi antimicrobial agents, it has been concluded that *cfr*-mediated methylation confers combined resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A (phenotype known as PhLOPSA) by interfering with the positioning of the drugs (Kehrenberg *et al.* 2005; Long *et al.* 2006).

The *cfr* gene was originally discovered on multi-resistance plasmids and isolated during a surveillance study for florfenicol resistance in *Staphylococcus* isolates of animal origin in 2000 (Schwarz *et al.* 2000; Kehrenberg and Schwarz 2006; Stefani *et al.* 2010; Long and Vester 2012). Subsequently, in 2005, the *cfr* gene was detected in a human *Staphylococcus* isolate (Toh *et al.* 2007). This was the first *cfr*-positive clinical strain of methicillin-resistant *S. aureus*, it had two rRNA methyltransferase genes (*cfr* and *ermB*) that were located on the same operon in the chromosome. The combined action of the two methyltransferases leads to modification of two specific residues A2058 and A2053 in 23S rRNA, and their co-expression confers resistance to all clinically relevant antibiotics that target the large ribosomal subunit (Smith and Mankin 2008; Long and Vester 2012). These strongly suggest that *cfr*, a natural resistance gene carried by mobile genetic elements such as plasmids and transposons, has the capability of disseminating among *Staphylococcus* pathogenic strains (Toh *et al.* 2007; Long and Vester 2012).

- iii) Resistance caused by mutations in ribosomal proteins L3 and L4

Linezolid resistance can also involve a less common mechanism: mutations in ribosomal protein L4 and L3 encoded by *rplD* and *rplC* genes, respectively. A loop ending in two tips of L3 extends into the PTC, even though the main part of ribosomal protein L3 is positioned on the surface of the 50S subunit (Long and Vester 2012). Similarly, part of ribosomal protein L4 is also placed relatively close to the PTC but in the tunnel through which nascent peptides exit the ribosome (Long and Vester 2012). Many studies and cases that are associated with L3 and L4 mutations have been reported in staphylococci (Locke *et al.* 2009; Locke *et al.* 2009; Locke *et al.* 2010; Mendes *et al.* 2010; Endimiani *et al.* 2011; Román *et al.* 2013; Rouard *et al.* 2017), however, the effects of the single and combined mutations L3 and L4 still need more specific information.

5.3 Inhibition of DNA biosynthesis

5.3.1 DNA replication

DNA replication is a well conserved process during which a DNA molecule is duplicated into two identical copies that are passed to daughter cells during cell division. Bacterial DNA replication is bi-directional and initiates at a single origin of replication (OriC) where a replication fork is created (Bird *et al.* 1972). During replication, each strand of unwind double helix DNA serves as a template for the production of its counterpart, structure stress are accommodated by helicases and topoisomerases. These enzymes are associated with the replication fork, called the replisome. They intervene in the initiation, the elongation and the termination of DNA synthesis. DNA polymerase is responsible for catalyzing the addition of nucleotides complementary to the template strand to the forming DNA from the 5' to the 3' end of the molecule. This process is semiconservative (Bussiere and Bastia 1999) and cellular proofreading and error-checking mechanisms enable replication fidelity.

5.3.1.1 DNA gyrase (topoisomerase II)

DNA gyrase and topoisomerase IV, which are homologous but have crucial distinct functions, are two type II topoisomerases present in bacteria (Levine *et al.* 1998; Aldred *et al.* 2014). DNA gyrase relieves the stress imposed when double-stranded DNA is being unwound by an helicase (Wigley *et al.* 1991; Cabral *et al.* 1997). It modulates the topological state of DNA by introducing negative supercoiling of the DNA or relaxing positive supercoils (Reece and Maxwell 1991). DNA gyrase is the only type II topoisomerase that can actively introduce negative supercoils into DNA (Kampranis and Maxwell 1996).

DNA gyrase is made up of two distinct functional subunits GyrA and GyrB, which in turn have two subunits forming an A₂B₂ tetramer (Watt and Hickson 1994; Levine *et al.* 1998; Champoux 2001). The right-handed DNA double helix is wrapped around the A₂B₂ tetramer, which affects the degree of supercoils. GyrA subunits carry out a double-stranded nick, a segment of DNA then passes through the nick to the opposite side of the DNA gyrase; this movement is performed by GyrB subunits, then broken double-stranded DNA is resealed by GyrA and the gyrase is released from the DNA (Watt and Hickson 1994; Roca 1995; Levine *et al.* 1998). Bacterial DNA gyrase is the target of many antibiotics, such as quinolones (Laponogov *et al.* 2009). Quinolones (including nalidixic acid and ciprofloxacin) bind to GyrA subunits and interfere with its strand cutting and resealing functions (Wohlkonig *et al.* 2010).

5.3.1.2 DNA topoisomerase IV

Topoisomerase IV (Topo IV) is one of two type II topoisomerases in bacteria. *parC* and *parE* that encode topo IV are homologous to GyrA and GyrB, respectively (Kato *et al.* 1990; Peng and Marians 1993). The major function of topoisomerase IV is unlinking or decatenating freshly synthesized DNA in an ATP-dependent reaction (Levine *et al.* 1998; Deibler *et al.* 2001). As a result of the double-helical nature of DNA and its semiconservative mode of replication, the two newly replicated DNA strands are to be interlinked. These links have to be removed for the chromosome (or plasmids) to segregate into daughter cells so that cell division can complete. Topo IV is able to pass one double-strand DNA through another double-strand DNA (Rawdon *et al.* 2016). During the reaction, topo IV first binds to a specific region of one of the double helices, makes a double-stranded cut, then drives the passage of unbroken helices through a transient gap with the energy of ATP hydrolysis; after the passage, the broken helix is resealed (Schoeffler and Berger 2008).

In addition to the role in the decatenation of post-replicative catenanes, topo IV also relaxes positive supercoils arising ahead of active replication forks and of transcribing RNA polymerases. It shares this role with DNA gyrase (Khodursky *et al.* 2000; Koster *et al.* 2010; Rawdon *et al.* 2016). However, the decatenation activity of Topo IV is far more effective (Hiasa and Marians 1996; Ullsperger and Cozzarelli 1996).

Topo IV is also a target of quinolones, which include ciprofloxacin.

5.3.2 Quinolones and Fluoroquinolones

Quinolones are one of the largest and most commonly prescribed classes of antibacterial agents used worldwide. Quinolones have been classified into four generations, mainly based on their

spectrum of activity followed by the development of their derivatives (Ball 2000). Nalidixic acid, the parent compound of the quinolones, was originally discovered in 1962 as a by-product of anti-malarial research (Leshner *et al.* 1962). Additional compounds, such as cinoxacin, norfloxacin and enoxacin became available clinically more than a decade after. These agents, considered as the first generation of quinolones, were mainly used for the treatment of urinary tract infection (Ball 2000). Fluoroquinolones, the second generation of quinolones, have a true fluorine atom in their chemical structure and are used to treat a wide variety of Gram-negative and partial Gram-positive bacterial infections. One typical example is ciprofloxacin, one of the most widely used antibiotics worldwide (Andersson and MacGowan 2003; Heeb *et al.* 2011). The third generation compared with the fourth generation, are less effective against Gram-negative and Gram-positive bacteria, and some anaerobes. The fourth generation can be used against respiratory agents and has a broad activity against Gram-positive bacteria (especially *Streptococcus pneumoniae*), atypical organisms and a variable activity against anaerobes. The first generation is rarely used. Frequently prescribed medications are second generation quinolones such as, ciprofloxacin, levofloxacin and moxifloxacin, or their generic equivalents.

Quinolones have been developed for more than 5 decades from drugs used primarily to treat urinary tract infections and have become the most commonly prescribed antibacterials in the world (Aldred *et al.* 2014). This achievement has been made possible by a clear understanding of the structure-activity relationships (Van Bambeke *et al.* 2005). Quinolones share a bicyclic core structure related to the compound 4-quinolone (Brighty and Gootz 2000). Ciprofloxacin, which was first synthesized in 1983, has been derived from nalidixic acid by the addition of piperazine at the C-7 position and the introduction of a cyclopropyl group to the N1 position (Figure 19). It displays considerably improved the activity against DNA gyrase and the entry into Gram-positive organisms. The clinical success of ciprofloxacin spawned an array of newer-generation quinolones that displayed an even broader spectrum of activity, especially against Gram-positive species involved in respiratory tract infections (Stein 1988; Emmerson and Jones 2003; Andriole 2005; Aldred *et al.* 2014).

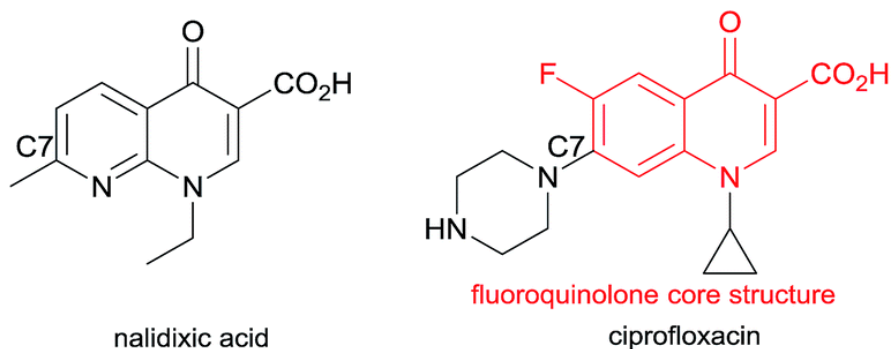


Figure 19 Structures of nalidixic acid and ciprofloxacin (Klahn and Bronstrup 2017).

5.3.2.1 Mechanism of action

Quinolones eradicate bacteria by blocking DNA replication through the conversion of their targets (DNA gyrase and topo IV) into toxic enzymes that fragment bacterial chromosome. They bind to complexes interface in the cleavage-ligation active site that is formed between DNA and gyrase or topo IV in a noncovalent manner (Laponogov *et al.* 2009; Bax *et al.* 2010; Laponogov *et al.* 2010; Wohlkonig *et al.* 2010). Shortly after binding, quinolones induce a conformational change in the enzyme. When the enzyme cuts the DNA, quinolones stabilize the enzyme-DNA complexes and prevent the religation of the broken DNA strands. The enzyme is trapped on the DNA resulting in the formation of a drug-enzyme-DNA ternary complex. The ternary complex perturbs the cleavage-religation equilibrium resulting in permanent chromosomal breaks, which is fatal to bacteria. In turn, the generation of these DNA breaks triggers the SOS response and other DNA repair pathways, ternary complexes are reversible, and the broken DNA strands can be religated (Chen and Liu 1994; Shiro *et al.* 1995).

Quinolones stabilizing cleavage complexes impair the overall catalytic functions of gyrase and topo IV (Aldred *et al.* 2014). It was demonstrated that gyrase is the primary toxic target of quinolones and topo IV is a secondary drug target in *E. coli* (Khodursky *et al.* 1995), which is consistent with the speculation that during DNA replication in *E. coli*, gyrase is located preferentially ahead of the advancing replication fork whereas topo IV acts behind (Khodursky *et al.* 1995; Khodursky *et al.* 2000). Exceptionally, in some Gram-positive bacteria including *S. aureus* and *S. pneumoniae*, it has been indicated that topo IV, rather than gyrase, is the primary target of quinolones (Ferrero *et al.* 1994; Muñoz and De La Campa 1996; Pan *et al.* 1996; Fournier *et al.* 2000). This gave rise to the concept that gyrase was the primary target for quinolones in Gram-negative bacteria, whereas topoisomerase IV was the preference for many Gram-positive species. However, subsequent studies showed that this paradigm does not fit in many cases, different quinolones have been shown to have different primary targets (Pan and

Fisher 1997; Pan and Fisher 1998; Fournier *et al.* 2000). The preference of quinolone for their targets is controversial and needs to be further evaluated in accordance with bacterial species and drugs.

5.3.2.2 Mechanism of resistance

Fluoroquinolones are potent, broad-spectrum agents that have been extensively used in human and veterinary medicine because of their effectiveness against both Gram-negative and Gram-positive bacteria (Aldred *et al.* 2013; Redgrave *et al.* 2014). However, the World Health Organization (WHO) now proposes that fluoroquinolones should be used restrictedly (Collignon *et al.* 2009), due to the rising issue of resistance. Resistance to fluoroquinolones is multifactorial. It includes i) target modifications, ii) horizontal acquisition of mobile genetic elements, iii) multidrug-resistance (MDR) efflux pumps.

i) Target alterations

The most common mechanism of high-level quinolone resistance is due to the mutated target genes, DNA gyrase (*gyrA* and *gyrB*) and topo IV (*parC* and *parE*). The region where mutations arise in these genes is a short DNA sequence known as the quinolone resistance-determining region (QRDR) (Yoshida *et al.* 1990; Yoshida *et al.* 1991; Redgrave *et al.* 2014). Spontaneous mutations cause amino acid substitutions in QRDR, modify the structure of target proteins and affect the fluoroquinolone-binding affinity of the enzyme, subsequently leading to drug resistance (Piddock 1999; Hooper 2000). The amino acids that are most frequently associated with quinolone resistance in clinical isolates are Ser83 and/or Asp87 in *gyrA*, Ser80 and/or Glu84 in *parC* (Yoshida *et al.* 1990; Friedman *et al.* 2001), as well as Ser458 and Glu460 in *parE* which are outside of the QRDR (*E. coli* numbering)(Sorlozano *et al.* 2007; Bansal and Tandon 2011). The appearance and high frequency of occurrence of mutations in *parE* gene (24 of 54 *E. coli* isolates, 44.4%) is alarming (Bansal and Tandon 2011). Mutations in *gyrB* occur but in a lower frequency in *E. coli* (Yamagishi *et al.* 1981; Yoshida *et al.* 1991; Heddle and Maxwell 2002).

A single mutation in DNA gyrase is sufficient to cause quinolone resistance; however, the accumulation of multiple mutations in and out of the QRDR regions of both gyrase and topo IV appears to be a major contributor to high-level quinolone resistance.

ii) Plasmid-mediated resistance

Mobile genetic elements, such as plasmids and transposons that carry antibiotic resistant determinants, can decrease susceptibility to antibiotics by horizontal transmission. Plasmid-mediated quinolones resistance has been identified as an emerging clinical problem that generally cause low-level resistance (Wang *et al.* 2003; Drlica *et al.* 2009; Carattoli 2013; Guan *et al.* 2013), occasionally high-level (Robicsek *et al.* 2006; Strahilevitz *et al.* 2009).

Three families of genes are relevant to plasmid-mediated quinolone resistance. The first, also the most common, are the *Qnr* genes that encode proteins sharing homology with McbG and MfpA, which are DNA mimics (Tran and Jacoby 2002; Robicsek *et al.* 2006; Strahilevitz *et al.* 2009; Aldred *et al.* 2014). They function either by decreasing the number of available enzyme-DNA complexes or by preventing quinolones access to the cleavage complexes by binding to gyrase and topo IV (Tran and Jacoby 2002; Xiong *et al.* 2011). The second plasmid-encoded enzyme is aac(6')-Ib-cr, which is a variant of an aminoglycoside acetyl transferase that acetylates the unsubstituted nitrogen of the C7 piperazine ring (found in norfloxacin and ciprofloxacin) decreasing drug activity (Robicsek *et al.* 2006; Guillard *et al.* 2013). The last group of mobile quinolone resistance genes is made up of efflux pumps, such as *oqxAB* and *qepA* (*qepA1* and *qepA2*), found in animal and human bacterial infections, respectively (Yamane *et al.* 2007; Cattoir *et al.* 2008; Strahilevitz *et al.* 2009).

iii) Multidrug-resistance efflux pumps

In addition to plasmid-encoded efflux pumps, chromosomal multidrug efflux pumps are capable of removing drugs from the bacterial cell, thus reducing the cellular concentration of antibiotics. Various classes of transporters are associated with this mechanism, for instance, NorA of *S. aureus*, belongs to major facilitator superfamily (MFS) pumps, and the resistance nodulation division (RND) family of tripartite transporters of Gram-negative pathogens (Kaatz and Seo 1995; Piddock 2006; Redgrave *et al.* 2014).

5.4 Inhibition of RNA biosynthesis

Transcription is the synthesis of RNAs from a DNA matrix. It is an underutilized target for antibiotics compared with DNA replication and protein translation, but it is an excellent antibacterial target: i) transcription is an essential process for cell viability, ii) RNA polymerase (RNAP) and transcription factors are highly conserved across bacteria, which allows the potential development of broad-spectrum anti-transcriptional agents (Perez and Groisman 2009), iii) low potential cytotoxicity due to the non-similarity of RNAP at the sequence level between eukaryotes and prokaryotes (Chopra 2007), iv) structure-based drug derivatives are

feasible thanks to the clear high-resolution structures of RNAP (Zhang et al. 1999; Murakami and Darst 2003; Ma et al. 2016). Rifamycins is one typical class of antibiotics that targets bacterial transcription, since the discovery and development of rifampicin in the 1960s, it has been widely used in clinical therapies.

5.4.1 Bacterial RNA polymerase

The first step of gene expression is transcription, the key enzyme responsible for transcription is DNA-directed RNA polymerase (RNAP or RNAPol), which is the direct or indirect target of most regulation of transcription (Chamberlin 1976; von Hippel 1998; Nudler 1999). Therefore, detailed knowledge of RNAP structure and function is required for thorough understanding of gene expression. In 1999, the determination of the crystallographic structure of bacterial RNAP from *Thermus aquaticus* was a milestone to understand the RNAP multi-subunit family (Zhang et al. 1999).

RNAP is a large multi-subunit protein that is conserved across bacterial species (Burgess 1969; Young 1991; Ebright 2000; Minakhin et al. 2001). The stereoscopic vision of bacterial RNAP is reminiscent of a crab claw with two “pincers” defining an active center Mg^{2+} at its base, for RNA synthesis (Ebright 2000). Bacterial RNAP core enzyme consists of five subunits, two α , β , β' and ω (~ 400 kDa):

- β' , the β' subunit encoded by *rpoC* gene is the largest subunit (Ovchinnikov et al. 1982). It constitutes one “pincer” which is part of the active center responsible for RNA synthesis.
- β , the β subunit is the second largest one, it is encoded by *rpoB* gene and makes up the other “pincer”, together with β' subunit to form a complete active center.
- α^I and α^{II} , they have distinct locations and functions even though they have identical sequences, α^I interacts with β subunit and is located closer to the active center, α^{II} interacts with β' subunit and is situated farther to the active center (Ebright 2000). Each α subunit has two domains i) α NTD (N-Terminal domain) is responsible for RNAP assembly (Zhang and Darst 1998), ii) α CTD (C-terminal domain) is connected to α NTD through a unstructured and flexible linker of 13-20 residues (Blatter et al. 1994; Jeon et al. 1995). This linker allows α CTD to interact with different DNA and transcription factors within the upstream region of DNA promoter, an A/T rich sequence upstream of the -35 element (Busby and Ebright ; Jeon et al. 1997; Ebright 2000; Gourse et al. 2000).

- ω , is the smallest subunit of RNAP. It has a structural role to maintain β' in a correct conformation and forms a functional core enzyme with $\alpha_2\beta\beta'$ (Ghosh *et al.* 2003; Mathew and Chatterji 2006).

The core enzyme ($\alpha_2\beta\beta'\omega$), is capable of binding DNA non-specific, so the requirement of a sigma factor (σ) is a prerequisite to initiate specific transcription from DNA promoter. Sigma factor assembles with the core enzyme to form the “holoenzyme” (or $E\sigma$) that contains 6 subunits ($\alpha_2\beta\beta'\omega\sigma$, ~ 450 kDa) (Burgess *et al.* 1969; Travers and Burgess 1969). Sigma factors recognize specific promoter DNA sequences, permit transcription initiation at correct sites, interact with transcription factors, participate in promoter DNA opening and affect the early phases of transcription (Figure 20) (Gruber and Gross 2003; Saecker *et al.* 2011).

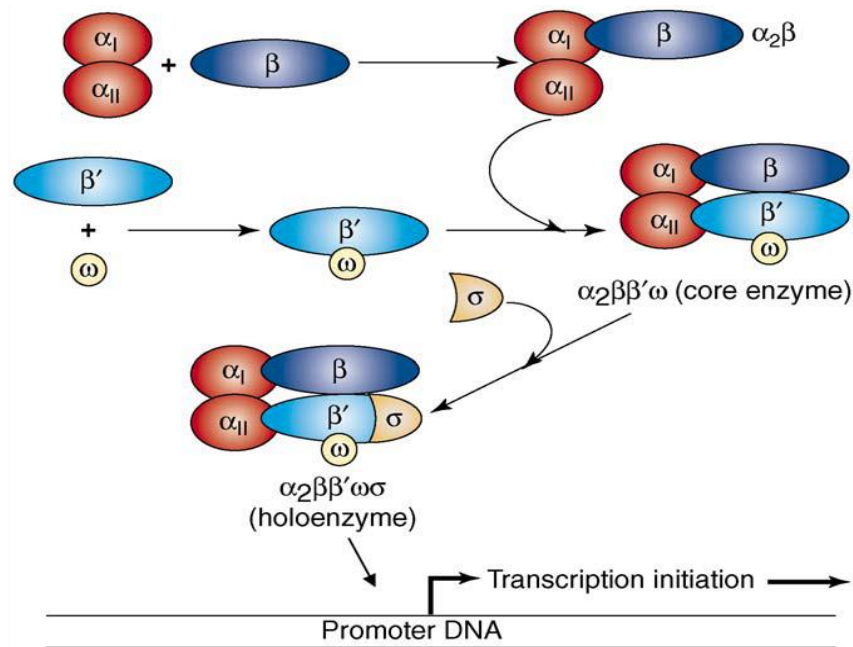


Figure 20 Schematic representation of the assembly process of RNA polymerase from different subunits (Mathew and Chatterji 2006).

5.4.2 Rifamycins

The rifamycins, a subclass of ansamycins family, were originally metabolised by the soil bacterium *Streptomyces mediterranei* (after renamed *Amycolatopsis rifamycinica*) which was first discovered in 1957 (Margalith and Beretta 1960; Bala *et al.* 2004). In 1963, chemical structure of rifamycins were determined (Prelog 1963) and it opened up the way to the synthesis of a vast number of semisynthetic derivatives (Wehrli and Staehelin 1971).

Among the numerous rifamycin derivatives, rifampicin (rifampin) is the most important and most widely used. It is orally active compared with other natural rifamycins. It is a broad

spectrum antibiotic against Gram-positive bacteria, some Gram-negative bacteria and it is particularly effective against mycobacteria (Tupin *et al.* 2010).

5.4.2.1 Mechanism of action

In 1970s, it was first demonstrated *in vitro* and in *E. coli*, that rifampicin acts on the DNA-dependent RNA polymerase rather than on the DNA-directed DNA polymerase at low concentration (0.02 µg/ml) (Calvori *et al.* 1965; Hartmann *et al.* 1967; Umezawa *et al.* 1968; Wehrli *et al.* 1968). Similar results have been obtained in various bacterial species, such as *S. aureus* (Wehrli *et al.* 1968).

The specificity of other potent inhibitors of nucleic acid synthesis (such as actinomycin, mitomycin and chromomycin) is different from rifampin and they have different toxic effects on human cells (Wehrli 1983). These antibiotics interact with DNA template and inhibit both DNA-dependent DNA and RNA synthesis (Kamiyama 1968; Zunino *et al.* 1972), in contrast, rifampin interacts directly with the RNA polymerase, which it inactivates at very low concentration (~ 0.01 µg/ml) (Wehrli *et al.* 1968). In view of its highly specific inhibition, rifampicin has become an important tool in the study of RNA biosynthesis and metabolism.

Further studies revealed that rifampicin binds in a pocket of the RNAP β subunit deep within the DNA/RNA channel, close to the RNAP active center. It blocks the path of the elongating RNA at the 5' end when the transcript becomes 2 to 3 nt long (Figure 21) (Korzheva *et al.* 2000; Campbell *et al.* 2001; Feklistov *et al.* 2008). The conformation of the β subunit within the core enzyme plays an important role in rifampicin binding and action since isolated β subunits do not bind rifampicin (Wehrli *et al.* 1968; Wehrli 1983; Williams and Piddock 1998). Rifampicin interrupts RNAP function by its ability to bind tightly to a relatively non-conserved part of the structure, and does not interfere directly with the catalytic activity of RNAP (Campbell *et al.* 2001).

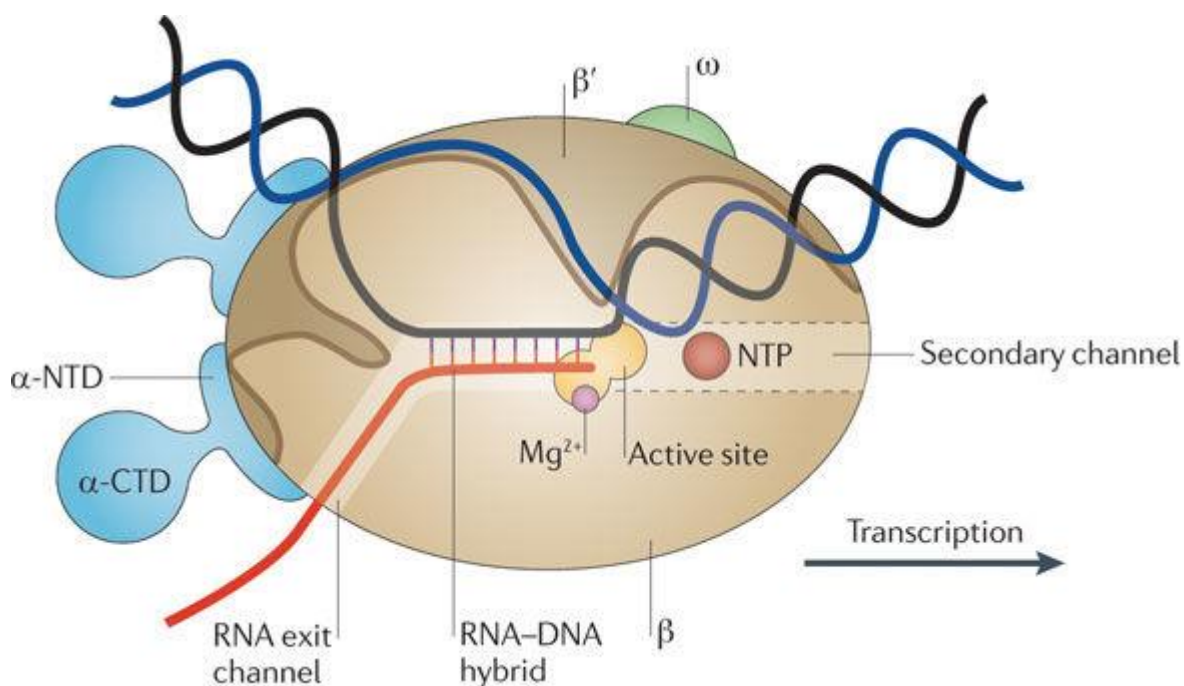


Figure 21 Model of DNA transcription (Santangelo and Artsimovitch 2011).

5.4.2.2 Mechanism of resistance

The potent antibacterial activity of rifampicin is due to the specific steric block of bacterial RNA polymerase. However, rifampicin resistance was reported shortly after its introduction in the medical practice (Atlas and Turck 1968; Alifano *et al.* 2015). Spontaneous resistance to rifampicin is mainly conferred by single point mutations resulting in amino acid substitutions in the center of the *rpoB* gene among Gram-negative (*E. coli*), Gram-positive (*S. aureus*, *B. subtilis* and *Mycobacterium*) (Campbell *et al.* 2001). It is not surprising that the mutations determining resistance are conserved across species since RNAP is highly conserved among eubacteria. The majority of the mutations are clustered within three distinct sites of *rpoB* gene: cluster I (covering amino acids 507 to 533), cluster II (amino acids 563 to 572), cluster III (amino acid 687 and 679) (*E. coli* numbering) (Jin and Gross 1988; Taniguchi *et al.* 1996; Tupin *et al.* 2010). These clusters were initially called the “rifampicin region”, but now are also known as the rifampicin resistance-determining regions (RRDR) (Campbell *et al.* 2001; Goldstein 2014). Mutations affecting residues 516, 526 and 531 in cluster I are particularly frequent among clinical rifampicin resistant isolates in many bacterial species and responsible for high-level of resistance.

Most mutations reported in *S. aureus* are covered by the ones in *E. coli* and *M. tuberculosis* (Morrow and Harmon 1979; Aubry-Damon *et al.* 1998; Wichelhaus *et al.* 1999; Wichelhaus *et*

al. 2002; O'Neill *et al.* 2006; Villar *et al.* 2011; Watanabe *et al.* 2011). For instance, substitution of the conserved H526 in *E. coli* corresponds to H481 in *S. aureus*.

Target point mutations conferring rifampicin resistance are widespread but there are other resistance mechanisms, such as duplication of the target gene *rpoB* (Vigliotta *et al.* 2005; Ishikawa *et al.* 2006), the action of RNAP-binding proteins (Newell *et al.* 2006), modification of rifampicin (Imai *et al.* 1999) and modification of cell permeability (Hui *et al.* 1977; Siddiqi *et al.* 2004).

The emergence of rifampicin resistance during therapy can generally be avoided with the use of sufficient combination therapy since resistant strains are rapidly observed when rifampin is applied independently (Strausbaugh *et al.* 1992).

5.4.2.3 Effects of *rpoB* mutations on VISA and rifampicin resistance

Notably, *rpoB* mutations do not only confer rifampicin resistance but also facilitate selection of vancomycin-intermediate *S. aureus* (VISA) (Matsuo *et al.* 2011; Gao *et al.* 2013; Saito *et al.* 2014). *rpoB* mutations are one of the major contributors to promote the hVISA-to-VISA phenotypic conversion. More than 70% of the VISA strains carry *rpoB* mutations (Matsuo *et al.* 2011; Watanabe *et al.* 2011; Alifano *et al.* 2015). *rpoB* (H481Y), one of the most frequent amino acid substitution in *S. aureus* mentioned above, is responsible for reduced vancomycin susceptibility. It has been shown that *rpoB* H481Y results in specific transcriptional effects including the up regulation of capsule production and *agr* (accessory gene regulator) gene expression, conversely with an attenuation of virulence and a reduced susceptibility to antimicrobial peptides and whole-blood killing, and leads to persistent infection (Gao *et al.* 2013). Among the transcriptional effects, the up regulation of *agr* gene expression is striking, because the *agr* quorum-sensing system controls i) metabolic operons involved in carbohydrate and amino acid metabolism, ii) a wide array of virulence genes in *S. aureus* such as phenol-soluble modulins (PSM) cytolysin genes that are regulated directly by the sRNA RNAIII (Queck *et al.* 2008; Alifano *et al.* 2015).

It is likely that the spontaneous occurrence of *rpoB* mutations plays dual functions on VISA and rifampicin resistance. The selection of hVISA with rifampin establishes rifampin-resistant *rpoB* mutant strains whose levels of vancomycin resistance are increased in various degrees, depending on the location of the mutations and the nature of amino acid substitutions (Matsuo *et al.* 2011). This implies that rifampin and vancomycin combination therapy of against MRSA

needs to be re-evaluated in view of the risk that rifampin promotes vancomycin-intermediate resistance (Matsuo *et al.* 2011).

Results

OUTLINES OF THESIS

S. aureus is a commensal and opportunistic pathogen, which is associated with various infections ranging from superficial infections to some life-threatening diseases. A notorious character of *S. aureus* is its possibility to be multidrug resistant, and therefore to contribute to high morbidity and mortality, a key challenge for clinicians and scientists. Bacterial regulatory RNAs of *S. aureus* have been studied for more than a decade and are now considered as an indispensable part of the complex genetic regulatory network. Hundreds of regulatory RNAs were detected thanks to the development of next generation sequencing (NGS) and bioinformatics. The main goal of this thesis is to identify regulatory RNAs that are related to antibiotic resistance in *S. aureus*.

- 1) The compilation of *S. aureus* regulatory RNAs studies suggests that they could be up to five hundreds. Of these, only a few are well identified and characterized. A difficulty in studying regulatory RNAs of *S. aureus* is to have an accurate annotation. Using published data and our own RNA-seq data, we revisited all putative sRNAs that could be expressed in *S. aureus* model strain HG003. We concluded that the number of *bona fide* sRNAs in *S. aureus* is well below than what is commonly stated (Chapter I).
- 2) We set up a methodology to determine phenotypes associated with *S. aureus* sRNAs genes by “competitive fitness experiments”. This strategy is used to evaluate the adaptive ability to various environmental conditions of sRNA gene mutants within a library of mutants. (Chapter II).
- 3) Three independent biological replicates of two sRNA mutant libraries were constructed using the strategy presented in Chapter II. The selection of sRNA gene mutants to disrupt was based on the *bona fide* sRNA list proposed in Chapter I. The sRNA mutant fitness was tested in twelve growth conditions (Chapter III).

1 Assessment of *Bona Fide* RNAs in *Staphylococcus aureus* (Chapter I)



Assessment of *Bona Fide* sRNAs in *Staphylococcus aureus*

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Bacterial regulatory RNAs have been extensively studied for over a decade, and are progressively being integrated into the complex genetic regulatory network. Transcriptomic arrays, recent deep-sequencing data and bioinformatics suggest that bacterial genomes produce hundreds of regulatory RNAs. However, while some have been authenticated, the existence of the others varies according to strains and growth conditions, and their detection fluctuates with the methodologies used for data acquisition and interpretation. For example, several small RNA (sRNA) candidates are now known to be parts of UTR transcripts. Accurate annotation of regulatory RNAs is a complex task essential for molecular and functional studies. We defined *bona fide* sRNAs as those that (i) likely act in *trans* and (ii) are not expressed from the opposite strand of a coding gene. Using published data and our own RNA-seq data, we reviewed hundreds of *Staphylococcus aureus* putative regulatory RNAs using the DETR'PROK computational pipeline and visual inspection of expression data, addressing the question of which transcriptional signals correspond to sRNAs. We conclude that the model strain HG003, a NCTC8325 derivative commonly used for *S. aureus* genetic regulation studies, has only about 50 *bona fide* sRNAs, indicating that these RNAs are less numerous than commonly stated. Among them, about half are associated to the *S. aureus* sp. core genome and a quarter are possibly expressed in other *Staphylococci*. We hypothesize on their features and regulation using bioinformatic approaches.

Keywords: *bona fide* sRNA, *Staphylococcus aureus* HG003, RNA-seq, transcription factors, gene regulation

INTRODUCTION

Bacterial regulatory RNAs are essential elements of complex genetic networks that tune gene expression according to growth conditions (Wagner and Romby, 2015). Most of them associate by base pairing to target sequences, and affect stability, structure and translation efficiency of target RNAs. Regulatory RNAs are divided into two categories, *cis*- and *trans*-acting RNAs. *Cis*-acting RNAs regulate expression of adjacent genes without reaching their substrate by diffusion (Mellin and Cossart, 2015). In contrast, *trans*-acting RNAs are expressed from loci not necessarily genetically linked to their target. RNAs and in some cases proteins are targets of *trans*-acting RNAs. When a *trans*-acting RNA is expressed from a complementary strand of another gene, it is often called antisense RNA (asRNA) (Georg and Hess, 2011); the predicted target of these asRNAs is the RNA transcribed from the complementary sequence. *Trans*-acting RNAs that are not asRNAs are often referred to as sRNAs because most of them are of small size. In bacteria, they are usually

50–300 nucleotides long, non-coding and conditionally expressed (i.e., depending upon specific stress and/or growth phase), although several sRNAs do not fit this description. Of interest, RNAIII, which is over 500 nucleotides long and encodes delta haemolysin, is an “exceptional” staphylococcal sRNA paradigm (Novick et al., 1993). This example alone underlines the difficulty of giving a straightforward definition of a *bona fide* sRNA.

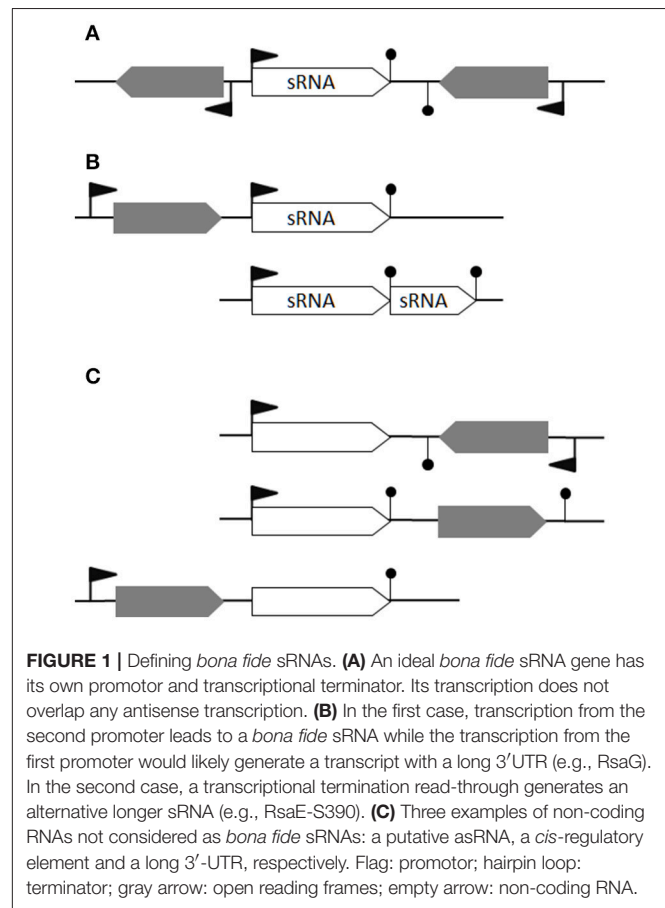
Since 2005, *S. aureus* non-coding RNAs have been searched by bioinformatics (Pichon and Felden, 2005; Geissmann et al., 2009; Marchais et al., 2009), DNA-arrays (Anderson et al., 2006; Roberts et al., 2006; Mäder et al., 2016), cDNA sequencing (Abu-Qatouseh et al., 2007, 2010), and RNA-seq methods (Beaume et al., 2010; Bohn et al., 2010; Howden et al., 2013; Broach et al., 2016; Carroll et al., 2016). The data are difficult to compare because of the different strains, growth conditions and experimental procedures used. In addition, many regulatory RNAs were renamed and in some cases, previously published work was overlooked. Data from different studies suggest that *S. aureus* may have hundreds sRNAs, but <10 have thus far been functionally characterized.

Despite recent releases of compilation and cross-comparison of available data in different *S. aureus* strains, (Felden et al., 2011; Sassi et al., 2015; Carroll et al., 2016; Mäder et al., 2016), it is still difficult to determine *bona fide* sRNAs from transcriptional background noise, asRNAs, and untranslated region (UTR) derived RNAs. We applied rigorous criteria to define sRNAs, and then used visual curation and bioinformatic approaches on compiled experimental data to assess *bona fide* sRNAs in *S. aureus*. *S. aureus* HG003 (Herbert et al., 2010), an NCTC8325 derivative, was used as the model strain to list *bona fide* sRNAs. Our main objectives were to identify sRNAs likely to act in *trans* and to clarify redundancies in the literature due to the use of different nomenclature. We then performed *in silico* analysis on these sRNAs to determine their phylogenetic conservation and to predict their putative regulators. The reassessment of the number of expressed sRNAs in *S. aureus* provided by this study may be applicable to other bacteria.

Bona Fide sRNA Definition

Bacterial genomes have complex organization with condensed information and flexible gene expression driven by multiple promoters with some internal to ORFs, operon organization, alternative premature termination, leader-less translation, and translational coupling (e.g., Mäder et al., 2016). The extent to which antisense RNA impacts gene expression in *S. aureus* is debatable, with reports of both high (Lasa et al., 2011) or more marginal (Mäder et al., 2016) effects. For these reasons, RNA boundaries are difficult to predict and may vary with strains and growth conditions.

We consider that a theoretical *bona fide* sRNA is (i) a gene not overlapping any other genes from the opposite strand, a definition excluding asRNAs, (ii) not a putative processed UTR and (iii) not a transcript derived from premature termination (i.e., riboswitch). It would therefore have its own promoter and a transcriptional terminator detected by computational predictions (Figure 1A), or interpreted as such because of clear expression



up- and down-shifts (Figure 1B). This restrictive definition excludes processed UTRs and short transcripts from premature transcription termination that could also act in *trans* (Loh et al., 2009); riboswitches and long UTRs are thus excluded as putative sRNAs (Figure 1C). Type I toxin-antitoxin systems comprising a small open reading frame post-transcriptionally controlled by an antisense RNA are also excluded; this concerns several *spr* genes located within pathogenicity islands (Pichon and Felden, 2005).

The first RNA-seq studies were performed with low read densities. Reads distant from coding sequences and not homologous to known non-coding RNAs (e.g., tRNAs, rRNAs, known UTRs) were first interpreted as putative *bona fide* sRNAs, and consequently, compiling results from different publications on *S. aureus* overestimated the number of sRNAs per bacterial strain. Indeed, recent high-density RNA-seq and tiling-array data reveal that many sequences previously considered as sRNAs are UTRs or premature termination products from longer transcripts.

Another consequence of sRNA identification with low read coverage was ambiguous identification of transcription start and termination sites. Even for well-studied sRNAs (e.g., RsaE), transcript boundaries differ according to studies, possibly because of strains, and growth conditions used for experiments. The recent high density transcriptome information is used here to define sRNA boundaries.

Staphylococcus aureus Regulatory RNA Data

Staphylococcus aureus genomes differ by the presence of variable elements (e.g., pathogenicity islands, SCCmec elements, prophages, transposable elements, insertion sequences, and plasmids). A recent study based on 64 *S. aureus* strains from different ecological niches reveals a core genome of 1,441 genes (not counting sRNAs genes, except tmRNA) and a pangenome of more than 7,400 genes, indicating a wide genetic diversity between strains (Bosi et al., 2016). Transcriptional patterns are influenced by these variable elements, which may affect virulence and antibiotic susceptibility. In addition, strain-specific elements have sRNA genes (Pichon and Felden, 2005) that can directly modulate core genome gene expression (Chabelskaya et al., 2010). sRNA expression has been experimentally studied by global approaches in different *S. aureus* strains, including NCTC8325 derivatives and methicillin resistant strains isolated in Japan, the USA and Europe (N315, USA300, MRSA252, respectively) (Felden et al., 2011; Sassi et al., 2015; Carroll et al., 2016; Mäder et al., 2016).

NCTC8325 (aka RN1) is a *S. aureus* strain isolated from a sepsis patient in 1960 widely used for genetic and physiological studies (Novick and Richmond, 1965). This strain is defective for two main regulators encoded by *rsbU* and *tcaR*: a positive activator of the general stress response regulator σ^B and a transcriptional activator of protein A-encoding gene, respectively. HG001, the strain used in the impressive transcriptional landscape study of 44 growth conditions published by Mäder et al. (2016), is a NCTC8325 derivative repaired solely for *rsbU*, whereas HG003, the strain we focus on in this analysis and that is now used as a model strain for *S. aureus* regulation studies (Herbert et al., 2010), is repaired for both *rsbU* and *tcaR* genes.

According to the compilation of data from several publications, *S. aureus* N315, NCTC8325, and Newman strains each could have over 500 putative regulatory RNAs (Sassi et al., 2015), the precise figure changing according to strains and sources (Carroll et al., 2016; Mäder et al., 2016). Most sRNAs were rediscovered in each independent analysis often under a different name. In order to compile an accurate list of *bona fide* sRNAs, we visually analyzed high-density coverage published data plus our own RNA-seq data (deep sequencing of pooled RNA extracts from cultures of HG003 strain grown in 16 different growth conditions; GEO GSE104971) as reported in Methods, and performed in-depth curation according to the rules defined in the previous chapter.

METHODS

RNA-seq for sRNA Detection

Experiments were performed with the *S. aureus* HG003 strain grown in different conditions: (i) eight samples in rich medium (BHI) at OD_{600nm} 0.6, 1.8, 3.3, 4.5, 7.2, 9.8, and 12.8, and stationary phase (24 h), (ii) seven samples under stress conditions (cold shock, heat shock, oxygen limitation, alkaline stress, oxidative stress, disulfide stress, iron-depleted condition and

(iii) one sample from colonies on BHI-agar plates (also see GSE10497 in GEO database). Total RNAs were extracted from these 16 growth conditions, pooled together and processed using the MICROBExpress kit (Ambion, AM1905) as recommended by the suppliers, to remove rRNAs. They were then sequenced using an Illumina Genome Analyzer Iix generating single-end 40-nt reads. After a FastQC (v0.10.1) quality control, reads from the stranded and single-end sequencing were mapped onto the reference genome (*S. aureus* subsp. aureus NCTC8325, CP000253.1 version) using Bowtie 2 with default parameters and an overlapping rate of 69%. DETR'PROK_2.1.2.sh pipeline was run to detect sRNAs (Toffano-Nioche et al., 2012, 2013); parameters are supplied as supplementary materials. The logarithm of the read coverage was computed for multiple or unique mapping reads on each strand; values were used to identify RNAs containing repeated regions (home-made shell scripts) and visualized with the Artemis genome viewer (Rutherford et al., 2000).

