Regulatory RNAs in Staphylococcus aureus : Function and Mechanism

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ABBREVIATIONS

σ^B	Sigma B
ADI	Arginine deiminase
agr	Accessory gene regulator
ahpCF	Alkyl hydroperoxide reductase
AIP	Autoinducing peptide
asRNAs	antisense RNAs
BCAA	Branched-chain amino acid
bp	Base pairs
CA-MRSA	Community-acquired MRSA
CcpA	Catabolite control protein A
CcpN	Control catabolite protein of gluconeogenic
ChiA	Chitinase
CHIPS	Chemotaxis inhibitory protein of *Staphylococcus aureus*
chp	Chemotaxis-inhibiting protein
clfB	Clumping factor B
coa	Coagulase
colA	Kappa-toxin or collagenase
cpd	2',3'-cyclic nucleotide phosphodi-esterase
CS	Cutting site
csp	Cold shock protein
Dcp	D-alanyl carrier protein
ds	Double-strand
EA	Ethanolamine
efb	Fibrinogen-binding protein
eut	Ethanolamine utilization
fbp	Fibronectin binding protein
FMN	Flavin mononucleotide
FNR	Fumarate and nitrate reductase regulatory
ftn	Ferritin
Fur	Ferric uptake repressor
Glc-6P	Glucosamine-6-phosphate
HA-MRSA	Hospital-acquired MRSA
Hfq	Host factor-I
hla	α-hemolysin
LTAs	Lipoteichoic acids
MRSA	Methicillin-resistant *Staphylococcus aureus*
narGHJI	Nitrate reductase
narT	Nitrate transporter
nir-operon	Nitrite reductase
NO	Nitrosative
NreBC	Nitrogen regulation
ORF	Open reading frame

PBP2	Penicillin binding protein 2
PC	Pyrimidine compound
PG	Plasminogen
PIA	Polysaccharide intercellular adhesin
PNAG	Poly-N-acetylglucosamine
PNPase	Polynucleotide phosphorylase
PSM	Phenol-soluble modulin
ptp	Protein tyrosine phosphatase
PVL	Panton-Valentine leukocidin
RBS	Ribosome-binding site
rot	Repressor of toxin
roxS	Related to oxidative stress
sak	Staphylokinase
SAM	S-adenosyl methionine
SCIN	Staphylococcal complement inhibitor
SCV	Small colony variant
SD	Shine-Dalgarno
sdhCAB	Succinate dehydrogenase
sec	Staphylococcal enterotoxin type C
sodA	Superoxide dismutase
Spr	Small pathogenicity island RNAs
SrhSR	Staphylococcal *resDE* homologues
sRNAs	*Trans*-encoded RNAs
SrrAB	Staphylococcal respiration response
SSRs	Small stable RNAs
TA	Toxin-antitoxin
TCA	Tricarboxylic acid
TCS	Two-component system
TPP	Thiamine pyrophosphate
TSST	Toxic shock syndrome toxin
UTR	Untranslated regions
VR-RNA	VirR-regulated RNA
VRSA	Vancomycin-resistant *Staphylococcus aureus*

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INTRODUCTION

I. *Staphylococcus aureus*

1. General features

In the second half of the nineteenth century, the Scotland surgeon Sir Alexander Ogston identified for the first time the bacterium *Staphylococcus* from a patient knee abscess joint (Ogston 1984). In 1884, based on pigmented colony types, the German physician and microbiologist Friedrich Julius Rosenbach renamed this bacterium *Staphylococcus aureus* to differentiate it from *Staphylococcus albus*, which is now called *Staphylococcus epidermidis*.

Staphylococcus aureus (in Greek *Staphylo* "bunch of grapes" and in Latin *aureus* "golden") is a Gram-positive coccal (spherical) bacterium member of the *Staphyloccaceae* family (Figure 1).

Scientific classification	
Domain:	Bacteria
Kingdom:	Eubacteria
Phylum:	Firmicutes
Class:	Bacilli
Order:	Bacillales
Family:	Staphylococcaceae
Genus:	*Staphylococcus*
Species:	*aureus*
Binomial name	
Staphylococcus aureus	
Rosenbach 1884	

Figure 1: *Staphylococcus aureus* also known "the golden Staph*"*.

This bacterium is non-motile, non-spore-forming, facultative anaerobic and grows in oxygenated conditions or ferments glucose to produce mainly lactic acid. Its colonies are fairly large, round, golden-yellow on rich medium and have a β-hemolytic activity on blood agar plates. *S. aureus* is positive for catalase (decomposes hydrogen peroxide to water and oxygen), reduces nitrate to nitrite and ferments mannitol (in contrast to *S. epidermidis*).

S. aureus produces a membrane-associated coagulase, with reacts with prothrombin in the blood to form staphylothrombin. This complex triggers blood clotting by converting soluble fibrinogen to insoluble fibrin and may protect the bacteria from phagocytosis (Tortora et al. 2013). *S. aureus* is negative for urease.

S. aureus reproduces by binary fission. After division, the daughter cells often remain attached, generating bacterial clusters. *S. aureus* has a circular chromosome of 2.8 M base pairs (bp) with a low GC composition (32.8%). The genome has about 2700 coding sequences of which approximately 38% have unknown function.

2. Role in disease

S. aureus is one of the most common causes of hospital-acquired (nosocomial) and community-acquired infection. It causes disease by three main mechanisms: (1) invasion of tissues and inflammation, (2) toxin production and (3) biofilm formation. *S. aureus* expresses a large number of virulence factors that include:

- Surface proteins, invasion factors (*e.g.*, leukocidin, kinases, hyaluronidase).
- Structures for evading phagocytes such as surface factors (*e.g.*, capsule, protein A), biochemical compounds and enzymes (*e.g.*, carotenoids, catalase, lipase, β-lactamase) or immunological disguises (*e.g.*, coagulase, protein A).
- Membrane-damaging toxins, exfoliatin toxins and superantigens.

S. aureus is an important cause of death and morbidity in humans. Carriage rates of *S. aureus* may vary between human populations and different studies but can be divided in three types of population: non-carriers (approximately 20% of the population); persistent carriers (20-25%) and intermittent carriers (55-60%) (Lindsay 2008). In human, *S. aureus* primarily colonizes the nasal passage and axillae and can occasionally be found as part of the flora of the digestive and vaginal tracts (Williams 1963). When the protective layer of the human epithelium is breached and the mechanisms of host immunity fail, the bacterium is able to colonize a wide range of different organs. Most common infections caused by *S. aureus* are skin and soft tissue lesions such as boils, styles, and furuncles. However, when this bacterium enters the bloodstream, it can cause more serious and life-threatening infections such as pneumonia (lung infection), endocarditis (inflammation of the heart inner

layer, the endocardium), osteomyelitis (infection and inflammation of bone or bone marrow) or thrombophlebitis (inflammation of veins caused by blood clots), urinary tract infections, bacteremia (presence of bacteria in the blood), sepsis (whole body inflammation cause by an infection) (Figure 2).

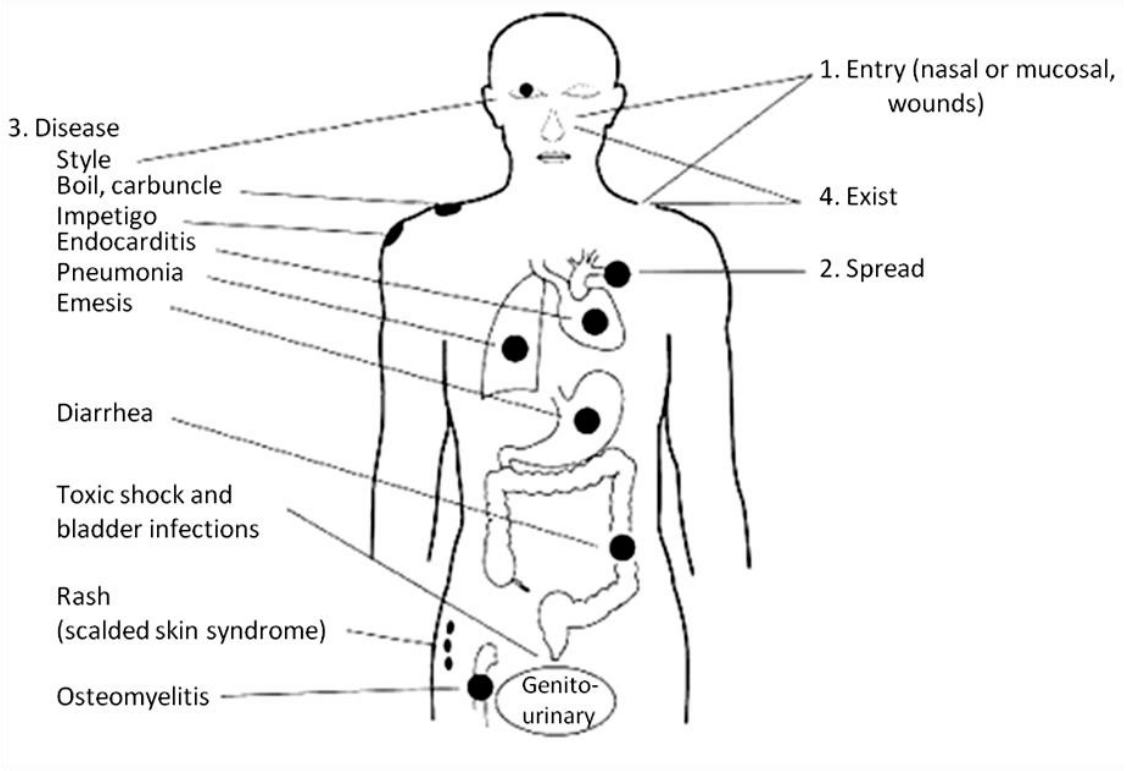


Figure 2: Sites of infection and diseases caused by S. *aureus.*

(From http://www.tjclarkinc.com/bacterial_diseases/hold/staphylococcus.htm).

S. aureus produces enzymes and toxins that are also responsible for food poisoning, toxic shock syndrome and scalded skin syndrome (Figure 2). Food poisoning is caused by eating food contaminated with enterotoxins released by the bacteria rather than by its infection. Toxic shock syndrome is caused by the release of a toxic shock syndrome toxin (TSST, 22 kDa superantigens) into the bloodstream leading to high fever, vomiting, diarrhea, low blood pressure and potentially to death. Scalded skin syndrome occurs mainly in children under 5-year-old, especially newborn babies; it is due to exotoxins (exfoliatin A and B) that cause skin damages. *S. aureus* is also a substantial pathogen for animals, transmitted between species, and consequently, a worrying zoonotic agent (McEvoy et al. 2013). *S. aureus* can overwhelmingly colonize a variety of animals leading to infections in about three percent of the cases (Schukken et al. 2009). This bacterium is one the main causes of mastitis

in cow (inflammation of udder) in the dairy industry with an infection rate up to 10 to 12 percent (Tenhagen et al. 2009).

3. Antibiotic resistance

S. aureus has the potential to become resistant to multiple antibiotics, complicating significantly its treatment (Uhlemann et al. 2014). In the 1940s, the first treatment against *S. aureus* infections was penicillin. Unfortunately, *S. aureus* developed quickly resistances to this antibiotic due to the presence of penicillinase (a form of β-lactamase) that degrades penicillin. Two decades later, more than 80% of hospital- and community-acquired *S. aureus* isolates were penicillin-resistant.