Literature and Experimental Data Integration

S. aureus global studies available in literature are summed up in Table S1. sRNA annotations (coordinates and strand) were collected from the following whole transcriptome analyses: 255 “indep” (transcripts with a promoter determined independently of annotated features) or “inter” (between two annotated regions transcribed from independent promoters) (Mäder et al., 2016), 286 sRNAs (Carroll et al., 2016), 352 NCTC8325 automatically annotated sRNAs (Sassi et al., 2015) using HG003 RNA-seq data (this work; GEO GSE104971), and 53 sRNAs (Beaume et al., 2010). These sRNA annotations were pooled together as a GFF file for the present expert analysis. sRNA expression profiles and reported annotations from different strains were compared with HG001 transcription profiles of *S. aureus* expression data browser (<http://genome.jouy.inra.fr/cgi-bin/aeb/index.py>). This manual expertise led us to draw up a *bona fide* sRNA list with their most probable positions as described in Tables 1, 2.

Transcription Factor Binding Sites in sRNA Promoter Regions

The 49 predictions of N315 Transcription Factors Binding Sites (TFBS) were downloaded (“reference regulons,” version 4.0, Fasta format) from the RegPrecise web site (Novichkov et al., 2013). Equivalences were searched for strain NCTC8325 as follows: When TFBS predictions are supported by only one promoter sequence in N315, we collected the predicted TFBS from other *Staphylococcus* species (using curl facilities of the RegPrecise web site). For each TFBS, a Position-Specific Scoring Matrix (PSSM) was computed with the MEME tool (Bailey and Elkan, 1994) using a background model built on the NCTC8325 genome sequence (fasta-get-markov in MEME suite, k-mer size of 3, -oops, -dna). The *S. aureus* NCTC8325 chromosome was scanned with the corresponding PSSM for each of the 49 TFBS with MAST (4.12.0) (Bailey and Gribskov, 1998). Only results on sRNA promoter regions (ranging from -100 nt to +50 nt from the 5' sRNA end) and with a statistical *E*-value < 0.01 were

TABLE 1 | Bona fide sRNAs expressed in HG003.

Name	Other names	Start	End	Strand	UCCC	Validations	Comments
srn_0335	Includes SAOUHSCs258, S35	115205	115614	-	+	NB ^{a,b}	Repeated region
RsaG	Teg93, sRNA31, srn_0510, SAOUHSCs054, S58	201738	201962	+	+	NB ^c RT ^c 5' ^c	Own promoter + read-through from SAOUHSC_00183
Sau-5971	srn_0880, SAOUHSCs073, S109	361904	362002	-	-	NB ^d	
srn_0890	sRNA71, SAOUHSCs205, SAOUHSC_A00354	367121	367211	-	-		Part of putative ORF SAOUHSC_A00354
Teg147	sRNA85, srn_0960, SAOUHSCs103	386294	386353	+	-		
srn_1505	SAOUHSCs189, S204	569615	569939	+	-	NB ^a	Own promoter and 3'UTR from SAOUHSC_00559
RsaA	Teg88, Sau-64, sRNA132, srn_1510, SAOUHSCs048, S210	575845	575987	+	+	NB ^{c,d} RT ^c 5' ^{c,e} 3' ^e	Sigma B regulation
RsaA _L	c.f. above + RsaA-Sau-76, srn_1520, SAOUHSCs164, S211	575845	576126	+	+	NB ^f	Sigma B regulation Processed into two sRNAs
RsaC	Teg90, sRNA135, srn_1590, SAOUHSCs050, S234	623360	624458	-	+	NB ^c 5' ^{3c}	Internal repeat. Own promoter and read-through from SAOUHSC_00634
RsaD	sRNA138, srn_1640, SAOUHSCs051, S243	639711	639872	-	+	NB ^c RT ^c	Antisense of putative SAOUHSC_00650 Antisense expression in some conditions
RsaH	Teg94, Sau-6059, sRNA162, srn_1910, SAOUHSCs055, S317	774294	774421	+	+	NB ^c RT ^c 5' 3' ^{c,e}	Antisense of SAOUHSC_00792 promoter
tmRNA	Teg150, ssrA, SAOUHSCs006, WAN014GIY, sRNA166, S329	788284	788675	+	+	NB ^g	Own promoter and read-through from SAOUHSC_00804
RsaE	Sau-20, Teg92, sRNA183, srn_2130, S389	911380	911481	+	+	NB ^c RT ^c 5' ^{c,e} 3' ^e	Own promoter and read-through from SAOUHSC_00937
RsaE-S390	srn_2130, S389 + S390, includes RsaF	911380	911739	+	+		Poor expression; long product from RsaE terminator read-through
sRNA195	sRNA195, srn_2320, SAOUHSCs226, S414	990586	990684	-	-		Possible antisense of SAOUHSC_01018 3'UTR
sRNA207	srn_2500, SAOUHSCs229	1078428	1078718	-	+		Internal repeat
Teg106	srn_2730, SAOUHSCs093, S540	1247774	1247925	+	+		Poor expression
Teg108	sRNA222, srn_2740, SAOUHSCs094	1248013	1248138	-	+		
srn_2975	SAOUHSCs275, S596	1362893	1363064	+	+	NB ^{a,b}	5' partly antisense of SAOUHSC_01422. Longer transcript with terminator read-through antisense of SAOUHSC_01423
S627	None	1462734	1462962	-	-		Own promoter and 3'UTR from SAOUHSC_01514. Repeated region. Antisense expression in some conditions
SprX2	Ssr6, RsaOR, Teg15, srn_3820.1, SAOUHSC_A01455	1464058	1464207	-	+	NB ^{e,h} 5' ^e	Repeated region; putative ORF SAOUHSC_A01455; Possibly associated with S629
S629	None	1464252	1464380	-	-		Possibly 5'UTR of SAOUHSC_A01455
6S RNA	Teg97, SsrS, Ssr80, WAN01CC8T, sRNA256, SAOUHSCs026, S685	1639003	1639243	-	-	NB ^g	Terminator read-through to SAOUHSC_01736
sRNA264	srn_3320, SAOUHSCs017, S706	1685428	1685667	-	-		Terminator read-through to SAOUHSC_01787
srn_3355	SAOUHSCs110, included in S713	1707679	1707781	-	-		
Sau-5949	Teg120, sRNA272, srn_3460, SAOUHSCs070	1771663	1771728	+	-	NB ^d	Possible antisense of SAOUHSC_01865 3'UTR
srn_3555	SAOUHSCs221	1821336	1821444	+	-		Repeated region
SprB	Teg9, srn_3600, SAOUHSCs030	1849001	1849117	-	-	NB ^{g,d}	Not detected in Mäder et al.
sRNA287	srn_9340, SAOUHSCs236, S774	1863800	1863899	-	-		Own promoter + possible terminator read-through from SAOUHSC_T00050

(Continued)

TABLE 1 | Continued

Name	Other names	Start	End	Strand	UCCC	Validations	Comments
srn_9345	S808	1923614	1923879	-	-	NB ^a	Own promoter + 3'UTR of SAOUHSC_02016
S810	None	1924486	1924611	-	-		Own promoter inside SAOUHSC_02019. Repeated regions.
Teg122	srn_3770, SAOUHSCs097	2027317	2027376	+	-		52 pb; in proximity of a putative type I TA system.
SprD	Teg14, sRNA300, srn_3800, SAOUHSCs032, S853	2033619	2033763	-	+	NB ^{g,d}	
Teg124	srn_3810	2033838	2033899	-	-		Not detected in Mäder et al.
SprX1	ssr6, RsaOR, Teg15, sRNA299, srn_3820, SAOUHSCs033, S854	2035228	2035378	-	+	NB ^{e,h} 5' ^e	Repeated regions; possible 5'UTR of SAOUHSC_02170
RNAIII	sRNA317, srn_3910, SAOUHSCs022, S871	2093158	2093673	-	+	NB ^{g,b,d} 5' ^{g,i}	SAOUHSC_02260 (<i>hld</i>) mRNA
RsaOG	RsaI, Teg24, sRNA356, srn_4390, SAOUHSCs047, S999	2377317	2377465	-	-	NB ^{b,c,j} RT ^{c,k} 5' ^c	Antisense expression in some conditions in Mäder et al. study.
Ssr42	RsaX28, Teg27, sRNA363, srn_4470, SAOUHSCs084, S1036	2446923	2448156	-	+		1252 pb; high constitutive transcription; terminator read-through antisense of SAOUHSC_02663
RsaX20	Teg128 + Teg130, srn_4520, SAOUHSCs100 (included), SAOUHSC_02702 + S1052	2484471	2484732	+	+		Contains putative ORF SAOUHSC_02702
Sau-19	Teg131, RsaX21, sRNA382, srn_4680, SAOUHSCs060	2556335	2556412	+	-	NB ^d	Not detected in Mäder et al.
Teg33	sRNA400, srn_5010, S1164	2721121	2721350	-	+		Own promoter; 3'UTR from SAOUHSC_02961; antisense of putative SAOUHSC_02960

sRNA names are given according to publication claim priority or most commonly used name. A list of previous analyses taken into account for this table is presented in Table S1. All sRNAs listed here were detected in at least 2 independent global expression studies performed in different strains. sRNA boundaries are given according to publications and GEO GSE104971 data. The presence of "UCCC" motif proposed by Geissmann et al. is indicated. NB: Northern blot; RT: RT-PCR; 5'-3': 5' 3' RACE.

^aMäder et al., 2016.

^bCarroll et al., 2016.

^cGeissmann et al., 2009.

^dAbu-Qatouseh et al., 2010.

^eBohn et al., 2010.

^fLiolou et al., 2012.

^gPichon and Felden, 2005.

^hEyraud et al., 2014.

ⁱNovick et al., 1993.

^jMarchais et al., 2010.

^kBeaume et al., 2010.

conserved in order to report only the most probable predictions. However, this high stringency may discard effective TFBSs.

sRNA Coregulation and Identification of Putative sRNA Targets

Genes coregulated with the sRNAs from Table 3 were identified using the web browser from Mäder et al. showing *S. aureus* expression data (Mäder et al., 2016). Relevant pages are indicated in Table S3. The RNApredator website (Eggenhofer et al., 2011) was used to predict sRNA-mRNA interactions between the sRNAs from Table 3 and the NCTC8325 genome (accession # NC_007795). In the absence of conservation data, RNAPlex program used by RNA predator is among the best predictor (Pain et al., 2015). Results are presented in Data Sheet 2.

sRNA Conservation

sRNA sequence similarities were searched against a nucleotide database (see Table S2 for the list of strains). Complete genomic

sequences were downloaded from the NCBI database. Similarity search parameters (blastall 2.2.26) were defined to report a maximum of hits (-e 1000 -W7) with specific scoring criteria (-r2 -G5 -E2) designed for sRNA identification (Ott et al., 2012). For each genome, only the blast hit with the best score was kept and divided by the score obtained in *S. aureus* NCTC8325. The resulting score ratios are represented by a color scale: the more the sequence of the hit is similar to the sRNA sequence, the darker the pixel is (R script). A 50% similarity ratio threshold was applied to define conserved sRNA genes.

RESULTS AND HYPOTHESIS

HG003 Bona Fide sRNAs

Based on a computational analysis of our HG003 RNA-seq data (GEO GSE104971), 88 UTRs, 22 antisense RNAs, 24 CDSs, 11 T-boxes, and riboswitches, and 3 toxin-antitoxin systems were annotated among the 527 putative regulatory RNAs found and

TABLE 2 | *Bona fide* sRNAs in HG003 with poor expression in the tested conditions of RNA-seq (this study) and tiling arrays (Mäder et al., 2016) datasets.

Name	Other names	Start	End	Strand	UCCC	Validations	Comments
Sau-27	srn_2690	1219192	1219282	+	-	NB ^d	No signal
Sau-85	srn_2760, SAOUHSCs165 (but longer)	1252254	1252305	+	-		Poor expression
RsaB	srn_3410, SAOUHSCs049, SAOUHSC_01844	1750160	1750216	+	+	RT ^c 5'3' ^c	Poor expression; possibly 3'UTR of SAOUHSC_01844
sRNA334	srn_9480, SAOUHSCs242	2214760	2214889	+	-		Poor expression
sRNA390	srn_9510, SAOUHSCs250	2629688	2629848	+	-		Poor expression

For explanations, see **Table 1** legend.

TABLE 3 | List of transcription factor motifs found by MAST analysis (E -value < 0.01) in the putative promoter region (-100 to +50 nts from transcription start sites) of *bona fide* sRNAs.

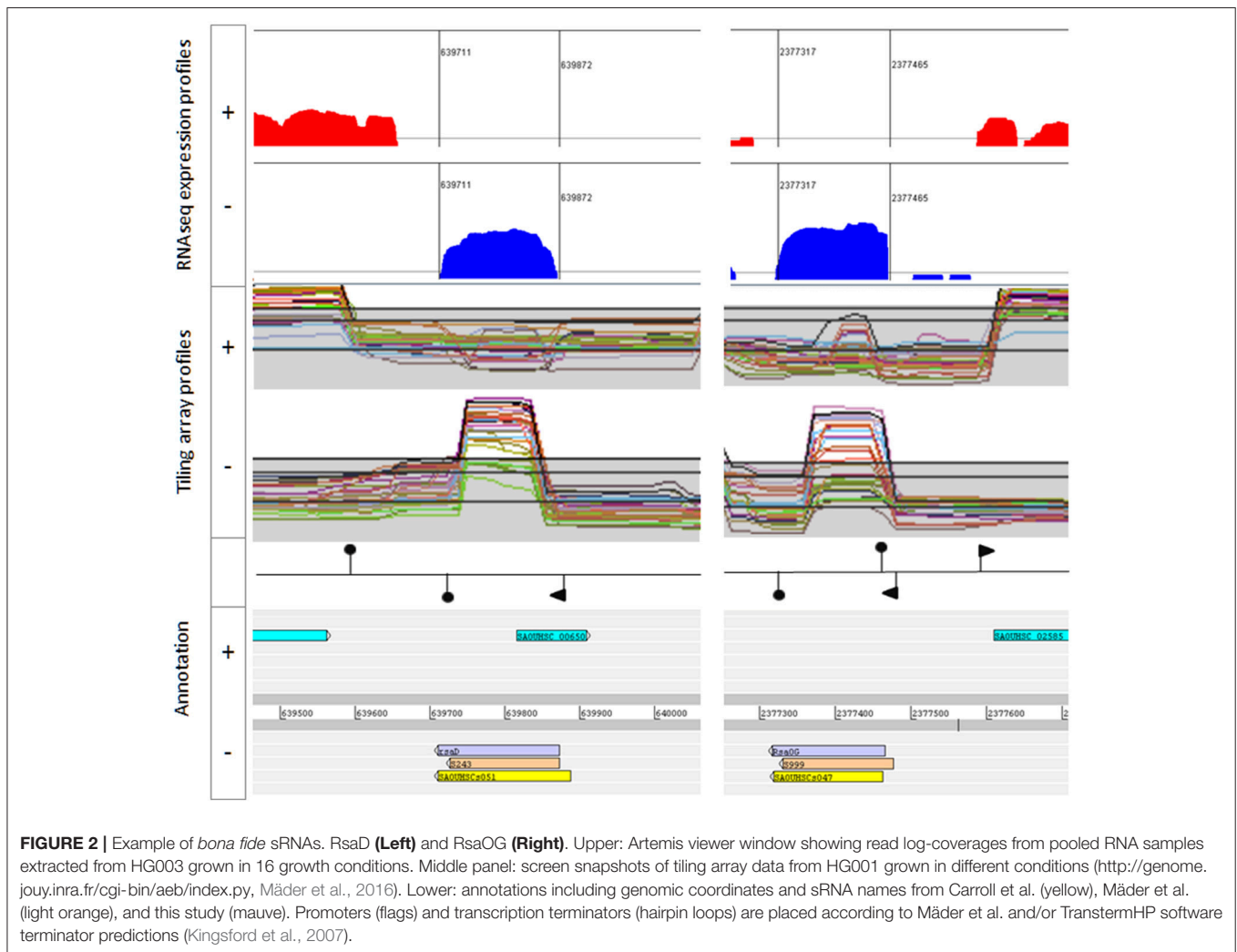
sRNA	TF	Effector(s)	TF function	References
RNAIII	AgrA*	Cell density (AIP)	Regulation of quorum sensing	Mäder et al., 2016; Novick and Geisinger, 2008; Yarwood et al., 2001
	BirA	Biotin	Biotin metabolism	
	SrrA*	NO, anaerobiosis	Anaerobic switch	
RsaB	Fur	Fe ²⁺	Iron homeostasis	This study
RsaD	CodY	Branched-chain amino acids	Amino acid metabolism	Mäder et al., 2016
RsaE	SrrA*	NO, anaerobiosis	Anaerobic switch	Durand et al., 2015, 2017
	Rex*	NAD	Anaerobic metabolism	
RsaOG	CcpA	HPr, phosphocarrier protein; Fructose-1,6-diphosphate	Carbon catabolism	Mäder et al., 2016
RsaX20	Zur	Zn ²⁺	Zinc homeostasis	Mäder et al., 2016
Sau-19	ArcR	Arginine	Arginine metabolism	This study
	Rex	NAD	Anaerobic metabolism	This study
srn_2975	Fur	Fe ²⁺	Iron homeostasis,	Mäder et al., 2016
	NanR	N-acetylmannosamine-6-P	Sialic acid catabolism	Mäder et al., 2016
sRNA207	BirA	Biotin	Biotin metabolism	This study
sRNA287	SarA*		Pathogenesis regulation	Mauro et al., 2016

Motifs were defined as given by Regprecise (Novichkov et al., 2013) for *S. aureus* N315 strain. We also search for GraRS, WalkR, and SrrA putative regulations using reported motifs (Sterba et al., 2003; Hartig and Jahn, 2012; Nicolas et al., 2012; Mäder et al., 2016; Durand et al., 2017); *corresponds to proposed or validated regulations reported elsewhere (discussed in the text) corresponding to motifs that did not pass the stringent E -value chosen or that are upstream of sequences selected for analysis. No DNA binding site was found for GraRS and WalkR.

indexed in the SRD database (Sassi et al., 2015). According to the definition given above, we considered a restricted list of 352 putative sRNA candidates to which we adjoined those of Carroll et al.'s and Mäder et al.'s sRNA lists. A gene-finding format (GFF) file including these putative sRNAs was generated (Data Sheet 1) and visually analyzed and compared to HG003 RNA-seq profiles using Artemis genome browser (Rutherford et al., 2000). In addition, HG001 tiling array profiles were scrutinized for each putative sRNA using the *S. aureus* expression data browser (<http://genome.jouy.inra.fr/cgi-bin/aeb/index.py>). From these inspections, we applied the *bona fide* criteria to compile a curated list of 41 *bona fide* sRNAs expressed in at least one biological condition in the HG003 strain (**Table 1**). We also added 5 *bona fide* sRNAs described in other strains but poorly expressed in HG003 and HG001 in the tested conditions. For instance, no expression was detected for Sau-27 in our HG003 RNA-seq or in HG001 tiling arrays data. As conditions might exist in which these sRNAs are expressed, we retained them in a separate table (**Table 2**).

Most of the rejected sRNAs were found to be part of UTRs, or displayed a strong antisense-transcription signal. We discarded from the *bona fide* sRNA list, most RNAs with antisense expression and those likely part of type I toxin-anti-toxin systems [e.g., Teg13, RsaOI, srn_2335, SprC and S929 (Figure S1)]. However, we retained sRNA genes transcribed in antisense of putative small ORFs with no reported expression [e.g., Teg33, S596 and RsaD (**Figure 2**)]. Either the peptide does not exist or the antisense decay activity on the mRNA is efficient and completely turns off peptide expression.

Many short transcripts may encode small ORFs (sORF); however, for most of them, their expression is not confirmed. To avoid considering sORF genes as *bona fide* sRNA genes, we discarded those with either high conservation or with a hydrophobic domain. Four sRNAs (srn_0890, SprX2, RsaB and RsaX20) with putative sORFs were retained as their translation was uncertain. We also kept RNAIII expressing the delta hemolysin as its main function and structural part are associated with *trans*-acting regulation (Novick et al., 1993). Small peptides



are also predicted by the microbial gene annotation platform MicroScope (Vallenet et al., 2017) for RsaA, Sau-76, 6S RNA, and sRNA264. Moreover, a ribosome profiling study suggests new ORFs corresponding to sRNA genes (e.g., RsaA_L, SprB, 6S RNA, and tmRNA) (Davis et al., 2014); however, a ribosome binding on RNAs is not sufficient to confirm protein expression. In the absence of further biological validation and because sRNAs can have a regulatory activity both through RNA targeting and *via* the expression of small peptides, we retained all of them but their status may change in the future.

As the number and the depth of deep-sequencing analyses increase, separated adjacent sRNA transcription units can be merged. Here, we consider that Teg128 and Teg130 likely do not exist *per se* and annotation should be merged to correspond to RsaX20 (Figure S2). In another example, RsaA and Sau-76 share the same promoter, and RNase III-dependent processing generates shorter transcripts (Lioliou et al., 2012); in Table 1, we considered, as previously published, the two transcriptional entities, the short transcript RsaA, and the longer form RsaA_L (Figure S3).

The transcriptional study of HG001 in multiple growth conditions indicates a transcript named S390 downstream of the *rsaE* transcriptional terminator (Mäder et al., 2016). S390 has a putative terminator but no obvious promoter. Its expression is low compared to that of *rsaE*, possibly suggesting that S390 may result from a transcriptional terminator read-through of RsaE. Weak conservation of S390 beyond *S. aureus*, as opposed to high conservation of RsaE, questions its functional importance. RsaF is a 105 nucleotide sRNA. *rsaF* transcription was proposed to initiate from a promoter embedded in the *rsaE* gene, with expression resulting from transcriptional terminator read-through (Geissmann et al., 2009). As RsaF and its promoter were not detected in the transcriptome databases, we chose to consider just two transcripts, RsaE and the RsaE/S390 fusion.

Also, many previously reported sRNAs are now known to be part of UTRs. One example is Teg49: initially characterized as a *bona fide* sRNA (Beaume et al., 2010), it is also within the 5'UTR of *sarA* mRNA, yet Teg49 plays a *trans*-acting role by modulating *sarA* expression (Kim et al., 2014; Manna et al., 2017). For two recently proposed sRNAs, S1077 (Figure S4) and S736,

which have their own terminators, authors showed that they are both part of longer transcripts that extend downstream of their terminators (Mäder et al., 2016) and are probably *cis*-acting elements. Alternatively, transcriptional terminator read-through from sRNA genes could generate longer regulatory RNAs.

The 4.5S RNA, which is the RNA component of the signal recognition particle ribonucleoprotein complex and is not a regulatory RNA was removed from the *bona fide* sRNA list. Two other sRNAs that interact with proteins, 6S RNA and tmRNA, were kept in the sRNA list as they may have regulatory functions (Makhlin et al., 2007; Cavanagh and Wassarman, 2014).

Our RNA-seq transcriptome data are similar to those produced by tiling arrays and presented in the *S. aureus* expression data browser (<http://genome.jouy.inra.fr/cgi-bin/aeb/index.py>). Indeed, many *bona fide* sRNAs listed in **Tables 1, 2** are independently detected using these two methodologies. Our transcriptome analysis contains 27 *bona fide* sRNAs not annotated as such in the Mäder et al. study, although reported elsewhere. Six are located in repeat regions not evaluated by the tiling array method (e.g., S627 and S629 Figure S5). Others have either no expression or an expression level that does not fulfill the cut-off selection imposed by the authors (Figure S6). The slight expression differences observed [namely for, *srn_0890*, T_{eg}147, T_{eg}108 (Figure S6), Sau-85, RsaB, Sau-5949, SprB, T_{eg}122, T_{eg}124, sRNA334, Sau-19 (Figure S6), and sRNA390] could be due to allelic variation of the *tcaR* regulator between the two sister strains HG001 and HG003 or to specific expression of sRNAs in at least one of the conditions tested only in our dataset (e.g., heat shock).

sRNA Features

S. aureus is a low guanine-cytosine (33% GC) content member among Firmicutes. Local variation within the genome of this percentage may reflect DNA acquisition by horizontal transfer (Garcia-Vallve et al., 2000). This could be the case for T_{eg}122, tmRNA, T_{eg}147, *srn_9345* and 6S RNA, whose GC content is above 40%. However, for tmRNA and 6S RNA, the composition is likely constrained by their interaction with proteins.

Base-pair associations between RNA molecules initiate with unpaired nucleotides; the pairing may then extend beyond these seed motifs. Using the MEME suite (Bailey et al., 2009), we searched for over-represented motifs within the 46 selected sRNAs, which may serve as seed of sRNA/RNA interactions. A conserved C-rich motif (UCCC) in unpaired regions was reported for several *S. aureus* non-coding RNAs (Geissmann et al., 2009). Impressively, this motif is present in 48% of HG003 *bona fide* sRNAs, often in multi-copy (from 1 to 5 motifs in HG003 RsaC; **Tables 1, 2**). sRNAs lacking this motif are often of small size. A stretch of C and/or G is possibly an efficient discriminating element since *S. aureus* is only 33% GC. As suggested by the authors, it also may indicate that sRNAs with GC-rich unpaired patches may share a mode of action (Geissmann et al., 2009). We have also looked for an alternative motif in sRNAs not featuring UCCC but found none, suggesting that each of these sRNAs would find their target with specific sequences.

HG003 sRNA Conservation

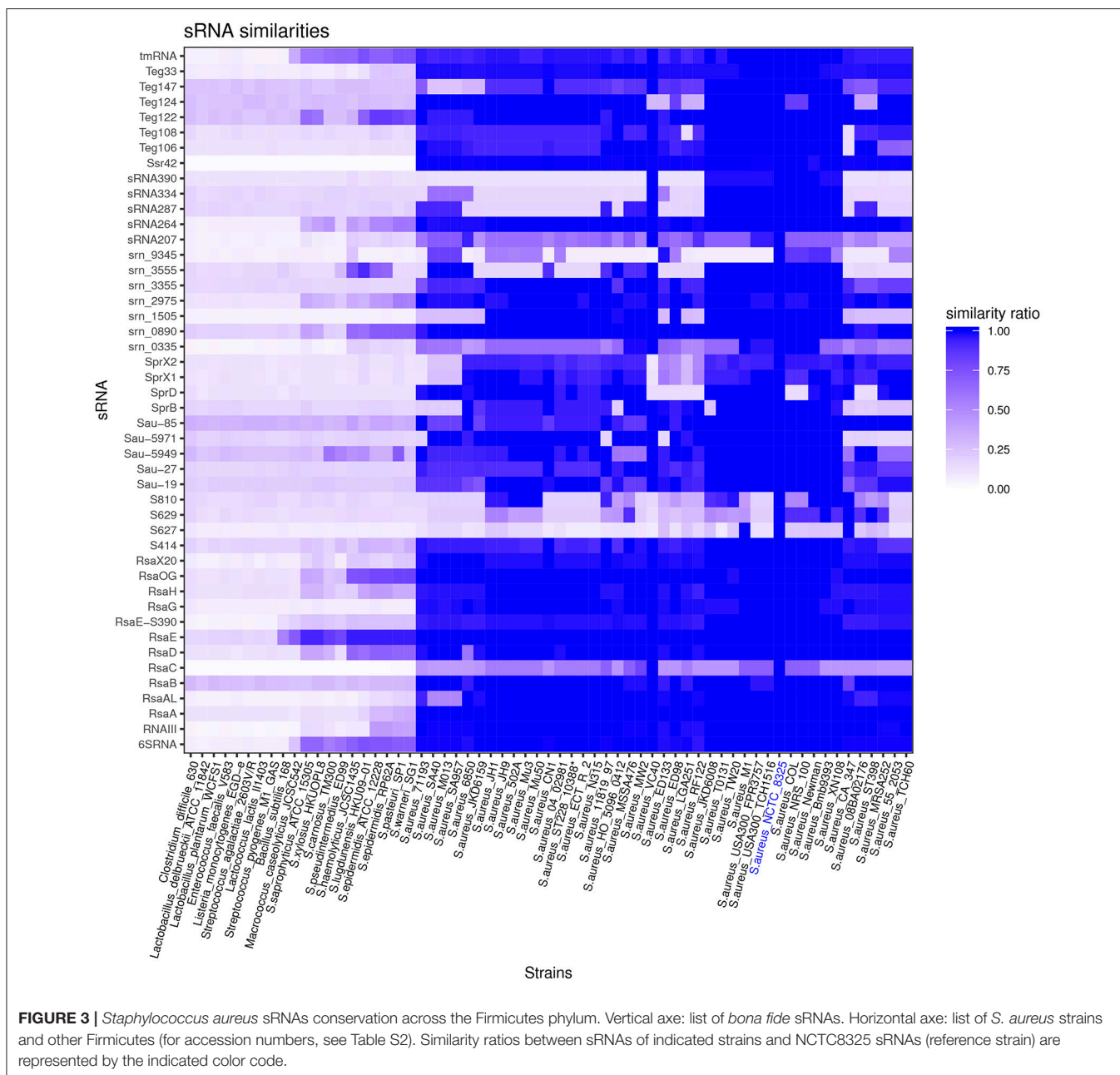
Among 46 HG003 *bona fide* sRNA genes, 54% are conserved in all tested *S. aureus* strains (**Figure 3**) and may be part of the core genome. 24% of the 46 *bona fide* sRNA genes are conserved among other species of the *Staphylococcaceae* family. However, most HG003 *bona fide* sRNAs are species specific. sRNA genes present on pathogenicity islands such as *sprX1*, *sprX2*, and *sprD* are *de facto* present solely in strains bearing these elements. S629, S810, and *srn_9345* genes are poorly conserved among the 43 *S. aureus* strains included in the analysis, and S627 was found in only three of these strains, M1, CA347 and NCTC8325. Of note, *srn_3555*, while absent in many *aureus* strains, is conserved in non-*aureus* staphylococci such as *S. lugdunensis*, *S. haemolyticus*, and *S. epidermidis* suggesting its acquisition by horizontal transfer. The phylogenetic study suggests *rsaC* is poorly conserved in *S. aureus*. However, its conservation is probably underestimated due to the presence of repeat sequences, whose number varies according to strains (Figure S7).

Transfer-messenger RNA (tmRNA) and 6S RNA, which interact with SmpB protein and RNA polymerase, respectively, are widely conserved in bacteria. Apart from these sRNAs, RsaE is the only HG003 *bona fide* sRNA conserved in distantly-related Firmicutes. It differs from *Bacillus subtilis* RsaE almost exclusively by its terminator region. This unusual conservation reveals an unexpected selective pressure to preserve RsaE sequence integrity; we hypothesize that in addition to its numerous mRNA targets (Geissmann et al., 2009; Bohn et al., 2010), RsaE may interact with a protein constraining the RNA sequence to ensure its regulatory activity.

HG003 sRNA Transcriptional Regulation

Transcription in *S. aureus* depends on four sigma (σ) factors: σ^A , the primary σ factor responsible for the transcription of most genes, σ^B , involved in the general stress response, σ^H , implicated in the competence state but cryptic in NCTC8325 (Morikawa et al., 2012), and σ^S , the extracytoplasmic function sigma factor (Burda et al., 2014).

In *S. aureus*, the σ^B regulon comprises about 249 coding genes expressed from 145 promoters (Bischoff et al., 2004; Mäder et al., 2016). The σ^B consensus recognition site was used to find small σ^B regulated genes called *sbr* (for σ^B -dependent small RNA) (Nielsen et al., 2011). Three were found in several strains including SH1000, a NCTC8325 σ^B+ derivative. However, *sbrA* and *sbrB* encode putative small basic peptides and are not regulatory RNA genes. The 3' end of *sbrC* overlaps with the 3' end of *mntC*, that codes a metal binding lipoprotein, and their corresponding RNAs interact *in vitro*, indicating that SbrC should be categorized as an asRNA. σ^B -mediated regulation has been proposed for RsaA, RsaF and RsaD, as their expression was enhanced in σ^B proficient strains, and a characteristic σ^B promoter was found upstream of *rsaA* (Geissmann et al., 2009). However, so far, σ^B regulation was confirmed only for *rsaA* and its derivative *rsaAL* (including *sau-76*) (Mäder et al., 2016). No σ^B promoter was found upstream of *rsaD* despite activation of the σ^B regulon by several growth conditions (Mäder et al., 2016). Hence, only one out of 46 *bona fide* sRNAs, *rsaA*, appears to be transcribed by σ^B . Remarkably, while often associated



with adaptation and stress responses, almost all sRNAs have σ^A promoters. Since they are usually modulated by specific growth conditions, their expression likely relies on additional regulatory factors.

Transcription factors (TFs) bind specific DNA sites that can be detected by biocomputing tools when consensus sequences are already described. We performed such analyses for the *bona fide* sRNAs using MAST (Bailey et al., 2009) and predicted TF regulation for 8 sRNA genes (Table 3). The putative regulatory targets, and those previously reported, are discussed below.

RNAIII activates virulence genes either directly or indirectly at high *S. aureus* cell density. It is positively regulated by the quorum sensing regulator AgrA (Novick and Geisinger,

2008). AgrA also activates its own operon and *psm* (phenol-soluble modulins) genes that encode toxins (Queck et al., 2008), sometimes inadvertently annotated as sRNA genes. A putative BirA binding motif is detected upstream of the RNAIII gene (Mäder et al., 2016). BirA is a biotin-dependent repressor that downregulates genes implicated in biotin synthesis and transport (Henke and Cronan, 2016). In addition, RNAIII is reportedly repressed by SrrAB, a two-component system involved in aerobic to anaerobic adaptation and energy metabolism similar to *B. subtilis* ResDE (Yarwood et al., 2001). SrrAB-dependent RNAIII repression may result from a direct interaction of SrrA with the *agr* P3 promoter (Pragman et al., 2004).

SrrAB has an opposite effect on RsaE expression compared to that on RNAlII. The absence of SrrAB results in a drastic reduction of RsaE and an SrrA binding motif is detected 125 nucleotides upstream *rsaE* transcriptional start site (Durand et al., 2015). In *B. subtilis*, expression of *roxS*, the *rsaE* ortholog, is submitted to a double regulation by the activator ResDE, the SrrAB functional homolog, and the redox sensing repressor of anaerobic metabolism Rex (Pagels et al., 2010; Durand et al., 2017). As an identical Rex binding motif is also present within the RsaE promoter region, this double regulation is likely conserved for *rsaE* in *S. aureus*. RNAlII and RsaE would both exert a role in response to impaired respiration and indeed, in *B. subtilis* the absence of RoxS, results in the modulation of genes related to redox homeostasis (Durand et al., 2015).

In anaerobiosis, ArcR, a Crp/Fnr family transcriptional activator, stimulates arginine utilization as an energy source (Makhlin et al., 2007). We found that *sau-19*, an sRNA gene poorly expressed in conditions thus far tested, has ArcR and Rex binding motifs; these motifs resemble each other and concern the same sequence. Full activation of *Sau-19* may need growth conditions in which Rex is inactive and ArcR is active, as observed for the arginine deiminase pathway (Makhlin et al., 2007).

S. aureus adapts to nutrient shifts with dedicated TFs. CcpA is a master regulator of carbon utilization in Gram-positive bacteria (Halsey et al., 2017). It binds to catabolite-response elements (*cre*) DNA sequences, and may act as an activator or a repressor. A *cre* box is detected within the promoter region of *rsaOG* (alias *rsal*). RsaOG regulation by CcpA is supported by its coregulation with other CcpA regulated genes such as *lip*, *putA*, *fadXEDB*, and *rocA* (Mäder et al., 2016) (Table S3). This sRNA with a predicted pseudoknot (Marchais et al., 2010) is strongly modulated by growth conditions, and is increased in oxidative stress, during stationary phase and in human serum (Howden et al., 2013; Carroll et al., 2016). Fructose-1,6-bisphosphate is an allosteric effector of CcpA function (Schumacher et al., 2007). Interestingly, in addition to sugar transporters (i.e., SAOUHSC_02520, SAOUHSC_02815), the fructose-1,6-bisphosphate aldolase (SAOUHSC_02926) is a putative RsaOG target (Data Sheet 2), which in turn may contribute to CcpA regulation.

In *S. aureus*, CodY is a pleiotropic regulator affecting expression of numerous metabolic and virulence genes in response to branched amino acid and GTP availability (Geiger and Wolz, 2014; Waters et al., 2016). The presence of a CodY box in the promoter region of *rsaD* suggests that this sRNA belongs to its regulon. This proposal is strongly supported by the observation that *rsaD* is expressed in the same condition as CodY-regulated genes such as SAOUHSC_00962, *mtnE-dhh*, and *oppBCDFA* (Mäder et al., 2016) (Table S3).

Iron starvation is known to limit bacterial development during infection, but at the same time, an excess of iron generates deleterious reactive oxygen radicals. Consequently, intracellular iron homeostasis is tightly controlled and in many bacteria, the iron-sensing regulator Fur is involved. RhyB is an important Fur-regulated sRNA conserved in many Gram-negative bacteria that represses numerous genes and contributes to virulence

(Oglesby-Sherrouse and Murphy, 2013). Iron-responsive sRNAs with a similar function are also present in Gram-positive bacteria (e.g., Gaballa et al., 2008). From the HG003 *bona fide* sRNA list, Fur boxes were detected in front of *rsaB* and *srn_2975* (S596), suggesting their implication in iron homeostasis. Regulation of *srn_2975* by iron is supported by (i) its co-expression with *isd* and *sbh* genes related to heme/hemin and iron uptake and utilization, respectively and (ii) predicted targets that are related to iron metabolism (Mäder et al., 2016). *Srn_2975* would be the *S. aureus* functional ortholog of RhyB. Two NanR binding motifs are also found upstream of *srn_2975*. NanR is a repressor controlling sialic acid (N-acetylneuraminic acid) catabolism enzymes that may play an important role during growth in the host (Olson et al., 2013). Like iron, the metal ion zinc is essential. Zur, a zinc-sensing Fur-like protein (Lindsay and Foster, 2001) regulates zinc intracellular concentration. One sRNA gene, *rsaX20*, is preceded by a Zur binding motif, and interestingly RsaX20 is co-expressed with genes from the Zur regulon (Mäder et al., 2016) (Table S3). Consequently, RsaX20 is possibly associated with metal homeostasis.

sRNA regulation can be directly linked to virulence and pathogenicity factors. Besides the Agr system, as an example, SarA is a transcriptional factor belonging to the core genome, which is implicated in infectivity and biofilm formation. SarA represses sRNA287 and SprC, two sRNAs located on the same pathogenicity island (Mauro et al., 2016).

The sRNA regulators discussed here are associated with quorum sensing, aerobic to anaerobic transition, carbon source availability, metal metabolism or infectivity. All these processes crucial for virulence and survival within the host indicate that functional studies of *S. aureus* sRNAs are essential for understanding the global regulatory network governing bacterial pathogenicity.

HG003 sRNA Transcriptional Termination

Bacterial transcription terminates either at secondary structures formed by nascent RNAs (intrinsic termination) (Ray-Soni et al., 2016) or *via* the activity of a termination factor such as Rho (Grylak-Mielnicka et al., 2016). Notably, while essential in several bacteria including *Escherichia coli*, Rho is dispensable in *S. aureus* (Washburn et al., 2001). Transcriptome data of HG001 *rho* in three different conditions is available (Mäder et al., 2016). Most intrinsic terminators are detected by bio-computing analysis. The presence of terminators within intergenic regions was initially used as an indication of the existence of sRNA genes (Wassarman et al., 2001) with the general belief that sRNA genes have Rho-independent terminators. Using TransTermHP with default parameters (<http://transterm.cbcb.umd.edu>) (Kingsford et al., 2007), intrinsic terminators were detected for 38 sRNAs among the 46 retained for HG003. By analyzing HG001 *rho* transcriptomic data for the nine sRNAs with no detected intrinsic terminator (Mäder et al., 2016), we conclude that they had no apparent Rho-dependent termination.

Several sRNAs have their own promoter but are also expressed because of a terminator read-through from upstream gene resulting in a longer RNA. In several cases, the expression of the sRNA gene and its upstream gene (or operon) is remarkably

co-regulated (e.g., *rsaG*, *srn_1505*) suggesting that both genes are associated with the same function. In this case, the sRNA promoter would be present to boost sRNA expression.

CONCLUSION

Most reported small transcripts correspond to UTRs and asRNAs. Our curated analysis led to a number of *bona fide* sRNAs in *S. aureus* that is smaller than what would be expected from the compilation of all sRNA studies. Even among this restricted list, the regulation, targets and functions of these sRNAs are still mostly unknown. Studying *bona fide* sRNA genes present the advantage that their deletion, in principal, has no polar effect on adjacent genes, thus facilitating genetic approaches to search for phenotypes (Le Lam et al., 2017). Putative proposed sRNA regulators (Table 3) are starting points to elucidate their function. Indeed, sRNAs often act as effectors of the transcription factors controlling their expression. They are the polishing regulators that would fine tune genetic regulation and refine bacterial adaptability. Described sRNAs are mostly negative regulators and often act as invertors of regulatory responses: induction of an sRNA by a given activator may lead to gene down-regulation. The same reasoning applies conversely for a repressed sRNA. For the above-discussed regulators, exploring genes repressed by inactivation of a repressor or induced by the absence of an activator is a good hint to discover sRNA-targets.

The number of *bona fide* sRNAs is lower than initially proposed, although we expect that new candidates will be added to this group. Publications based on high-throughput sequencing data indicate dense transcription with numerous so far uncharacterized transcripts that are putative regulatory elements. This pervasive transcription is hidden and probably not selected in a natural environment; and mutations such as *rnc* and *rho* are required to unmask it (Lasa et al., 2011; Lioliou et al., 2012; Mäder et al., 2016). Active *S. aureus* RNA processing generates numerous alternative RNA species (Lioliou et al., 2013; Bonnin and Boulouc, 2015) and many transcripts have long UTRs with

a regulatory role demonstrated only in a few cases (e.g., de Los Mozos et al., 2013; Boulouc and Repoila, 2016). It is likely that besides *bona fide* sRNAs, *S. aureus* has a plethora of RNA-based regulations nesting within these non-translated RNAs.

AUTHOR CONTRIBUTIONS

TR, PB, and CM: Conception, design of the work. WL, TR, and TL: Data acquisition. CT-N, PB, and CM: Data analysis. TR, PB, and CM: Drafting of the work and revision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2018.00228/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Data

Assessment of *bona fide* sRNAs in *Staphylococcus aureus*

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Supplementary Data

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Table S1: *S. aureus* sRNA global studies

Strain	Main data type	sRNA number	Reference
Mu50	<i>in silico</i>	191	(Pichon and Felden, 2005)
UAMS-1 MSSA from osteomyelitis	Affymetrix microarrays	126	(Anderson et al., 2006)
RN6390	<i>in silico</i>	110	(Geissmann et al., 2009)
N315	<i>in silico</i>	250	(Marchais et al., 2009)
Clinical isolates: A3878I A3878III	Cloning and sequencing	142	(Abu-Qatouseh et al., 2010)
N315	RNA-seq	30	(Bohn et al., 2010)
N315	RNA-seq	195	(Beaume et al., 2010)
JKD6008 ; JKD6009	RNA-seq	409	(Howden et al., 2013)
N315 ; Newman ; 18 <i>S. aureus</i> + 10 other Staphylococci	RNA-seq + <i>in silico</i>	575	(Sassi et al., 2015)
NCTC8325 ; USA300 ; MRSA252	RNA-seq	~300	(Carroll et al., 2016)
HG001	Tiling array	~300	(Mader et al., 2016)
HG003	RNA-seq	501	This study

Table S2: Strains used to study the sRNA conservation across the Firmicute phylum

Genebank	Strain	Genebank	Strain
CP001844.2	<i>S.aureus</i> _04_02981	AL009126.3	<i>Bacillus_subtilis</i> _168
CP003808.1	<i>S.aureus</i> _08BA02176	AM180355.1	<i>Clostridium_difficile</i> _630
CP003194.1	<i>S.aureus</i> _11819_97	AE016830.1	<i>Enterococcus_faecalis</i> _V583
CP007454.1	<i>S.aureus</i> _502A	CR954253.1	<i>Lactobacillus_delbrueckii</i> _ATCC_11842
CP002388.1	<i>S.aureus</i> _55_2053	AL935263.2	<i>Lactobacillus_plantarum</i> _WCFS1
CP006706.1	<i>S.aureus</i> _6850	AE005176.1	<i>Lactococcus_lactis</i> _II1403
CP003045.1	<i>S.aureus</i> _71193	AL591824.1	<i>Listeria_monocytogenes</i> _EGD-e
CP005288.1	<i>S.aureus</i> _Bmb9393	AP009484.1	<i>Macrococcus_caseolyticus</i> _JCSC542
CP006044.1	<i>S.aureus</i> _CA_347	AM295250.1	<i>S.carnosus</i> _TM300
CP003979.1	<i>S.aureus</i> _CN1	AE015929.1	<i>S.epidermidis</i> _ATCC_12228
CP000046.1	<i>S.aureus</i> _COL	CP000029.1	<i>S.epidermidis</i> _RP62A
FR714927.1	<i>S.aureus</i> _ECT_R_2	AP006716.1	<i>S.haemolyticus</i> _JCSC1435
CP001996.1	<i>S.aureus</i> _ED133	CP001837.1	<i>S.lugdunensis</i> _HKU09-01
CP001781.1	<i>S.aureus</i> _ED98	CP004014.1	<i>S.pasteuri</i> _SP1
HE681097.1	<i>S.aureus</i> _HO_5096_0412	CP002478.1	<i>S.pseudintermedius</i> _ED99
CP000736.1	<i>S.aureus</i> _JH1	AP008934.1	<i>S.saprophyticus</i> _ATCC_15305
CP000703.1	<i>S.aureus</i> _JH9	AE009948.1	<i>Streptococcus_agalactiae</i> _2603V/R
CP002120.1	<i>S.aureus</i> _JKD6008	AE004092.2	<i>Streptococcus_pyogenes</i> _M1_GAS
CP002114.2	<i>S.aureus</i> _JKD6159	CP003668.1	<i>S.warneri</i> _SG1
FR821779.1	<i>S.aureus</i> _LGA251	CP007208.1	<i>S.xylosus</i> _HKUOPL8
CP003166.1	<i>S.aureus</i> _M013		
HF937103.1	<i>S.aureus</i> _M1		
BX571856.1	<i>S.aureus</i> _MRSA252		
BX571857.1	<i>S.aureus</i> _MSSA476		
AP009324.1	<i>S.aureus</i> _Mu3		
BA000017.4	<i>S.aureus</i> _Mu50		
BA000033.2	<i>S.aureus</i> _MW2		
BA000018.3	<i>S.aureus</i> _N315		
CP000253.1	<i>S.aureus</i> _NCTC_8325		
AP009351.1	<i>S.aureus</i> _Newman		
CP007539.1	<i>S.aureus</i> _NRS_100		
AJ938182.1	<i>S.aureus</i> _RF122		
CP003604.1	<i>S.aureus</i> _SA40		
CP003603.1	<i>S.aureus</i> _SA957		
HE579059.1	<i>S.aureus</i> _ST228_10388*		
AM990992.1	<i>S.aureus</i> _ST398		
CP002643.1	<i>S.aureus</i> _T0131		
CP002110.1	<i>S.aureus</i> _TCH60		
FN433596.1	<i>S.aureus</i> _TW20		
CP000255.1	<i>S.aureus</i> _USA300_FPR3757		
CP000730.1	<i>S.aureus</i> _USA300_TCH1516		
CP003033.1	<i>S.aureus</i> _VC40		
CP007447.1	<i>S.aureus</i> _XN108		

Table S3: sRNA Web access to coregulation data from *S. aureus* Expression Data Browser from (Mader et al., 2016)

sRNA	<i>S. aureus</i> Expression Data Browser link
RNAIII	http://genome.jouy.inra.fr/cgi-bin/aeb/viewdetail.py?id=S871_2093169_2093503_-1
RsaB	http://genome.jouy.inra.fr/cgi-bin/aeb/viewdetail.py?id=NA_1750105_1750194_1
RsaD	http://genome.jouy.inra.fr/cgi-bin/aeb/viewdetail.py?id=S243_639727_639872_-1
RsaE	http://genome.jouy.inra.fr/cgi-bin/aeb/viewdetail.py?id=S389_911368_911465_1
RsaOG	http://genome.jouy.inra.fr/cgi-bin/aeb/viewdetail.py?id=S999_2377331_2377476_-1
RsaX20	http://genome.jouy.inra.fr/cgi-bin/aeb/viewdetail.py?id=S1052_2484599_2484726_1
Sau-19	None
srn_2975	http://genome.jouy.inra.fr/cgi-bin/aeb/viewdetail.py?id=S596_1362874_1363048_1
sRNA207	Internal repeat
sRNA287	http://genome.jouy.inra.fr/cgi-bin/aeb/viewdetail.py?id=S774_1863829_1863923_-1

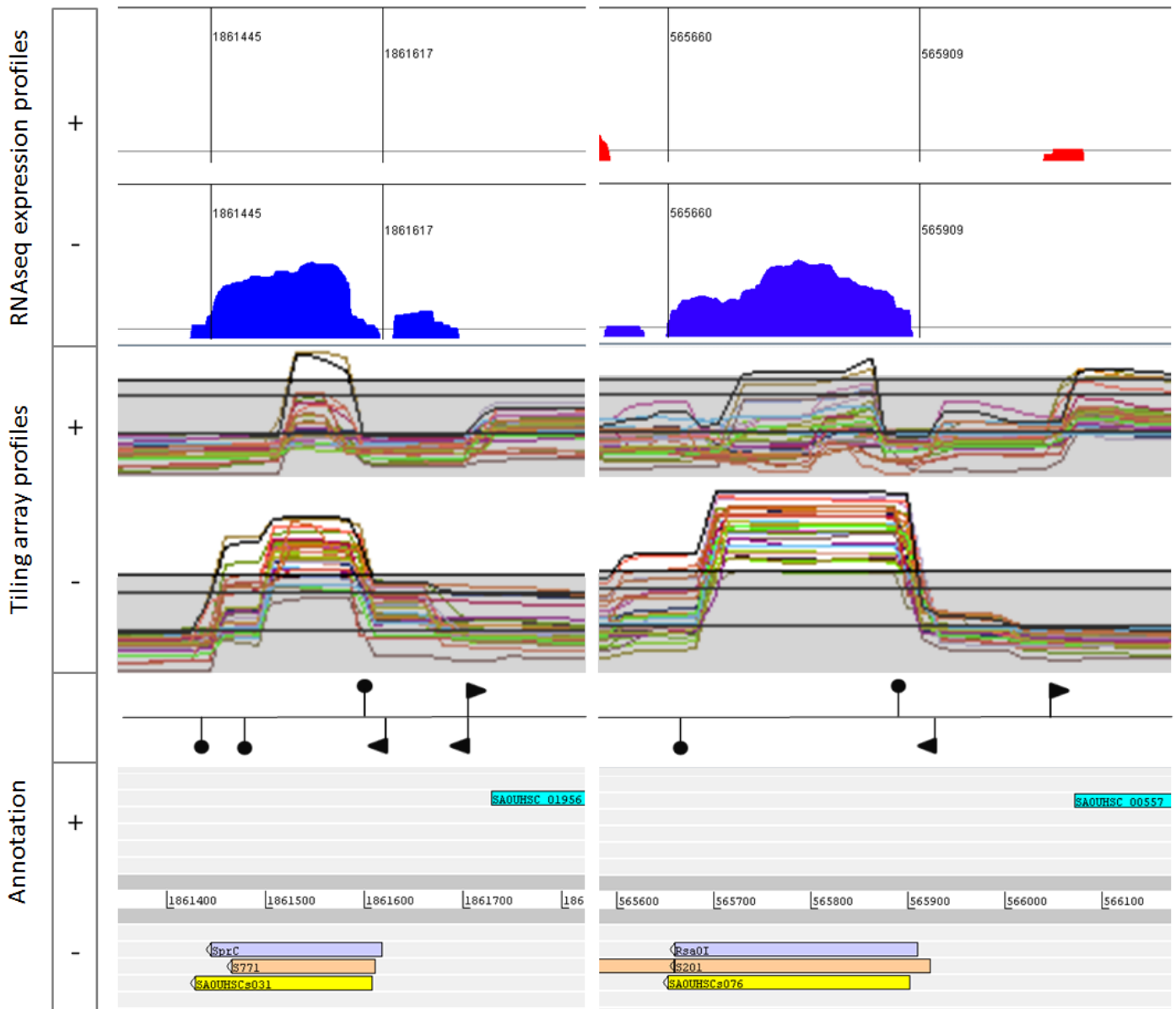


Figure S1: Putative toxin-antitoxin systems excluded from the *bona fide* list. SprC and RsaOI were excluded from the *bona fide* sRNA list because of high transcription levels on their opposite coding strand, in particular in RPMI and plasma (Mader et al., 2016).

Upper panel: Artemis viewer window showing read log-coverages from pooled RNA samples extracted from HG003 grown in 16 growth conditions. Middle panel: screen snapshots of tiling array data from HG001 grown in different conditions (<http://genome.jouy.inra.fr/cgi-bin/aeb/index.py>, Mäder et al. 2016). Lower panel: annotations including genomic coordinates and sRNA names from Carroll et al. (yellow), Mäder et al. (light orange) and this study (mauve). Promoters (flags) and transcription terminators (hairpin loops) are placed according to Mäder et al. and/or TranstermHP software terminator predictions (Kingsford et al., 2007).

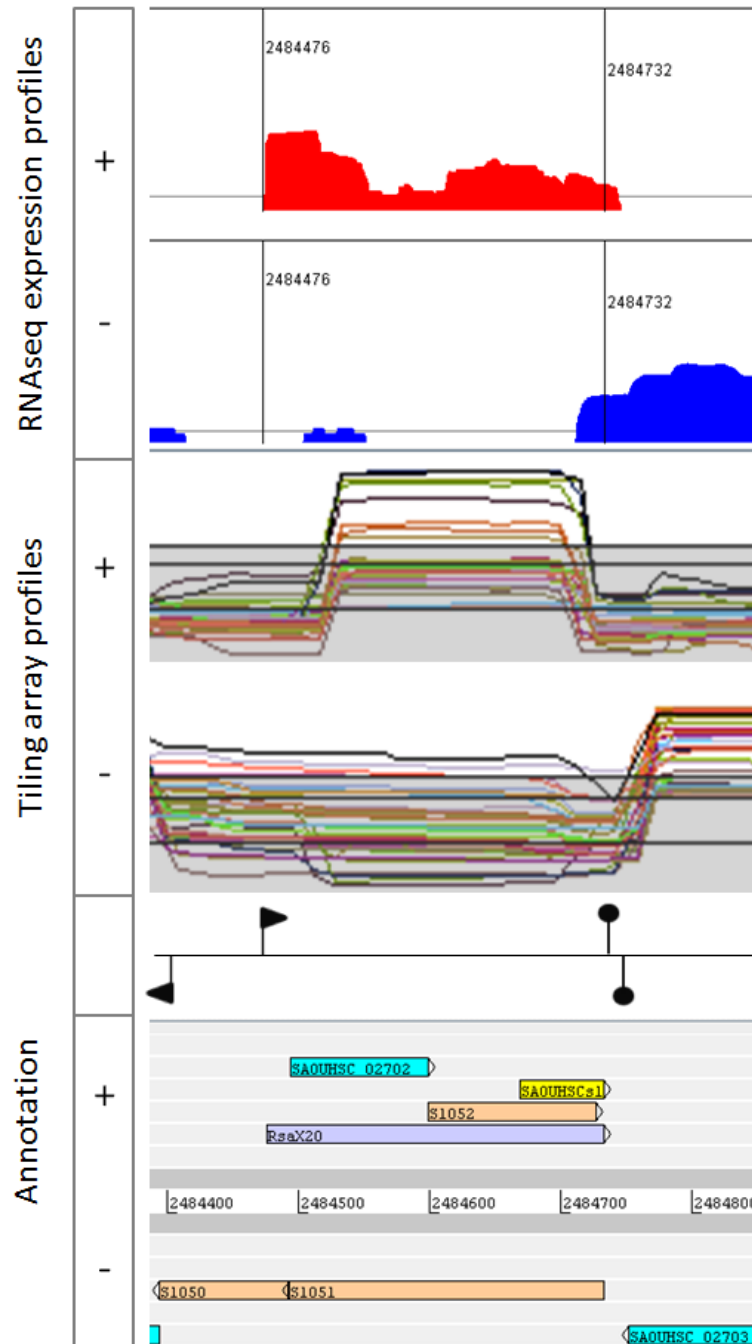


Figure S2: Data compilation reduces the number of putative sRNAs. In this example, Teg128 (SAOUHSC_02702) and Teg130 are considered as parts of RsaX20. For figure legend, see Figure S1.

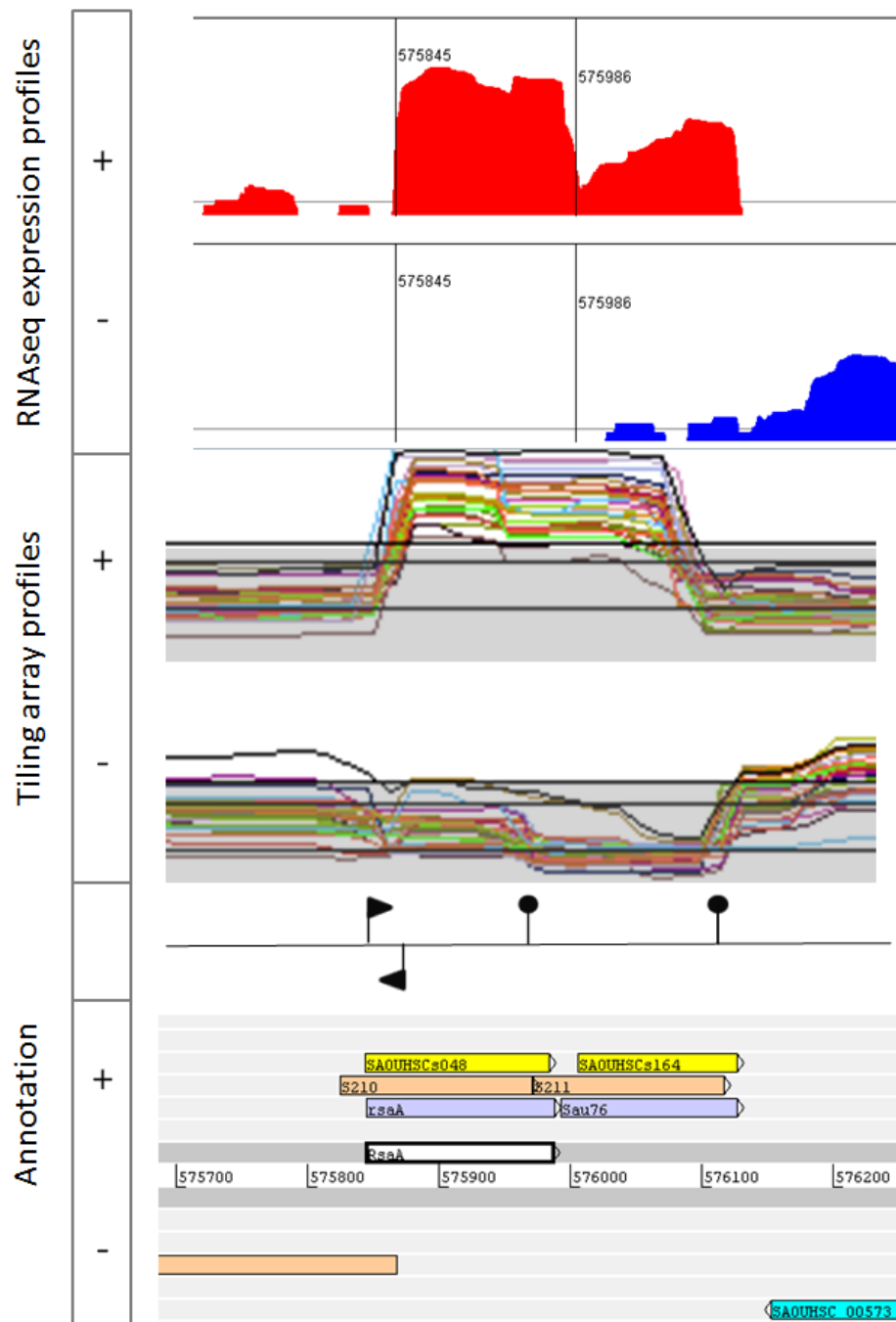


Figure S3: Alternative sRNAs due to transcriptional termination read-through. Transcription from the *rsxA* promoter leads to i) RsaA and ii) RsaA_L after a transcriptional termination read-through. RsaA_L includes Sau76. (see also (Lioliou et al., 2012)). For figure legend, see Figure S1.

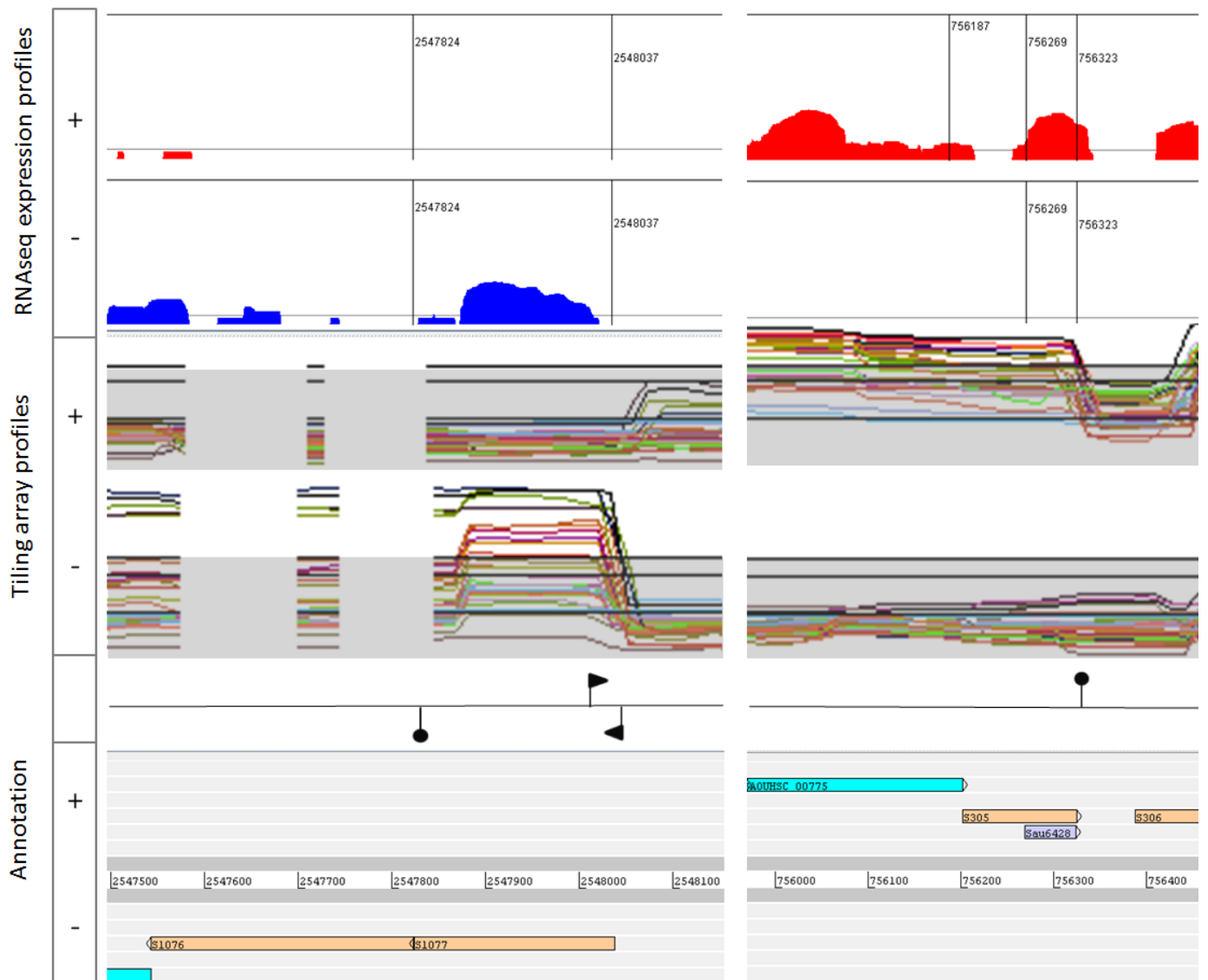


Figure S4: UTRs excluded from the *bona fide* sRNA list. A deep sequence coverage allows to conclude that S1077 is a 5'-UTR and may act as a *cis* regulatory element and that Sau6428, a former sRNA, is a 3'-UTR. For figure legend, see Figure S1.

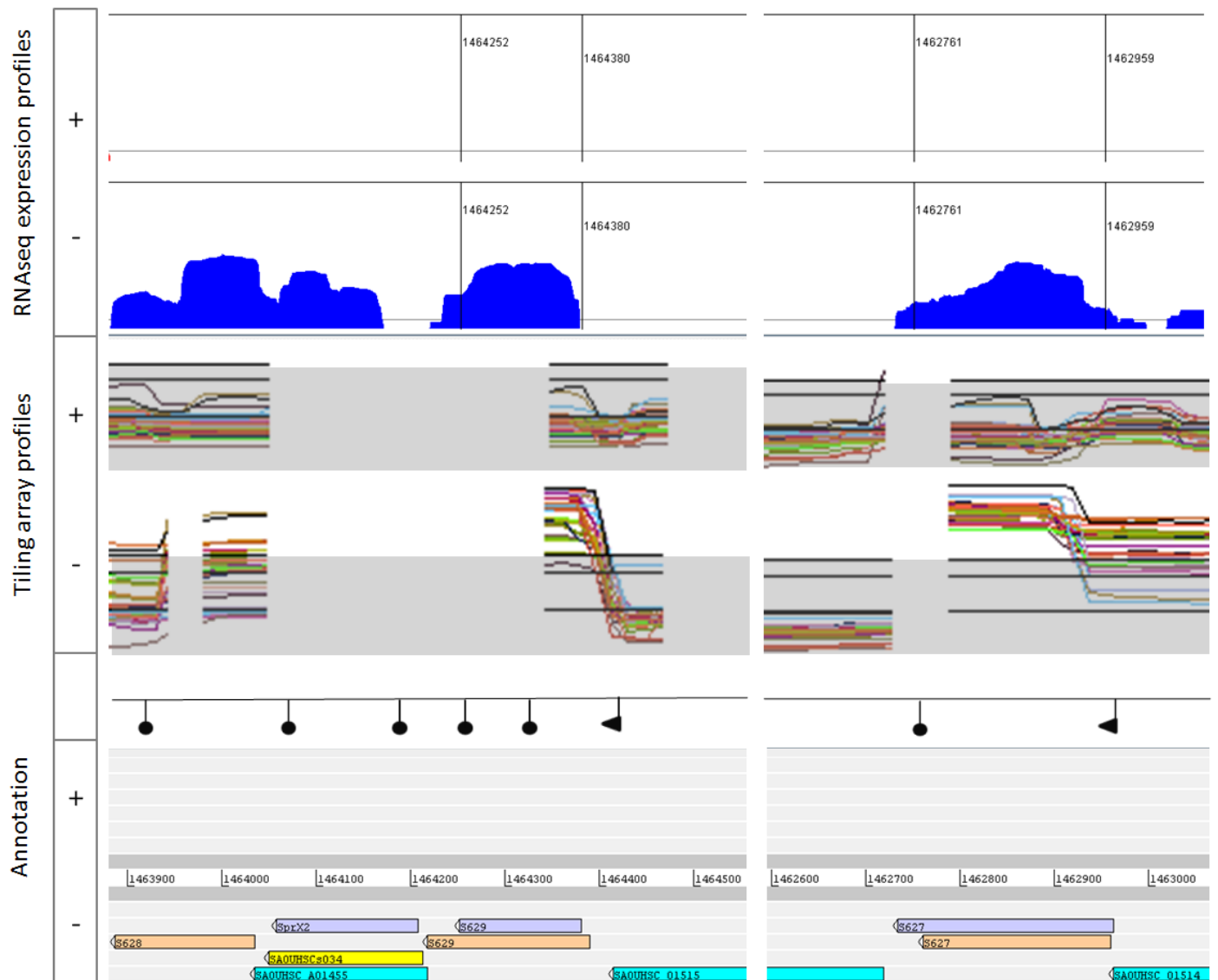


Figure S5: *Bona fide* sRNAs. Example of HG003 *bona fide* sRNAs, S627 and S629, not considered by Mader *et al.* study because of a partial sequence duplication (absence of read coverage in middle panel). For figure legend, see Figure S1.

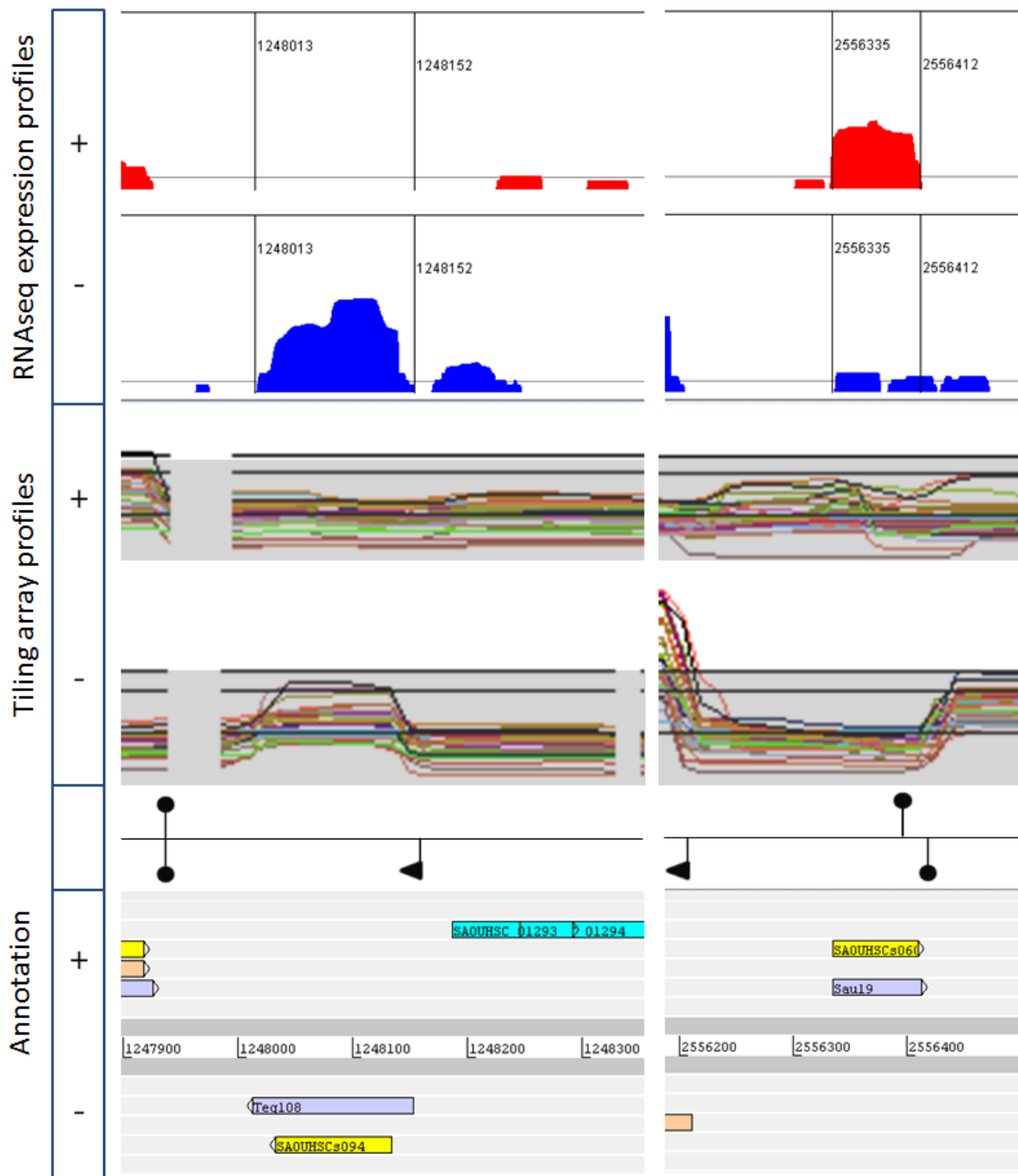


Figure S6: *Bona fide* sRNAs. Example of unfamiliar HG003 *bona fide* sRNAs because of low expression level in other analyses: Teg108 and Sau-19. For figure legend, see Figure S1.

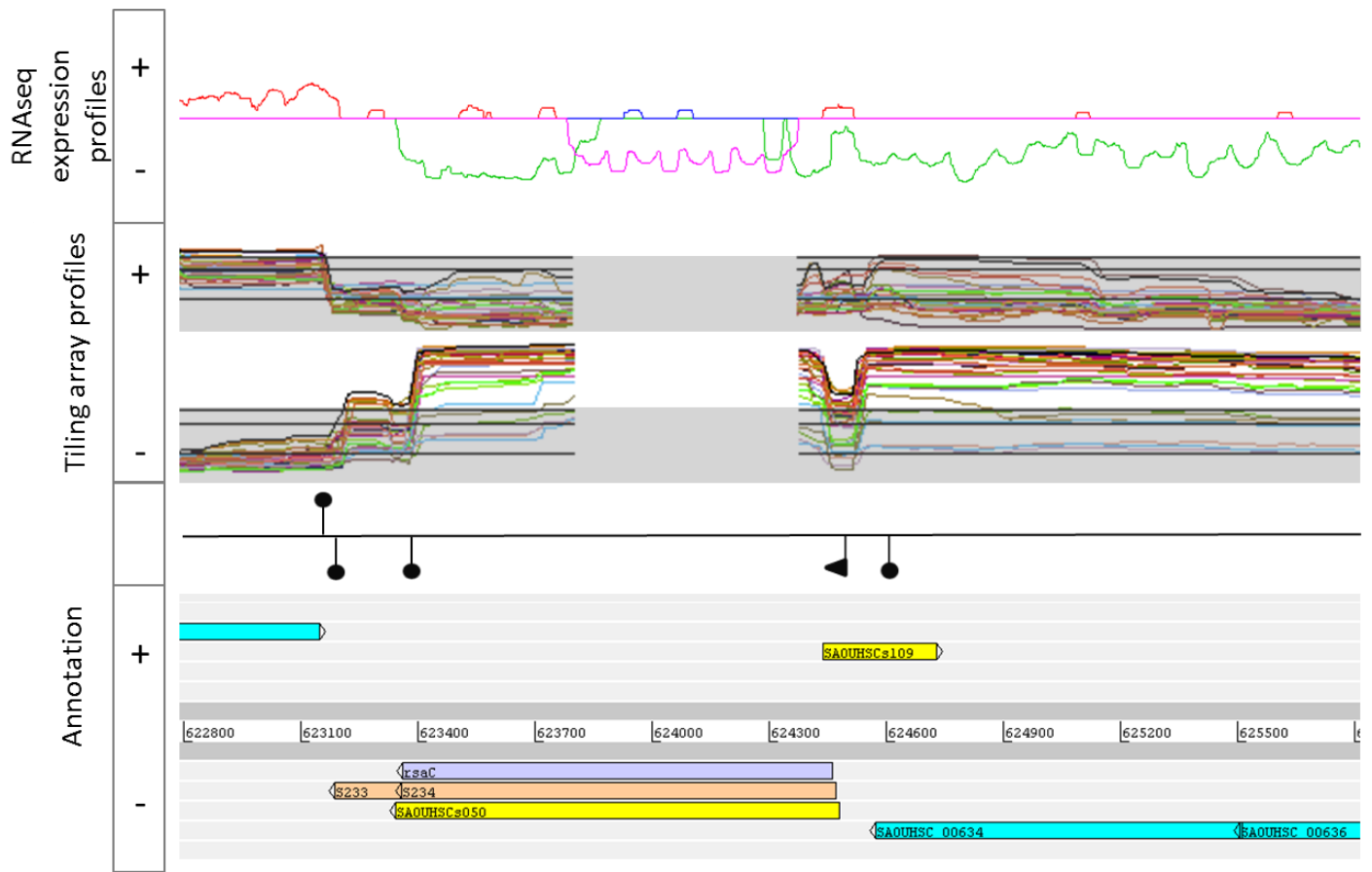


Figure S7: Expression profile of *RsaC* in HG003. Upper panel: Artemis viewer window showing read log-coverages from pooled RNA samples extracted from HG003 grown in 16 growth conditions. Red and blue lines for the + strand, green and pink lines for the minus strand. Red and green lines correspond to the mapping of reads with unique matches while blue and pink lines are reads with multiple matches spread over the genome map. As *RsaC* has internal repeats, this latter representation allows the visualization of *RsaC* expression. For middle and lower panels legend see Figure S1.

DETRPROK analysis:

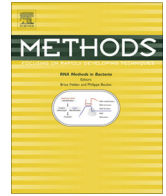
Parameters used with DETRPROK_2.1.2.sh (Toffano-Nioche et al., 2012; Toffano-Nioche et al., 2013) on *Staphylococcus aureus* GENBANK CP000253.1 annotation without SAOUHSC_A.

```
-read_len 40
-features_list "CDS|rRNA|tRNA"
-op_gap 0
-clust_gap 0
-RNA_gap 20
-RNA_merge 50
-5utr_min_reads 10
-5utr_min_size 10
-5utr_coverage 0
-asRNA_min_reads 20
-asRNA_min_size 50
-asRNA_coverage 20
-sRNA_min_reads 10
-sRNA_min_size 50
-sRNA_coverage 4.4
-sRNA_inclusion 0.000000001
-all_feature false
-rm_tmp false
-verbose true
```

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2 Finding sRNA-associated phenotypes by ompetition assays: An example with *Staphylococcus aureus* (Chapter II)



Finding sRNA-associated phenotypes by competition assays: An example with *Staphylococcus aureus*



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ABSTRACT

Bacteria optimize their fitness in response to a changing environment by tight regulation of gene expression. Regulation can be controlled at both transcriptional and post-transcriptional levels via key players such as sigma factors, regulatory proteins and regulatory RNAs. The identification of phenotypes associated with gene deletions is the established method for finding gene functions but may require testing many conditions for each studied mutant. As regulatory RNAs often contribute to fine-tuning gene expression, phenotypes associated with their inactivation are often weak and difficult to detect. Nevertheless, minor phenotypes conferring modest advantages, may allow bacteria to emerge after some generations under selective pressure. A strategy employing DNA barcodes can be used to perform competition experiments between mutants and to monitor fitness associated with mutations in different growth conditions. We combined this strategy with deep sequencing to study regulatory RNAs in *Staphylococcus aureus*, a major opportunistic pathogen.

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1. Introduction

Bacterial regulatory RNAs are divided into two categories, *cis*- and *trans*-acting: *cis*-acting RNAs exert their regulatory activities on associated or interdependent adjacent RNA sequences, while *trans*-acting RNAs pair with independent RNAs or bind to proteins. *trans*-acting RNAs targeting RNAs that are expressed from a complementary strand of another RNA (usually an mRNA) are called asRNAs (for antisense RNAs) [1]; those expressed from a DNA sequence with no transcript on the complementary strand are usually referred to as sRNAs (for small RNAs). Bacterial sRNAs are often 50–300 nucleotides in length, non-coding, and conditionally expressed (e.g., depending upon specific growth stresses or the growth phase [2]). Their association to mRNA targets by base-pairing affects the stability and translation of targets [3,4]. As sRNAs often contribute to the “fine-tuning” of gene expression, their associated phenotypes are difficult to determine. Determining their function requires at the least, for each mutant, the construction of sRNA gene deletion and the test of many conditions, with no assurance of success. However, minor sRNA-mediated phenotypes conferring modest advantages may affect bacterial fitness and emerge as dominant traits after some generations under selective pressure. To investigate sRNA functions in bacteria, we

use a method based on the detection of barcoded deletions first developed in yeast [5] and then applied to enterobacteria [6–8], which allows single mutants to be followed within a population. We adapted the protocol to *Staphylococcus aureus* and coupled it to deep sequencing technology.

S. aureus is a major animal (including human) opportunistic pathogen causing syndromes ranging in severity from minor skin infections to life threatening diseases such as infective endocarditis and necrotizing pneumonia [9]. Its proliferation and pathogenicity are due to rapid adaptations to environmental conditions and controlled expression of virulence factors [10–12]. Numerous elements orchestrate the adaptive regulatory networks in bacteria. Among them, sigma factors, regulatory proteins contribute to transcriptional regulation. A second line of control is posttranscriptional for which sRNAs are essential contributors [13–15]. *S. aureus* has hundreds of regulatory RNAs for which the functions and mechanisms are mostly unknown [13]. To determine their biological roles, we constructed a collection of *S. aureus* sRNA tagged mutants and performed competition experiments by growing mutants together in different growth conditions. In each of these conditions, the relative amount of each mutant was determined thanks to tags and a multiplexing procedure described below. We identified sRNA deletion mutants that accumulated or disappeared in the different tested conditions, leading to preliminary functional assignments of previously unknown regulators. The strategy we developed can be instrumental in identifying sRNA-dependent phenotypes and to unmask sRNA functions.

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2. Approach

2.1. General method

A set of *S. aureus* mutants is constructed. In each mutant, referred to as Δ sRNA-tag, one sRNA gene is substituted with a mutation specific barcode sequence. The mutants are pooled and grown together under different growth conditions. For each tested culture, total DNA is extracted and tag sequences are PCR-amplified. The relative amount of each barcode is determined for each condition by high throughput DNA sequencing (DNA-seq) (Fig. 1). As specific primer pairs are used for each condition, many samples can be analyzed in one DNA-seq run.

2.2. Mutant construction

2.2.1. Design of gene disruptions

Targeted genes are replaced by unique DNA barcode sequences. An essential feature of the design is that the substitution removes sRNA activities with minimized consequences on adjacent genes.

When possible, we design sRNA gene deletions that remove promoters and keep terminators. Transcription may initiate from alternative unknown promoters, and some terminators are bidirectional; keeping the terminators may prevent polar effects that would be generated by complete sRNA gene deletions.

Data from transcriptomic profiles with tiling arrays or RNA deep sequencing (e.g. [16]) should be used to design deletions. The deletion boundaries are nevertheless constrained by the design of efficient primers, which may be problematic for organisms with low GC content such as *S. aureus*.

2.2.2. Generation of DNA tags

A library of DNA tags is generated by PCR-amplification of an oligonucleotide containing a 40-mer random sequence sandwiched between two non-random regions (Fig. 2A). The PCR products are inserted into a cloning vector (e.g. pJET, Fermentas) and transformed into *Escherichia coli* (e.g., DH5 α). Each clone has a different insert. Plasmids are extracted and inserts are sequenced. Plasmids with *ad hoc* sequences are used as DNA barcode source. Hundreds of them are generated in one experiment. Examples of tags are presented (Table S1).

2.2.3. Plasmid assembly

Locus replacements in *S. aureus* are classically performed by two-step homologous recombination (integration and excision) of conditionally replicative plasmids at targeted loci (Fig. 2B). We use two plasmids, a pMAD derivative [17] and pIMAY [18]. Upstream and downstream regions (800–1000 nt) of deleted genes are PCR-amplified from genomic DNA. A specific DNA barcode sequences is assigned to each gene deletion, and is PCR-amplified from plasmids obtained as described in Section 2.2.2. Primers are designed with overlapping sequences to perform Gibson assemblies [19] with upstream sequences – DNA barcodes – downstream sequences and chosen vectors (Table S2).

2.2.4. Gene replacement in *S. aureus*

2.2.4.1. HG003 transformation. Restriction modification systems in *S. aureus* are strong barriers for incoming DNA. Improperly modified DNA is degraded. For this reason, plasmids are routinely constructed in *E. coli* and then transformed into RN4220, a *S. aureus* strain permissive for foreign DNA [20]. Plasmids extracted from RN4220 can then be transferred in the strain of interest (e.g. HG003). Recently, *E. coli* strains with modified restriction-modification system have been released to facilitate staphylococcal cloning. First, DC10B, a strain deleted for its DNA cytosine methyltransferase reportedly allows plasmid transformation directly into different strains of *S. aureus* and *S. epidermidis* [18]. More recently, a set of *E. coli* strains containing the type I adenine methylation system of different *S. aureus* clonal complexes (1, 8, 30 and ST93) has improved the method [21]. We use the IM08B strain to then directly transform our model strain HG003.

2.2.4.2. Mutagenesis with pMAD. Experiments are performed according to the published protocol [17] with a few modifications. The first step is HG003 transformation with pMAD derivatives constructed as described in Section 2.2.3.

Transformants are selected on BHI plates containing erythromycin and incubated at 28 °C, a permissive temperature for pMAD replication in *S. aureus*. As pMAD carries the *bgal* gene encoding β -galactosidase, X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) is included in solid medium during the whole inactivation process to detect the presence of plasmids *via* the formation of blue colonies. The second step is the integration of the plasmid within the host chromosome by homologous

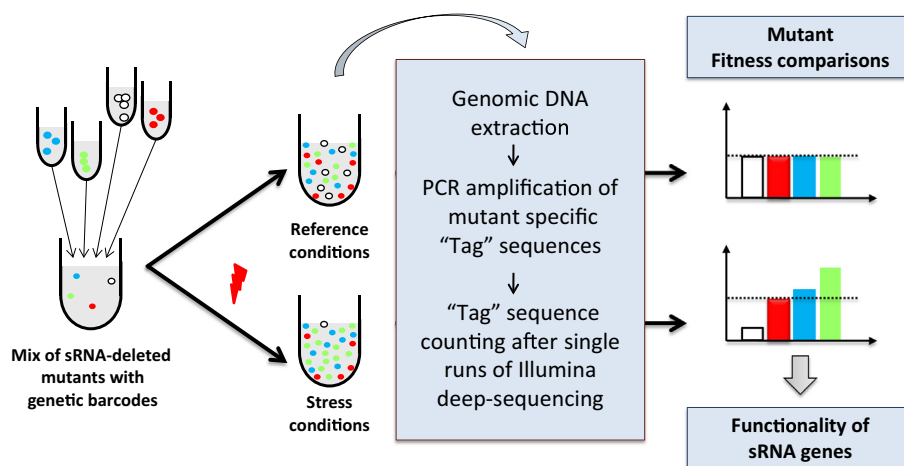


Fig. 1. Competition experiments with barcoded deletion mutants analyzed by DNA deep sequencing. A set of Δ sRNA-tag mutants, each with a unique DNA barcode, is challenged to different growth conditions. DNA retrieved from each condition is PCR-amplified with primers having “experiment identifiers”, allowing many experiments to be sequenced in a single Illumina deep-sequencing run. Sequences are counted and the number of each tag-mutant in each condition is compared to reference conditions (in the given example, the reference is a BHI aerobic culture grown at 37 °C). Overrepresentation of mutants suggests a detrimental role of the sRNAs for survival in the tested condition; underrepresentation suggests a beneficial effect.

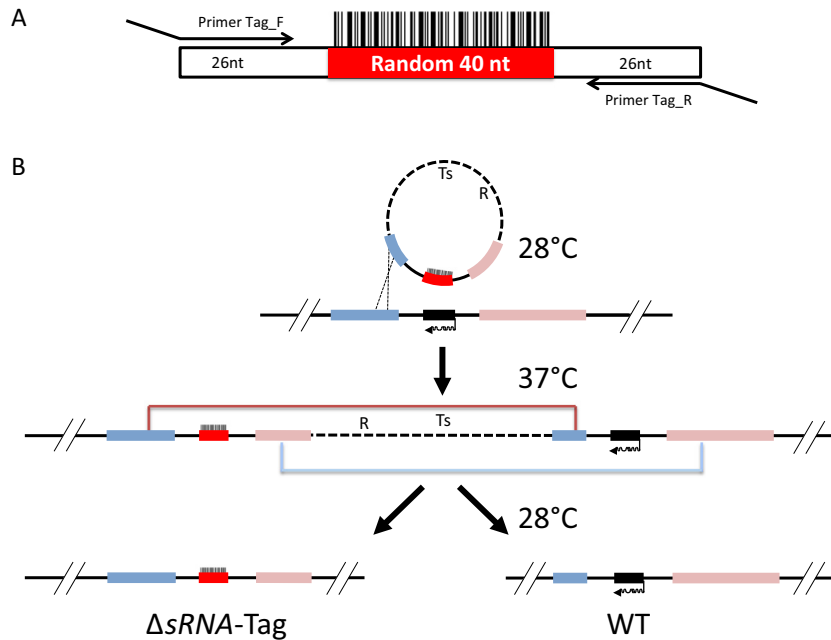


Fig. 2. Construction procedure of barcoded deletion mutant in *S. aureus*. A) Construction of a DNA tag barcode library. It is generated by PCR amplification of an oligonucleotide containing 26nt –40nt random –26nt. PCR products are cloned and sequenced; the resulting plasmids are used to generate unique 40nt sequences flanked by 36 common nt. B) Schematic view of the gene inactivation process by double crossing over with a *rep*(Ts) plasmid (see also [17,18]).

recombination. It is achieved by raising the culture temperature to 37 (or 42) °C. Since plasmid DNA replication is deficient at temperature above 37 °C, erythromycin induces the selection of bacteria with plasmid integrated into the chromosome. In a third step, strains with an integrated plasmid are shifted back to 28 °C. This low temperature leads to activation of plasmid replication which is deleterious for growth if the plasmid remains integrated in the genome. Plasmid replication favors growth of clones that undergo a second recombination event allowing the accumulation of strains with plasmid excision from the chromosome accumulates. At this step, clones are tested on X-Gal and erythromycin to identify white sensitive clones that have lost the plasmid. Among them, for most constructions, the second recombination event leads, in 50% of the clones, to the chromosomal desired event.