In 1959, methicillin, the first semi-synthetic penicillinase-resistant penicillin, was used in clinical treatments. Two years later, the first case of methicillin-resistant *S. aureus* (MRSA) was reported. Other antibiotics from the same group such as oxacillin, cloxacillin, dicloxacillin, flucloxacillin and nafcillin were developed to replace methicillin, but *S. aureus* strains became extensively resistant to all β-lactam antibiotics due to the emergence of a penicillin binding protein 2 (PBP2) having a decrease binding affinity for penicillin. Glycopeptide antibiotics, including vancomycin, are the most efficient weapons against Gram-positive infections, including the problematic MRSA strains. The existence of vancomycin-resistant *S. aureus* (VRSA) that are already MRSA is a serious threat in human health since it could lead to a therapeutic dead-end (Zetola et al. 2005). In industrialized nations, 20-60% of all hospital *S. aureus* strains are methicillin-resistant (Hospital-acquired MRSA, HA-MRSA), and newly emerging community-acquired MRSA (CA-MRSA) combine antibiotic resistance with hyper-virulence. In 1999, it was reported that about 500,000 patients of United States hospitals were infected every year by *S. aureus* (Bowersox 1999).

The genome of MRSA strains carry *SSCmec* mobile genetic elements containing the *mecA* gene that confer resistance to methicillin and all other β-lactam antibiotics (Katayama et al. 2000). In addition, CA-MRSA strains not only have a short *SCCmec* but also a Panton-Valentine leukocidin locus (Vandenesch et al. 2003).

S. aureus can acquired resistance against virtually all antimicrobial agents available in hospitals and communities (Deleo et al. 2010). However, a new cell wall inhibitor named

teixobactin was recently reported as an efficient antibiotic for Gram-positive pathogens including drug-resistant strains (Ling et al. 2015). Teixobactin binds to motifs of lipid II and lipid III, which are precursor of peptidoglycan and cell wall teichoic acid, respectively and hence inhibits cell wall synthesis. So far, no resistant *S. aureus* strains with teixobactin were observed.

II. Overview of adaptation and virulence in *S. aureus*

1. Some examples in adaptation

Adaptation to environmental changes is a crucial step for survival and development. Most bacteria adapt to new conditions by changing genes expression including those encoding structural proteins, transporters and metabolic enzymes. The success of *S. aureus* as a virulent pathogen is due to its ability to respond to change in different environments. It tolerates dry conditions, nutrient deprivation and survives on different external surfaces (Oie & Kamiya 1996; O'Connell & Humphreys 2000). It can also grow in a variety of media within a broad range of temperatures (from 15^{o}C to 45^{o}C, optimal 37^{o}C), pHs (acidic to alkaline) and high salt conditions (concentration up to 15 percent) (Bore et al. 2007).

Dissecting the transcriptional adaptation of *S. aureus* is central for understanding how this pathogen interacts with its various hosts and is able to cause life-threatening diseases. This chapter will present a few examples of *S. aureus* adaptation, which are reminiscent of conditions found during host infection.

1.1. Oxygen limitation

S. aureus transiting between hosts or when living on the skin is in an aerobic environment but when growing in an abscess, the oxygen concentration is limited. *S. aureus* which is a facultative anaerobe that respires with or without oxygen has to sense the oxygen level and adapts its response accordingly. Transcriptomes and proteomes were performed in anaerobic conditions in strain COL to investigate the influence of oxygen on *S. aureus* global gene expression (Fuchs et al. 2007). In limited oxygen conditions, 130 genes were upregulated and 77 genes were downregulated. As expected, many genes belonging to the glycolysis, fermentation and anaerobic pathways showed an increase of expression.

Moreover, some virulence factor genes were also upregulated such as *pls, hlY, splCD, epiG, isaB*. On the other way, many genes encoding ribosomal proteins, tRNA synthesis, elongation factor G and enzymes involved in the tricarboxylic acid (TCA) cycle, decrease their transcription.

SrrAB (**S**taphylococcal **r**espiration **r**esponse) [or **Srh**SR (**S**taphylococcal ***r**esDE* **h**omologues)] was the first oxygen sensor system discovered in *S. aureus*. It is a two-component system (TCS) in which SrrB is a membrane sensor and SrrA is a cytoplasmic response regulator (Throup et al. 2001; Yarwood et al. 2001). It is homologous to the ResDE aerobic/anaerobic regulation system from *Bacillus subtilis*. The *srrAB* mutant had a growth defect in anaerobiosis while no phenotype was observed in aerobic condition. The transcriptome analysis of wild-type and *srrAB* strains grown in aerobic and micro-aerobic condition indicate that the expression of regulatory RNA RNAIII and *spa* (encoding the protein A) genes (see chapter IV.3 for more details) increase in *srrAB* mutant under micro-aerobic conditions. In addition, the SrrAB system is involved in nitrosative (NO) and hypoxia stress response (Kinkel et al. 2013). Various *srrAB*-required genes that were found to vary during two stress conditions are involved in cytochrome and heme biosynthesis, anaerobic metabolism and NO-detoxification.

SrrAB depletion affects the expression of genes involved in the TCA cycle, in fermentation and energy, arginine catabolism, xanthine catabolism and cell morphology (Throup et al. 2001). It also impacts biofilm formation and increases cell death. It has been proposed that SrrAB links the oxygen response to the regulation of virulence factors (Yarwood et al. 2001). Indeed, the *srrAB* mutant has an attenuated virulence in a murine model for hematogenous pyelonephritis infection as compared to a wild-type strain (Throup et al. 2001).