2.2.4.3. Mutagenesis with pIMAY. Experiments are performed according to the published protocol [18]. As with pMAD, the first step is HG003 transformation with the constructed plasmid as described in Section 2.2.3. Transformants are selected on BHI plates containing chloramphenicol incubated at 28 °C, a permissive temperature for pIMAY replication in *S. aureus*. The integration step is obtained by maintaining antibiotic selection and raising the culture temperature to 37 °C. Strains with an integrated plasmid are shifted back to 28 °C to select plasmid excision events. At this step, bacteria are plated on anhydrotetracycline to induce expression of an anti-SecY gene carried by pIMAY. If pIMAY is still present, expression of anti-SecY leads to bacterial death and thus selects for the second recombination event and the deleted strains [18]. Note that pIMAY-Z, a pIMAY with LacZ white-blue selection is available and would be useful for such disruption [21].

For constructed strains, see Table S3.

2.3. Competition experiments

All mutant strains are first grown overnight individually in a rich medium. They are then diluted (1:1000), grown until

$OD_{600} \sim 0.6-0.7$, and pooled so that each mutant is present in the same amount according to OD_{600} normalization. This culture mix comprises the starting culture of a Δ sRNA-tag set. Three independently prepared starting cultures are generated in this way. Samples are aliquoted and stored at -80 °C as stocks for experiments. As OD_{600} measurements may not closely correlate with the number of bacteria, the ratio of each tag is determined by DNA sequencing for the starting cultures. A maximum variation of two-fold to the expected proportion was observed for one mutant. A CFU-OD relationship for each mutant could be determined to optimize the starting mix assembly. The storage at -80 °C may affect differentially the viability of specific mutants. For this reason, we also measured the tag ratio after growing the starting culture in rich media at 37 °C to OD_{600} 0.6. The ratio of each mutant remained unchanged except for one which was growing more slowly than the others in all tested conditions. The starting culture storage at -80 °C did not affect selectively the constructed mutants.

Competition experiments are performed using 1000-fold dilutions of the three tag deletion sets into fresh culture medium and growing them in the desired test conditions (Fig. 3A). A first sampling is performed during exponential phase when cultures reach OD_{600} 0.6–0.7 (Sampling 1). The remaining cultures are left to grow and the following day, the cultures are diluted 1000-fold into a fresh culture medium and grown again in the same test conditions. A second sampling is performed when the cultures reach OD_{600} 0.6 to 0.7 (Sampling 2) (Fig. 3B). As many sRNA genes are expressed during stationary phase, Sampling 2 is more appropriate for detection of corresponding mutant phenotypes. In addition, phenotypes detected in Sampling 1 may be more pronounced in Sampling 2. We normalize the proportion of each mutant in the tested condition to two different control conditions: the starting inoculum and growth in aerobic rich standard medium at 37 °C. These two controls avoid any overlook of storage condition impact and of mutants with general growth defect. Growth conditions, samplings and control conditions must be adapted to the biological questions being addressed.

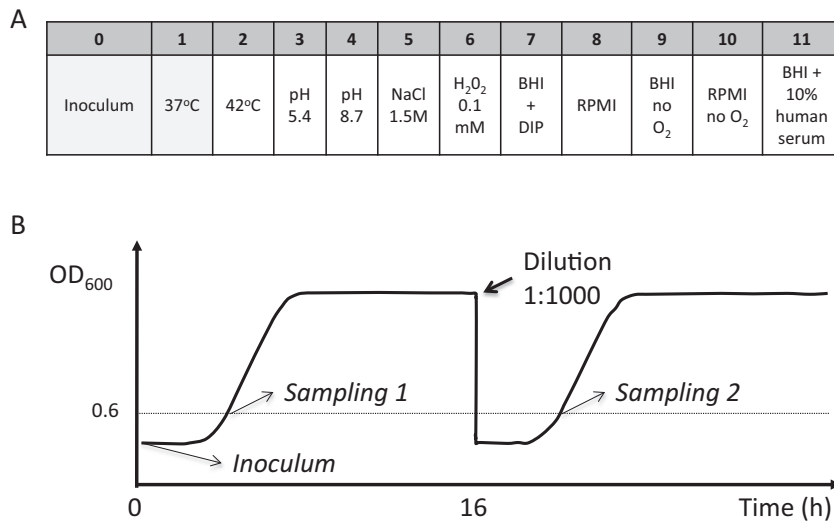


Fig. 3. Growth conditions and samplings. A) Summary of the tested conditions. In light grey, proposed reference conditions. B) Scheme indicating the sampling steps. In italic are the sequenced samples.

2.3.1. DNA-seq library preparation

In order to evaluate the amount of each mutant within a mixed population, the representation of each DNA barcode needs to be determined. In most previously published fitness protocols, genomic DNA from mixed populations was extracted, the tags were PCR-amplified and the proportion of each specific tag was determined by hybridizing the labeled PCR products on dedicated DNA arrays [8]. These experiments are tedious and expensive, as each test condition requires at least one array. We decided to count the PCR products by deep sequencing rather than by arrays. However, as all growth conditions (including triplicates) have to be discriminated, in principle, these experiments would require constructing as many DNA-seq libraries as there are tested conditions, increasing significantly the cost of the method. We therefore adapted the protocol as follows: PCR products of each experiment were obtained with two primers having 5'-extensions of 5 nucleotides; these “experiment identifiers” were specific to each counted sample (Table S4, Fig. S1). Warning: Illumina libraries require variability on the first four nucleotides sequenced for proper system calibration. The same quantity of PCR products from the different conditions were mixed together. In a pilot experiment, a DNA-seq library was made from forty different conditions and a deep-sequencing experiment was performed. Unexpectedly, about 80% of the DNA barcode sequences were associated with experiment identifiers (forward compared to reverse) coming from two independent experiments. As amplified tags of each experiment differ only by their 5 terminal nucleotides, the denaturation steps and PCR-amplification during DNA-seq library construction likely led to illegitimate pairing of identical barcodes coming from different experiments and artifactual results (data not shown). We solved this technical issue by removing the amplification step from the standard DNA-seq library construction protocol. The resulting protocol adapted to deep sequencing technology is time saving, increases the response linearity, and is cheaper if several conditions are pooled, as compared to previously used array technology.

2.3.2. Sequencing data analysis

In the given example, we performed a 100 nt paired-end sequencing to get the complete tag sequence with its forward and reverse experiment identifiers. As a general rule, the sequence length should cover the two experiment identifiers. 100 nt paired-end sequences are compiled to obtain full length barcodes (SI § 2). Only assemblies with the best quality scores are further processed.

Briefly, each experimental condition is demultiplexed using the 5-nucleotide experiment identifiers, both in the 5' and 3' ends of PCR amplicons (Table S4) with no mismatch allowed. Chimeric sequences with identifiers from different experiments are discarded. Tag sequences are retrieved from the whole amplicon sequences by fastx_trimmer and mutants are discriminated thanks to fastx_barcode_splitter with 5 mismatches allowance (http://hannonlab.cshl.edu/fastx_toolkit/commandline.html; a flowchart of the procedure is presented Fig. S2). Barcodes are then counted for each experiment set. The frequency of each mutant compared to the others and in the different growth conditions is determined by calculating the ratio of the frequency of the barcode in the tested condition on the frequency in a reference condition. Standard deviation is determined on the ratios obtained from three independent biological experiments.

3. Results and discussion

3.1. Construction of 39 tagged *sRNA*-gene deletions

sRNA genes selected for disruption were chosen based on data available when the project started [13]. Deleted regions comprised, as per available information, promoter region and *sRNA* gene. The recent release of *S. aureus* transcriptomic data in many growth conditions will be valuable for optimizing deletion design in future experiments [16]. Thirty-nine tagged deletion mutants (Table S3) were constructed according to the protocol described above (Section 2.2) in HG003, an NCTC8325 derivative in which *rsbU* and *tcaR* mutations were repaired and that is infective and used as a model strain for staphylococcal regulation studies [22]. Disruptions presented here were performed using pMAD2 (plasmid #67682 at Addgene, <https://www.addgene.org/>), a replication thermo-sensitive plasmid derived from pMAD [17]. As high temperature and erythromycin selection stimulate mutations in the *saeS* gene [23], we performed the pMAD2 integration step at 37 °C instead of 42 °C. We also used an alternative plasmid, pIMAY, which carries a highly temperature sensitive replicon in staphylococci and which is proposed to alleviate problems that arise with pMAD-like plasmids [18]. However, we noticed that several pIMAY derivatives constructed in *E. coli* could not transform staphylococcal strains. These plasmids were partially sequenced, and found to contain mutations affecting the *repA* gene: a deletion from nucleotide 1034 to 1167 (pIMAY position), a deleted A and an additional A

within a stretch of A (pIMAY position 913–920). We suggest that mutations in the pIMAY *repA* gene appear due to activity of the two replication origins in *E. coli* [24]. *repA* mutations are prevented by growing *E. coli* containing pIMAY at 37 °C only, and by not leaving cultures on the bench.

To optimize the mutant construction, we tested two new *E. coli* strains that enable a direct transformation of certain *S. aureus* strains with their extracted plasmids [18,21]. pMAD and pIMAY derivatives extracted from IM08B, but not from DC10B, transform HG003. However, the transformation efficiency of IM08B-extracted plasmids is dependent on plasmid types (either because of size or antibiotic resistance). Our observations and tips are summed up (Table 1); the use of pIMAY plasmid in IM08B for one-step *S. aureus* transformation is a time-saving method as proposed [21].

3.2. Conditions of competition experiments

Each Δ sRNA-tag mutant was inoculated separately in BHI broth and grown aerobically (180 rpm) for 16 h at 37 °C. Overnight cultures were diluted 1:1000 in fresh BHI broth and grown to OD₆₀₀ ~ 0.6–0.7. The cultures were then pooled together in the same amount (normalized so that each culture was at a final OD₆₀₀ of 0.6) and stored in aliquots at –80 °C. The procedure was repeated three times to generate three independent sets. The consequences of sRNA gene deletions were tested in eleven growth conditions (Fig. 3A): For the first 8, these were done in BHI with aeration 180 rpm 1) at 37 °C, 2) at high temperature (42 °C), 3) at acid pH (5.4 adjusted with 1 M HCl), 4) at alkaline pH (8.7 adjusted with 1 M NaOH), 5) at high osmolarity (1.5 M NaCl), 6) in oxidative condition (0.1 mM H₂O₂), 7) in iron depletion (1.4 mM 2,2'-Bipyridyl), 8) in synthetic medium containing amino acids, vitamins, inorganic salts and glucose (RPMI 1640 medium, Sigma-Aldrich). As *S. aureus* is a facultative anaerobe, we also tested the Δ sRNA-tag mutant sets in oxygen-limited conditions. Growth was performed in a Falcon tube (50 mL) completely filled with 9) BHI or 10) RPMI medium. In addition, the mix of mutant strains was grown aerobically 11) in BHI medium containing human serum (10%).

Competition experiments were initiated by diluting the thawed out tagged mutant sets 1000-fold into fresh culture media in the

chosen growth conditions. For each set, one aliquot was prepared directly for sequencing as the control sample. For each competition assay, bacteria cultures are sampled at OD ~ 0.6 (Sampling 1). The remaining cultures are grown overnight, diluted 1:1000 the day after in the same medium, grown in the same condition and harvested at OD ~ 0.6 (Sampling 2) (Fig. 3B).

3.3. Data processing

Barcode sequences from Samplings 1 and 2 were identified for all experiments and counted for the three Δ sRNA-tag sets as described. Results from growth at 42 °C are presented (Fig. 4). The proportion of each mutant within the remaining population was normalized to the same culture grown at 37 °C. Mutants were considered to either accumulate or disappear in the tested condition when a minimum five-fold difference was observed compared to the reference condition. At 42 °C, in Sampling 1, only Δ sau30 was underrepresented. In Sampling 2, in addition to Δ sau30, Δ sau6836 and Δ rsaH were also underrepresented (Fig. 4). Mutants with a growth disadvantage at 42 °C revealed by these competition experiments were grown individually in BHI at 37 °C and 42 °C, and compared to the parental strain. The Δ sau30 mutation led to a growth defect at 42 °C but not at 37 °C as compared to the wild type strain. However, no growth difference was observed between Δ sau6836, Δ rsaH and the parental strain at 42 °C, likely because growth differences are minor and difficult to observe on standard growth curve or require passing through stationary phase (data not shown).

Results from the 11 growth conditions (summarized in Fig. 5) reveal that several constructed mutants were affected by the tested growth conditions and that sRNAs are possibly involved in these phenotypes. While standard deviations give support for the experimental data, additional experiments are required to confirm that observed phenotypes are linked directly to studied genes since secondary mutations might arise during mutant construction [23], and/or mutations might have polar effects on adjacent genes. Complementation studies can address these issues. As a rule of thumb, for each gene studied, we now construct three independent mutants that are used separately to constitute the triplicate sets, thus reducing chances of obtaining unlinked secondary mutations in the three independent constructions.

Table 1

Comparison of *S. aureus* gene deletion tools used for this project. In red are the drawbacks and in green the advantages.

Plasmid Properties in <i>E. coli</i>	Transformation in <i>S. aureus</i> HG003	2 step crossing over	
		Integration	Excision
<i>Plasmids</i>			
pMAD 9.7 kb Amp 100	Ery 0.5; 28 °C	Ery 0.5; 37 to 42 °C High temperature increases secondary mutations	At least three temperature shifts Blue/white screen Anti-SecY counter-selection
pIMAY 5.7 kb High frequency of mutations in <i>repA</i> Cm 20	Cm 5; 28 °C	Cm 5; 37 °C	
<i>E. coli</i> strains			
DH5 α	Via RN4220 i.e. 2 steps		
DC10B	Via RN4220 i.e. 2 steps		
IM08B	Direct Poor efficiency with pMAD		
<i>Tips for pIMAY utilization</i>			
Maintain <i>E. coli</i> (IM08B) cultures of pIMAY at 37 °C and store plates at 4 °C (don't leave them on the bench) Stock pIMAY in <i>S. aureus</i>	Maintain <i>S. aureus</i> strain transformed with plasmid at 28 °C in liquid for at least 3 h before plating	Check for the absence of free plasmid Keep cultures at 37 °C	Test at least 10 antibiotic-sensitive clones

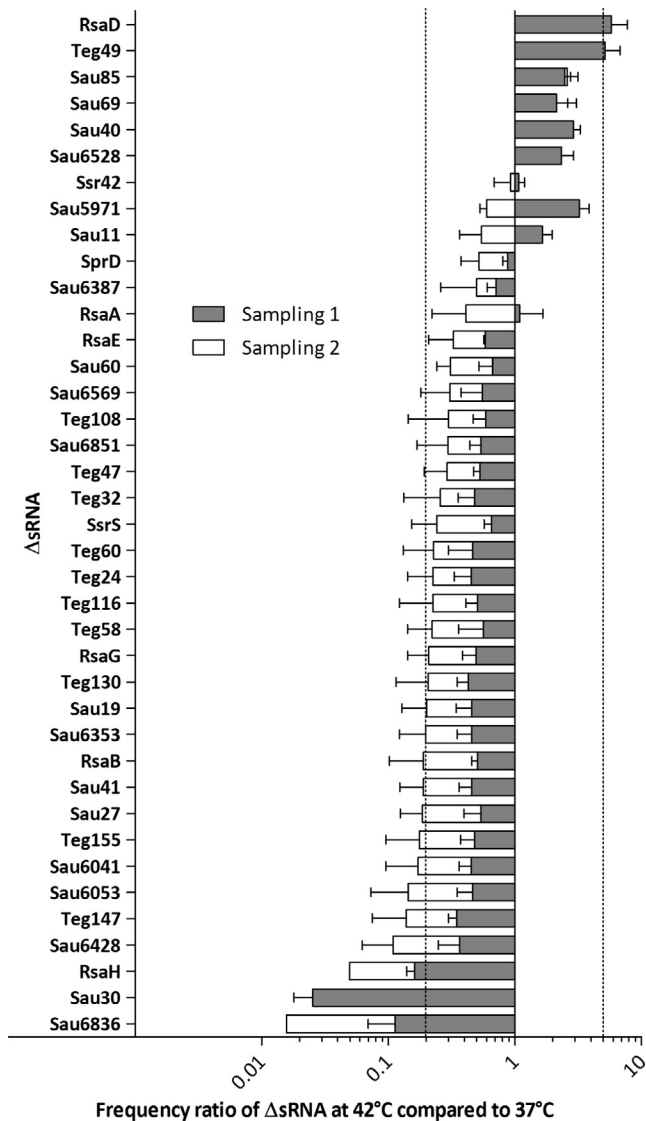


Fig. 4. Competition assay at 42 °C. Thirty-nine Δ sRNA-tag mutants were inoculated in the same amount and grown at 42 °C. They were sampled as shown (Fig. 3B). Total DNA was extracted, barcode sequences were PCR amplified and their relative amount was counted via the analysis of DNA-Seq experiments. The histograms represent the disappearance (left bars) or accumulation (right bars) of indicated deletion mutants (vertical-axis) at 42 °C compared to 37 °C in sampling 1 (grey bars) and 2 (white bars). Data are shown as average values and the standard deviation of triplicate samples is indicated. Three Δ sRNA-tag mutants have no standard deviation in sampling 2 as in at least one of the replicates the mutant disappeared totally leading to unrepresentable error-bars. The presented values correspond to the higher positive value obtained in the other replicate(s). The dotted lines correspond to a five-fold difference threshold.

4. Conclusions

A documented method to assay mutant competitiveness is to label strains with different antibiotic resistance or fluorescent markers. However, the number of available markers is limited, and antibiotic markers impact strain behavior; hence fitness assays have been difficult to perform on a large scale. The introduction of DNA barcodes opens possibilities for assessing mutant fitness in a competitive biotope without affecting the outcome. We worked out the bottlenecks in utilization of DNA-barcodes in deep sequencing. We optimized multiplex deep sequencing to study sRNA gene functions in *S. aureus* which are notoriously difficult

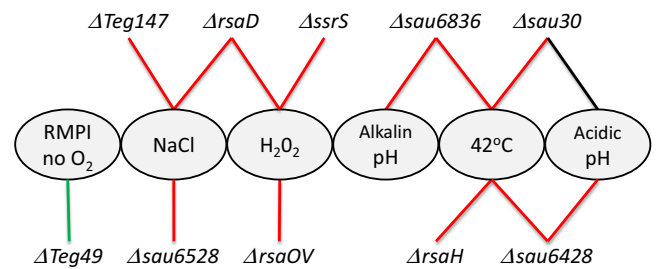


Fig. 5. sRNA competitive fitness experiments: summary. Diagram linking sRNA-gene deletions with altered fitness to their corresponding tested growth conditions (sampling 2). Green, over-representation; red, under-representation; black, disappearance. Experiments were performed in triplicate as indicated in Fig. 4 legend and Section 3.2. Only mutants with at least a five-fold difference compared to a reference at 37 °C are presented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to identify. Competition experiments as described here can be performed in any kind of growth conditions. For example, our next step will be to challenge Δ sRNA-tag sets to *ex-vivo* and *in vivo* animal models to identify sRNA genes related to virulence.

Growth in the presence of other mutants could reveal patterns of interaction or epistasis between different bacterial subpopulations and possibly whether particular bacterial combinations interact synergistically or antagonistically [25]. Interestingly, variants leading to improved fitness in one growth condition can lead to altered fitness in another condition [26,27]. Competition studies using the methodology described here will give the best chance of identifying the elusive functions of regulatory sRNAs.

Disclosure of potential conflicts of interest

None to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ymeth.2016.11.018>.

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3 *Staphylococcus aureus* regulatory RNAs driving fitness upon antibiotic exposure (Chapter III)

3.1 Material and Methods

3.1.1 Mutant constructions

3.1.1.1 Principle of gene disruptions

An essential feature of the design is to remove the sRNA activity with minimized consequences on adjacent genes. When possible, we design deletions that remove promoters and keep terminators. Transcription may initiate from alternative unknown promoters, and some terminators are bidirectional; for these reasons, keeping terminators may prevent polar effects that would be generated by complete sRNA gene deletions. Promoter regions were kept in few cases when we considered that the sRNA gene could part of a 5'UTR. Transcriptomic profiles from tiling arrays (Mader *et al.* 2016) and RNA-Seq were carefully analyzed to design the deletions. Boundaries were nevertheless constrained by the selection of efficient primers, which is often problematic for organisms with low GC content such as *S. aureus*. Disrupted genes were replaced by unique DNA barcode sequences: tags.

3.1.1.2 Generation of DNA tag library

DNA tags were generated by the PCR-amplification of an oligonucleotide containing a 40-mer random sequence sandwiched between two non-random regions. The PCR product was cloned into pJET vector and transformed into *E. coli* DH5 α . Plasmids were extracted and their inserts were sequenced. A collection of plasmids, each one containing a different 40 nt sequence, was obtained and stored providing a source of *ad hoc* DNA tags for barcoded deletions. Hundreds of tags were generated in one experiment (Table S1).

3.1.1.3 Plasmid assembly for gene inactivation

Locus replacement in *S. aureus* is classically performed by a two-step homologous recombination with an integration step and an excision step at a targeted locus, using a conditionally replicative plasmid carrying the desired sequence. We used pIMAY, a Gram⁺/Gram⁻ shuttle plasmid with a thermo-sensitive replication origin, inactive in Gram⁺ at 37°C. Efficient recombination of pIMAY in *S. aureus* requires 800-1000 nt-long homologous sequences. Consequently, for gene disruption, about 900 nt-long sequences upstream and downstream the genes to be deleted were PCR-amplified from genomic DNA. A specific DNA barcode sequence is assigned to each gene deletion, and is PCR-amplified from plasmids obtained as described in Section 3.1.1.2. Primers are designed with overlapping sequences to perform Gibson assembly (Gibson *et al.* 2009). The cloning in pIMAY includes an upstream

sequence, a DNA barcode, and a downstream sequence (Table S2). The integrity of inserted sequences was verified by plasmid DNA sequencing.

3.1.1.4 Transformation in *S. aureus*

Restriction modification systems in *S. aureus* are strong barriers for the entry of exogenous DNA. Improperly modified DNA is degraded. For this reason, plasmids are routinely constructed in *E. coli* and then transformed into RN4220, a *S. aureus* strain permissive for foreign DNA (Kreiswirth *et al.* 1983). Plasmids extracted from RN4220 can then transform strains of interest (*e.g.* HG003). Recently, a set of *E. coli* strains containing the type I adenine methylation system of different *S. aureus* clonal complexes (1, 8, 30 and ST93) facilitating staphylococcal cloning were reported (Monk *et al.* 2015). Plasmids were constructed in one of them, IM08B, permitting to isolate plasmids that directly transform the pathogenic model strain HG003.

3.1.1.5 Gene mutagenesis with pIMAY.

Experiments were performed as described (Monk *et al.* 2012; Le Lam *et al.* 2017). The first step was HG003 transformation with pIMAY derivatives constructed as described in Section 3.1.1.3. Transformants were selected on BHI plates supplemented with chloramphenicol incubated at 28°C, a permissive temperature for pIMAY replication in *S. aureus*. Plasmids integrated in the chromosome were selected by maintaining the antibiotic selection and raising the culture temperature to 37°C. Strains with integrated pIMAY derivatives were identified by PCR. They were shifted back to 28°C in the absence of antibiotic to select strains having excised their plasmid from the chromosome. During this excision step, the bacteria were plated on BHI plates containing aTc (anhydrotetracycline), an inducer of an anti-*secY* RNA gene carried by pIMAY. The toxicity of the anti-*secY* allows the selection of bacteria that lost pIMAY (Bae and Schneewind 2006; Monk *et al.* 2012). Note that, all constructed mutants shown in Table S3 are in independent triplicates.

3.1.2 Fitness experiments

3.1.2.1 Preparation of sRNA mutant libraries

One fresh colony of all constructed sRNA mutants (Table 2) was inoculated and grown individually overnight in liquid TSB medium. Cultures were then diluted (1:1000) into fresh and pre-warmed TSB medium, grown until $OD_{600} = 1$ and pooled together in stoichiometric proportions to form a Δ sRNA-tag library. The library was aliquoted and stored at -80°C.

The libraries were made in independent triplicates (set 1, 2 & 3) using, for each one, the biological constructed triplicate mutants (*c.f.* Section 3.1.1.5). A first version of the library, v1, was assembled (Table 2), including 30 reported tagged-mutants (No.1~30) (Le lam *et al.* 2017) and 48 additional ones (No.31~78) (Liu *et al.* 2018) Based on the results of fitness experiments obtained with library v1, a second library (v2) was assembled with the remove of a few mutants and the addition of new ones (Table 3 and 4).

Table 2 library v1 sRNA mutant list

No.	Mutants	Other names	Barcode	Reference
1	Δ sau60	srn_1420, SAOUHSCs139,S185,ssr155	tag003	Abu-Qatouseh <i>et al.</i> , 2010
2	Δ RNAIII	sRNA317, srn_3910, SAOUHSCs022, S871	tag004	Novick <i>et al.</i> , 1993
3	Δ rsaD::tag6	sRNA138, srn_1640, SAOUHSCs051, S243	tag006	Geissmann <i>et al.</i> , 2009
4	Δ rsaOG	teg24, rsaI, sRNA356, srn_4390, SAOUHSCs047, S999	tag009	Marchais <i>et al.</i> , 2009
5	Δ rsaG	teg93, sRNA31, srn_0510, SAOUHSCs054, S58	tag011	Geissmann <i>et al.</i> , 2009
6	Δ sau6041	srn_2680, SAOUHSCs166,S527	tag014	Abu-Qatouseh <i>et al.</i> , 2010
7	Δ sau6428	teg109, srn_1870, S305	tag016	Abu-Qatouseh <i>et al.</i> , 2010
8	Δ sau6836	srn_1620, S240	tag017	Abu-Qatouseh <i>et al.</i> , 2010
9	Δ teg147	sRNA85, srn_0960, SAOUHSCs103	tag018	Beaume <i>et al.</i> , 2010
10	Δ teg49	srn_1550, SAOUHSCs089, S227	tag020	Beaume <i>et al.</i> , 2010
11	Δ teg58	SAOUHSCs090, S457	tag022	Beaume <i>et al.</i> , 2010
12	Δ teg60	srn_2520, SAOUHSCs091, S467	tag023	Beaume <i>et al.</i> , 2010
13	Δ rsaB	srn_3410, SAOUHSCs049, SAOUHSC_01844	tag025	Geissmann <i>et al.</i> , 2009
14	Δ rsaD::tag26	sRNA138, srn_1640, SAOUHSCs051, S243	tag026	Geissmann <i>et al.</i> , 2009
15	Δ sau69	srn_3630, S784	tag027	Abu-Qatouseh <i>et al.</i> , 2010
16	Δ teg116	srn_3130, SAOUHSCs095	tag030	Beaume <i>et al.</i> , 2010
17	Δ sau6851	teg53, srn_2070, S365	tag032	Abu-Qatouseh <i>et al.</i> , 2010
18	Δ sau11	SAOUHSCs008, S416	tag033	Abu-Qatouseh <i>et al.</i> , 2010
19	Δ teg108	sRNA222, srn_2740, SAOUHSCs094	tag033	Beaume <i>et al.</i> , 2010
20	Δ sau85	srn_2760, SAOUHSCs165	tag038	Abu-Qatouseh <i>et al.</i> , 2010
21	Δ sau6353	srn_3110	tag042	Abu-Qatouseh <i>et al.</i> , 2010
22	Δ rsaE	sau20, teg92, sRNA183, srn_2130, S389	tag045	Geissmann <i>et al.</i> , 2009
23	Δ RNAIII- <i>agr</i>		tag047	
24	Δ rsaH::tag49	srn_1910, SAOUHSCs055, S317	tag049	Geissmann <i>et al.</i> , 2009
25	Δ ssr42	rsaX28,teg27,sRNA363,srn_4470,SAOUHSCs084, S1036	tag050	Roberts <i>et al.</i> , 2006
26	Δ teg155	SAOUHSCs107	tag053	Beaume <i>et al.</i> , 2010

27	<i>ΔrsaOI</i>	teg47, srn_1490, sau6477,ssr156, SAOUHSCs076, S201	tag151	Marchais <i>et al</i> , 2009
28	<i>Δsau19</i>	teg131, rsaX21, sRNA382, srn_4680, SAOUHSCs060	tag152	Abu-Qatouseh <i>et al</i> , 2010
29	<i>Δsau27</i>	srn_2690	tag153	Abu-Qatouseh <i>et al</i> , 2010
30	<i>Δsau6053</i>	srn_2200, SAOUHSCs074,S399	tag154	Abu-Qatouseh <i>et al</i> , 2010
Red: two different mutants sharing one tag.				
31	<i>ΔsprF3</i>	srn_4090, SAOUHSCs036, S929	tag070	Pichon and Felden, 2005
	<i>ΔsprG3</i>	teg19b, srn_4100, SAOUHSCs039, S930		Pichon and Felden, 2005
32	<i>Δteg146</i>	sau63, srn_0950, SAOUHSCs068,S122	tag071	Abu-Qatouseh <i>et al</i> , 2010
33	<i>ΔsRNA37</i>	srn_0590, SAOUHSCs203, S72	tag072	Howden <i>et al</i> , 2013
34	<i>ΔsRNA334</i>	srn_9480, SAOUHSCs242	tag073	Howden <i>et al</i> , 2013
35	<i>ΔrsaA</i>	teg88, sau64, sRNA132, srn_1510, SAOUHSCs048, S210	tag075	Geissmann <i>et al</i> , 2009
36	<i>Δsau76</i>	srn_1520, SAOUHSCs164, S211	tag076	Abu-Qatouseh <i>et al</i> , 2010
37	<i>ΔrsaOI::tag77</i>	teg47, sau6477, srn_1490, SAOUHSCs076, S201,ssr156	tag077	Marchais <i>et al</i> , 2009
38	<i>Δteg149</i>	srn_1060, S128	tag078	Beaume <i>et al</i> , 2010
39	<i>Δteg7</i>	srn_3390, SAOUHSCs019, S727	tag079	Beaume <i>et al</i> , 2010
40	<i>Δteg16</i>	srn_3950, SAOUHSCs149, S883	tag080	Beaume <i>et al</i> , 2010
41	<i>ΔsRNA287</i>	srn_9340, SAOUHSCs236, S774	tag085	Howden <i>et al</i> , 2013
42	<i>ΔsRNA71</i>	srn_0890, SAOUHSCs205, SAOUHSC_A00354	tag086	Howden <i>et al</i> , 2013
43	<i>ΔsRNA207</i>	srn_2500, SAOUHSCs229	tag087	Howden <i>et al</i> , 2013
44	<i>Δteg140</i>	srn_0380, S49	tag090	Beaume <i>et al</i> , 2010
45	<i>Δteg55</i>	srn_2370, S436	tag092	Beaume <i>et al</i> , 2010
46	<i>ΔsRNA209</i>	srn_2530, S469	tag093	Howden <i>et al</i> , 2013
47	<i>ΔsRNA219</i>	srn_2660, S520	tag094	Howden <i>et al</i> , 2013
48	<i>Δteg106</i>	srn_2730, SAOUHSCs093, S540	tag095	Beaume <i>et al</i> , 2010
49	<i>ΔsRNA260</i>	srn_3280, S695	tag096	Howden <i>et al</i> , 2013
50	<i>ΔsRNA345</i>	srn_4220, SAOUHSCs083, S960	tag097	Howden <i>et al</i> , 2013
51	<i>ΔncRNA2</i>		tag099	This study
52	<i>ΔncRNA3</i>	SAOUHSCs110,S713	tag100	This study
53	<i>ΔncRNA4</i>	S945	tag101	This study
54	<i>ΔncRNA5+6</i>	SAOUHSCs114, S1065, srn_4610, sRNA377	tag102	Howden <i>et al</i> , 2013
55	<i>ΔncRNA7</i>	srn_4635, S1077	tag106	Sassi <i>et al</i> , 2015
56	<i>ΔssrS</i>	teg97, 6S, ssr80, WAN01CC8T, sRNA256, SAOUHSCs026, S685	tag107	Roberts <i>et al</i> , 2006
57	<i>ΔsprC</i>	srn_3610, SAOUHSCs031, S771	tag109	Pichon and Felden, 2005
58	<i>ΔsprF1</i>	srn_3830, SAOUHSCs035, S857	tag110	Pichon and Felden, 2005

	<i>ΔsprG1</i>	srn_3840, SAOUHSCs038, S856		Pichon and Felden, 2005
59	<i>ΔsprX2::tag111</i>	ssr6, rsaOR, teg15, srn_3820.1, SAOUHSC_A01455	tag111	Pichon and Felden, 2005
60	<i>ΔsprY2</i>	S629	tag112	Mader <i>et al</i> , 2016
61	<i>ΔsprY3</i>	S810	tag113	Mader <i>et al</i> , 2016
62	<i>Δsau41</i>		tag115	Abu-Qatouseh <i>et al</i> , 2010
63	<i>ΔsRNA258</i>	srn_9320, SAOUHSCs231, S693	tag116	Howden <i>et al</i> , 2013
64	<i>Δsau5949</i>	teg120, sRNA272, srn_3460, SAOUHSCs070	tag117	Abu-Qatouseh <i>et al</i> , 2010
65	<i>ΔsprF2</i>	srn_2230, SAOUHSCs040, S402	tag118	Pichon and Felden, 2005
	<i>ΔsprG2</i>	teg102, srn_2240, SAOUHSCs037, S401		Pichon and Felden, 2005
66	<i>ΔsprB</i>	teg9, srn_3600, SAOUHSCs030	tag121	Pichon and Felden, 2005
67	<i>ΔsprD</i>	teg14, sRNA300, srn_3800, SAOUHSCs032, S853	tag122	Pichon and Felden, 2005
68	locus3(M)		tag139	This study
69	locus2(T)		tag140	This study
70	locus1(O)		tag141	This study
71	<i>Δsau5971</i>	srn_0880, SAOUHSCs073, S109	tag142	Abu-Qatouseh <i>et al</i> , 2010
72	<i>Δhfq</i>		tag143	
73	<i>ΔsprA1</i>	srn_3580, SAOUHSCs027, S764	tag144	Pichon and Felden, 2005
	<i>ΔsprAs1</i>	teg152, srn_3590, SAOUHSCs105, S765		Pichon and Felden, 2005
74	<i>ΔsprX2::tag145</i>		tag145	Pichon and Felden, 2005
75	<i>ΔsprX1::tag146</i>	ssr6, rsaOR, teg15, sRNA299,	tag146	Pichon and Felden, 2005
76	<i>ΔrsaH::tag147</i>	teg94, sau6059, sRNA162,	tag147	Geissmann <i>et al</i> , 2009
77	<i>ΔsprY1</i>		tag148	Mader <i>et al</i> , 2016
78	<i>ΔsprX1::tag149</i>	srn_3820, SAOUHSCs033, S854	tag149	Pichon and Felden, 2005

Table 3 Five sRNA mutants removed from library v1

sRNA mutants	Barcode	Comments
<i>ΔrsaD::tag6</i>	tag006	Disruption affecting the adjacent gene
<i>Δteg49</i>	tag020	5'UTR of <i>sarA</i> gene
<i>Δsau11</i>	tag033	Same tag as <i>Δteg108</i>
<i>ΔrsaH::tag49</i>	tag049	Disruption affecting the adjacent gene
<i>ΔsprX1::tag149</i>	tag149	Construction increasing the difficulty of the interpretation of <i>sprX2::tag145 sprX1::tag149</i> double mutant

Table 4 Five sRNA mutants added to library v2

mutants	Others names	Barcode	Comments
Δ rsaC	srn_1590, SAOUHSCs050, S234	tag133	<i>Bona fide</i> sRNA
Δ S204	srn_1505, SAOUHSCs189,	tag134	<i>Bona fide</i> sRNA
Δ S596	srn_2975, SAOUHSCs275	tag135	Involved in Fur regulation
Δ S627		tag136	<i>Bona fide</i> sRNA
Δ S808	srn_9345	tag137	<i>Bona fide</i> sRNA

3.1.2.2 Fitness experiments

All cultures were performed in aerobic conditions at 37°C, 180 rpm. Experiments were initiated by the dilution of the thawed out tagged mutant library (v1 or v2) 1000-fold into a fresh TSB medium without or with antibiotics. For each experiment, an aliquot of the initiating culture was kept as a control sample. Briefly, cells were sampled at OD = 1, OD = 7.5 and 24 h. The overnight culture was then diluted in the same medium 1/1000 times and sampled at OD = 1 twice (Figure 26). Alternatively, to test the impact of long stationary phase on the mutant libraries, cells were maintained in the same medium up to 11 days with sampling every 24 h (Figure 29). Sampled cells were centrifuged briefly and kept at -80°C before further analysis. Triplicate samplings (*i.e.* set 1, 2 & 3) were performed at the same time.

3.1.2.3 DNA-seq libraries

The proportion of each DNA barcode reflecting the proportion of each mutant in the mix was determined by DNA sequencing by NGS technology as described in (Le Lam *et al.* 2017). Briefly, DNA was extracted from sampled pellets, PCR amplified with multiplexing primers to allow the pool of 50 experiments in one NGS run. Each tested sample was PCR amplified with a couple of primers that are different on their last 5' end nucleotides to subsequently associate tags to given conditions (*c.f.* Chapter II Section 2.1). We designed 50 different pairs of primers.

3.1.3 Analysis of deep sequencing data

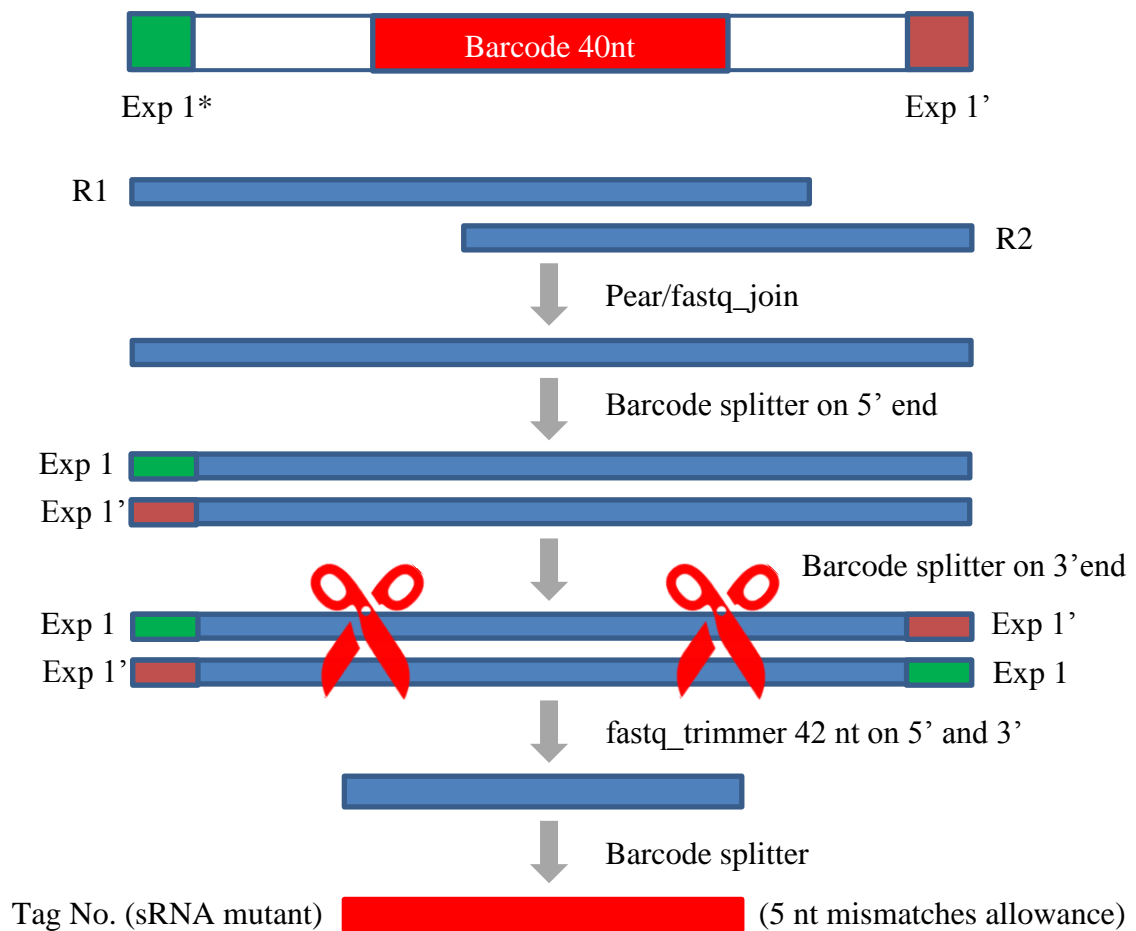
Barcodes counting. We performed paired-end DNA-seq (75 to 90 nt) to get a complete tag sequence with forward and reverse experiment identifiers (Figure 22). The following processes were performed with Galaxy platform (<https://usegalaxy.org/>). Forward and reverse paired-end sequences were merged with “Pear” (Zhang *et al.* 2014) or “fastq-join” (Aronesty 2013) to obtain full length barcode sequences. Multiplexed experiments were sorted first, by the five nt experimental identifiers with “barcode splitter” set as “no mismatch” (http://hannonlab.cshl.edu/fastx_toolkit/) and second, by the 3' ends of PCR amplicons. At this step it was checked that 5' and 3' identifiers were coherent. Chimeric sequences with identifiers

from different experiments were discarded. Barcodes with matched experimental identifiers were further processed. Tag sequences were retrieved from the whole amplicon sequences by “fastq_trimmer” (Blankenberg *et al.* 2010) and mutants were sorted with a 5 nt mismatch allowance with “barcode splitter” (http://hannonlab.cshl.edu/fastx_toolkit/).

Ratio calculation. The proportion of each mutant in the different tested conditions was determined by calculating the ratio of the frequency of the barcode in the tested condition on the frequency in a reference condition, *e.g.*, growth in aerobic TSB medium at 37°C. Standard deviation is determined on the ratios obtained from three independent biological experiments.

DESeq2. DESeq2 is a statistical approach for differential analysis of count data (Love *et al.* 2014). It utilizes shrinkage estimation for dispersions and fold changes to improve stability and interpretability of estimates, and therefore, enables a quantitative analysis focused on the strength rather than the mere presence of differential expression (Love *et al.* 2014). We performed a DESeq2 analysis using SARTools on Galaxy platform with default parameters.

Both analyses were performed with the two calculation approaches. In the following chapters, data will be presented with the latest. We present mutants differentially enriched compared to the reference with a fold change enrichment > 3 in the given condition.



Exp 1* → Experimental identifier Primer 1

Figure 22 Barcodes counting procedure with Galaxy tools

3.1.4 Determination of sub-lethal/sub-inhibitory concentration of antibiotics

Overnight culture of library v1 was prepared. Antibiotic sub-lethal concentrations of HG003 library v1 were determined by plating 10^3 CFU on BHI plates with 2-fold serial dilutions of the antibiotics; colonies were counted on each plate and the concentration leading to ~ 50% reduction was chosen.

3.2 Results

3.2.1 sRNA::tag mutant libraries.

To study the sRNA mutant adaptation to different growth conditions and antibiotic stress, we used a set of reported tagged mutants (Le Lam *et al.* 2017) complemented with 53 additional ones (*c.f.* material and methods). The selection of the sRNA genes included in this study was based on the expert analysis presented in Chapter I. For most constructions, the promoter region and a large portion of the sRNA gene were deleted. In few cases, the sRNA promoters were kept because they could possibly contribute to the expression of downstream genes. The recent release of *S. aureus* transcriptomic data in 44 growth conditions was used for optimizing deletion design of our study (Mader *et al.* 2016). The 53 additional mutants were constructed in HG003 using pIMAY (*c.f.* Section 3.1.1.5). All new mutants were constructed in triplicate to generate three biological sets (Table S3b). The deletions of Δ *rsaD*, Δ *rsaH* and Δ *rsaOI* mutants reported in Le Lam *et al.* (2017) were overlapping with adjacent UTRs and therefore were reconstructed. The mutants were assembled in two libraries, v1 and v2; their composition is indicated in Table 2 and Table 4, respectively.

Mutant controls were constructed:

- i) Three strains with tag insertions in “neutral” loci (with no detected transcription) located either next to the origin of replication, to the terminus or in between, named locus 1 origin (O), locus 2 terminus (T) and locus 3 middle (M), respectively.
- ii) Identical sRNA gene deletions with two different tags (*i.e.* Δ *sprX1* and Δ *sprX2*). In addition, a double-mutant was constructed with tags allowing the discrimination of the single and double mutants (Figure 23).

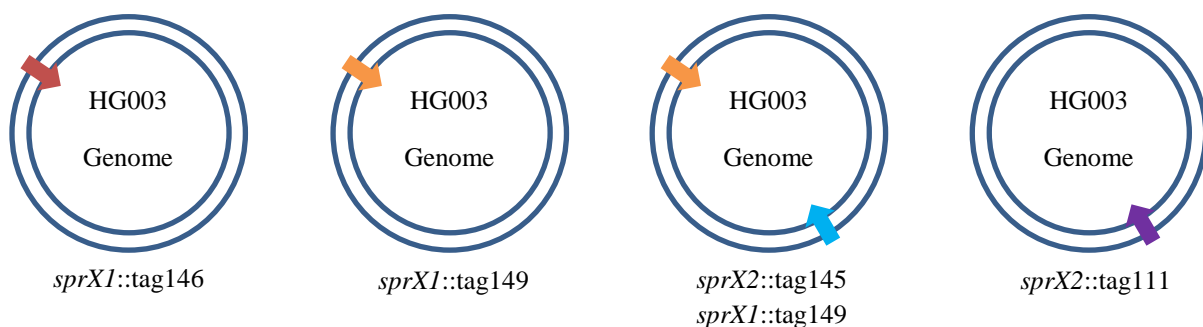


Figure 23 Δ *sprX1* and Δ *sprX2* constructions

3.2.2 Library validations

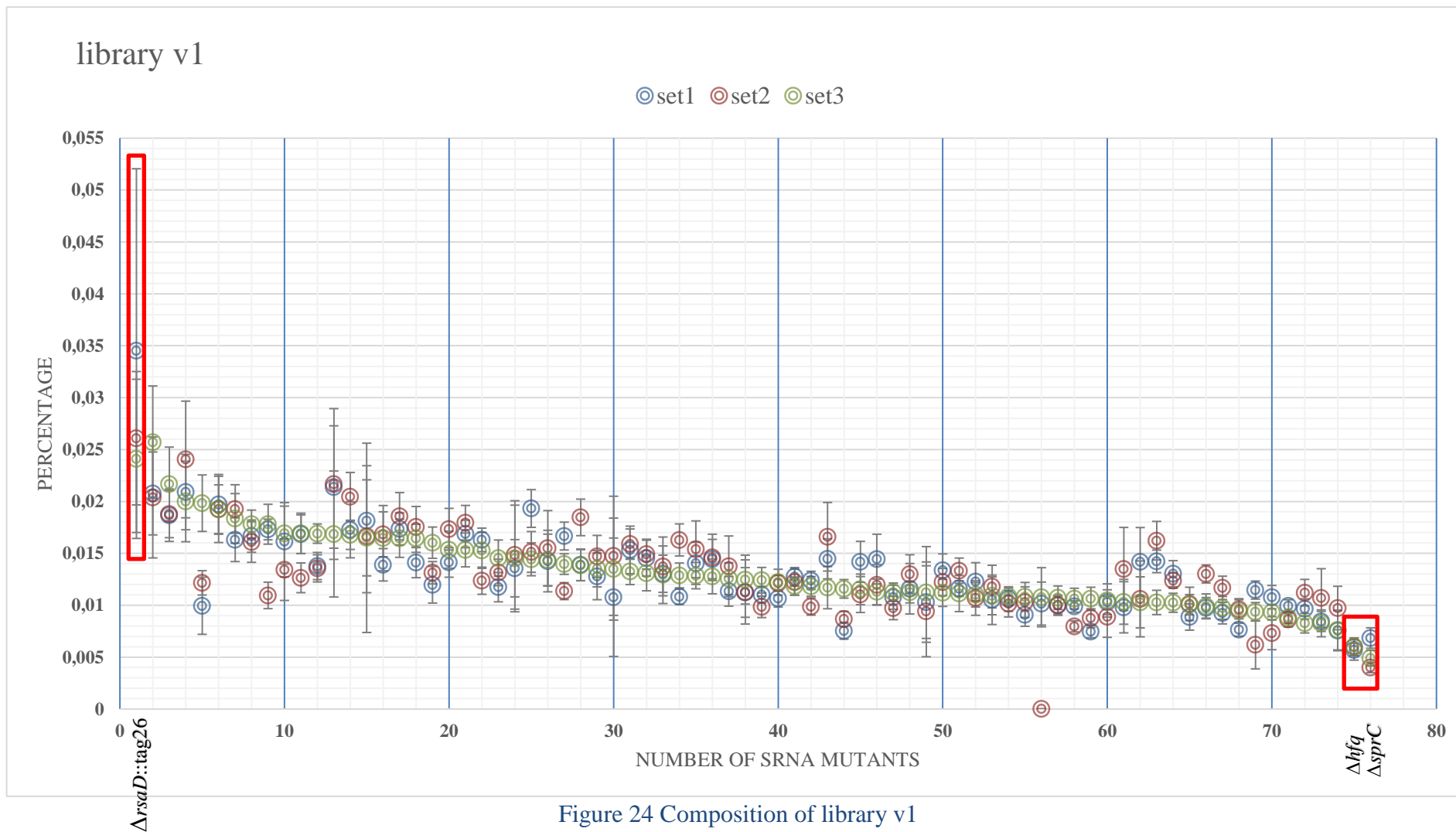
The methodology for fitness experiments is described in details in Section 3.1.2.2. Twelve conditions (control, starvation and 10 antibiotics) were tested with library v1. In addition, the control was also tested with library v2.

The proportion of each mutant in the control and starvation conditions were normalized to the starting inoculum. Antibiotics conditions were normalized i) to the starting inoculum, and ii) to samples grown in the same medium and temperature without the antibiotic (control condition, aerobic TSB medium at 37°C). These two controls avoid any overlook of storage condition impact and of mutants with general growth defect or advantage.

3.2.2.1 Library composition

The libraries contain about 80 mutants that were assembled by putting the same quantity of each mutant (normalized to the same optical density). The ratio of each mutant within the library should be between 0.0125~0.013. Each time libraries were used for fitness experiments, the mutant proportions from the starting inoculum were determined. Consequently, the composition of each set (*i.e.* 1, 2 & 3) for libraries v1 and v2 were determined eleven and two times, respectively (Figure 24 and Table S4 for library v1, and Figure 25 and Table S5 for library v2). The variation between independent triplicates (set 1, 2 & 3) illustrated by error bars are low except for $\Delta rsaD::tag26$ in library v1 (mark in red frame) due to a technical imprecision, it was added post-assembly. For the same reason, the proportion of $\Delta rsaD::tag26$ was higher than expected (2.6 fold). This issue was solved in v2 library (Figure 25).

Interestingly, the proportions of $\Delta sprC$ and Δhfq were significantly reduced both in v1 and v2 libraries (mark in red frame). We suggest that the optical density measured for these two mutants does not reflect the usual bacterial cell number; morphological changes, aggregation or general composition alterations with these mutants could affect the measures. A second hypothesis is a partial lysis happening possibly during the storage, reducing the tag count of $\Delta sprC$ and Δhfq in comparison to other mutants. Thus, apart from these anomalies, libraries are composed of mutants in stoichiometric proportions.



library v2

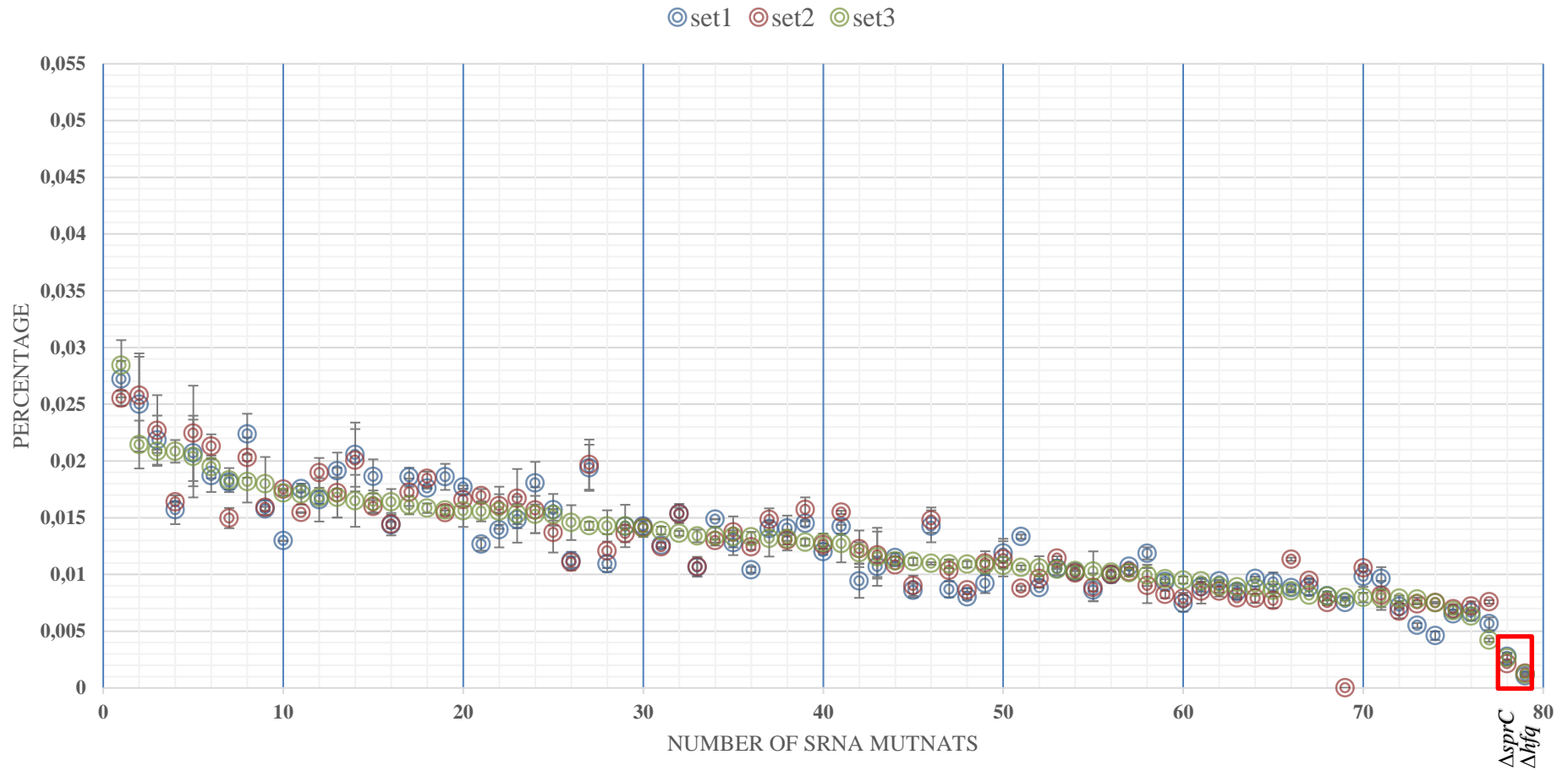


Figure 25 Composition of library v2

3.2.2.2 Mutants affected by growth in the control condition.

Prior testing the effect of stress conditions on mutant libraries, we questioned the behavior of each mutant in “control” growth conditions. We therefore grew the library v1 (set 1, 2 & 3) in TSB medium at 37°C. Samples were withdrawn at early exponential phase (OD₆₀₀=1), late exponential phase (OD₆₀₀=7.5) and overnight (24 h). Then, the overnight cultures were diluted 1000-fold into fresh TSB medium and samples were collected at OD₆₀₀=1. This operation was repeated on day 3 (Figure 26). This experiment addresses the impact of stationary phase on mutant fitness, and the successive dilution increase the contrast between mutant ratios, they serve as internal controls.

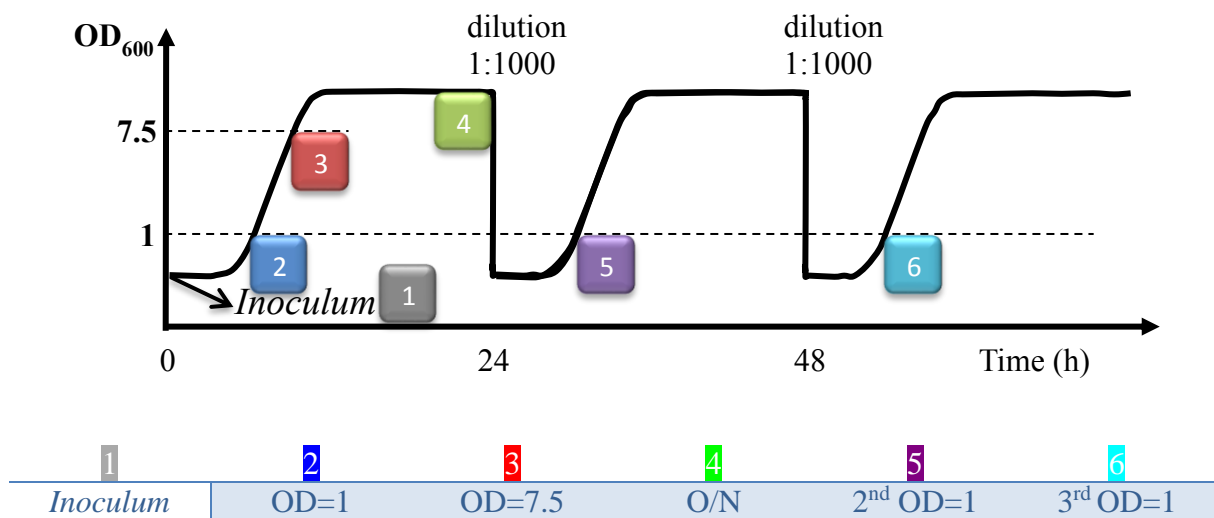


Figure 26 Serial dilution fitness experiments

Barcode sequences from collected samples were counted for library v1 as indicated (*c.f.* Section 3.1.3). Mutants were considered to be significantly affected by growth conditions when they either accumulate or decrease at least 3 times compared to the rest of the population. Mutants leading to significant variations are presented Figure 27.

Δ *rsaD::tag6* had a significant growth disadvantage in the control growth condition. However, it was not the case for Δ *rsaD::tag26*. Δ *rsaD::tag6* was constructed prior having high quality transcriptomic data. We noticed lately that the Δ *rsaD::tag6* deletion interfered with the promoter region of SAOUHSC_00651; we therefore constructed Δ *rsaD::tag26*. We conclude that the growth disadvantage in TSB of Δ *rsaD::tag6* is likely due to the alteration of SAOUHSC_00651 promoter and UTR region. The SAOUHSC_00651 gene encode a putative membrane protein of unknown function.

$\Delta sau69$ mutant accumulates more than the others with time in the control condition. As transcriptomic data with higher resolution were obtained, we found that $\Delta sau69$ removes the 5'UTR of *prsA*, which encode a lipid-anchored protein conserved in all Gram-positive species. PrsA assists the post-translocational folding between the outer surface of the cytoplasmic membrane and cell wall (Jousselin *et al.* 2012).

The same experiment was performed with a second version of the library (v2) in which $\Delta rsdD::tag6$ was removed (Figure 28). $\Delta sau85$, $\Delta sau6428$, $\Delta sau6836$, $\Delta sau60$ were under-represented after growing in the control condition using libraries v1 and v2. However, for those accumulating, only $\Delta sau69$ was in both experiments. With library v2, $\Delta S810$ ($\Delta sprY3$) and $\Delta rnaIII$ had a significant growth advantage that was reproduced with the three independent sets (1, 2 & 3). Differences between the two libraries are possibly due to their different composition, which in turn may affect the growth of specific mutants.

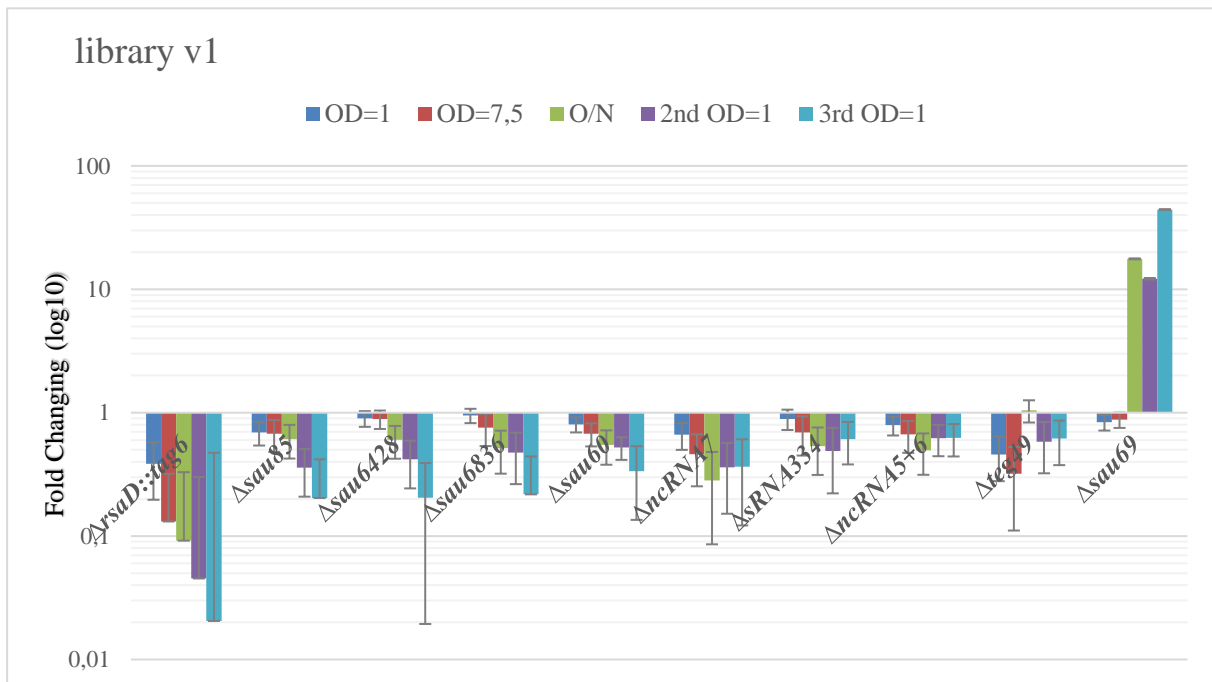


Figure 27 Changing fold of sRNA mutants of library v1 in control condition

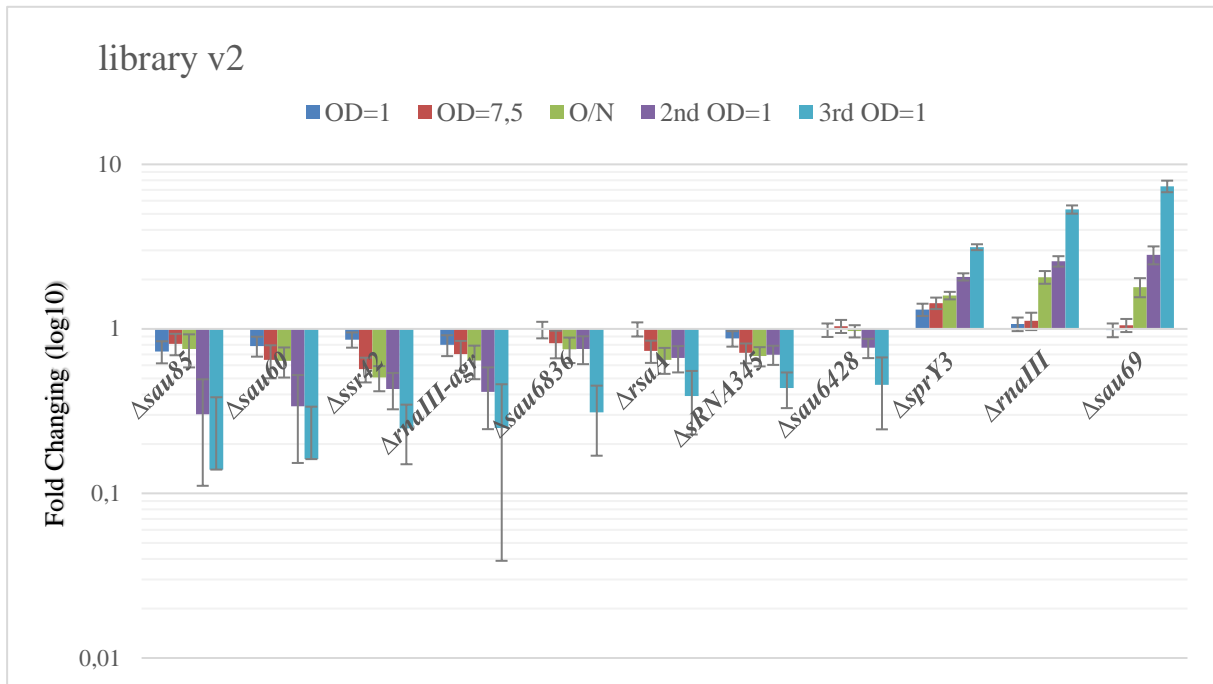


Figure 28 Changing fold of sRNA mutants of library v2 in control condition

3.2.2.3 Mutants affected by long term stationary phase

During the control growth condition, bacteria underwent repeated stationary phases. We considered that some mutants might accumulate or disappear because of transient growths in stationary phase. To address this question, we grew the library aerobically in liquid TSB at 37°C and kept the culture for 11 days in the same medium. Samples were withdrawn at day 1, 3, 5, 7, 9 and 11 (Figure 29). Mutants leading to significant variations are presented (Figure 30).

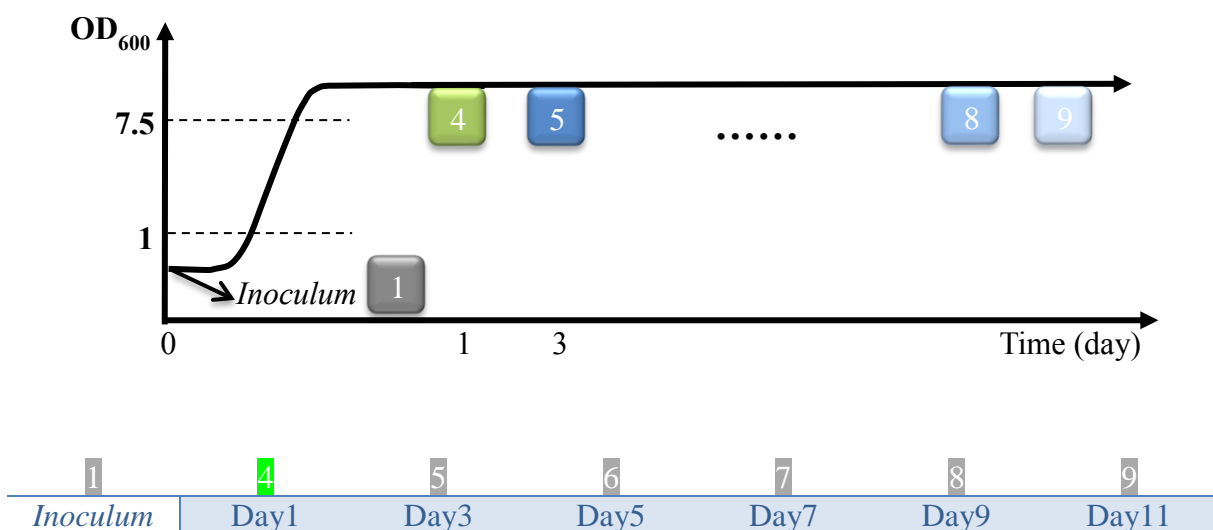


Figure 29 Starvation fitness experiments.

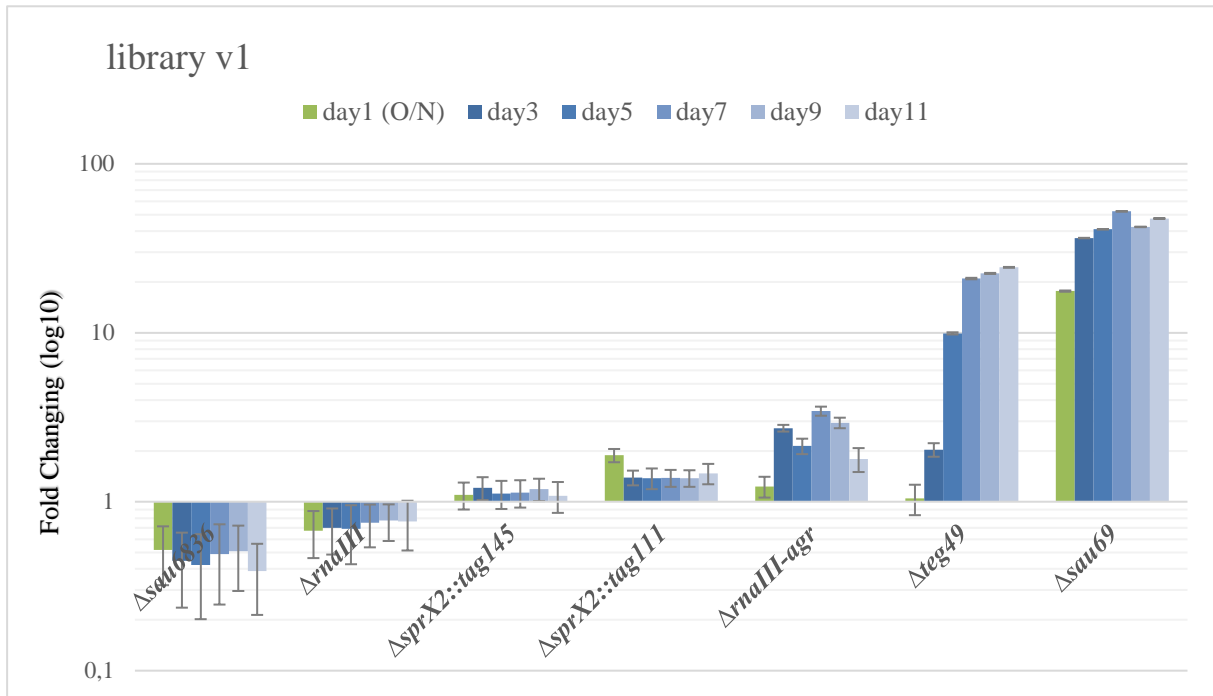


Figure 30 Proportions of sRNA mutants in library v1 after a long stationary phase.

The $\Delta rnaD::tag6$ mutant is not underrepresented in the stationary phase suggesting that its disappearance in the control experiments takes place during the exponential phase.

$\Delta sau69$ mutant was overrepresented in the control but also in prolonged stationary phase experiments. Alteration of *prsA* provides a growth advantage during stationary phase. $\Delta teg49$ tends to disappear in the control condition (present only in v1) but was strongly overrepresented in prolonged stationary phase, suggesting that its accumulation is associated with stationary phase. *teg49* is at same time a sRNA and a 5'UTR of *sarA* gene (Kim *et al.* 2014). *sarA* has three promoters, P1, P3 and P2 and encodes a staphylococcal global regulator playing a critical role in virulence, antibiotic resistance and biofilm formation. The $\Delta teg49$ deletion prevents the expression of Teg49 but also alters *sarA* expression. It is likely that the $\Delta teg49$ accumulation in stationary phase is SarA-dependent. Consequently, we suggest that the deregulation of SarA and PrsA increases greatly staphylococcal fitness in stationary phase.

The $\Delta sau6836$ mutant tends to disappear but the effect is not due an sRNA as the deletion that we constructed alters the promoter region of two genes:

- *abcA*, encoding an ATP binding cassette transporter-like protein, a component of a multidrug efflux system against various antibiotics and chemicals (Yoshikai *et al.* 2016).

- *pbp4*, encoding the transpeptidase and DD-alanine carboxypeptidase penicillin-binding protein 4, involved in secondary cross-linking of the peptidoglycan layers (Kozarich and Strominger 1978; Wyke *et al.* 1981; Henze and Berger-Bachi 1996).

3.2.3 Fitness experiments in the presence of antibiotics.

The library v1 of tagged sRNA deletions was challenged with sub-lethal concentration of antibiotics. Growth condition, samplings and data analysis were performed as for the control growth condition except that the TSB medium was supplemented with sub-lethal concentration of antibiotics. Ten antibiotics clinically relevant for *S. aureus* were chosen for the study. These antibiotics target different processes including bacterial cell wall and membrane formation, protein synthesis, DNA and RNA biosynthesis. Their sub-lethal concentration for the fitness experiments were determined as described (Material and Methods) (Table 5).

Table 5 sub-lethal concentration of antibiotics measured for HG003 and compared to published MIC.

Mechanism	Envelope synthesis inhibitors				
Targets	cell wall				cell membrane
Antibiotics	vancomycin	flucloxacillin	cloxacillin	cefazolin	daptomycin
MIC against MSSA* (µg/ml)	0.25-2	0.25-0.5‡	0.125-0.25†	0.5-4	0.125-0.25
HG003 sub-lethal concentration (µg/ml)	1.0	0.1	0.0625	0.1	2.75

Mechanism	Protein biosynthesis inhibitors			DNA replication	RNA synthesis
Targets	ribosomal 30S	ribosomal 50S		DNA gyrase/topoisomerase	RNA polymerase
Antibiotics	gentamicin	linezolid	clindamycin	ciprofloxacin	rifampicin
MIC against MSSA* (µg/ml)	0.06-16	0.5-1.0	0.06-256	0.03-4.0	0.01-1.0
HG003 sub-lethal concentration (µg/ml)	0.8	0.3	4.5	0.1	0.006

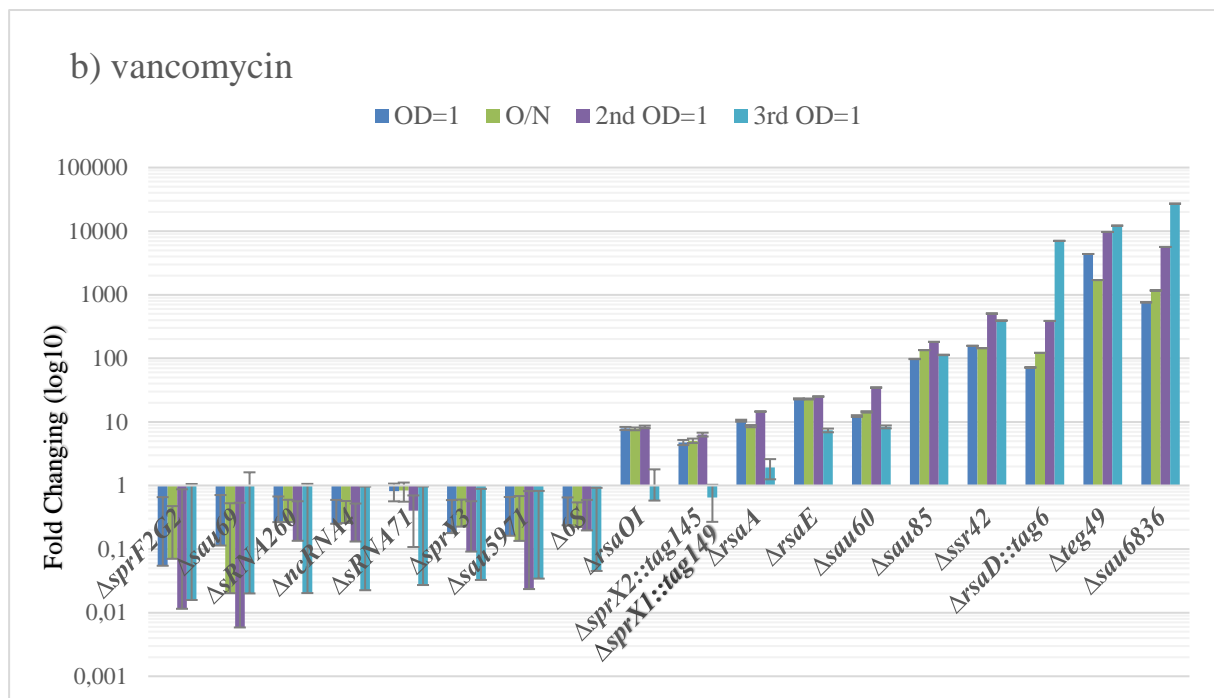
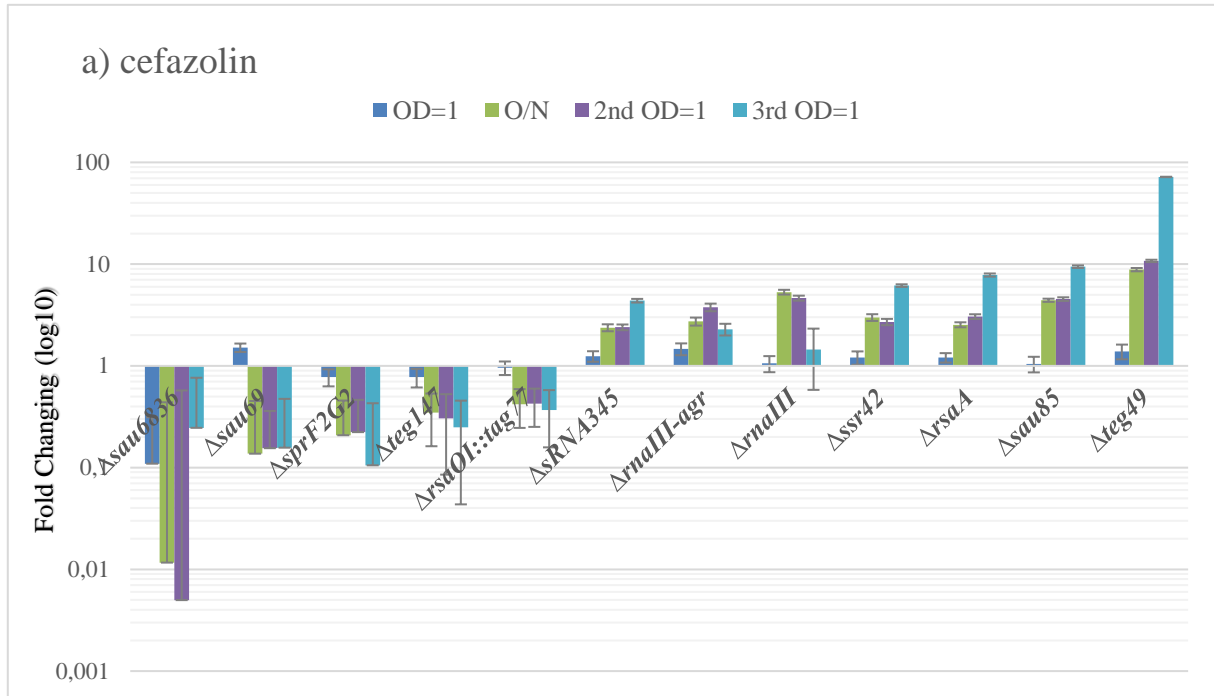
* Reference from <http://www.antimicrobe.org/b237tabrev.htm> except ‡ and †.

‡ Reference from (Sutherland *et al.* 1970; Rijnders *et al.* 2009)

† Reference from (Baumgartner and Glauser 1983; Fernández Guerrero and Górgolas 2006)

3.2.3.1 Antibiotics targeting bacterial envelope

The sRNA mutant fitness (library v1) was tested in the presence of cefazolin (Figure 31a), vancomycin (Figure 31b), flucloxacillin (Figure 31c), cloxacillin (Figure 31d), and daptomycin (Figure 34); only results with sRNAs leading to significant variations are shown.



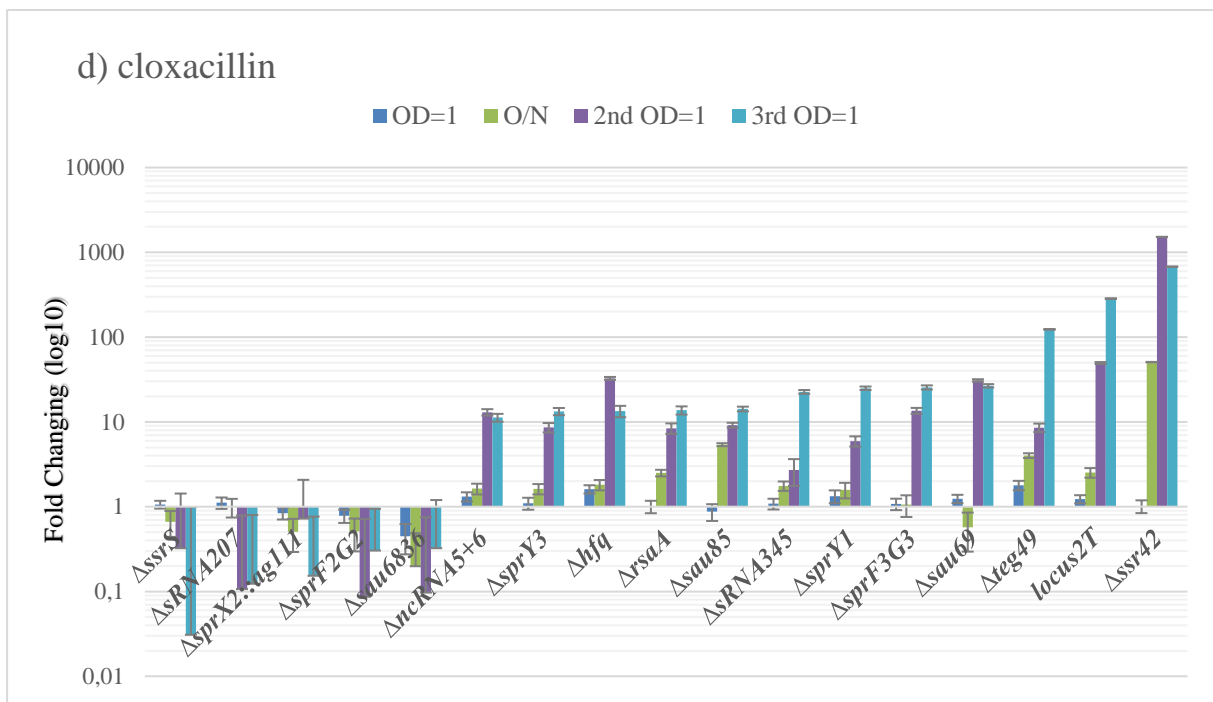
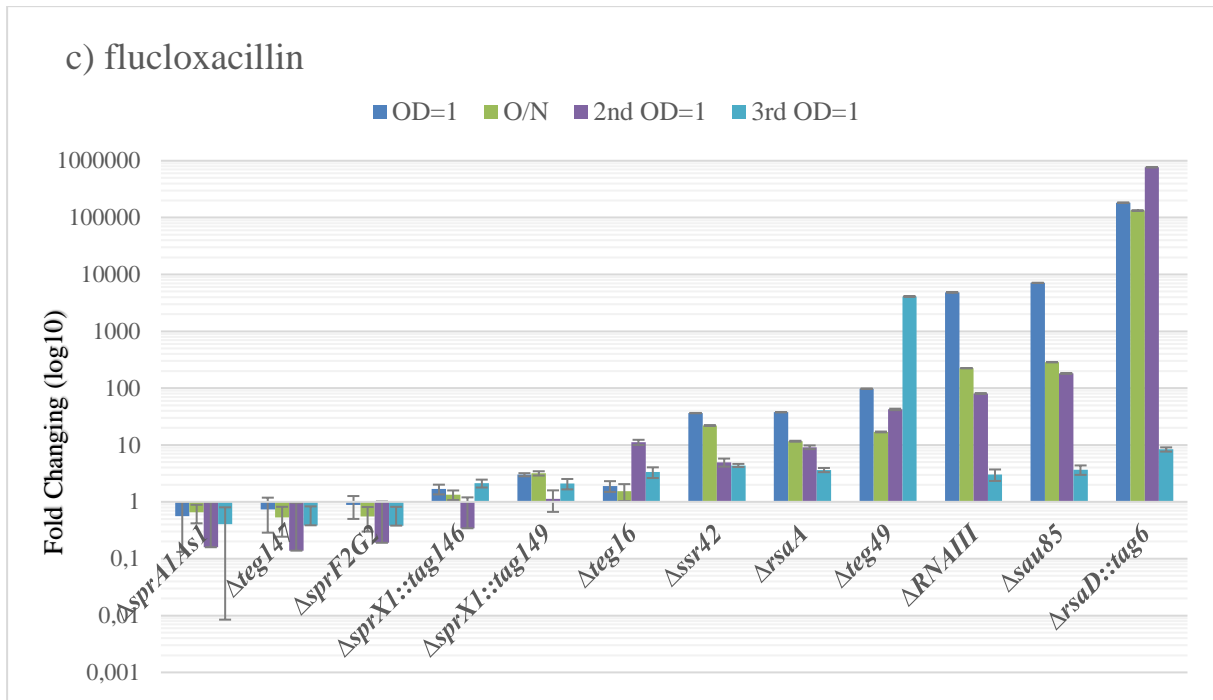


Figure 31 sRNA mutants of library v1 affected by sub-lethal concentrations of the indicated antibiotics

In the presence of cefazolin, $\Delta sRNA345$, $\Delta rnaIII$, $\Delta ssr42$, $\Delta rsaA$, $\Delta sau85$, and $\Delta teg49$ mutants accumulate. It is interesting to note this observation is shared for i) $\Delta ssr42$, $\Delta rsaA$, $\Delta sau85$ and $\Delta teg49$ with vancomycin, ii) $\Delta rnaIII$, $\Delta ssr42$, $\Delta rsaA$, $\Delta sau85$ and $\Delta teg49$ with flucloxacillin, and iii) $\Delta sRNA345$, $\Delta ssr42$, $\Delta rsaA$, $\Delta sau85$, and $\Delta teg49$ with cloxacillin (however, see after for comments on vancomycin and flucloxacillin) suggesting that these deletions are likely related to general cell-wall perturbations.

In contrast, the $\Delta sau6836$ mutant tends to disappear in cefazolin, but accumulates strongly in vancomycin. However, the $sau6836$ deletion, as indicated above, alters the promoter region of *abcA* and *pbp4*. *abcA* confers intrinsic resistance to β -lactams (Villet *et al.* 2014) and the disruption of *pbp4* reduces peptidoglycan cross-link, resulting in an increase of free D-Ala-D-Ala residues, which is vancomycin target (Peleg *et al.* 2009). It is supposed that vancomycin binds to these free D-Ala-D-Ala residues in the outer layers of the cell wall and is unable to reach its site of action at the cell membrane (Howden 2005). The trapped vancomycin molecules within the cell wall clog the peptidoglycan meshwork and form a physical barrier towards further incoming vancomycin molecules (Howden 2005; Cui *et al.* 2006). The increased resistance to β -lactams associated with a decrease glycopeptide resistance is called the “seesaw effect”. As mentioned above, the deletion of $sau6836$ influences the expression of *abcA* and *pbp4* (Figure 32) and the “seesaw effect” is observed (Figure 33). While the $\Delta sau6836$ mutant does not affect an sRNA, the results obtained with this deletion support the fitness approach.

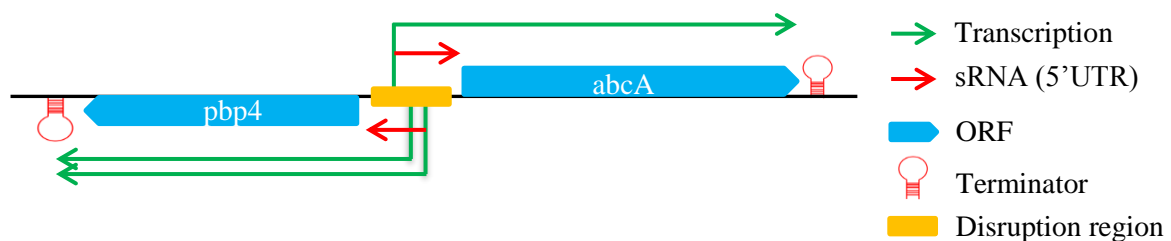


Figure 32 Schematic presentation of *sau6836*, *pbp4* and *abcA*.

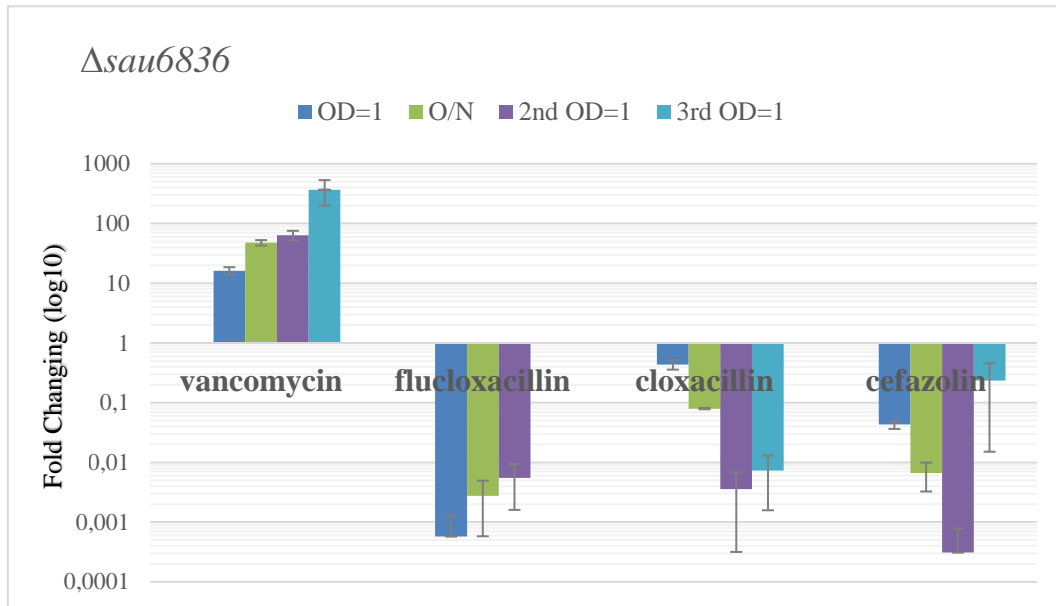


Figure 33 “Seesaw effect” observed with beta-lactams and the glycopeptide vancomycin.