Under anaerobic condition, the response regulator SrrA-P binds to a 100 bp DNA sequence located in the upstream region of the *ica* gene to activate the production of polysaccharide intercellular adhesin (PIA). PIA is an important cell surface factor that protects *S. aureus* against human neutrophils (Ulrich et al. 2007). Therefore, the SrrAB TCS plays a positive role in PIA production helping bacterial survival against human defense mechanisms. A model of SrrAB activity is presented in figure 3.

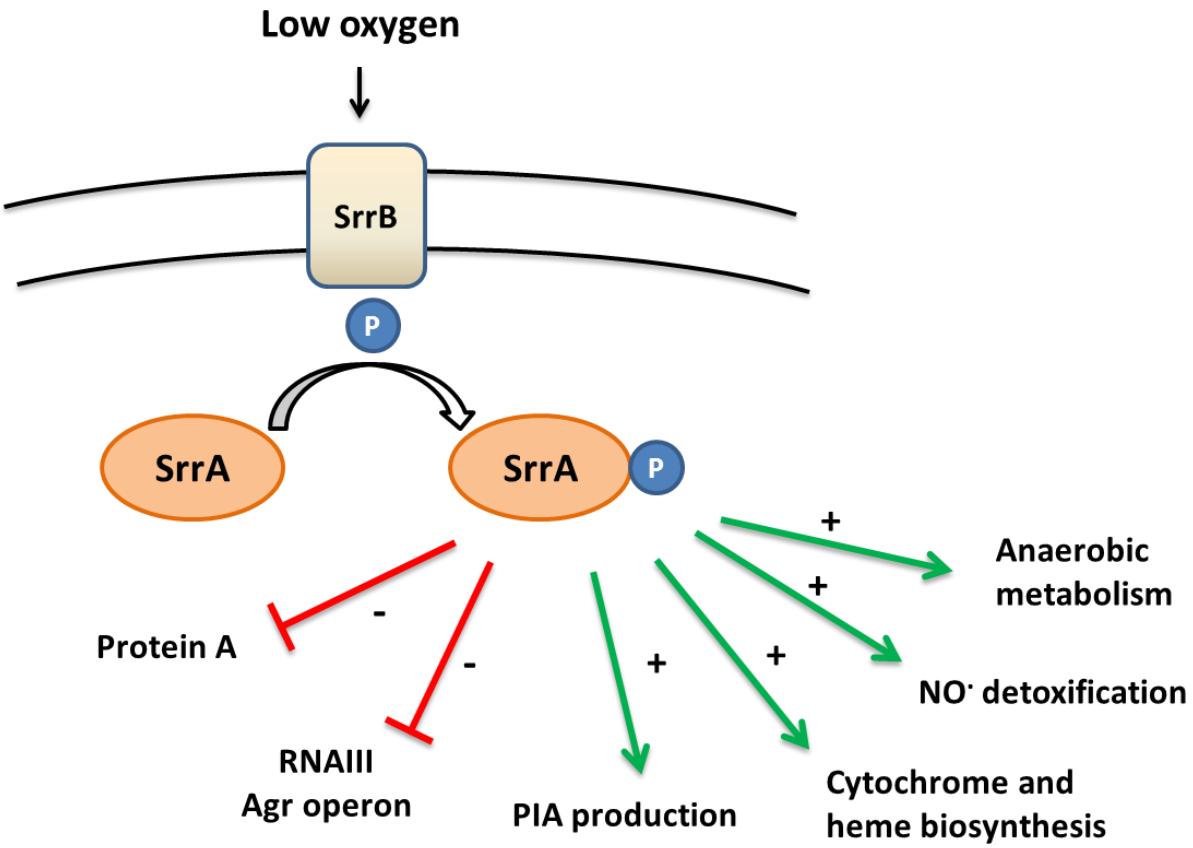


Figure 3: Model of SrrAB system. PIA: Polysaccharide intercellular adhesin production.

The second oxygen sensor system discovered was the TCS NreBC (**N**itrogen **re**gulation) present in some staphylococci such as *S. aureus*, *S. epiderminis, S. carnosus* (Kamps et al. 2004). NreB is a cytoplasmic oxygen sensor containing an O_2-labile iron-sulfur cluster considered as equivalent to the FNR (Fumarate and Nitrate reductase Regulatory) sensor, while NreC is a response regulator that controls gene expression involving in nitrogen regulation. In the presence of iron and low oxygen level, iron-sulfur cluster is formed and NreB autophosphorylates itself. Then, the active form NreB-P transfers its phosphoryl group to the response regulator NreC and activates it. NreC-P then positively controls the expression of the nitrite reductase (*nir*) operon , nitrate reductase (*narGHJI*) operon and *narT* (nitrate transporter) gene. On the contrary, under aerobic condition, NreB is dephosphorylated and its iron-sulfur cluster is destroyed (Figure 4).