RsaA deletion accumulates in presence of all tested antibiotics targeting bacterial cell wall. RsaA targets autolysins and the global regulator MgrA, which among others, regulates AbcA transporter (Truong-Bolduc and Hooper 2007; Romilly *et al.* 2014; Tomasini *et al.* 2017). RsaA implication in cell-wall turn-over was shown but so far, no phenotype in presence of beta-lactams or glycopeptides had been demonstrated.

The regulatory RNAs Srr42 (Morrison *et al.* 2012) and RNAIII (Novick *et al.* 1993) that are considered as virulence factors are also contributing to a better survival in the presence of antibiotics targeting the envelope. An increase cell wall turnover, a change of surface charge, a reduced *agr* activity are known to be implicated in glycopeptide resistance (Park *et al.* 2012). RNAIII targets different cell-wall-associated protein mRNAs such as *spa*, *ltaS* and *lytM*. LtaS is part of lipoteichoic acid biosynthesis pathway and LytM is an autolysin. Their translation is inhibited by RNAIII (Yan *et al.* 2014; Lioliou *et al.* 2016) and we can hypothesize that RNAIII deletion increases their expression level, modifies bacterial envelope homeostasis decreasing beta-lactams and glycopeptides sensitivity. In this experiment, $\Delta RNAIII$ is over-represented only in cefazolin and flucloxacillin both targeting PBPs so that the proposed fitness experiments might be a mean to investigate antibiotic specificities and compare mechanistic.

The concentration of vancomycin and flucloxacillin used for the fitness experiments resulted in slower growth rates, lower growth yields and more dispersed results. The population composition was dominated by the accumulation of mutants *rsaD::tag6*, *teg49*, *sau85*, *ssr42*

and *sau6836*, amplifying the disappearance of the others. Consequently, these experiments should be interpreted with caution, and redone with lower antibiotic concentrations.

While the three above-discussed antibiotics affect peptidoglycan synthesis, daptomycin targets the membrane. Mutant proportions were less affected by daptomycin than by the other antibiotics (Figure 34). Only Δ *sau5971* had an almost 10-fold under-representation at the end of the experiment. The mutant was also affected by alkaline pH (Le Lam *et al.* 2017). It was proposed that Δ *sau5971*, may come from the processing of SA0355, in strain N315 (Lioliou *et al.* 2012) but recent data in strain HG001 suggest that it is expressed from its own promoter. Δ *sau5971* is likely a *bona fide* sRNA of about 100 nt. Interestingly, the expression of two putative Δ *sau5971* targets (best binding score around the SD using RNAPredator (Eggenhofer *et al.* 2011)) SAOUHSC_02596 and SAOUHSC_01353, are induced by colistine (Mader *et al.* 2016), an antibiotic that, like daptomycin, alters the membrane. Of note, despite big standard deviations, Δ *sprF2G2* is negatively affected by all antibiotics that targeting bacterial envelope. *sprF2G2* is part of a toxin-antitoxin system which may contribute to survival to antibiotic targeting bacterial envelope.

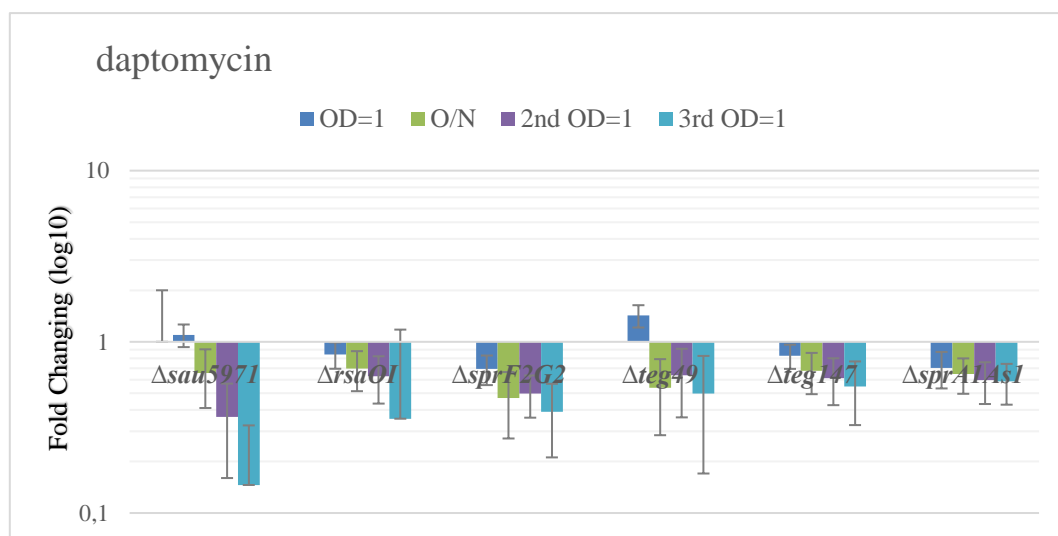


Figure 34 sRNA mutants of library v1 affected by sub-lethal concentration of daptomycin.

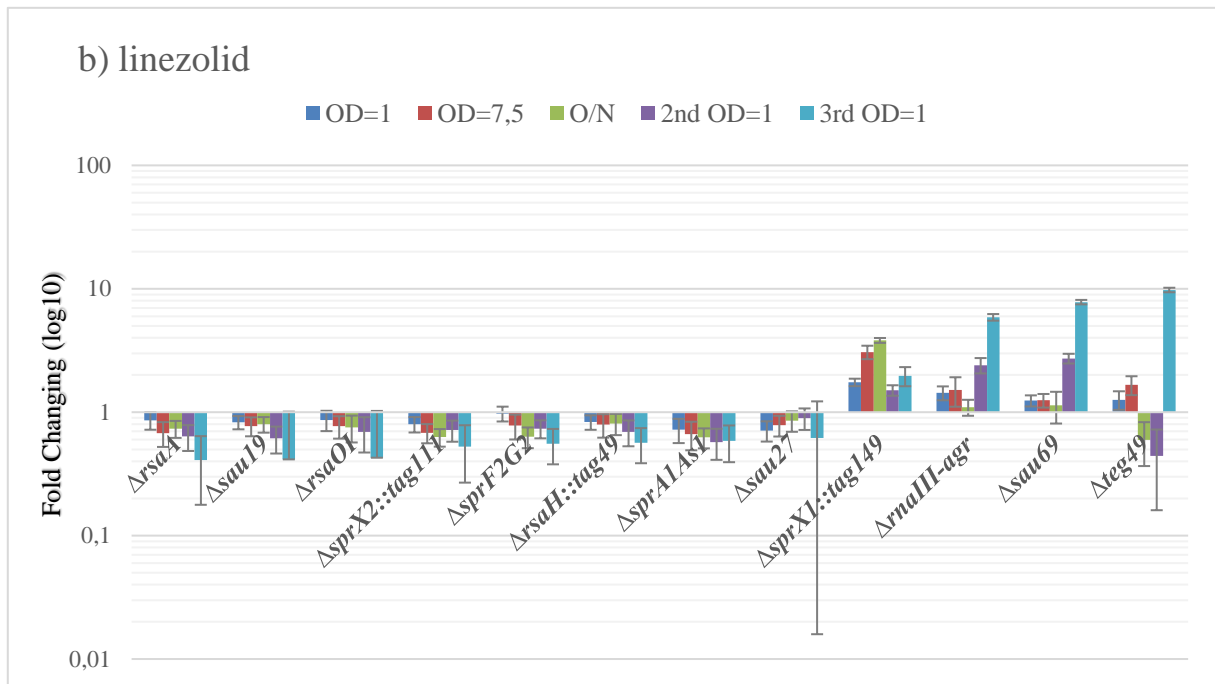
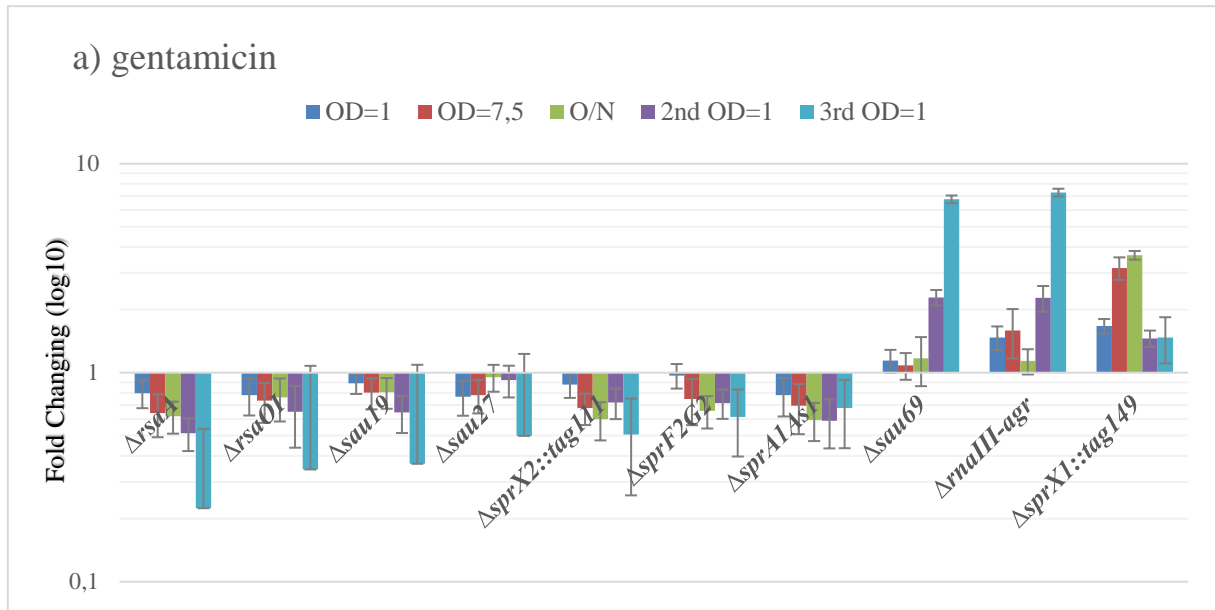
3.2.3.2 Antibiotics inhibiting protein synthesis

The sRNA mutant fitness (library v1) was tested in the presence of gentamicin (Figure 35a), linezolid (Figure 35b) and clindamycin (Figure 35c); only results with sRNAs leading to significant variations are shown.

It is striking that the inhibition of protein synthesis by gentamicin and linezolid results in largely overlapping results despite their two different mode of action. In both cases, the Δ *rsaA* strain was the most negatively affected, but Δ *sprF2G2*, Δ *sprA1S1* and Δ *sprX2::tag111* strains were also progressively eliminated. Also, strains Δ *sau69* and Δ *rnaIII-agr* were strongly accumulated during growth in presence of gentamicin and linezolid. Δ *sprF2G2*, as well as Δ *sau69* and Δ *rnaIII-agr* were also affected by clindamycin, while Δ *ssr42*, Δ *ssrS*, Δ *sRNA345* and Δ *sau5971* were accumulating only in clindamycin.

As discussed above, phenotypes associated with Δ *sau69* are likely due an altered expression of the PrsA foldase (Jousselin *et al.* 2015). The proportion of *rnaIII-agr* double mutant shows a gradual accumulation among gentamicin, linezolid and clindamycin. The absence of any effect on the *rnaIII* mutant suggests that the observed result is *agr*-dependent. The effect of linezolid on *agr* is unclear. The expression of RNAIII was shown to be reduced upon linezolid exposure (Tsuji *et al.* 2012; Soon *et al.* 2016), which would be coherent with a selective advantage for the *rnaIII* mutant although the relationship with *agr* has not been drawn. Clindamycin at sub-inhibitory concentrations inhibits differentially exoproteins and cytoplasmic proteins; the latest being unaffected. Clindamycin changes the expression of RNAIII targets at the transcription level: *spa* and *hla* are inhibited but *coa* and *fbpB*, are stimulated. *agr* and *sar* did not seem to be affected (Herbert *et al.* 2001). As a consequence, clindamycin exposure mimics RNAIII deletion and/or a relative overproduction of Rot. Clindamycin effect on Agr/RNAIII system is thus indirect and must be primarily on an upstream regulator. Nevertheless, the fitness experiment shows *agr* deletion favors bacterial development upon ribosome targeting antibiotics.

The putative targets proposed for the unknown sRNAs by RNAPredator do not provide any explanation for the observed phenotypes; they would need further characterization.



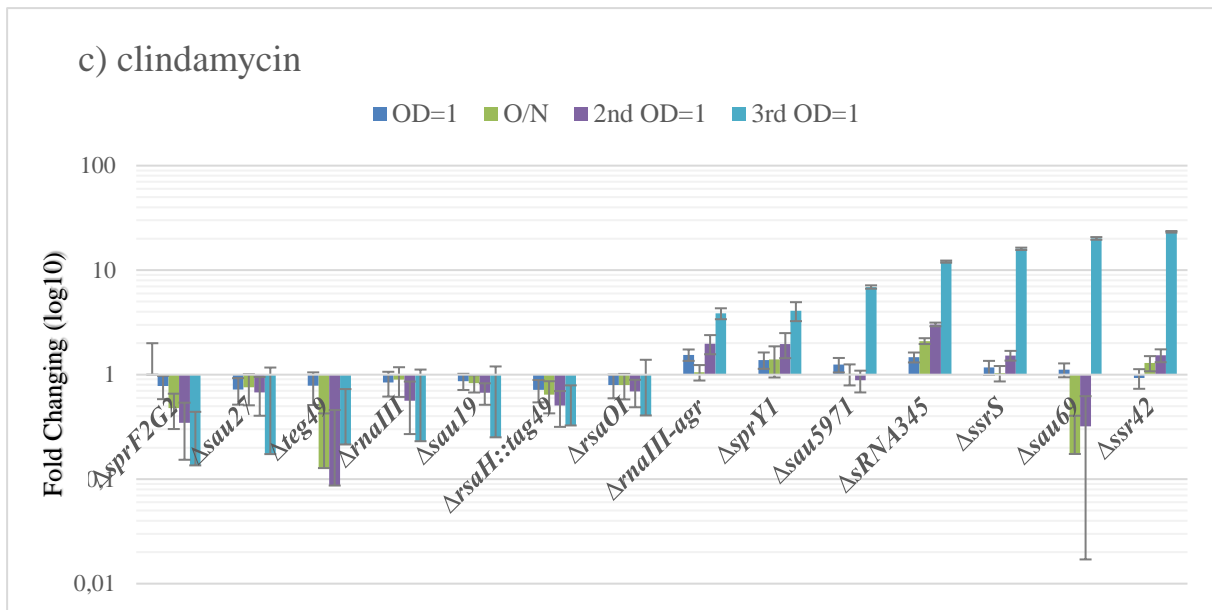


Figure 35 sRNA mutants of library v1 affected by sub-lethal concentration of the indicated antibiotics

3.2.3.3 Ciprofloxacin, an antibiotic inhibiting DNA replication

The sRNA mutant fitness (library v1) was tested in the presence of ciprofloxacin. Results with sRNAs leading to significant variations are shown (Figure 36).

Several mutants affecting *bona fide* sRNAs ($\Delta rsaA$, $\Delta rsaE$ and $\Delta rsaH$), progressively disappear in the presence of ciprofloxacin, a second-generation quinolone inhibiting DNA gyrase. In contrast, beside $\Delta teg49$ and $\Delta sRNA345$, the proportion of several *spr* mutants strongly increase in the presence of ciprofloxacin. ($\Delta sprY3$, $\Delta sprY1$, $\Delta sprX1::tag149$ and $\Delta sprX2::tag145$). *spr* genes are located on genomic pathogenicity islands and most of them encode putative toxin-antitoxin (TA) systems. We suggest that ciprofloxacin by affecting the DNA supercoiling may stimulate the expression of toxins; consequently, the deletion of TA system would provide a selective advantage.

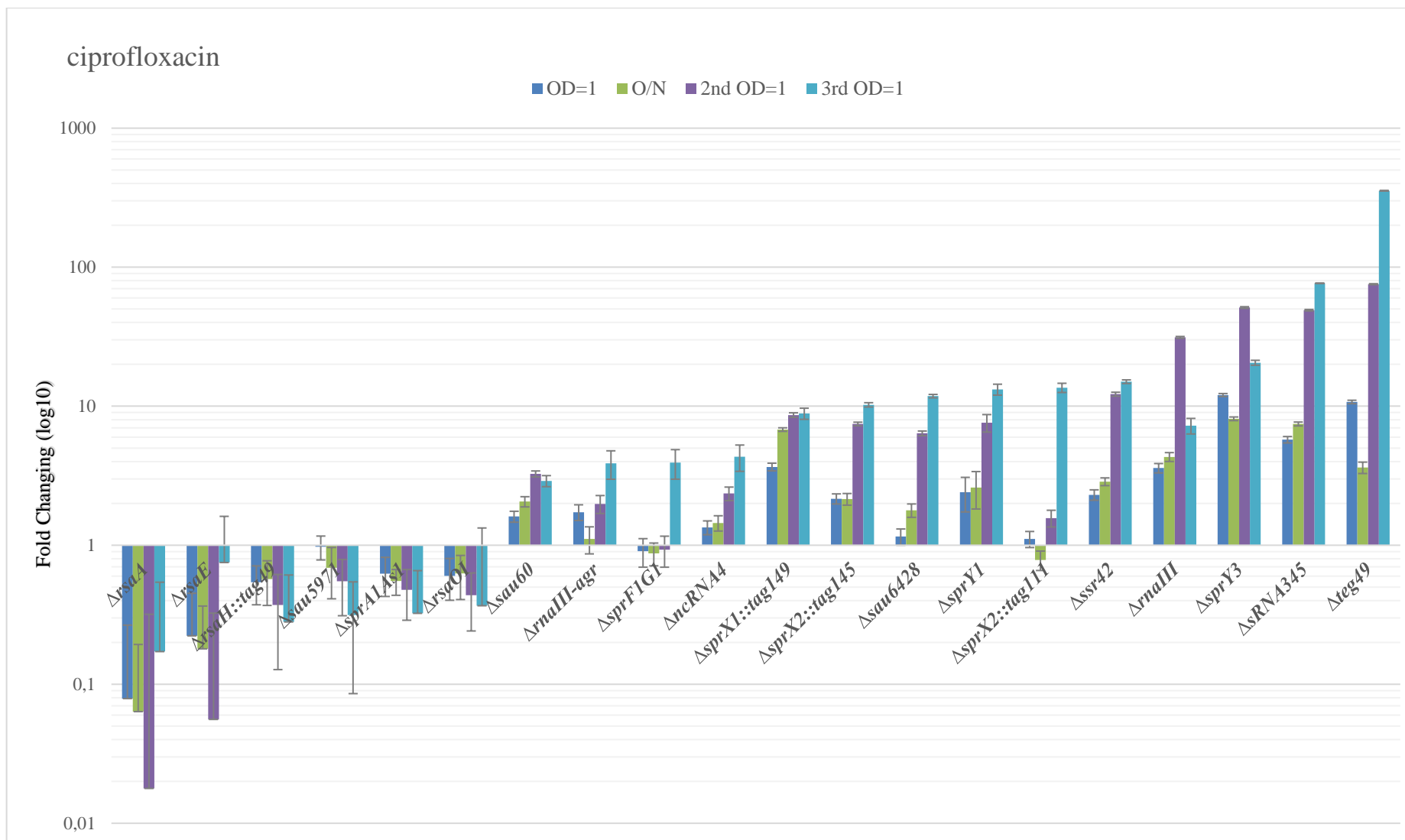


Figure 36 sRNA mutants of library v1 affected by sub-lethal concentration of ciprofloxacin.

3.2.3.4 Effect of rifampicin on regulatory RNA mutants

The work presented in this section was performed in collaboration with Marick Esberard during a Master internship in our team.

Rifampicin is an RNA polymerase inhibitor, which binds to its β subunit in the DNA/RNA tunnel, blocking the transcript elongation during the initiation step (Campbell *et al.* 2001). Competition experiments between the different mutants were performed in presence of sub-lethal concentration of rifampicin (6 ng/ml) as indicated (Table 4). Mainly, two strains were progressively underrepresented: $\Delta ssrS::tag107$ and $\Delta sau60::tag3$. After about 24 generations, the proportion of $\Delta ssrS::tag107$ and $\Delta sau60::tag3$ was reduced 100 and 10 times compared to other competing strains, respectively (Figure 37). In order to test whether this loss of fitness corresponds to a greater individual sensitivity of strains to rifampicin, the efficiency of plating (EOP) of mutants and parental strains were tested on BHI agr plates containing different concentrations of rifampicin (from 0 to 12.5 ng/ml). The EOP on plates containing between 3.13 and 6.25 ng/ml of rifampicin was 100 times lower for mutant $\Delta ssrS::tag107$ than is parental (Figure 38). The phenotype was visible only at concentrations close to or below the MIC. However, we did not detect any rifampicin susceptibility for $\Delta sau60::tag3$ in the tested conditions (Figure 39).

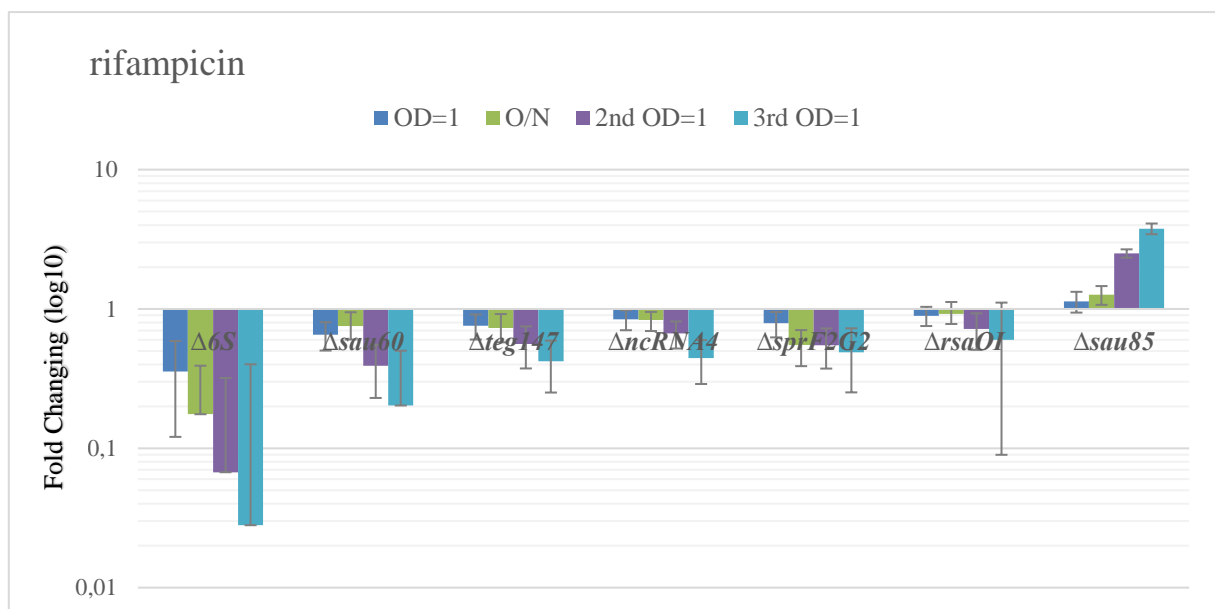


Figure 37 sRNA mutants of library v1 affected by sub-lethal concentration of rifampicin.

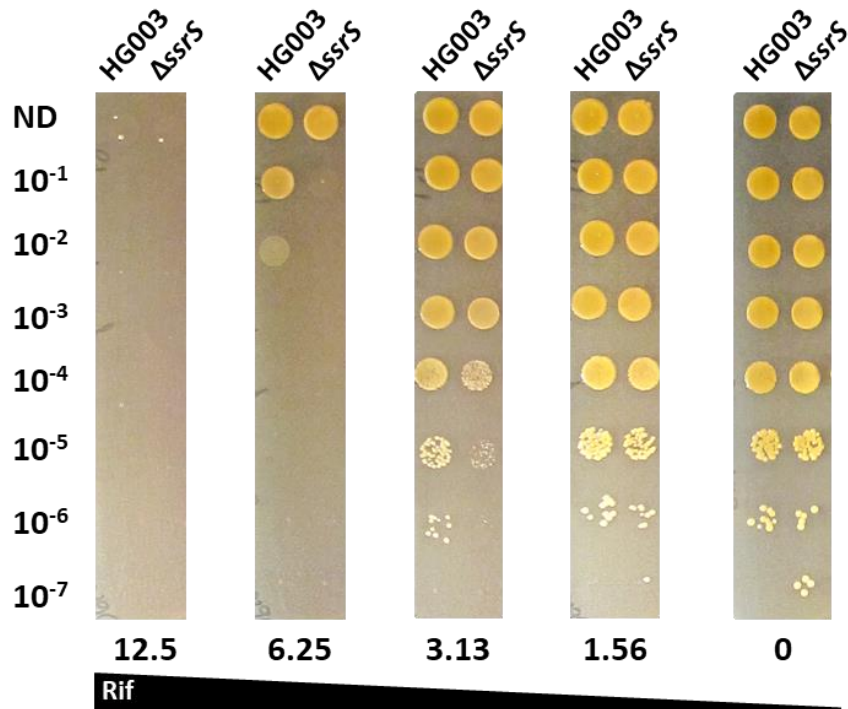


Figure 38 *ssrS* mutant phenotype confirmation. Cultures are 10-fold diluted (serial dilutions in y axis). Rifampicin concentrations used are indicated in $\mu\text{g.L}^{-1}$ in x axis. 5 μl drops are spotted on BHI+/-rifampicin plates and incubated at 37°C during 24 h. Experiment realized 5 times. HG003: parental strain; ΔssrS : *ssrS*::tag107 mutant; ND: Non-Diluted culture; Rif: Rifampicin.

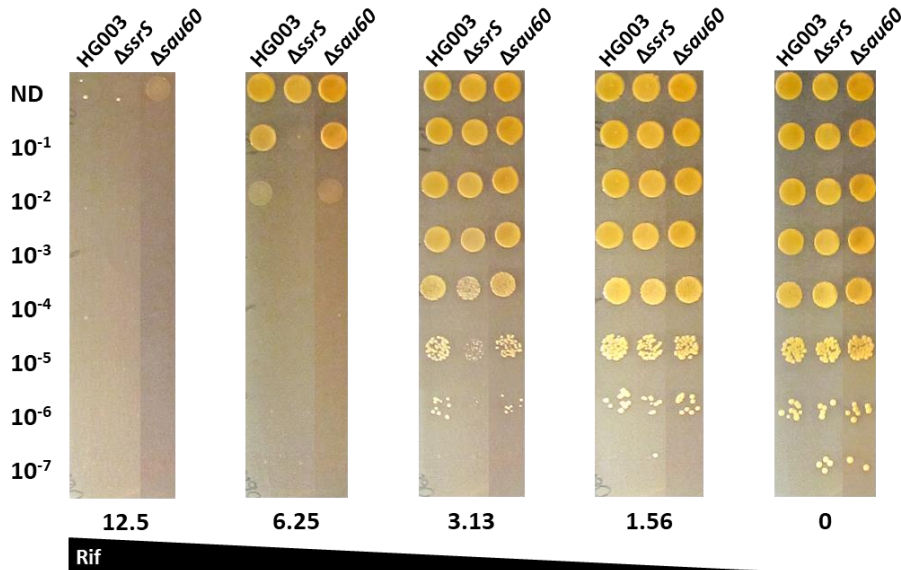


Figure 39 No Δsau60 phenotype with rifampicin. Cultures are 10-fold diluted (serial dilutions in y axis). Rifampicin concentrations used are indicated in $\mu\text{g.L}^{-1}$ in x axis. 5 μl drops are spotted on BHI+/-rifampicin plates and incubated at 37°C during 24 h. Experiment realized 1 time with biological duplicates. HG003: parental strain; ΔssrS : *ssrS*::tag107 mutant; Δsau60 : *sau60*::tag3 mutant; ND: Non-Diluted culture; Rif: Rifampicin.

Complementation of the $\Delta ssrS::tag107$ phenotype

To confirm that rifampicin susceptibility was due to the *ssrS* deletion, a complementation test was performed. The *ssrS* transcribed sequence (from the Transcription Start Site (TSS) to its transcriptional terminator) was cloned into the plasmid pRMC2, generating pRMC2-6S. With this plasmid, the expression of *ssrS* is under the control of the Ptet-O promoter inducible by the addition of aTc to growth media. The complementation of the sensitivity phenotype of $\Delta ssrS::tag107$ was tested by spot test with $3.13 \mu\text{g}\cdot\text{L}^{-1}$ of rifampicin (Figure 40). In the presence of aTc, the $\Delta ssrS::tag107$ strain pRMC2-6S had a 1000-fold greater EOP than the $\Delta ssrS::tag107$ pRMC2 strain suggesting that induction of *ssrS* contributes to resistance to low concentrations of rifampicin. The same observation was made for the strain HG003 pRMC2-6S, which has an EOP 100 times greater than the control strain HG003 pRMC2. We conclude that an induction of *ssrS* above its natural level contributes to an increased resistance to rifampicin. The mutation $\Delta sau60$ and $\Delta ssrS$ affect products related to the RNA polymerase, the target of rifampicin. Indeed, *sau60* is an inter-operon sequence located upstream of *rpoB* gene, which encodes the β subunit of the RNA polymerase; its deletion likely affects the amount of *rpoB*. *ssrS* expresses 6S RNA, a regulatory RNA interacting with the RNA polymerase. 6S RNA is conserved in prokaryotes (Wehner *et al.* 2014; Burenina *et al.* 2015). In *E. coli*, it is stable, weakly expressed in exponential phase but accumulating up to 10,000 times in stationary phase (Wassarman and Storz 2000). The 6S RNA binds to the σ^{70} -coupled RNA polymerase and thus inhibits transcription at many σ^{70} promoters. Consequently, the 6S RNA promotes the transcription dependent on alternative σ factors. In *E. coli*, 6S RNA is therefore at the center of a regulatory network. In *S. aureus*, besides the structure, little is known on 6S RNA (Pichon and Felden 2005). In contrast to *E. coli*, there is a constitutive and high expression of 6S RNA in *S. aureus* (Mader *et al.* 2016).

The mutations conferring resistance to rifampicin described in the literature are localized in the gene encoding the β subunit of RNA polymerase; in *S. aureus*, these mutations confers a high resistance level (Aubry-Damon *et al.* 1998). We show here that 6S RNA confers a resistance to rifampicin at low concentration. It may protect by steric hindrance by forming a complex with the RNA polymerase, it would partially prevent the interaction between rifampicin and the β subunit of the RNA polymerase.

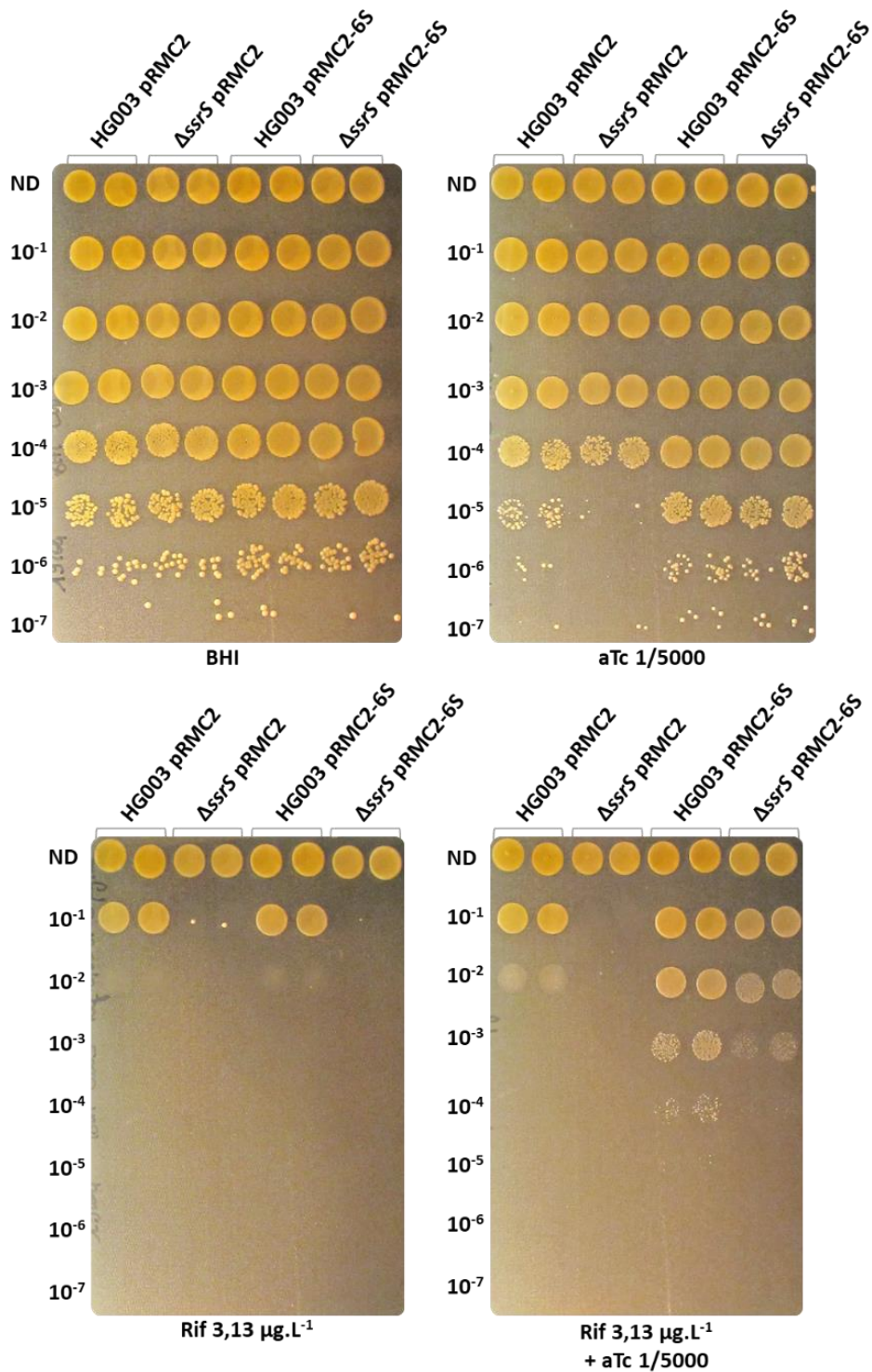


Figure 40 $\Delta ssrS$ phenotype complementation. Cultures are 10-fold diluted (serial dilutions in y axis). Rifampicin concentrations used are indicated below in $\mu\text{g.L}^{-1}$. 5 μl drops are spotted on BHI/chloramphenicol 5 mg/L plates to maintain the plasmid +/- aTc (0.2 mg/L) and then incubated at 37°C during 24 h. Experiment realized 2 times with biological duplicates. HG003: parental strain; $\Delta ssrS$: *ssrS*::107 mutant; pRMC2: empty plasmid with aTc inducible promoter; pRMC2-6S: plasmid with aTc inducible promoter containing *ssrS* gene (from TSS); ND: Non-Diluted culture.

Discussion

Bacterial regulatory RNAs have been gradually considered as the indispensable part of the complicated genetic regulation network, together with sigma factors, two component systems and regulatory proteins. The use of NGS and bioinformatics suggested that *S. aureus* could have more than five hundreds non-coding regulatory RNAs, with so far, only few of them well characterized. The lack of regulatory RNA accurate annotations is a general and common difficulty to study their function. We therefore decided to select the sRNAs of interest in the model strain HG003 using a strict definition of *bona fide* sRNAs. For this study, we used published transcriptomic data and our own RNA-seq data, which were analyzed by visual curation and bioinformatics approaches. We concluded that HG003 has about 50 sRNAs (*c.f* Chapter I). This number is within the range of reported results for the well-characterized bacterium *E. coli* (about 80) (Raghavan *et al.* 2011).

Like other species, the *S. aureus* sRNAs are mainly species specific. Those, which are not conserved within the *S. aureus* species, are expressed from variable genetic elements (*e.g.* sRNA390, sRNA334, sRNA287). The sRNAs known to target proteins (*i.e.* tmRNA and 6S RNA) are more conserved than other sRNAs; this is probably due to the slowest evolution of protein sequences compared to UTR sequences, which are usually the targets of sRNA genes. Interestingly, RsaE, one of the most studied sRNAs in Firmicutes, is the most conserved sRNA of our selection, from *S. aureus* to *B. subtilis*. This could be due to its numerous targets related to central metabolism (Rochat *et al.* 2018), which would constrain its evolution or due to an interaction with an unknown protein. RsaOG (Marchais *et al.* 2010) also known as RsaI (Geissmann *et al.* 2009) remains conserved within the *Staphylococcus* genus. It was recently shown that RsaOG (RsaI) act as a sponge sRNAs against RsaG (Bronesky *et al.* 2018) (BioRxiv: <https://doi.org/10.1101/278127>). However, RsaG seems present only in *S. aureus*. RsaOG also associates with RsaE *in vitro* (Bronesky *et al.* 2018; Rochat *et al.* 2018) (BioRxiv: <https://doi.org/10.1101/278127>), if this interaction is relevant *in vivo*, it may explain the RsaOG (RsaI) conservation.

The bioinformatics search for sRNA putative regulators may provide clues to decipher the sRNA functions and consequently our laboratory investigated some sRNA regulations that we proposed (Liu *et al.* 2018). The expression of S596 was reported as possibly controlled by the ferric uptake regulator (Fur) (Mader *et al.* 2016; Liu *et al.* 2018). Rodrigo Coronel-Tellez confirmed that the S596 sRNA strongly accumulates in a Δfur *S. aureus* strain (personal communication). The association of S596 to the iron metabolism is also supported by the following observations: i) the absence of S596 and a multicopy plasmid carrying the S596 gene

confer a sensitivity to dipyrindyl, an iron chelator (Claire Morvan, Aurélie Jaffrenou and Rodrigo Coronel-Tellez, personal communication), and ii) the S596 predicted targets are often associated with the iron metabolism.

Regulatory RNAs are often involved in the adaptation to changing conditions. However, in many cases, they contribute solely to the "fine-tuning" of gene expression and therefore sRNA-associated phenotype are difficult to identify; the example of S596 remains an exception. Nevertheless, minor sRNA-mediated phenotypes conferring modest advantages, may affect bacterial fitness and emerge as dominant traits after some generations under selective pressure.

We set up a strategy to determine the fitness of sRNA mutants grown under different conditions. Deleted sRNA loci were replaced by DNA barcodes. This method was first developed in yeast (Shoemaker *et al.* 1996), and then applied to enterobacteria (Mazurkiewicz *et al.* 2006; Hobbs *et al.* 2010; Hobbs and Storz 2012). It allows to follow the proportion of single mutants within a sRNA::tag library. We improved this strategy by coupling it to the DNA-seq technology and applied to study regulatory RNAs in *S. aureus* (Le Lam *et al.* 2017) (*c.f.* Chapter II).

The main difficulty for this method is the construction of mutants in *S. aureus*. The sRNA gene replacement by DNA specific tags is performed by a two-step crossing-over strategy with the chromosomal intergration and excision of replication thermosensitive plasmids. A first set of mutants was constructed using pMAD (Arnaud *et al.* 2004). However, the pMAD integration step at 42°C in the presence of erythromycin was reported to be mutagenic (Traber *et al.* 2008). We improved the method using pIMAY which allows to perform the integration step at 37°C and carries a positive selection for the plasmid excision (Monk *et al.* 2012; Monk *et al.* 2015). 53 additional tagged sRNA mutants were constructed with pIMAY. Each mutant was constructed in biological triplicate. By constructing these independent triplicates, we could possibly detect phenotypes due to secondary mutations, as they would likely be revealed by only one strain.

To test the robustness of our approach, we constructed an identical sRNA gene (*sprX2*) deletion but substituted with two different tags (*tag111* and *tag145*).

Results from different fitness experiments performed with library v1 reveal a remarkable homogeneity between *sprX2::tag111* (Figure 41a) and *sprX2::tag145* (Figure 41b). The trend of changing fold of construction is nearly identical and the amplitude of error bars between triplicates are reasonable. This comparative analysis supports the fitness approach we developed. Even if not understood, most observed differences are likely meaningful.

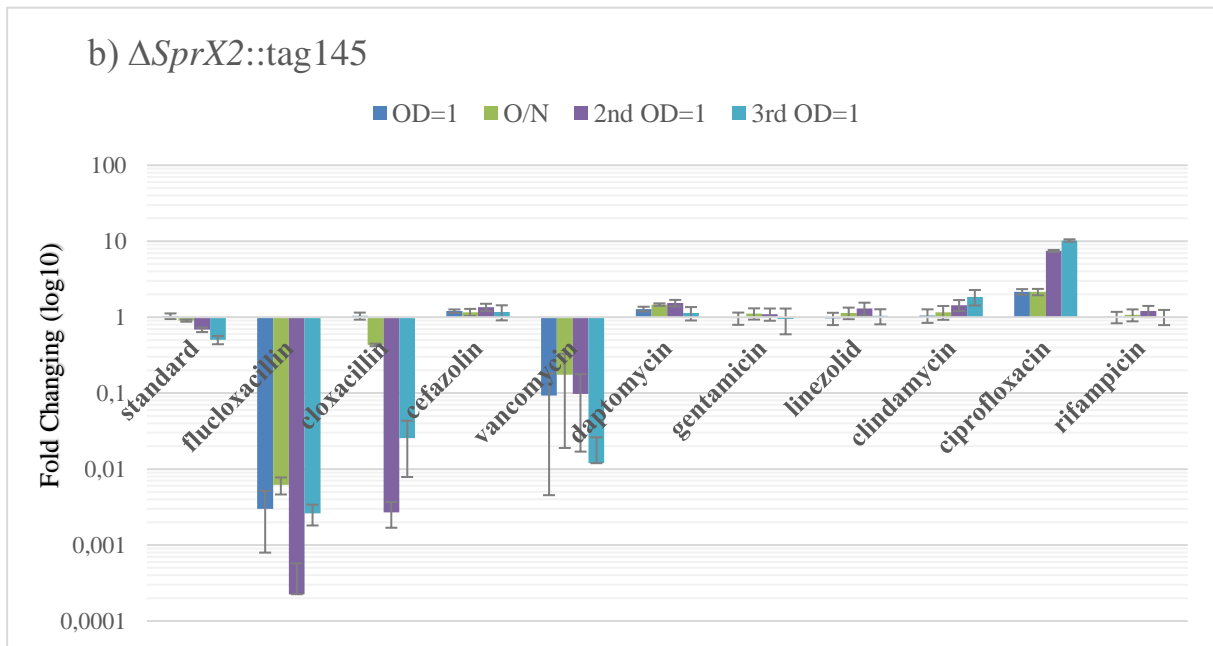
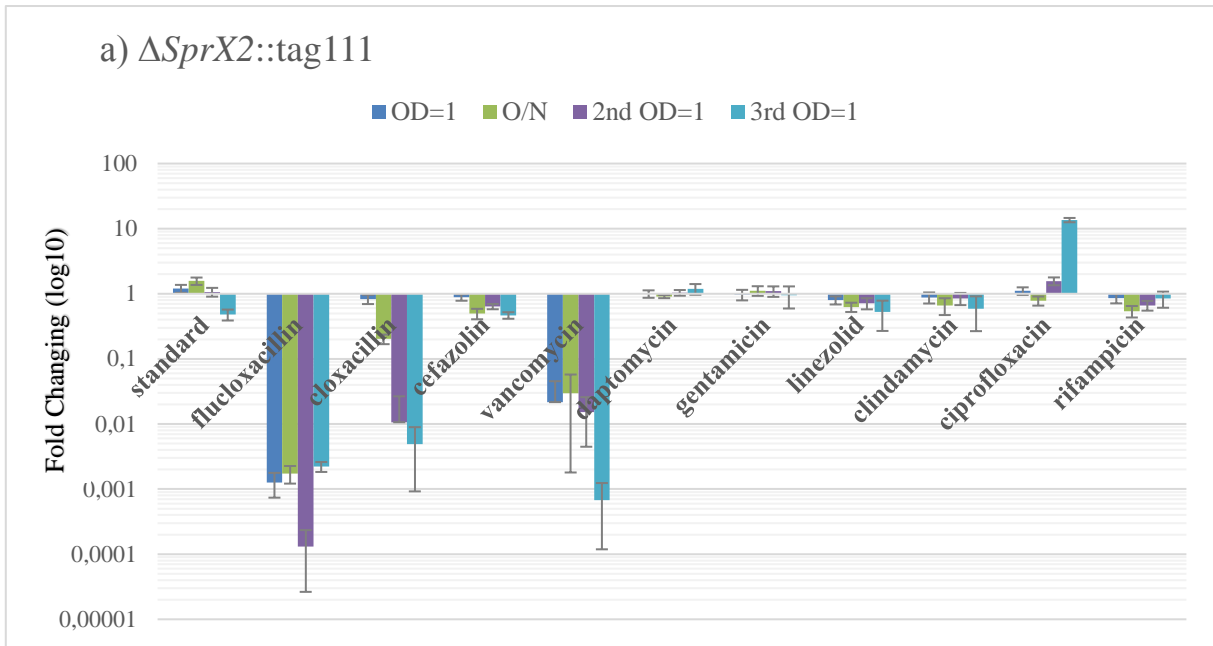


Figure 41 $\Delta sprX2::tag111$ and $\Delta sprX2::tag145$ of library v1 affected by 11 growth conditions

Additionally, samplings were taken to look for the accumulation or diminishing of sRNA mutants over time (*c.f.* Section 3.2.2.2 Figure 26). With these conditions, it is easier to discover and visualize the changing ratio of sRNA mutants both during one growth curve (OD=1, OD=7.5 and overnight), and in the process of serial dilution (OD=1, 2nd OD=1 and 3rd OD=1) (*e.g.* Figure 42), unless the stressful conditions applied are not appropriate. For example, the sub-lethal concentrations of vancomycin and flucloxacillin retain for this study were too high, causing difficulties to interpret the data since almost all sRNA mutants were diminishing. Besides, samplings OD=7.5 were not harvested in several experiments since this OD was not reached.

Some mutant behaviors could be explained by results from the literature as indicated below with three examples:

- The *sau6836* deletion does not affect a sRNA as initially expected but the *abcA* 5'UTR. The *abc* gene encodes a multidrug efflux systems acting on a wide range of antibiotics and its disruption confers an antibiotic sensitivity (Schrader-Fischer and Berger-Bachi 2001) and, indeed, the fitness of Δ *sau6836* is affected by sub-lethal concentration of cefazolin (Figure 42 and 43).
- The regulatory RNA RsaA targets the *mgrA* mRNA (Romilly *et al.* 2014) and MgrA is a global regulator controlling the expression of surface proteins (Tomasini *et al.* 2017). These results give a rational to the impact of *rsaA* deletion on the antibiotic resistance that we observed.
- Teg49 is considered as a sRNA originating from the *sarA* 5'UTR (Kim *et al.* 2014). Consequently, the Δ *teg49* deletion that we constructed prevents the expression of *teg49* but also affects the *sarA* expression. We observed that Δ *teg49* affects drastically the bacterial fitness in several conditions; however, we cannot discriminate between an Δ *teg49* or *sarA* effect. It would be interesting to dissociate the effect of SarA from that of its 5'UTR processed sRNA.

Teg49. *rnaIII* and *agr* are also intimately regulated (Novick and Geisinger 2008). Thanks to two different constructions that we made, affecting either *rnaIII* or *rnaIII* and *agr*, the effects of RNAIII and *agr* can be distinguished. The data obtained from fitness experiments also reveals some underlying regulation. For instance, RNAIII may regulate directly drug transporter and autolysins.

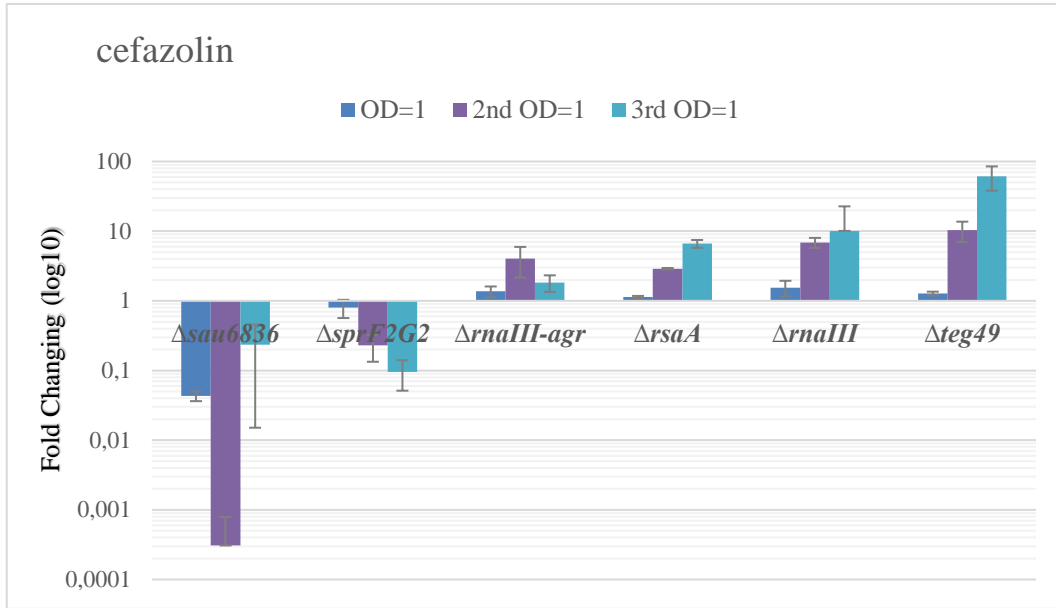


Figure 42 Example of cefazolin in serial dilution

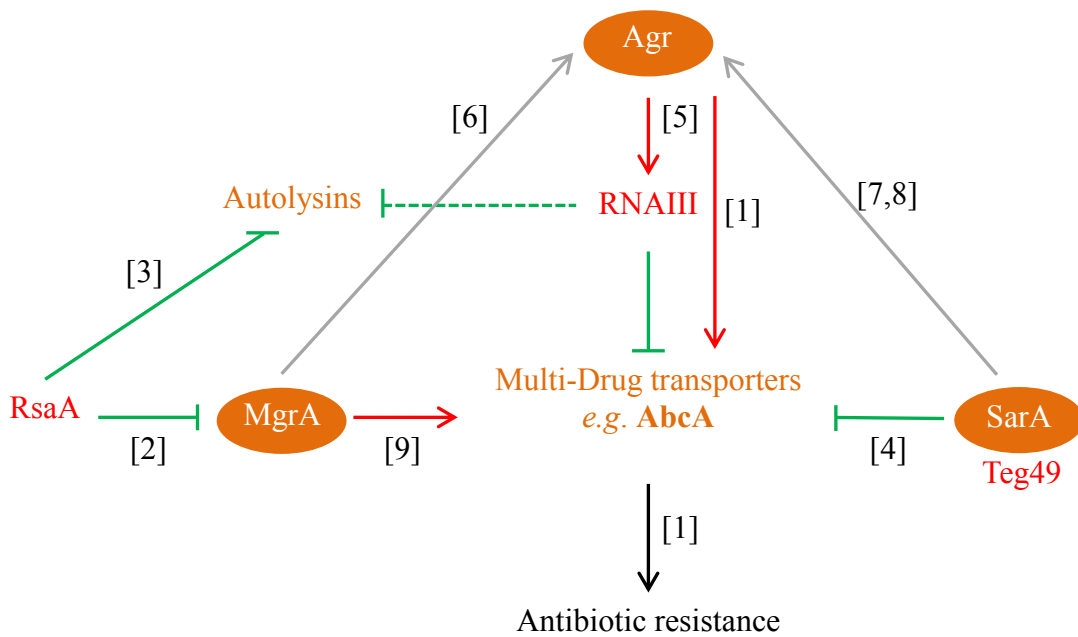


Figure 43 Example of sRNA regulation network

- [1] (Schrader-Fischer and Berger-Bachi 2001)
- [2] (Romilly *et al.* 2014)
- [3] (Tomasini *et al.* 2017)
- [4] (Kim *et al.* 2014)
- [5] (Novick and Geisinger 2008)
- [6] (Gupta *et al.* 2015)
- [7] (Reyes *et al.* 2011)
- [8] (Manna *et al.* 2018)
- [9] (Truong-Bolduc and Hooper 2007)

With the exception of ciprofloxacin, $\Delta sprF2G2$ is affected by all tested antibiotics in our fitness experiments (Figure 44). $sprF2G2$ is a paralog of $sprFG$ and both loci are encoding putative TA systems (Pichon and Felden 2005). SprFG are implicated in *S. aureus* virulence (Pichon and Felden 2005). The fact that SprF2G2 is associated with antibiotic resistance is an original observation; how the presence of a TA system could improve antibiotic resistance remains to be explored.

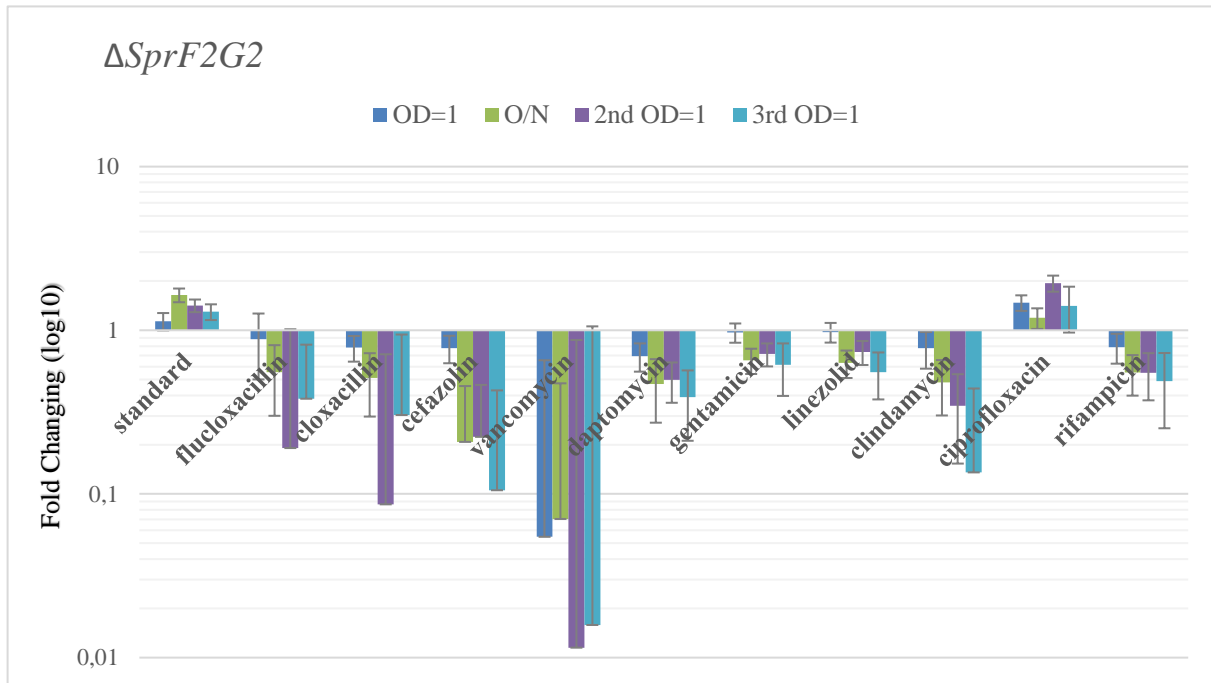


Figure 43 $\Delta sprF2G2$ affected by tested antibiotics

The phenotype of $\Delta srrS$ discovered by exposure to sub-lethal concentration of rifampicin was unexpected. We confirmed the phenotype by spot plating on a solid medium containing rifampicin. The direct implication of 6S RNA in the phenotype was confirmed by complementation studies. Mutation conferring the resistance to rifampicin are classically found in the *rpoB* gene (encoding the β subunit of the RNA, the rifampicin target). In contrast to *rpoB* mutations, the presence of 6S RNA confers an increased viability only to a low level of rifampicin concentration. We propose that 6S RNA alters the rifampicin activity by steric hindrance. In *E. coli*, 6S RNA is induced in stationary phase and it contributes to improve stationary phase survival by stimulating σ^s -dependent transcription. In contrast, the *S. aureus* 6S RNA expression seems constitutive (Mader *et al.* 2016). Consequently, the *S. aureus* 6S RNA may have a different function than in *E. coli*, contributing to improve adaptability in the present on low concentration of RNA polymerase inhibitors.

The antibiotic resistance of *S. aureus* is a growing severe issue. The project presented here reveals that several regulatory RNA genes are important for the adaptation to antibiotics, at least at low concentration. The antibiotics chosen for this study are targeting different processes and are often used to fight *S. aureus*. This study gives clues to understand the gene regulatory network associated with antibiotic resistance and in a long term may contribute to improve therapy against *S. aureus*.

Supplementary data

Table S1: DNA tag library

Name	Sequence*
tag70	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCATCCCGCACAACCGAGCAC ACCCACCCACACCATCGAAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag71	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCGCCCCGCGGAGAGAGACCTA ACTCGCGCGACCTCCCGCGAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag72	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTACACATCACTCCATCGACATAA AGCTCAAAACCCAACGAAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag73	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTACCACCAAAGAAACACCACAA AAGCCCGCTCTAAACTCGAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag74	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTCTCTCGCTCGCTCTCTAGCC CGCTACCTCTTACCTAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag75	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTATCGATCTAACCCGCGCCACCT CGCCACAGACATAACCACCAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag76	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAGCCCTACACCACTAGCTCCCA CAAGACACCCCAAACCTAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag77	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTCACACCAGCGAAAGCCCTC ACCAGCAACCCCCAGAGCTAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag78	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTACCTCCAACCCAAAACATCAC CAGCACCCGAACACTATCCAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag79	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCACACTACCACTCGCGCTAACG AACGAACGCCACCCCGCAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag80	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTAGCCCGCTAGCTCGAACCC CCATACCGACCTCCAACAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag81	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAGCGAGATCCATACACATCAA AAGAGCTCAAGACCTAGCGAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag82	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTCCCACAAACACACTACAAC CAACCTATAAATCCCTCCAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag83	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAGCGCAAAAAAATACCCCA TCAAACGCTCCCACGACACAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag84	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTCCCACAAACTCACTACAactca acctataaatccctccAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag85	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTATCAACATAGCCCACACGCCC CCTCGATCCCCGCGCACAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT
tag86	ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAGACCGATCTCAAGCTCTATAC CTATCACAAAATATCTCCAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag87 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTATCTACACCACCAACAAACAA
TCCAACGAACAAAACCCATAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag88 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTACCCACATCCCCACTAGCA
ACCTCCCGCTACCAAGACAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag89 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAACCACACCACCAACCAACCC
AGCGCCACCgCTAACTCAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag90 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTACCACAAGATCAAGCAACAAA
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tag91 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAGCACGCACCCACAAACTCTAG
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tag92 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTATCGCCATCTATCACCAAAC
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tag93 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCAATCGACAGACCAATCAGC
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tag94 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCGCCCATATCTCTATCTACCTC
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tag96 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCGCTACCAAGCAACCACTCCAG
CTCGAAAGAGACCGCCAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag97 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCAAGCCCACCCAATCGACCGCA
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tag98 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAAAAATCCCACGCACACACAAG
CGACAACAAACTACAAACAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag99 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCACCCTATCCACCTCTCACACC
ACCTCCCTAGCCC GCCATAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag100 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCCTATAGCGAACCCGCACTCC
CCAAAAAATCACGACATAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag101 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTATCTCCCAAACCTCGAAACCC
CACCCAAGACCGACATATAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag102 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCAATCGAAATAGCCCACCAC
CCACATATATATCTCTACAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag103 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCACCACACCACCTCCCACGCT
AACCACCGCGCACCATCCAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag104 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTCCACAAACTCTCTACAACCT
CAACCTATAAATCCCTCCAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag105 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCCGGATCAATCCACCACCCG
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tag106 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCCAATCAATATCTCCATACAG
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tag107 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAACTCCCACACTAGACCGAGAA
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tag108 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTCTACAAACATACACCGCAGC
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tag109 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCCGCCACCCCCAAATACAACG
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tag111 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTACCAATCACCATCCAAACCAGC
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tag112 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTACCCACGCCCTCTATCTACCC
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tag113 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTATCTCAATACACCAAGATCAAC
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CATACCAACCTCCATCAAAGCTTGTCATTGCTGTACCCACAACCTAGGTATAT

tag115 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTACCTACCCCCGCTCTCCAGCA
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tag117 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCGAGCTATATAGAACAACCTAG
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accccccaatcaagaaAAGCTTGTCATTGCTGTACCCACAACCTAGGTATAT

tag125 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCCCATAGCAACCTCAACAAAC
CTCGACACCCCTAGACACAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag126 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTACCACCACAACACCACCCAGAC
CGATACCTCCCCCAATCAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag127 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAGCGACCGCGCCAACCATCAAC
AAATCAACCTCGCACACGAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag128 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCAACAGAGCTCACTCTCACAAT
CCACAAACAACGCCATACAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag129 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTCGACAGATACCCCAATCTCC
ATCCACCACACGACCCAAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag130 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTACCGCTAACCAACCCCGGATAC
ACCCCGACCGAAAAACCAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag131 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCCTATCACGACCTACAGCTAG
ATCCCCGAACCTAACTAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag132 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAACTCCAGAAAGAACCCCGAG
AACCCACAGCAAACACTAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag133 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCACACGCGAGACCCAGACACCC
CCAGCTCCCCAGAAAGAAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag134 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTACCTCCAACACCACATACCCCA
AGAACGAACCTCCCTATATAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag135 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTCGAAAGCACTCCCCATAACC
ACAGAACACACGCCAAACAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag136 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCAAAACCTCACGACATCCATAT
AGACACCCACCACTAAAAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag137 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTCCCCAAAACACAACCTAAAA
CAAAAGATCTATCGATAAAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag138 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAGACCACAAAAGAGCGCCATCC
CGCCAAAGCAACAGCACCAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag139 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCACACACATCCAGCCACCCCT
CCACCCCAACCTCAAACCAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag140 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCCCACCACTCCCTACCTAGCC
CCACACACCACAATAAAAAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

Tag141 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCATCAAGACCTAGATCGCAAA
CCCTCAAAGAAAACCTCAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag142 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTCTATCACTAGCGCCCGAGAC
CACTCGATAAATCCACAAAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag143 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCCCCCCCCACCTCGCAATCG
CGAGACAAACCACCCTATAAGCTTGTTCATTGCTGTACCCCAACCTAGGTATAT

tag144 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAAACCACTCGACAACGACCACG
CACGATAACTAACCCTCCAAGCTTGTCATTGCTGTACCCCAACCTAGGTATAT

tag145 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCGCGAGCCAGAGATCCCTCCAG
AGATAACCACGCCATCCAGAAGCTTGTCATTGCTGTACCCCAACCTAGGTATAT

tag146 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAAACCCTCTCCCCCTCCACCCCG
AACTCTCAAGAGCCCTCTAAGCTTGTCATTGCTGTACCCCAACCTAGGTATAT

tag147 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAAACAAGCCCTCGACAACCACCA
AGACAGACCGAGCTAAACAAGCTTGTCATTGCTGTACCCCAACCTAGGTATAT

tag148 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCCATAGCCCCCTCTCTCACA
ATCTATCTCGCCCCATCTAAGCTTGTCATTGCTGTACCCCAACCTAGGTATAT

tag149 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTCTCGACACCACTCAAAAGAGCA
CGTCCCGCCCCACCAGAAGCTTGTCATTGCTGTACCCCAACCTAGGTATAT

tag150 ATATACCTAGGTCCATACGCAGCTATGCAATAAGCTTAGATCCAACCATCCATAAACAT
ACCAAGATAGCGCCCTCCAAGCTTGTCATTGCTGTACCCCAACCTAGGTATAT

* Grey highlighted sequences are 40nt barcodes.

Table S2: Plasmids

Name	Relevant genotype	Reference
pJET	rep (pMB1), bla (Amp ^R), eco47IR, PlacUV5, T7 promoter	Fermentas
pIMAY	rep (p15A), rep (pWV01 ^{ts}), pBluescript MCS, Phelp-driven Cm ^R , inducible secY antisense from pKOR1, RP4 conjugative origin of transfer	(Monk <i>et al.</i> 2012)
pDEsprF3G3::tag70	pIMAY derivative for chromosomal substitution of <i>sprF3G3</i> locus with tag70 sequence	This study
pDEteg146::tag71	pIMAY derivative for chromosomal substitution of <i>teg146</i> locus with tag71 sequence	This study
pDEsRNA37::tag72	pIMAY derivative for chromosomal substitution of <i>sRNA37</i> locus with tag72 sequence	This study
pDEsRNA334::tag73	pIMAY derivative for chromosomal substitution of <i>sRNA334</i> locus with tag73 sequence	This study
pDErsaA::tag75	pIMAY derivative for chromosomal substitution of <i>rsaA</i> locus with tag75 sequence	This study
pDEsau76::tag76	pIMAY derivative for chromosomal substitution of <i>sau76</i> locus with tag76 sequence	This study
pDErsaOI::tag77	pIMAY derivative for chromosomal substitution of <i>rsaOI</i> locus with tag77 sequence	This study
pDEteg149::tag78	pIMAY derivative for chromosomal substitution of <i>teg149</i> locus with tag78 sequence	This study
pDEteg7::tag79	pIMAY derivative for chromosomal substitution of <i>teg7</i> locus with tag79 sequence	This study
pDEteg16::tag80	pIMAY derivative for chromosomal substitution of <i>teg16</i> locus with tag80 sequence	This study
pDEsRNA287::tag85	pIMAY derivative for chromosomal substitution of <i>sRNA287</i> locus with tag85 sequence	This study
pDEsRNA71::tag86	pIMAY derivative for chromosomal substitution of <i>sRNA71</i> locus with tag86 sequence	This study
pDEsRNA207::tag87	pIMAY derivative for chromosomal substitution of <i>sRNA207</i> locus with tag87 sequence	This study
pDEteg140::tag90	pIMAY derivative for chromosomal substitution of <i>teg140</i> locus with tag90 sequence	This study
pDEteg55::tag92	pIMAY derivative for chromosomal substitution of <i>teg55</i> locus with tag92 sequence	This study
pDEsRNA209::tag93	pIMAY derivative for chromosomal substitution of <i>sRNA209</i> locus with tag93 sequence	This study

pDE <i>sRNA219</i> ::tag94	pIMAY derivative for chromosomal substitution of <i>sRNA219</i> locus with tag94 sequence	This study
pDE <i>teg106</i> ::tag95	pIMAY derivative for chromosomal substitution of <i>teg106</i> locus with tag95 sequence	This study
pDE <i>sRNA260</i> ::tag96	pIMAY derivative for chromosomal substitution of <i>sRNA260</i> locus with tag96 sequence	This study
pDE <i>sRNA345</i> ::tag97	pIMAY derivative for chromosomal substitution of <i>sRNA345</i> locus with tag97 sequence	This study
pDE <i>ncRNA2</i> ::tag99	pIMAY derivative for chromosomal substitution of <i>ncRNA2</i> locus with tag99 sequence	This study
pDE <i>ncRNA3</i> ::tag100	pIMAY derivative for chromosomal substitution of <i>ncRNA3</i> locus with tag100 sequence	This study
pDE <i>ncRNA4</i> ::tag101	pIMAY derivative for chromosomal substitution of <i>ncRNA4</i> locus with tag101 sequence	This study
pDE <i>ncRNA5+6</i> ::tag102	pIMAY derivative for chromosomal substitution of <i>ncRNA5+6</i> locus with tag102 sequence	This study
pDE <i>ncRNA7</i> ::tag106	pIMAY derivative for chromosomal substitution of <i>ncRNA7</i> locus with tag106 sequence	This study
pDE <i>ssrS</i> ::tag107	pIMAY derivative for chromosomal substitution of <i>ssrS</i> locus with tag107 sequence	This study
pDE <i>sprC</i> ::tag109	pIMAY derivative for chromosomal substitution of <i>sprC</i> locus with tag109 sequence	This study
pDE <i>sprFIG1</i> ::tag110	pIMAY derivative for chromosomal substitution of <i>sprFIG1</i> locus with tag110 sequence	This study
pDE <i>sprX2</i> ::tag111	pIMAY derivative for chromosomal substitution of <i>sprX2</i> locus with tag111 sequence	This study
pDE <i>sprY2</i> ::tag112	pIMAY derivative for chromosomal substitution of <i>sprY2</i> locus with tag112 sequence	This study
pDE <i>sprY3</i> ::tag113	pIMAY derivative for chromosomal substitution of <i>sprY3</i> locus with tag113 sequence	This study
pDE <i>sau41</i> ::tag115	pIMAY derivative for chromosomal substitution of <i>sau41</i> locus with tag115 sequence	This study
pDE <i>sRNA258</i> ::tag116	pIMAY derivative for chromosomal substitution of <i>sRNA258</i> locus with tag116 sequence	This study
pDE <i>sau5949</i> ::tag117	pIMAY derivative for chromosomal substitution of <i>sau5949</i> locus with tag117 sequence	This study

pDE <i>sprF2G2</i> ::tag118	pIMAY derivative for chromosomal substitution of <i>sprF2G2</i> locus with tag118 sequence	This study
pDE <i>sprB</i> ::tag121	pIMAY derivative for chromosomal substitution of <i>sprB</i> locus with tag121 sequence	This study
pDE <i>sprD</i> ::tag122	pIMAY derivative for chromosomal substitution of <i>sprD</i> locus with tag122 sequence	This study
pDE <i>rsaC</i> ::tag133	pIMAY derivative for chromosomal substitution of <i>rsaC</i> locus with tag133 sequence	This study
pDE <i>S204</i> ::tag134	pIMAY derivative for chromosomal substitution of <i>S204</i> locus with tag134 sequence	This study
pDE <i>S596</i> ::tag135	pIMAY derivative for chromosomal substitution of <i>S596</i> locus with tag135 sequence	This study
pDE <i>S627</i> ::tag136	pIMAY derivative for chromosomal substitution of <i>S627</i> locus with tag136 sequence	This study
pDE <i>S808</i> ::tag137	pIMAY derivative for chromosomal substitution of <i>S808</i> locus with tag137 sequence	This study
plocus3M::tag139	pIMAY derivative for chromosomal insertion tag139 sequence between SAOUHSC_01263 and SAOUHSC_01264	This study
plocus2T::tag140	pIMAY derivative for chromosomal insertion tag140 sequence between SAOUHSC_03030 and SAOUHSC_03031	This study
plocus1O::tag141	pIMAY derivative for chromosomal insertion tag141 sequence between SAOUHSC_00009 and SAOUHSC_00010	This study
pDE <i>sau5971</i> ::tag142	pIMAY derivative for chromosomal substitution of <i>sau5971</i> locus with tag142 sequence	This study
pDE <i>hfq</i> ::tag143	pIMAY derivative for chromosomal substitution of <i>hfq</i> locus with tag143 sequence	This study
pDE <i>sprA1As1</i> ::tag144	pIMAY derivative for chromosomal substitution of <i>sprA1As1</i> locus with tag144 sequence	This study
pDE <i>sprX2</i> ::tag145	pIMAY derivative for chromosomal substitution of <i>sprX2</i> locus with tag145 sequence	This study
pDE <i>sprX1</i> ::tag146	pIMAY derivative for chromosomal substitution of <i>sprX1</i> locus with tag146 sequence	This study
pDE <i>rsaH</i> ::tag147	pIMAY derivative for chromosomal substitution of <i>rsaH</i> locus with tag147 sequence	This study
pDE <i>sprY1</i> ::tag148	pIMAY derivative for chromosomal substitution of <i>sprY1</i> locus with tag148 sequence	This study

pDE*sprXI*::tag149

pIMAY derivative for chromosomal substitution of *sprXI*
locus with tag149 sequence

This study

Table S3a: Bacterial strains

Name	Relevant genotype	Reference
<i>E. coli</i> IM08B	F-mcrA Δ (mrr-hsdRMS-mcrBC) Φ 80dlacZ Δ M15 Δ lacX74 endA1 recA1 deoR Δ (ara,leu)7697 araD139 galU galK nupG rpsL λ - Δ dcm Phelp-hsdMS (CC8-2) (SAUSA300_1751) of NRS384 integrated between the atpI and gidB genes; PN25-hsdS (CC8-1) (SAUSA300_0406) of NRS384 integrated between the essQ and cspB genes	(Monk <i>et al.</i> 2015)
<i>S. aureus</i> HG003	NCTC8325 derivative, <i>rsbU</i> and <i>tcaR</i> repaired, <i>agr+</i>	(Herbert <i>et al.</i> 2010)

Table S3b: Bacterial strains

Relevant genotype	Strains			Reference
	Set1	Set2	Set3	
HG003 Δ <i>sprF3G3::tag70</i>	SAPhB960 clone2	SAPhB961 clone3	SAPhB999 clone4	This study
HG003 Δ <i>teg146::tag71</i>	SAPhB880 clone7	SAPhB881 clone2	SAPhB882 clone1	This study
HG003 Δ <i>sRNA37::tag72</i>	SAPhB886 clone4	SAPhB887 clone6	SAPhB888 clone9	This study
HG003 Δ <i>sRNA334::tag73</i>	SAPhB862 clone1	SAPhB863 clone1	SAPhB864 clone2	This study
HG003 Δ <i>rsaA::tag75</i>	SAPhB943 clone1	SAPhB944 clone3	SAPhB945 clone1	This study
HG003 Δ <i>sau76::tag76</i>	SAPhB890 clone3	SAPhB891 clone1	SAPhB892 clone1	This study
HG003 Δ <i>rsaOI::tag77</i>	SAPhB883 clone1	SAPhB884 clone3	SAPhB885 clone1	This study
HG003 Δ <i>teg149::tag78</i>	SAPhB877 clone3	SAPhB878 clone2	SAPhB879 clone1	This study
HG003 Δ <i>teg7::tag79</i>	SAPhB868 clone3	SAPhB869 clone1	SAPhB870 clone1	This study
HG003 Δ <i>teg16::tag80</i>	SAPhB865 clone7	SAPhB866 clone3	SAPhB867 clone7	This study
HG003 Δ <i>sRNA287::tag85</i>	SAPhB871 clone4	SAPhB872 clone3	SAPhB873 clone1	This study
HG003 Δ <i>sRNA71::tag86</i>	SAPhB874 clone3	SAPhB875 clone5	SAPhB876 clone3	This study
HG003 Δ <i>sRNA207::tag87</i>	SAPhB913 clone1	SAPhB914 clone3	SAPhB915 clone2	This study
HG003 Δ <i>teg140::tag90</i>	SAPhB896 clone2	SAPhB897 clone6	SAPhB898 clone2	This study
HG003 Δ <i>teg55::tag92</i>	SAPhB904 clone2	SAPhB905 clone3	SAPhB906 clone2	This study
HG003 Δ <i>sRNA209::tag93</i>	SAPhB907 clone1	SAPhB908 clone3	SAPhB909 clone1	This study
HG003 Δ <i>sRNA219::tag94</i>	SAPhB916 clone1	SAPhB917 clone12	SAPhB918 clone1	This study
HG003 Δ <i>teg106::tag95</i>	SAPhB899 clone3	SAPhB900 clone1	SAPhB946 clone1	This study
HG003 Δ <i>sRNA260::tag96</i>	SAPhB921 clone3	SAPhB922 clone3	SAPhB947 clone1	This study

HG003 Δ <i>sRNA345</i> ::tag97	SAPhB910 clone1	SAPhB911 clone2	SAPhB912 clone3	This study
HG003 Δ <i>ncRNA2</i> ::tag99	SAPhB932 clone1	SAPhB933 clone1	SAPhB934 clone4	This study
HG003 Δ <i>ncRNA3</i> ::tag100	SAPhB940 clone4	SAPhB941 clone7	SAPhB942 clone14	This study
HG003 Δ <i>ncRNA4</i> ::tag101	SAPhB935 clone2	SAPhB936 clone4	SAPhB937 clone3	This study
HG003 Δ <i>ncRNA5+6</i> ::tag102	SAPhB929 clone3	SAPhB930 clone1	SAPhB931 clone1	This study
HG003 Δ <i>ncRNA7</i> ::tag106	SAPhB968 clone8	SAPhB969 clone1	SAPhB970 clone4	This study
HG003 Δ <i>ssrS</i> ::tag107	SAPhB954 clone4	SAPhB955 clone1	SAPhB956 clone4	This study
HG003 Δ <i>sprC</i> ::tag109	SAPhB963 clone1	SAPhB964 clone2	SAPhB965 clone6	This study
HG003 Δ <i>sprFIG1</i> ::tag110	SAPhB1006 clone3	SAPhB1007 clone11	SAPhB1008 clone12	This study
HG003 Δ <i>sprX2</i> ::tag111	SAPhB974 clone4	SAPhB975 clone1	SAPhB997 clone3	This study
HG003 Δ <i>sprY2</i> ::tag112	SAPhB978 clone5	SAPhB979 clone2	SAPhB980 clone3	This study
HG003 Δ <i>sprY3</i> ::tag113	SAPhB957 clone2	SAPhB958 clone2	SAPhB959 clone4	This study
HG003 Δ <i>sau41</i> ::tag115	SAPhB901 clone2	SAPhB902 clone2	SAPhB903 clone5	This study
HG003 Δ <i>sRNA258</i> ::tag116	SAPhB893 clone3	SAPhB894 clone1	SAPhB895 clone5	This study
HG003 Δ <i>sau5949</i> ::tag117	SAPhB948 clone1	SAPhB949 clone2	SAPhB950 clone4	This study
HG003 Δ <i>sprF2G2</i> ::tag118	SAPhB966 clone1	SAPhB967 clone1	SAPhB998 clone6	This study
HG003 Δ <i>sprB</i> ::tag121	SAPhB1031 clone1	SAPhB1032 clone1	SAPhB1033 clone1	This study
HG003 Δ <i>sprD</i> ::tag122	SAPhB1000 clone4	SAPhB1001 clone6	SAPhB1002 clone4	This study
HG003 Δ <i>rsaC</i> ::tag133	SAPhB1242 clone3	SAPhB1243 clone1	SAPhB1244 clone5	This study
HG003 Δ <i>S204</i> ::tag134	SAPhB1234 clone1	SAPhB1235 clone2	SAPhB1236 clone2	This study
HG003 Δ <i>S596</i> ::tag135	SAPhB1231 clone4	SAPhB1232 clone6	SAPhB1233 clone2	This study
HG003 Δ <i>S627</i> ::tag136	SAPhB1228 clone1	SAPhB1229 clone1	SAPhB1230 clone1	This study
HG003 Δ <i>S808</i> ::tag137	SAPhB1239 clone6	SAPhB1240 clone6	SAPhB1241 clone3	This study
HG003 locus3M::tag139	SAPhB1015 clone3	SAPhB1016 clone2	SAPhB1017 clone3	This study
HG003 locus2T::tag140	SAPhB1012 clone1	SAPhB1013 clone2	SAPhB1014 clone3	This study
HG003 locus1O::tag141	SAPhB1009 clone1	SAPhB1010 clone7	SAPhB1011 clone10	This study
HG003 Δ <i>sau5971</i> ::tag142	SAPhB1018 clone3	SAPhB1019 clone2	SAPhB1020 clone4	This study
HG003 Δ <i>hfq</i> ::tag143	SAPhB1024 clone9	SAPhB1025 clone2	SAPhB1026 clone3	This study
HG003 Δ <i>sprA1AsI</i> ::tag144	SAPhB976 clone2	SAPhB977 clone2	SAPhB996 clone3	This study
HG003 Δ <i>sprX2</i> ::tag145	SAPhB1027 clone2	SAPhB1028 clone3	SAPhB1029 clone4	This study

HG003 $\Delta sprXI::tag146$	SAPhB1003 clone3	SAPhB1004 clone11	SAPhB1005 clone5	This study
HG003 $\Delta rsaH::tag147$	SAPhB971 clone1	SAPhB972 clone2	SAPhB973 clone2	This study
HG003 $\Delta sprYI::tag148$	SAPhB1021 clone3	SAPhB1022 clone1	SAPhB1023 clone2	This study
HG003 $\Delta sprXI::tag149$	SAPhB981 clone1	SAPhB982 clone1	SAPhB983 clone2	This study

Table S4: Statistical analysis of library v1

No.	Gene ID	Tag	set1	set2	set3	set1-stderr	set2-stderr	set3-stderr
1	<i>ΔrsaD::tag26</i>	tag026	0,034529965	0,026090204	0,024101844	0,017524284	0,006424581	0,007669398
2	<i>ΔsprX1::tag149</i>	tag149	0,020767511	0,020382747	0,025709677	0,003993314	0,005824491	0,005416338
3	<i>Δteg140</i>	tag090	0,018670924	0,018785357	0,021670353	0,002525242	0,002276911	0,003563472
4	<i>ΔrsaA</i>	tag075	0,020906628	0,024043791	0,01999364	0,003632545	0,00562136	0,003879254
5	<i>ΔsprY1</i>	tag148	0,009946664	0,012156637	0,01983164	0,002749872	0,001173275	0,002721091
6	<i>Δteg149</i>	tag078	0,019718457	0,019233503	0,019251273	0,002880355	0,003193153	0,002345389
7	<i>ΔrsaOI::tag77</i>	tag077	0,016314562	0,019270665	0,018364372	0,002109629	0,002338634	0,002392131
8	<i>ΔsRNA345</i>	tag097	0,016655398	0,016090726	0,017811983	0,001554993	0,001958368	0,001356388
9	<i>Δteg155</i>	tag053	0,017297597	0,010946075	0,017804597	0,000995095	0,001275793	0,001921708
10	<i>Δssr42</i>	tag050	0,016137149	0,013422565	0,016927499	0,003410804	0,002960272	0,002957772
11	<i>Δteg147</i>	tag018	0,016843429	0,012642682	0,016919918	0,00186031	0,001433802	0,001949332
12	<i>ΔrsaH::tag147</i>	tag147	0,013778362	0,013550647	0,016898826	0,001308211	0,001306279	0,00093287
13	<i>Δteg49</i>	tag020	0,021390682	0,021680494	0,016859936	0,005894164	0,007261793	0,006067431
14	<i>Δsau41</i>	tag115	0,017262378	0,020444384	0,016782813	0,002695648	0,002343612	0,001406055
15	<i>Δsau69</i>	tag027	0,01813635	0,016645423	0,016493821	0,005307325	0,005438415	0,009120411
16	<i>ΔsprA1As1</i>	tag144	0,013895364	0,016834822	0,01646163	0,001559824	0,002164299	0,00314262
17	<i>ΔrsaG</i>	tag011	0,017352156	0,018570429	0,016453234	0,00154303	0,002280337	0,001843152
18	<i>ΔsprF3G3</i>	tag070	0,014104641	0,017552417	0,016450302	0,001448757	0,001960301	0,001355952
19	<i>ΔsprF1G1</i>	tag110	0,011972736	0,01312956	0,016026541	0,001763626	0,001078301	0,00150789
20	<i>ΔsRNA37</i>	tag072	0,014123832	0,017325492	0,015345014	0,001424686	0,002007763	0,001777472
21	<i>Δsau19</i>	tag152	0,016871217	0,017948224	0,015328055	0,001324405	0,00166594	0,00163013
22	<i>Δteg7</i>	tag079	0,01629639	0,012362371	0,015289979	0,001132228	0,00130626	0,0017359
23	<i>ΔncRNA2</i>	tag099	0,011742105	0,013161964	0,014538795	0,001413111	0,001274568	0,00174619
24	<i>Δsau6836</i>	tag017	0,013563404	0,014847455	0,014505239	0,002759748	0,005219351	0,005135912
25	<i>ΔrsaOG</i>	tag009	0,019321438	0,015137519	0,014424095	0,001817414	0,001961925	0,001583727
26	<i>Δsau6851</i>	tag032	0,014255797	0,015499018	0,014344269	0,002965182	0,003618416	0,002501742
27	<i>ΔrsaOI</i>	tag151	0,016664265	0,011358358	0,013965571	0,001339958	0,000814142	0,000993526
28	<i>ΔsRNA334</i>	tag073	0,013891251	0,018464618	0,013855153	0,001520163	0,001760463	0,00149966
29	<i>ΔsprF2G2</i>	tag118	0,012782445	0,014701369	0,013515688	0,002249612	0,002028208	0,00174856
30	<i>ΔrsaD::tag6</i>	tag006	0,01076942	0,014761515	0,013481636	0,005703203	0,005739134	0,00491523
31	<i>Δsau60</i>	tag003	0,015325171	0,015887519	0,013244112	0,00229408	0,001459213	0,001254237
32	<i>ΔncRNA5+6</i>	tag102	0,014539275	0,014922915	0,013079339	0,001727313	0,001437922	0,001689944
33	<i>ΔsRNA71</i>	tag086	0,013032909	0,013738394	0,012936807	0,001792529	0,002834169	0,00277485
34	<i>Δsau76</i>	tag076	0,010822332	0,016292111	0,012870689	0,000746565	0,001544359	0,001196629
35	<i>ΔsRNA287</i>	tag085	0,014033403	0,015390631	0,012851515	0,002029069	0,002734091	0,001268715
36	<i>ΔncRNA4</i>	tag101	0,014447985	0,014612385	0,012782751	0,001899029	0,002230843	0,00168179
37	<i>ΔncRNA7</i>	tag106	0,011335353	0,01374733	0,012549307	0,001428501	0,002924853	0,001544757
38	<i>ΔssrS</i>	tag107	0,011231161	0,011279413	0,012470146	0,002393161	0,003091651	0,002353721
39	<i>ΔTeg116</i>	tag030	0,011016314	0,009844755	0,012409687	0,000797804	0,001033204	0,001229729
40	<i>ΔsRNA219</i>	tag094	0,010642218	0,012122193	0,012218327	0,000819407	0,000757237	0,001247478
41	<i>ΔsprX::tag146</i>	tag146	0,012741096	0,012232594	0,011841335	0,000887379	0,001250473	0,000882812
42	<i>ΔrsaE</i>	tag045	0,012395864	0,009874247	0,011812556	0,000875165	0,000825618	0,000952192
43	<i>Δteg16</i>	tag080	0,01447208	0,016594799	0,011692572	0,001927145	0,003304106	0,002033007
44	<i>ΔsprX2::tag145</i>	tag145	0,007538008	0,008681105	0,011579238	0,000809995	0,000735256	0,000894212
45	<i>ΔRNAIII-agr</i>	tag047	0,014182781	0,011028751	0,011525643	0,001971355	0,001729541	0,00147534
46	<i>Δsau6428</i>	tag016	0,014447052	0,011959294	0,011285794	0,002395706	0,001952607	0,001234881
47	<i>ΔsprX2::tag111</i>	tag111	0,010769427	0,009729496	0,011257763	0,001158333	0,001121614	0,000881917
48	<i>Δteg55</i>	tag092	0,011590248	0,012998028	0,011237325	0,002431161	0,001868055	0,001013426
49	<i>ΔsprD</i>	tag122	0,010301936	0,009418576	0,011225394	0,003870456	0,004379947	0,004423021

50	<i>ΔrsaB</i>	tag025	0,01336224	0,012232392	0,011191078	0,001580116	0,001312528	0,001306425
51	<i>Δlocus2(T)</i>	tag140	0,011606337	0,013330991	0,011124832	0,000751809	0,00121421	0,001738271
52	<i>Δsau27</i>	tag153	0,012267301	0,010689806	0,011006397	0,001832503	0,001661522	0,0011735
53	<i>ΔsprY3</i>	tag113	0,010521868	0,011798573	0,010910384	0,002381562	0,002053617	0,001825082
54	<i>ΔrsaH::tag49</i>	tag049	0,010597532	0,010143105	0,010844418	0,001181537	0,001272223	0,000826991
55	<i>ΔsprB</i>	tag121	0,009080111	0,010299405	0,010828627	0,001106429	0,001478822	0,001368445
56	<i>Δteg146</i>	tag071	0,010159696	1,65616E-05	0,010769412	0,002073449	2,04502E-05	0,002847737
57	<i>Δlocus3(M)</i>	tag139	0,00999523	0,009997804	0,010723853	0,000970336	0,000849036	0,001131352
58	<i>Δsau6353</i>	tag042	0,009884519	0,007964154	0,01063895	0,000552868	0,000535177	0,00099711
59	<i>ΔsprY2</i>	tag112	0,007462095	0,008757447	0,010622963	0,000599792	0,000903816	0,001111597
60	<i>Δteg108</i>	tag033	0,010295483	0,008882136	0,010457941	0,001192422	0,001974745	0,001601415
61	<i>Δsau5971</i>	tag142	0,009802105	0,01349853	0,010329432	0,002465451	0,003997454	0,002160693
62	<i>ΔRNAIII</i>	tag004	0,014170444	0,010611236	0,010275049	0,003316848	0,003657053	0,002461008
63	<i>Δsau6053</i>	tag154	0,014225398	0,016218532	0,010269393	0,001086037	0,001855431	0,001169988
64	<i>ΔncRNA3</i>	tag100	0,013035673	0,012380495	0,010236563	0,001258571	0,000741557	0,000917184
65	<i>ΔsRNA209</i>	tag093	0,008866442	0,010130813	0,010049265	0,001268889	0,001604088	0,000978002
66	<i>ΔsRNA260</i>	tag096	0,009747331	0,013015531	0,009891594	0,000993942	0,000867196	0,001200858
67	<i>ΔsRNA258</i>	tag116	0,009240159	0,011675679	0,00974376	0,001039	0,001124043	0,001097688
68	<i>Δteg106</i>	tag095	0,007650478	0,009602255	0,009456949	0,000627446	0,000921483	0,0011747
69	<i>Δsau5949</i>	tag117	0,011460217	0,006195893	0,009365925	0,00086813	0,002334687	0,000890455
70	<i>Δteg60</i>	tag023	0,010779561	0,007307314	0,009308451	0,001124125	0,001590295	0,000729163
71	<i>Δteg58</i>	tag022	0,009948599	0,008626191	0,00881333	0,000581195	0,000717087	0,000537607
72	<i>Δsau6041</i>	tag014	0,009641378	0,011211399	0,008274003	0,000977904	0,001276797	0,000944962
73	<i>ΔsRNA207</i>	tag087	0,008440913	0,010692125	0,008257708	0,000969301	0,002827913	0,001294233
74	<i>Δsau85</i>	tag038	0,007546316	0,009731578	0,007615057	0,001930128	0,00209437	0,001915499
75	<i>Δhfq</i>	tag143	0,005685233	0,005976531	0,005999989	0,000977557	0,000836637	0,000855489
76	<i>ΔsprC</i>	tag109	0,006839305	0,00398979	0,004908058	0,000982707	0,000483148	0,000753455