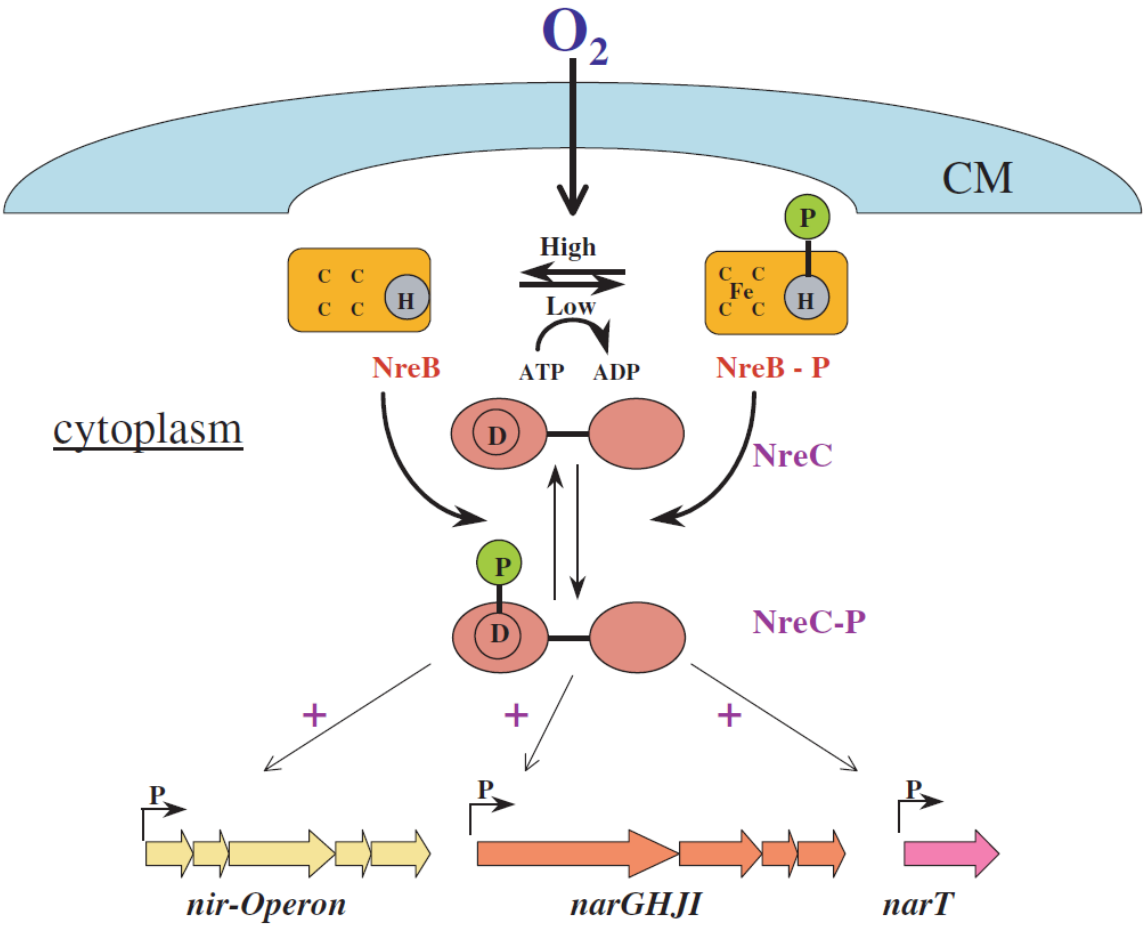


Figure 4: Model for the NreBC sensor and regulation system in staphylococci.

[From (Kamps et al. 2004)].

1.2. Iron restriction

Metal ions are crucial elements for life processes including virulence. In bacteria, some metal ions like iron, magnesium and manganese are essential elements required for growth and play important roles in metalloproteins.

Iron (Fe) is mainly found in two common oxidation state, ferrous (Fe^{2+}) and ferric (Fe^{3+}). Ferrous irons are solube while ferric irons are insoluble and also the most stable form of iron. The difference in solubility of Fe^{2+} and Fe^{3+} leads to difficulties for bacteria to acquire iron. Therefore, bacteria not only develop mechanisms to uptake iron from the environment by solubilizing and assimilating it; but also have to compete for iron with other microorganisms or with the host. On the other hand, the quantity of intracellular iron has to be strictly controlled since an excess is toxic.

In *S. aureus*, there are three Ferric uptake repressors (Fur) homologues called Fur, PerR and Zur. When iron is present in bacteria, it binds to Fur. The Fe-Fur complex then binds to inverted repeat motifs called Fur boxes located in promoter regions of Fur-regulated genes. A Fur depletion in *S. aureus* generates a growth defect due to an excessive acquisition of iron and a decrease of the *katA* expression (Horsburgh, Ingham, and Foster 2001). There is a coupling between iron and oxidative regulations via the Fenton reaction. When peroxide or superoxide reacts with iron, they form harmful hydroxyl radicals which are reduced by catalases or peroxidases. The murine abscess model was used to test the role of Fur in virulence; *fur* mutant had a reduced virulence compared to the wild-type strain. A proteomic study revealed that in iron-limiting conditions or after *fur* depletion, genes involved in glycolysis, iron acquisition and transport were upregulated while genes in the TCA cycle and *rsbU* were downregulated (Friedman et al. 2006).

PerR is a second Fur homologue in *S. aureus*. PerR was shown to sense peroxide level inside the cell and control iron storage proteins (Horsburgh et al. 2001). The PerR-dependent regulon comprises not only oxidative stress resistance genes such as catalase (*katA*), alkyl hydroperoxide reductase (*ahpCF*) but also iron storage related genes such as ferritin (*ftn*), *mgrA* and *fur*. Like *fur*, the *perR* mutant had a reduced survival in murine abscess model infection (Horsburgh et al. 2001). PerR is also involved in the pathogenesis of *Streptococcus pyogenes, Listeria monocytogenes* and *Enterococcus faecalis.*

The third Fur-like protein in *S. aureus* is Zur. Its gene is within an operon encoding two putative membrane proteins with homology to zinc and other metal transporters (Lindsay & Foster 2001). In *S. aureus*, the depletion of Zur did not affect Zn^{2+} uptake but its overexpression was shown to affect the whole operon in a Zn^{2+}-dependent manner. *zur* homologues were shown to regulate zinc uptake and ribosomal protein paralogs in *B. subilis* (Panina et al. 2003) and were involved in virulence in *Salmonella enterica* (Campoy et al. 2002) and *Xanthomonas campestris* (Tang et al. 2005). In contrast, *S. aureus zur* did not play a role in pathogenicity in a mouse skin infection model (Lindsay & Foster 2001).

1.3. Temperature

1.3.1. Cold shock

In *E. coli*, CspA is a major cold shock protein (Goldstein et al. 1990). In *S. aureus*, *cspA* was found to positively regulate the yellow pigment 4,4'-diaponeurosporene (Katzif et al. 2005). Transcriptome and mRNA turnover rates in response to cold shock were studied by Affymetrix *GeneChips* (Anderson et al. 2006). The *cspA* gene was moderately induced (2-fold) by a cold shock, while for two other cold shock genes, *cspB* was upregulated 9-fold and no change was observed for *cspC* expression. In addition, 46 genes upregulated and 416 genes downregulated by cold shock were identified. Many virulence factor genes [*i.e. seo* (enterotoxin), *lip* (lipase), *srtA* (sortase)], and regulatory genes [*i.e. lexA* (a SOS repressor) and SACOL0958 (general stress protein)] were also induced in cold shock condition. Surprisingly, *cspC* was strongly induced in oxidative stress (with hydrogen peroxide), salt condition by arsenate and various antibiotics such as ciprofloxacin, rifampicin, ampicillin, and cephalothin (Chanda et al. 2009).

1.3.2. Heat shock

CtsR and HrcA, SarA and Sigma B are regulators involved in heat shock adaptation in *S. aureus* (Clements & Foster 1999).

HcrA is a repressor of class I heat shock genes encoding *dnaK* and the *groESL* operons. CtsR has a dual function: it is a repressor of class III heat shock genes (encoding Clp ATP-dependent proteases) but also works together with HcrA to repress chaperon protein genes (Chastanet et al. 2003) (Figure 5). Sigma B (σ^B) is a transcriptional factor that associates with RNA polymerase to initiate transcription. In *S. aureus*, σ^B is an alternative "stress" sigma factor that modulates many stress response genes.

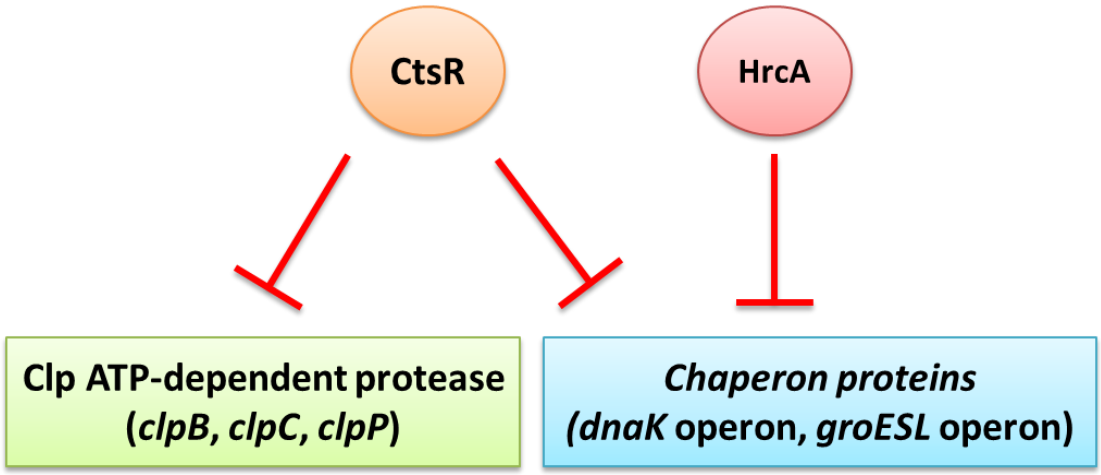


Figure 5: The CtsR, HrcA regulon of *S. aureus*

[Adapted from (Chastanet et al. 2003)].

Studies of the transcriptomic profile in response to heat shock revealed that 98 genes increased and 42 genes decreased (Anderson et al. 2006). Like for a cold shock, the viability of bacteria was not affected. The three heat shock genes, *ctsR, clpB* and *clpC* were significantly increased. In addition, many putative virulence genes [*i.e. hla* (α-hemolysin), pathogenicity island genes, *urea-ureG* (urease system)] were upregulated. Interestingly, 11 genes were induced in both heat and cold shock conditions; they may belong to the same family of temperature-mediated response genes.

1.4. pH

Electron transport chain is the last process of aerobic respiration to generate the energy. The main function of this chain is transferring electrons from donors to acceptors and associated with proton pumps that transfer protons (hydrogen ions) across a membrane to create an electrochemical proton gradient that powers ATP production. pH or the hydrogen ion concentration is the main factor affecting the cytoplasmic pH homeostasis of bacteria. Therefore, to maintain the growth, bacteria need to develop pH sensing and mechanisms to keep balance their cytoplasmic pH homeostasis.

1.4.1. Acid shock

Many bacteria, including pathogens, need to adapt to acidic conditions, *i.e*. in dairy food like yogurts, fermented milk or inside the gastrointestinal system. For example, the Gram-positive pathogen *L. monocytogenes* survives in the human stomach and even inside

the phagosome. The ability to tolerate acidic pH is considered as a virulence factor for bacteria.

There are 8 known mechanisms for acid resistance in Gram-positive bacteria (Figure 6). They can maintain their intracellular pH either by proton pumps (GAD system, F1F0ATPase) that increase the uptake of hydrogen ion or by generating alkaline products such as NH_3/ NH_4^+ (via an arginine deiminase (ADI) pathway) and an urease system that counteracts acid pH. Other mechanisms are protein and DNA repair systems. Some genes involved are *dnaK*, *groEL*, *htr*, *clp* ATPases and *lo18* for protein repair systems and *recA*, *uvr* and *smn* for DNA repair systems.