Table S5: Statistical analysis of library v2

No.	Gene ID	Tag	set1	set2	set3	set1-stderr	set2-stderr	set3-stderr
1	<i>ΔrsaG</i>	tag011	0,027211298	0,025514145	0,028428367	0,001614288	0,000652353	0,002220571
2	<i>Δteg140</i>	tag090	0,025007242	0,02577428	0,021449965	0,004178676	0,003707138	0,002108574
3	<i>Δlocus1(O)</i>	tag141	0,021840026	0,0226829	0,020851481	0,002161635	0,003118432	0,000130304
4	<i>Δsau5971</i>	tag142	0,015699444	0,016386996	0,020850203	0,00126497	5,42833E-05	0,000998626
5	<i>ΔrsaC</i>	tag133	0,020709103	0,022433531	0,020390702	0,002935855	0,004203608	0,003602309
6	<i>ΔsRNA37</i>	tag072	0,018702169	0,021304947	0,019477961	0,001435	0,001045289	0,000996228
7	<i>Δteg149</i>	tag078	0,018119609	0,014968298	0,018316941	0,000627933	0,000890146	0,001061734
8	<i>ΔrsaA</i>	tag075	0,022367883	0,020300297	0,018178036	0,00179732	0,001773309	0,001836985
9	<i>ΔncRNA5+6</i>	tag102	0,015778785	0,015898939	0,017981478	0,00018522	0,00066224	0,002371378
10	<i>Δsau6836</i>	tag017	0,012966372	0,017501506	0,017175115	3,72181E-06	1,99759E-05	0,000201695
11	<i>Δteg147</i>	tag018	0,017556252	0,015455721	0,017087156	0,000257752	4,19125E-05	0,000922438
12	<i>ΔsRNA345</i>	tag097	0,016577849	0,018956713	0,016798902	0,001911938	0,001311046	0,000570139
13	<i>ΔrsaD::tag26</i>	tag026	0,019140908	0,017205091	0,016789549	0,001600209	0,002193727	0,001766013
14	<i>ΔsRNA71</i>	tag086	0,020556668	0,020067699	0,01649037	0,002826711	0,002743425	0,002290348
15	<i>Δsau6053</i>	tag154	0,018637591	0,015973086	0,016439311	0,001504736	0,000580859	0,000964186
16	<i>ΔsRNA287</i>	tag085	0,01435144	0,014432425	0,016390295	0,000272238	0,000989973	0,001140033
17	<i>Δlocus2(T)</i>	tag140	0,018571122	0,01724257	0,01611088	0,000835134	0,0012069	0,000812228
18	<i>Δsau19</i>	tag152	0,017609194	0,018451881	0,015841604	5,32747E-05	0,000608087	0,000423324
19	<i>ΔrsaOG</i>	tag009	0,018600616	0,015426123	0,015663654	0,001149519	0,000206108	0,000456343
20	<i>Δssr42</i>	tag050	0,017695388	0,016568306	0,015607376	0,000142244	0,000526103	0,00141572
21	<i>Δsau60</i>	tag003	0,012662776	0,0169377	0,015561521	0,000564337	0,000585381	0,000884203
22	<i>ΔrsaOI::tag77</i>	tag077	0,013921615	0,016059818	0,015547188	0,0015369	0,00167518	0,001528108
23	<i>ΔncRNA7</i>	tag106	0,014825848	0,016686265	0,015323299	0,002020229	0,002609416	0,000895242
24	<i>Δsau41</i>	tag115	0,018046439	0,015665173	0,015282222	0,001877418	0,002016714	0,001644109
25	<i>ΔrsaOI</i>	tag151	0,015699327	0,013698326	0,015261741	0,001395332	0,001766743	0,000510528
26	<i>ΔsprY3</i>	tag113	0,011180318	0,01107765	0,014570878	0,000816492	0,000480837	0,001538214
27	<i>Δteg155</i>	tag053	0,019400716	0,019680528	0,014282536	0,00203473	0,002206722	0,000363316
28	<i>Δsau76</i>	tag076	0,010923771	0,012060062	0,014276968	0,000704572	0,001495701	0,001377709
29	<i>ΔsprD</i>	tag122	0,01424258	0,013587121	0,014273523	0,000138552	0,000749614	0,001869072
30	<i>ΔrsaH::tag147</i>	tag147	0,014261428	0,014121364	0,014085012	0,000479309	0,000675736	0,000784551
31	<i>ΔsprFIG1</i>	tag110	0,01256157	0,01241788	0,013870619	0,00025645	7,49709E-06	0,000339772
32	<i>Δteg16</i>	tag080	0,015368137	0,015331383	0,01362145	0,000848576	0,000553342	0,000212584
33	<i>Δteg146</i>	tag071	0,010639195	0,010676969	0,013389779	0,000786798	0,000879273	0,000555077
34	<i>ΔncRNA4</i>	tag101	0,014865438	0,012966123	0,01338389	7,11475E-06	0,000157723	0,000841812
35	<i>ΔsprF3G3</i>	tag070	0,012792207	0,013750745	0,013331938	0,001088256	0,001366899	0,000336933
36	<i>ΔsprXI::tag146</i>	tag146	0,010411962	0,01243307	0,013307889	0,00025375	0,000266908	0,000447608
37	<i>ΔRNAIII-agr</i>	tag047	0,014026293	0,014833874	0,013160902	0,00056552	0,000990534	0,001584184
38	<i>ΔsprX2::tag149</i>	tag149	0,014080156	0,013029549	0,013137877	0,001110979	0,000912163	0,000493531
39	<i>Δsau69</i>	tag027	0,014507473	0,015730749	0,012844399	0,000265593	0,001059404	0,000294826
40	<i>ΔS204</i>	tag134	0,011997557	0,012612931	0,012812562	0,000264319	0,000100394	0,00079669
41	<i>ΔsRNA334</i>	tag073	0,014225405	0,015470377	0,012715854	0,000615934	0,000148665	0,001648185
42	<i>ΔS808</i>	tag137	0,009433181	0,012261897	0,011903175	0,001495104	0,001613621	0,000927594
43	<i>Δteg108</i>	tag033	0,010688698	0,011705995	0,011557698	0,000910508	0,002072121	0,002555698
44	<i>Δsau6428</i>	tag016	0,011463447	0,010826262	0,011199573	0,000421898	6,37124E-05	0,000369743
45	<i>ΔncRNA2</i>	tag099	0,008579536	0,009007557	0,011136254	0,000602801	0,000897893	0,000310388
46	<i>Δsau6851</i>	tag032	0,014241716	0,01479189	0,010969492	0,001409085	0,001110128	0,00010691
47	<i>ΔssrS</i>	tag107	0,008695159	0,010361894	0,010917145	0,000712145	0,000960856	0,000378437
48	<i>ΔsRNA207</i>	tag087	0,008020856	0,008647303	0,01089593	0,00025497	0,00015438	0,000274409
49	<i>ΔsprF2G2</i>	tag118	0,009218033	0,01099169	0,010836963	0,000861582	0,001051795	0,000383777

50	<i>ΔrsaE</i>	tag045	0,011886276	0,011376829	0,010791589	0,001264988	0,001548916	0,000260977
51	<i>ΔrsaB</i>	tag025	0,01332744	0,008778613	0,010607547	0,000188652	0,000191873	0,000186058
52	<i>ΔsprB</i>	tag121	0,008830506	0,009635416	0,010580962	3,27944E-06	0,000229837	0,001027954
53	<i>ΔsRNA260</i>	tag096	0,010517452	0,011438796	0,010446306	5,45996E-05	0,000164494	0,000500054
54	<i>Δteg7</i>	tag079	0,010211863	0,010106798	0,010326981	0,000583353	0,000557755	5,01352E-05
55	<i>ΔsRNA209</i>	tag093	0,008615796	0,008862886	0,010281503	0,000945467	0,001234163	0,001754419
56	<i>Δsau27</i>	tag153	0,009974689	0,010006568	0,010198662	0,000705494	0,000201755	0,000525729
57	<i>ΔncRNA3</i>	tag100	0,010714418	0,010237062	0,010130187	0,000143838	0,000378798	0,000272374
58	<i>ΔsRNA258</i>	tag116	0,011861116	0,009017654	0,009915712	0,000530282	0,001545839	0,000937432
59	<i>Δteg55</i>	tag092	0,00937889	0,008234023	0,009616841	0,000189986	0,000322836	0,000357079
60	<i>ΔsprY1</i>	tag148	0,007455593	0,007893422	0,009506918	0,000735624	0,000490974	0,000305134
61	<i>Δlocus3(M)</i>	tag139	0,008917957	0,00851589	0,009417938	0,00046192	0,001078737	0,000274871
62	<i>ΔsprX2::tag145</i>	tag145	0,009435301	0,008506788	0,008954255	8,61825E-05	0,000455119	0,000544269
63	<i>Δsau6041</i>	tag014	0,008485148	0,007922044	0,008896598	0,0006855	7,48348E-05	0,000132166
64	<i>Δteg116</i>	tag030	0,009648676	0,007892002	0,008843286	0,000163625	0,000406991	0,000131199
65	<i>ΔsprX2::tag111</i>	tag111	0,009334729	0,007706769	0,008601921	0,000846501	0,000668447	0,000534915
66	<i>ΔRNAlII</i>	tag004	0,008829316	0,011343847	0,008551117	0,000258067	0,000140942	0,000133752
67	<i>ΔsRNA219</i>	tag094	0,00886972	0,009480772	0,008162873	0,000686093	0,000146404	3,71184E-05
68	<i>Δteg58</i>	tag022	0,008118073	0,007517764	0,008098982	3,63817E-05	0,000214402	0,000398989
69	<i>Δsau5949</i>	tag117	0,007507031	0	0,007980772	0,000224795	0	6,99312E-05
70	<i>ΔsprAlAs1</i>	tag144	0,009802715	0,010557799	0,0079613	0,000893894	0,000280135	0,000424963
71	<i>Δteg60</i>	tag023	0,009633733	0,008154147	0,007925359	0,001012198	0,001281515	0,000805864
72	<i>Δsau6353</i>	tag042	0,007193511	0,006757259	0,007903115	0,000186501	0,00062005	0,000235305
73	<i>ΔS596</i>	tag135	0,005508636	0,007407887	0,007822949	0,000203273	4,63558E-05	0,00013524
74	<i>ΔS627</i>	tag136	0,00459695	0,007513327	0,007496963	0,000380627	8,95065E-05	3,87235E-05
75	<i>ΔsprY2</i>	tag112	0,006525553	0,006963584	0,006793028	1,4533E-05	0,000375464	9,07227E-05
76	<i>Δsau85</i>	tag038	0,006667815	0,007211768	0,00631348	9,66372E-05	0,000412161	0,000396787
77	<i>Δteg106</i>	tag095	0,005659807	0,007578856	0,004210627	0,00055666	0,000150201	0,000155612
78	<i>ΔsprC</i>	tag109	0,00276283	0,002118222	0,002658012	0,000343461	9,92371E-05	0,000379836
79	<i>Δhfq</i>	tag143	0,001048665	0,001295608	0,001226593	7,02488E-05	0,000114922	3,58131E-05

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ARN régulateurs et adaptation aux antibiotiques chez *Staphylococcus aureus*

Staphylococcus aureus est un agent pathogène opportuniste responsable d'infections communautaires et nosocomiales pour lesquelles les traitements sont compliqués du fait de l'émergence de souches multi-résistantes. L'adaptation rapide de *S. aureus* à de multiples conditions de croissance contribue à sa virulence ; elle dépend de nombreux facteurs incluant la régulation des petits ARN (ARNrég).

Les ARN régulateurs bactériens ont été largement étudiés pendant plus d'une décennie et sont progressivement intégrés dans les réseaux de régulation. Les expériences de transcriptomique (avec puces à ADN ou séquençages massifs) et analyses bioinformatiques suggèrent que les génomes bactériens produisent des centaines d'ARN régulateurs. Cependant, si certains ont été identifiés, l'existence d'autres peut dépendre des souches, des conditions de croissance et du mode de détection utilisés pour l'acquisition et l'interprétation des données. Par exemple, il est maintenant connu que des petits ARN régulateurs (sRNA) putatifs sont en réalité les séquences non-traduites d'ARN messagers (séquence UTR). L'annotation précise des ARN régulateurs est une tâche complexe essentielle pour les études moléculaires et fonctionnelles. Nous avons défini les sRNA authentiques (*bona fide*) comme ceux qui (i) agissent probablement en *trans* et (ii) ne sont pas exprimés sur le brin opposé d'un gène codant. En utilisant les données publiées et nos propres données de RNA-seq, nous avons examiné des centaines d'ARN régulateurs putatifs de *S. aureus* en utilisant le pipeline de calcul DETR'PROK et une inspection visuelle des données d'expression pour déterminer quels signaux transcriptionnels correspondent aux sRNA. Nous concluons que la souche modèle HG003, un dérivé de NCTC8325 couramment utilisé pour les études de régulation génétique de *S. aureus*, ne contient qu'environ 50 *bona fide* sRNA, ce qui indique que ces ARN sont moins nombreux que couramment admis. Parmi eux, environ la moitié sont associés avec le core génome de *S. aureus* et le quart sont probablement exprimés dans d'autres staphylocoques (Liu et al, 2018).

Comme la plupart des ARNrég contribuent à une « régulation fine » de l'expression génique, les phénotypes dépendants des ARNrég sont généralement difficiles à détecter. Cependant, ces phénotypes peuvent apparaître comme un caractère important après plusieurs générations

sous une pression sélective. Nous avons développé une stratégie expérimentale pour mesurer l'évolution de la quantité de mutants d'ARNrég dans une population de mutants de *S. aureus* (Le Lam et al, 2017). Nous avons construit une collection de quatre-vingts mutants d'ARNrég dans la souche HG003. Chaque gène d'ARNrég est remplacé par une séquence d'ADN « code-barres » spécifique pour l'identification des mutants. La bibliothèque de mutants est cultivée dans différentes conditions de croissance, les codes-barres sont amplifiés par PCR et comptés par séquençage massif. Nous pouvons ainsi déterminer les mutants qui diminuent ou s'accumulent pendant une condition de stress et inférer une fonction à certains ARNrég. L'utilisation d'amorces spécifiques permet de multiplexer 50 conditions expérimentales.

Nous nous sommes posés la question suivante : les ARNrég de *S. aureus* participent-ils à la résistance aux antibiotiques ? J'ai obtenu des données en utilisant la méthode décrite ci-dessus. La bibliothèque de mutants d'ARNrég a été testée en présence de 10 antibiotiques ciblant les enveloppes, la synthèse des protéines, la répllication de l'ADN ou la synthèse de l'ARN. Plusieurs mutants sont affectés par les conditions de croissance testées. Par exemple, la proportion du mutant sau6836 augmente considérablement en présence de vancomycine et est réduite en présence de flucloxacilline, cloxacilline ou céfazoline. La proportion du mutant ARNIII-*agr* augmente progressivement en présence de gentamicine, de linézolide et de clindamycine. La proportion de mutant d'ARN 6S diminue significativement en présence de rifampicine. Il est important de noter que l'ARN 6S et la rifampicine ciblent l'ARN polymérase. L'ARNrég RsaA est un régulateur des autolysines dont l'absence affecte la survie en présence de ciprofloxacine. Ces exemples illustrent la puissance des expériences de compétition pour identifier les phénotypes dépendants des ARNrég et révèlent que plusieurs ARNrég contribuent à moduler la résistance aux antibiotiques.

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Introduction

1 *Staphylococcus aureus*

1.1 Caractéristiques générales

Staphylococcus aureus (*S. aureus*), souvent appelé le staphylocoque doré, a été isolé pour la première fois du pus d'une plaie chirurgicale dans une articulation du genou en 1880 à Aberdeen (Écosse) par le chirurgien Sir Alexander Ogston (Ogston 1881; Ogston 1882). Sous un microscope, *S. aureus* forme des grappes de bactéries en forme de sphère, ce qui l'a amené à nommer les staphylocoques de l'organisme en les distinguant des streptocoques formant des chaînes qui sont également associés aux infections des plaies chirurgicales (Ogston 1882). En 1884, Rosenbach a différencié les staphylocoques isolés de l'homme en fonction de la pigmentation des colonies et a proposé la nomenclature de *Staphylococcus aureus* pour les colonies pigmentées jaune orangé ou or et *Staphylococcus albus* (maintenant désigné *Staphylococcus epidermidis*) pour les colonies blanches. La pigmentation jaune est produite par la staphyloxanthine, un caroténoïde lié à la membrane.

S. aureus est une bactérie à Gram positif ayant un diamètre de 0,5 à 1,5 μm (Figure 1). Il est non motile, ne forme pas de spore et est un anaérobie facultatif qui peut se développer par respiration aérobie ou par fermentation. *S. aureus* est tolérant à de fortes concentrations de sel et présente une résistance à la chaleur (Harris *et al.* 2002). Il se reproduit asexuellement par fission binaire; la séparation complète des cellules filles est assurée par l'autolysine de *S. aureus* (Varrone *et al.* 2014). Le genre auquel il appartient, nommé *Staphylococcus*, est positif pour la catalase et négatif pour l'oxydase, ce qui le différencie du genre *Streptococcus*, qui est négatif pour la catalase; en outre, ils ont des compositions de parois cellulaires différentes (Harris *et al.* 2002).

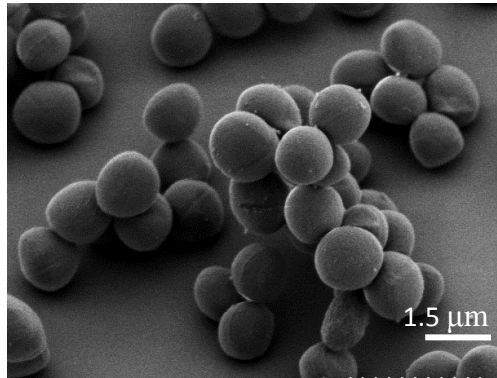


Figure 1 *Staphylococcus aureus* au microscope électronique.

(Thierry Meylheuc, Claire Morvan and David Halpern, INRA, Micalis, Jouy-en-Josas)

La paroi cellulaire de *S. aureus* est une couche protectrice épaisse et dure (G D Shockman and Barren 1983). En général, la paroi cellulaire de *S. aureus* présente les caractéristiques suivantes: i) couche de peptidoglycane épaisse, elle représente 50% de la masse de la paroi cellulaire et est capable de résister à une pression osmotique interne élevée; ii) les acides téichoïques, un groupe de polymères contenant des phosphates contribuant à environ 40% de la masse de la paroi cellulaire (Knox and Wicken 1973). Deux types d'acides téichoïques sont présents, l'acide téichoïque de la paroi cellulaire lié par covalence au peptidoglycane et à l'acide lipotéichoïque associé à la membrane inséré dans la bicouche phospholipidique des bactéries, qui servent d'agents de chélation et certains types d'adhérence; iii) protéines de surface, exoprotéines et peptidoglycanes hydrolases (autolysines), qui constituent les 10% restants du poids de la paroi cellulaire. Certains de ces composants sont impliqués dans l'adhésion et sont des déterminants de la virulence (Harris *et al.* 2002). Sous la paroi cellulaire se trouve le cytoplasme qui est entouré par la membrane cytoplasmique. À terme, il a été démontré que certaines souches cliniques de *S. aureus* possèdent des polysaccharides capsulaires (Fournier 1990; Thakker *et al.* 1998); il est rapporté que la production de capsules diminue la phagocytose *in vitro*, augmentant ainsi la virulence de *S. aureus* dans un modèle de bactériémie chez la souris (Wilkinson and Holmes 1979; Thakker *et al.* 1998).

1.2 Pathogénicité et maladies infectieuses

S. aureus fait partie de la flore animale normale et possède une capacité d'adaptation remarquable aux différentes niches (van Belkum *et al.* 2009). Il colonise préférentiellement les narines antérieures (Williams 1963) mais se trouve dans les sites extra-nasaux, y compris la peau, le pharynx (Ridley 1959), le tractus gastro-intestinal (Rimland and Roberson 1986), le tractus uro-génital féminin (Guinan *et al.* 1982) et les aisselles (Dancer and Noble 1991). Environ 30% de la population humaine est asymptomatique et colonisée de manière persistante (Wertheim *et al.* 2005; van Belkum *et al.* 2009). Cependant, ce microorganisme commensal est maintenant considéré dans le monde entier comme un agent pathogène opportuniste important lié à un large éventail d'infections associées à la communauté et acquises en milieu hospitalier, des infections superficielles aux maladies invasives et potentiellement mortelles. Une transition épidémiologique remarquable a été observée au cours des deux dernières décennies: i) un nombre croissant d'infections associées aux soins de santé en particulier des endocardites et des prothèses, ii) une épidémie d'infections cutanées et des tissus mous associées aux souches avec des facteurs de virulence particuliers (Tong *et al.* 2015); ce qui entraîne une morbidité et une mortalité considérables dans le monde entier.

❖ Infections de la peau et des tissus mous

S. aureus a traditionnellement été la principale cause d'infections bronchiques généralisées, avec l'apparition d'une épidémie mondiale d'infections sexuellement transmissibles associées au SARM (CA-MRSA) (Tong *et al.* 2015). La peau et les muqueuses sont d'excellentes barrières naturelles contre l'invasion des tissus locaux par *S. aureus*. Cependant, les brèches dans les barrières cutanées consécutives à un traumatisme et les interventions chirurgicales favorisent l'entrée de *S. aureus* dans les tissus sous-cutanés, créant ainsi des abcès locaux (Elek 1956; Elek and Conen 1957). La peau et les muqueuses sont d'excellentes barrières naturelles contre l'invasion des

tissus locaux par *S. aureus*. Cependant, les brèches dans les barrières cutanées consécutives à un traumatisme et les interventions chirurgicales favorisent l'entrée de *S. aureus* dans les tissus sous-cutanés, créant ainsi des abcès locaux (David and Daum 2010). La principale défense contre l'infection à *S. aureus* est la réponse des neutrophiles et des macrophages (Tong *et al.* 2015). Cependant, *S. aureus* peut échapper à cette réponse immunitaire de nombreuses façons, notamment en bloquant la chimiotaxie des leucocytes, en séquestrant les anticorps de l'hôte, en se détachant par la capsule polysaccharidique ou la formation de biofilms et en résistant à (Tong *et al.* 2015).

❖ Infections sanguines

La dissémination de *S. aureus* dans le sang est connue sous le nom de bactériémie, qui peut être classée en trois groupes en fonction de son apparition: i) acquis hospitalier (HA) (Klevens *et al.* 2008), communauté acquise (CA), iii) HA avec apparition dans la communauté (infection chez un patient ambulatoire ayant eu un contact prolongé avec le système de santé) (Thomer *et al.* 2016). La bactériémie est une maladie potentiellement mortelle qui peut entraîner une septicémie et un choc aigu. L'endocardite est une infection sanguine typique due à la colonisation à long terme du système vasculaire par *S. aureus* (Dastgheyb and Otto 2015). Il est largement associé aux utilisateurs de drogues par voie intraveineuse, qui introduisent *S. aureus* directement dans la circulation sanguine à travers des aiguilles contaminées ou une mauvaise stérilisation du site d'injection (Miro *et al.* 2005; Shrestha *et al.* 2015). De plus, les prothèses, y compris les cathéters veineux centraux, les implants chirurgicaux et les prothèses orthopédiques, servent de conduit direct dans l'espace intravasculaire et constituent des facteurs de risque de bactériémie (Jensen *et al.* 1999).

1.3 Adaptabilité et résistance aux antibiotiques

Les infections à *S. aureus* peuvent être à la fois fréquentes et graves, notamment en raison des vagues d'augmentation de la résistance aux antimicrobiens et de l'évolution du spectre clinique (Chambers and DeLeo 2009; Tong *et al.* 2015). *S. aureus* est connu pour être hautement adaptable.

L'adaptabilité innée de *S. aureus* a conduit à l'émergence d'une résistance à de multiples classes d'antibiotiques par l'acquisition d'éléments génétiques mobiles (MGE) codant des déterminants de résistance ou des mutations de locus influençant la sensibilité aux antibiotiques (DeLeo and Chambers 2009; Jensen and Lyon 2009; Fitzgerald 2014). *S. aureus* résistant à la méthicilline (SARM) a été documenté à un rythme rapide et croissant depuis son lancement en 1959. Les clones de SARM associés à l'hôpital (HA-SARM) sont désormais reconnus comme la principale cause d'infections nosocomiales dans le monde entier (Carleton *et al.* 2004; Fridkin *et al.* 2005; Nickerson *et al.* 2009). L'émergence de SARM associées à la communauté (CA-SARM) au cours des dernières décennies est également devenue préoccupante, car les souches virulentes de SARM-CA se propagent rapidement et peuvent affecter des individus apparemment en bonne santé (Kourbatova *et al.* 2005; Giersing *et al.* 2016). Le traitement des isolats de SARM nécessite l'utilisation de vancomycine, de clindamycine, de linézolide ou de daptomycine (Liu *et al.* 2011). L'émergence de *S. aureus* résistant à la vancomycine (VRSA) et de *S. aureus* intermédiaire contre la vancomycine (VISA) est préoccupante, car la vancomycine est considérée comme le traitement de dernier recours contre le SARM. Dans le même temps, le développement du vaccin *S. aureus* n'a jusqu'à présent pas été couronné de succès, ni l'utilisation d'anticorps contre le polysaccharide staphylococcique (Shinefield *et al.* 2002) ou contre les facteurs de virulence sécrétés (Kernodle 2011; Fowler *et al.* 2013; Thomer *et al.* 2016).

1.4 Facteurs de virulence de *S. aureus*

Les staphylocoques pathogènes sont généralement identifiés par leur capacité à produire de la coagulase et du caillot de sang humain et animal (Kloos and Musselwhite 1975). Cela permet de distinguer les souches positives pour la coagulase, y compris *S. aureus*, des souches à coagulase négative (CoNS), telles que *S. epidermidis*.

La virulence est définie comme la capacité d'un agent pathogène à réduire l'aptitude de l'hôte, en d'autres termes, la capacité d'un organisme à établir une infection et à provoquer une maladie chez un hôte. *S. aureus* code une grande variété d'adhésines et de facteurs de virulence impliqués dans divers mécanismes de virulence tels que l'adhésion, la colonisation, la formation de biofilms, l'évasion immunitaire, la stimulation immunitaire ou la lyse cellulaire et la résistance à la phagocytose (Dastgheyb and Otto 2015). *S. aureus* a trois régulateurs mondiaux de la virulence bien documentés: *agr* (Recsei *et al.* 1986; Morfeldt *et al.* 1988), *sar* (Cheung *et al.* 1992) and *sae* (Giraud *et al.* 1994), qui régulent l'expression de protéines de surface et exoprotéines (par exemple, toxines) (Harris *et al.* 2002). Par exemple, la famille des peptides phénol-solubles de la moduline (PSM), qui est la cytotoxine la plus puissante (Peschel and Otto 2013), est sous le contrôle du réseau de régulation *agr* qui contrôle également de nombreuses autres toxines telles que les hémolysines. toxine) (*e.g.* α -toxin) (Queck *et al.* 2008) et les leucotoxines (Recsei *et al.* 1986). *S. aureus* peut produire toute une gamme de toxines extracellulaires au cours du processus d'évasion immunitaire, notamment la toxine-1 du syndrome de choc toxique (TSST-1), les entérotoxines et les toxines exfoliatives (Harris *et al.* 2002). TSST-1 est la toxine responsable du syndrome de choc toxique (TSS) qui n'est causé que par des souches portant le gène TSST-1 (Jamart *et al.* 2005). L'ingestion d'entérotoxine produite par *S. aureus* dans des aliments contaminés peut provoquer une intoxication alimentaire (Argudín *et al.* 2010; Hennekinne *et al.* 2012). Les toxines exfoliatives sont associées au syndrome de la peau échaudée staphylococcique (MSSRA) (Mishra *et al.* 2016).

Certains facteurs de virulence sont codés par des gènes situés sur MGE, tels que plasmides, transposons, éléments d'insertion, îlots de pathogénicité (certaines entérotoxines associées à une intoxication alimentaire) (Dinges *et al.* 2000) or lysogenic bacteriophages (*e.g.* Panton-Valentine leucocidin) (Narita *et al.* 2001), ou bactériophages lysogènes (par ex. (Narita *et al.* 2001), et des facteurs interfèrent ou même suppriment clairement l'immunité innée de l'hôte, telle que la staphylokinase (Rooijackers *et al.* 2005).

2 Aperçu des petits ARN régulateurs chez

S. aureus

S. aureus est souvent exposé à un large éventail de contraintes dans ses environnements naturels en constante évolution, tels que la famine, la température, le pH, le taux d'oxygène et les antibiotiques. Elle s'est développée en réponse à ces changements, une pléthore de voies de signalisation qui détectent l'environnement et coordonnent les altérations temporelles de l'expression génique et de l'activité protéique qui favorisent la survie et la prolifération. Par conséquent, la compréhension et l'exploration de réseaux de régulation complexes et de leur dynamique qui sous-tendent des réponses adaptatives rapides et la production de facteurs de virulence sont une condition préalable pour trouver des stratégies alternatives pour lutter contre les infections à *S. aureus*. Les ARN régulateurs, associés à des systèmes à deux composants et à d'autres protéines régulatrices, sont impliqués dans ces circuits régulateurs.

Les ARN régulateurs, souvent appelés petits ARN (ARNs), sont généralement non codants et courts (50-500 nts) (Waters and Storz 2009). Leur fonction principale est souvent la régulation post-transcriptionnelle de l'expression des gènes (Mandin and Guillier 2013). A ce jour, de nombreux ARNs ont été prédits et identifiés chez *S. aureus* par bioinformatique (Pichon and Felden 2005; Geissmann *et al.* 2009; Marchais *et al.* 2009), ADN-tableaux (Anderson *et al.* 2006; Roberts *et al.* 2006; Mader *et al.* 2016), séquençage de l'ADNc (Hüttenhofer and Vogel 2006) et méthodes ARN-seq (Bohn *et al.* 2010; Howden *et al.* 2013; Broach *et al.* 2016; Carroll *et al.* 2016). Les ARNs ont différents modes d'action, ils agissent soit par appariement de bases avec des cibles d'acides nucléiques (par exemple, ARNm), en modifiant leur stabilité et leur efficacité de traduction; ou par la modulation de l'activité protéique en imitant leurs substrats (Mandin and Guillier 2013).

2.1 La diversité des interactions ARNs-ARNm

Les ARNs agissent en *cis* ou en *trans* en fonction de leur relation structurelle avec leurs gènes cibles et affectent les gènes aux niveaux transcriptionnel ou post-transcriptionnel:

- i) les ARN régulateurs agissant en *cis* sont généralement situés dans la région non traduite (UTR) 5' ou 3' de l'ARNm. Ils régulent la transcription des gènes adjacents en réagissant aux agents transactionnels ou aux indices environnementaux, tels que la température (thermosensors), la concentration intracellulaire de métabolites (riboswitches), les ARNt non chargés (boîtes T) ou les protéines. (Romby and Charpentier 2010).
- ii) les ARN régulateurs agissant en *trans* sont souvent situés dans des régions intergéniques et éloignés de leurs cibles d'ARNm. Ils présentent généralement des complémentarités d'appariement de bases partielles avec leurs cibles (Waters and Storz 2009; Richards and Vanderpool 2011; Jagodnik *et al.* 2017). Ils peuvent avoir plusieurs cibles (Romby and Charpentier 2010).
- iii) Les ARN antisens (asARN) sont transcrits à partir du brin opposé du gène cible. La plupart d'entre eux agissent en tant qu'ARNs *trans*-agissant et présentent souvent un degré élevé ou une complémentarité complète avec l'ARNm ciblé (Romby and Charpentier 2010). Un exemple spécifique a montré que les asRNA régulaient l'expression de l'opéron *ubiG* cible en *cis* par interférence transcriptionnelle au locus *ubiG* (Andre *et al.* 2008).

2.1.1 ARN régulateurs agissant de la sorte

Les régions non traduites (UTR) de l'ARNm contiennent des caractéristiques importantes affectant la régulation post-transcriptionnelle et traductionnelle de l'expression des gènes (Pesole *et al.* 2001; Ren *et al.* 2017).

Les ARNs agissant en *cis* font généralement partie des 5'UTR ou 3'UTR d'un ARNm, dont l'expression est régulée par les ARNs (Cho and Kim 2015). La longueur de 5'ou 3' UTR varie de quelques centaines à plusieurs centaines de nucléotides (Bouloc and Repoila 2016). Ils contiennent des sites de régulation dédiés qui peuvent non seulement être reconnus par divers régulateurs agissant en trans (métabolites, ARNt non chargés, protéines), mais fonctionnent également comme capteurs directs des signaux environnementaux (température, ions divalents, pH) (Breaker 2009; Narberhaus 2010; Ramesh and Winkler 2010; Smith *et al.* 2010). Certains ARN agissant en *cis* sont bien connus pour altérer l'expression des facteurs de virulence (Somerville and Proctor 2009; Caldelari *et al.* 2013).

2.1.1.1 5'UTR en tant que source d'ARN régulateurs

- i) Les ARNs actifs dans la détection des métabolites utilisés comme cibles potentielles d'antibiotiques

Un élément d'ARN à action *cis* très répandu dans 5'UTR des ARNm est constitué par les riboswitches qui détectent les métabolites et régulent les gènes associés (Breaker 2011). Les riboswitches comprennent deux domaines fonctionnels: un aptamère et une plate-forme d'expression (Nudler and Mironov 2004; Coppins *et al.* 2007; Dambach and Winkler 2009; Henkin 2009). L'aptamère ou le domaine du capteur est un récepteur conservé et structuré qui reconnaît spécifiquement par un ligand défini; la plate-forme d'expression subit des changements structurels significatifs, puis interrompt généralement l'expression de l'ORF en aval à la suite de la liaison du ligand (Figure 2), mais certains l'allument (Serganov 2010). Cette régulation se produit aux niveaux transcriptionnel ou post-transcriptionnel en fonction des positions de chargement du riboswitch sur l'ARNm, entraînant la répression ou l'activation de l'expression génique (Breaker 2011).

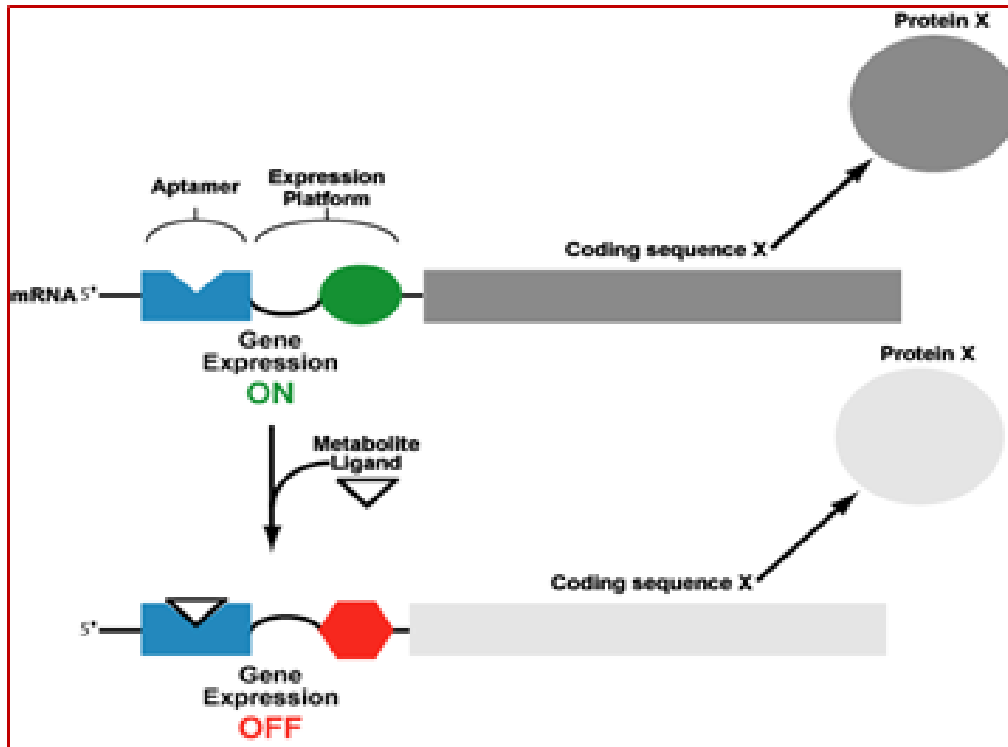


Figure 2 Mécanisme commun des commutateur. (Edwards and Batey 2010)

Les riboswitches sont des cibles attrayantes pour le développement de nouveaux composés antibactériens, qui offrent peut-être une solution alternative aux agents pathogènes nosocomiaux multi-résistants (Breaker 2009; Mulhbacher *et al.* 2010). A titre d'exemple, PC1 (2,5,6-triaminopyrimidin-4-one), un composé dérivé de pyrimidine, se lie aux riboswitches guanine et coupe constitutivement le gène *guaA* essentiel, qui code pour la synthase monophosphate de guanosine (GMP). Il a donc été démontré que PC1 exerce une activité bactéricide contre *S. aureus* et réduit l'infection chez les souris (Mulhbacher *et al.* 2010). Fait important, le PC1 a une activité à spectre étroit car il cible exclusivement les bactéries contenant le riboswitch à purine, ce qui devrait réduire la pression sélective pour la résistance des bactéries non ciblées (Caldelari *et al.* 2013); en revanche, il s'agit également de la principale limitation pour valider cliniquement PC1, car elle ne cible pas toutes les bactéries contenant des riboswitches à guanine, mais uniquement celles dans lesquelles le *guaA* est sous le contrôle d'un riboswitch. De plus, il n'existait pas de cytotoxicité apparente

chez les mammifères (souris) car les riboswitches étaient généralement absents chez l'hôte eukaryote (Mulhbacher *et al.* 2010).

ii) les ARNs à action Cis détectant des antibiotiques

La stabilisation induite par les antibiotiques des transcrits des gènes de la famille des *erm* chez *S. aureus* est un exemple bien caractérisé de la région régulatrice non traduite en 5'. Il s'agit d'un mécanisme d'atténuation de la translation contrôlé par le décrochage du ribosome spécifique d'un site et utilisé pour l'expression inductible des gènes de résistance aux antibiotiques *erm* (méthyltransférase) (Gryczan *et al.* 1980; Horinouchi and Weisblum 1980). La famille des *erm* spécifie les méthylases d'ARNr qui confèrent une résistance aux antibiotiques macrolides, lincosamides et streptogramines B (MLSB) en réduisant l'affinité entre ces antibiotiques et les ribosomes (Sandler and Weisblum 1989). En bref, l'ARNc d'*ermC* est transcrit de manière constitutive mais traduit à un niveau extrêmement bas en raison du masquage du site de liaison au ribosome et du codon d'initiation dans la structure secondaire de l'ARNm; en présence de concentrations sous-inhibitrices d'un inducteur (érythromycine ou lincosamides similaires) (Weisblum *et al.* 1971), l'antibiotique se lie aux ribosomes en provoquant un blocage des ribosomes. Le blocage pendant la traduction du peptide leader déclenche le changement de conformation qui libère le RBS *ermC* et active l'expression du gène de la méthylase (Vazquez-Laslop *et al.* 2008). Un mécanisme similaire a été proposé pour le gène *S. aureus ermA* qui a une structure plus complexe et code pour deux peptides, contrairement à celui de l'*ermC* (Murphy 1985).

2.1.1.2 3'UTR comme source d'ARN régulateurs

Les UTR bactériennes 5' sont plus ciblées que les 3'UTR. Cependant, les 3'UTR bactériens particulièrement longs 3'UTR ont récemment émergé comme une nouvelle classe d'éléments régulateurs post-transcriptionnels (Ren *et al.* 2017), ii) sont considérés comme un riche réservoir de petits ARN régulateurs soit par traitement de

le long 3'UTR ou par transcription de novo d'un promoteur interne (Kawano *et al.* 2005; Chao *et al.* 2012), iii) régulent la dégradation de l'ARN, iv) peuvent être ciblés par les ARNs régulateurs, et v) interagissent avec 5 'UTR pour réguler l'initiation de la traduction (Ruiz de los Mozos *et al.* 2013).

Titre : ARN régulateurs et adaptation aux antibiotiques chez *Staphylococcus aureus*

Mots clés : *Staphylococcus aureus*, ARN régulateurs, Adaptation, antibiotiques

Résumé : *Staphylococcus aureus* est un agent pathogène opportuniste responsable d'infections communautaires et nosocomiales pour lesquelles les traitements sont compliqués du fait de l'émergence de souches multi-résistantes. L'adaptation rapide de *S. aureus* à de multiples conditions de croissance contribue à sa virulence ; elle dépend de nombreux facteurs incluant la régulation des petits ARN (ARNrég).

Nous avons réalisé une étude précise de tous les petits ARN de la souche modèle HG003. Nous avons trouvé environ 50 authentiques (*bona fide*) petits ARN, un nombre beaucoup plus faible que précédemment rapporté.

Comme la plupart des ARNrég contribuent à une « régulation fine » de l'expression génique, les phénotypes dépendants des ARNrég sont généralement difficiles à détecter. Cependant, ces phénotypes peuvent apparaître comme un caractère important après plusieurs générations sous une pression sélective.

Nous avons développé une stratégie expérimentale pour mesurer l'évolution de la quantité de mutants d'ARNrég dans une population de mutants de *S. aureus*.

Nous nous sommes posés la question suivante : les ARNrég de *S. aureus* participent-ils à la résistance aux antibiotiques ? Dans ce mémoire, nous présentons des données en utilisant la méthode décrite ci-dessus. La bibliothèque de mutants d'ARNrég a été testée en présence de 10 antibiotiques ciblant les enveloppes, la synthèse des protéines, la réplication de l'ADN ou la synthèse de l'ARN. Plusieurs mutants sont affectés par les conditions de croissance testées. Ces exemples illustrent la puissance des expériences de compétition pour identifier les phénotypes dépendants des ARNrég et révèlent que plusieurs ARNrég contribuent à moduler la résistance aux antibiotiques.

Title: *Staphylococcus aureus* regulatory RNAs driving fitness upon antibiotic exposure

Keywords: *Staphylococcus aureus*, Regulatory RNA, fitness, antibiotics

Abstract: *Staphylococcus aureus* is an opportunistic pathogen and one of the major bacteria responsible for community-acquired and nosocomial infections for which the treatment is complicated by the emergence of multidrug resistant strains. Its adaptation to multiple growth conditions, which contributes to its virulence, is under the control of numerous factors including regulatory RNAs, often called sRNAs for small RNAs.

We performed an accurate survey of all sRNAs from the model strain HG003. We found about 50 bona fide sRNAs, a number much lower than previously reported.

As most sRNAs contribute to the "fine-tuning" of gene expression, sRNA-dependent phenotypes are generally difficult to detect. However, sRNA-mediated phenotypes may

emerge as dominant traits after a few generations under selective pressure.

We set up an experimental strategy to evaluate the fitness of *S. aureus* sRNA mutants within a population of sRNA mutants.

We questioned whether sRNAs could affect the antibiotic resistance in *S. aureus*. Here, we present data using the above-described method with the sRNA mutant library tested in the presence of 10 antibiotics (targeting the cell wall and cell membrane, inhibiting the biosynthesis of protein, interrupting DNA replication and RNA synthesis of bacteria). Several mutants were affected by the tested growth conditions. These examples illustrate the power of fitness experiments to identify phenotypes and reveals that several sRNAs contribute to modulate the resistance to antibiotics.