During an acidic challenge, bacteria alter their general energy and metabolism, and cell envelope, a switch that is needed for the adaptation. The role of the cell membrane is demonstrated by changes in membrane fatty acid profiles (Cotter & Hill 2003). A *Streptococcus mutants* strain with a *dltC* (encoding the D-alanyl carrier protein, Dcp) deletion was more sensitive to acid with a longer doubling-time and reduced growth yield than the parental strain (Boyd et al. 2000). The inactivation of *dltC* prevents the D-alanylation of lipoteichoic acids (LTAs), a main cell wall compound in Gram-positive bacteria.

Bacteria also resist to media acidification via global regulators such as TCSs and sigma factors. For example, in *L. monocytogenes* the depletion of *lisRK* encoding a TCS generates an altered acid shock response. The *lisRK* mutant was more sensitive to long time exposure to acid in stationary phase while it was more resistant to short time exposure during pre-stationary phase compared to the wild-type strain (Cotter et al. 1999).

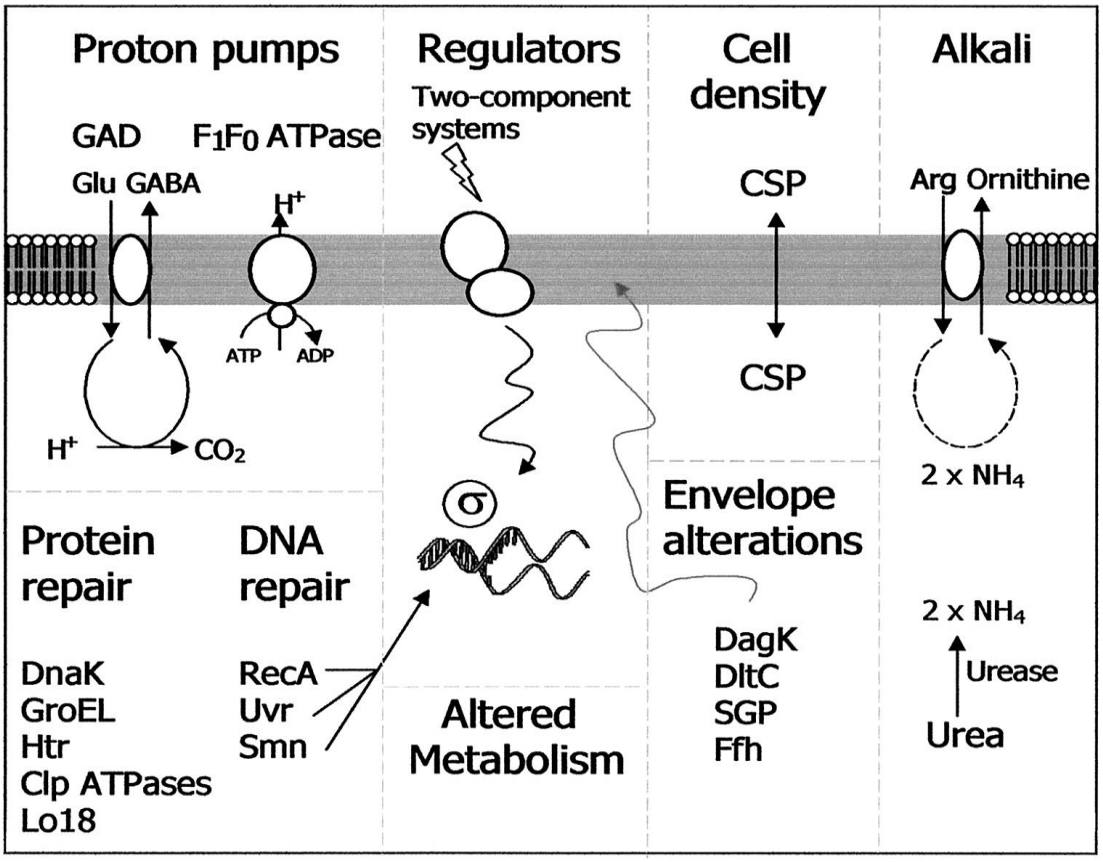


Figure 6: Mechanism of acid resistance of Gram-positive bacteria

[Adapted from (Cotter & Hill 2003)].

During its life cycle, *S. aureus* can undergo various pH conditions within or outside the host. It can survive in human body, colonize and cause infection in different places which have acidic to alkaline pH such as external labia (pH 3.8 - 4.5), lysosomal compartments (pH 4.5 - 5.5) and wound sites (pH 8.9).

The σ^B mutant is more sensitive to acid stress with a rapid loss of viability. *S. aureus* can be killed by a pH 2 but its resistance to acid increases if it is first pre-incubated at pH4, a non-lethal condition (Chan et al. 1998). In addition, the *sodA* (encoding a major superoxide dismutase) mutant has a reduced viability at low pH as compared to the wild-type strain (Clements et al. 1999). These results demonstrated the role of σ^B and SodA to adapt to acid stress. However, the mechanism of *sodA* involved in acid resistance is still not clear.

Several studies were performed to determine the gene expression under different acidic conditions, such as growth in mild acidic condition (pH~5.5) (Weinrick et al. 2004); acid shock at pH 4.5 during 20 min (Bore et al. 2007), acid shock at pH 4 during 30 min

(Anderson et al. 2010)**.** The results from Anderson *et al* not only confirmed many genes and overlapped with previous studies but also extended the network of genes related to acid stress adaptation. Interestingly, a total of 15 virulence genes were found downregulated in acidic condition including 7 known genes involved in acidic adaptation from (Bore et al. 2007). Five new additional virulence factors i.e. *spa,* chemotaxis-inhibiting protein CHIPS (*chp*), clumping factor B (*clfB*), fibrinogen-binding protein (*efb*) and staphylokinase precursor (*sak*) were revealed in this study. Moreover, 4 TCS such as SaeRS, LytSR, ArlSR and GdpS were also observed to be downregulated in response in acidic condition.

S. aureus is also a major cause of food poisoning. During food conservation, it undegoes stress associated with organic acids like lactic and acetic acids. *S. aureus* responses to medium containing these acids was explored by microarrays (Rode et al. 2010). First, a large variation in growth patterns was observed: bacterial growth was inhibited in medium containing acetic acid whereas bacteria exposed to lactic acid had a longer lag phase than when growing in medium containing HCl. Interestingly, only the pH of the culture containing lactic acid increased up to pH 7.5 during growth. Thus, compared with HCl induction, the response to lactic acid stress induced a specific mechanism to increase pH by accumulating ammonium and removing acid groups with production of diacetyl (2,3-butane dione) and pyrazines .

1.4.2. Alkaline shock

The first study on alkaline effect in *S. aureus* was reported in 1992 (Regassa & Betley 1992). The expression of the *agr* (accessory gene regulator) quorum sensing system (see chapter IV) examined in alkaline condition (pH from 6.5 to 8) revealed that the expression of RNAIII, one of the transcripts of *agr* locus, was higher at pH 7 but mostly vanished at pH 8. The expression of *sec* (staphylococcal enterotoxin type C), a target of the *agr* system, was also reduced in alkaline stress. Alkaline stress was found to strongly induce σ^B transcription and 122 σ^B-dependent genes (involved in capsule biosynthesis, Na+/H+ antiporter system and autolysin) were upregulated during this stress (Pané-Farré et al. 2006).

A microarray assay was also performed in alkaline condition (pH10 during 30 min) (Anderson et al. 2010). Cell viability in alkaline stress was not affected, but 128 transcripts

increased while 773 transcripts decreased. Downregulated genes were involved in nucleotide biosynthesis, amino acid metabolism and translation while upregulated were involved in amino acid biosynthesis (lysine, valine, isoleucine, histidine and threonine) and virulence (capsule biosynthesis). Interestingly, the alkaline shock stimulon induces the expression of (p)ppGpp, the activator of the stringent response.

2. Host-pathogen interaction

S. aureus is a versatile human pathogen, hence this bacterium needs to develop efficient mechanisms to survive in host defense and resist to the host immune system.

Briefly, the human immune system comprises a non-specific (innate) and specific (adaptive or acquired) immunity systems (Figure 7). The non-specific system includes two lines of defense. The first line consists of physical and chemical barriers, natural flora and mechanical barriers. If bacteria can pass the first line, they will face the second line of defense that includes defensive molecules, phagocytosis, complement and protective mechanisms. The specific immune system involves lymphocytes and antibodies which will recognize and eliminate pathogens, their toxins products, and also confers long-term protection by developing immunological memories.

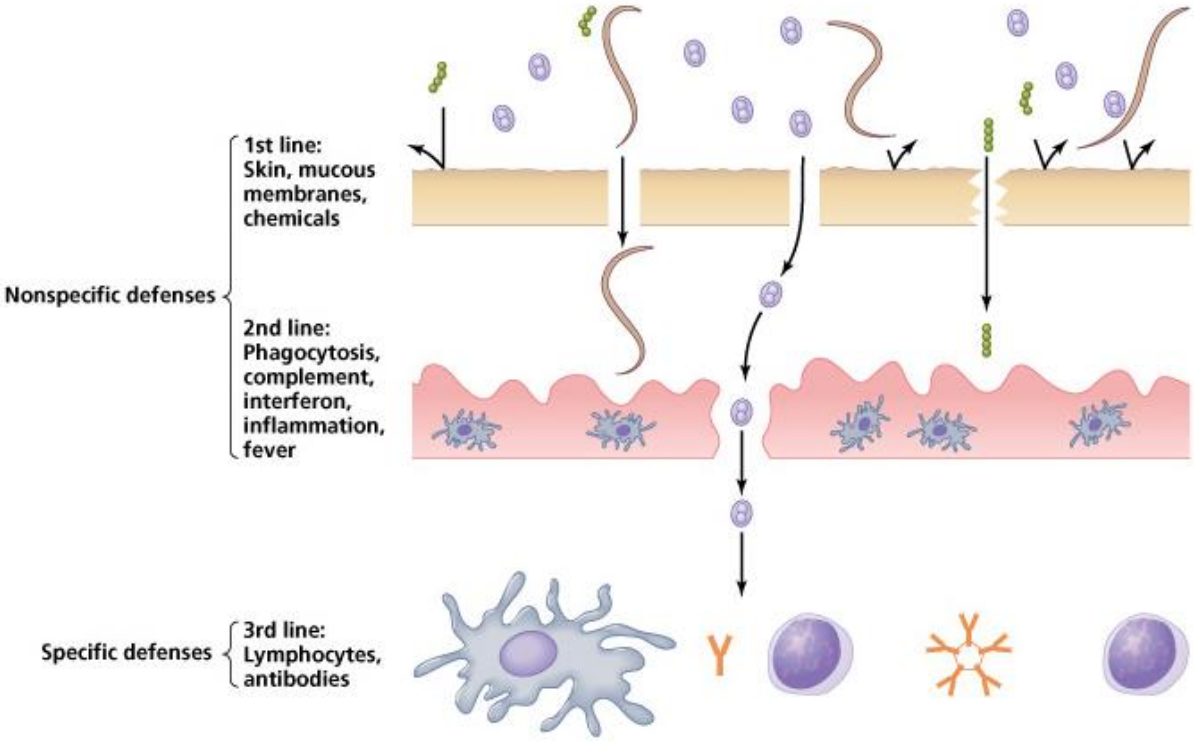


Figure 7: Human lines of defense against pathogens

http://www2.bakersfieldcollege.edu/bio16/15_innate_immune.htm

S. aureus developed different mechanisms to evade the innate immune system by inhibiting phagocyte functions, blocking complement activation, resisting antimicrobial peptides, lysing neutrophils (Nizet 2007) (Figure 8).

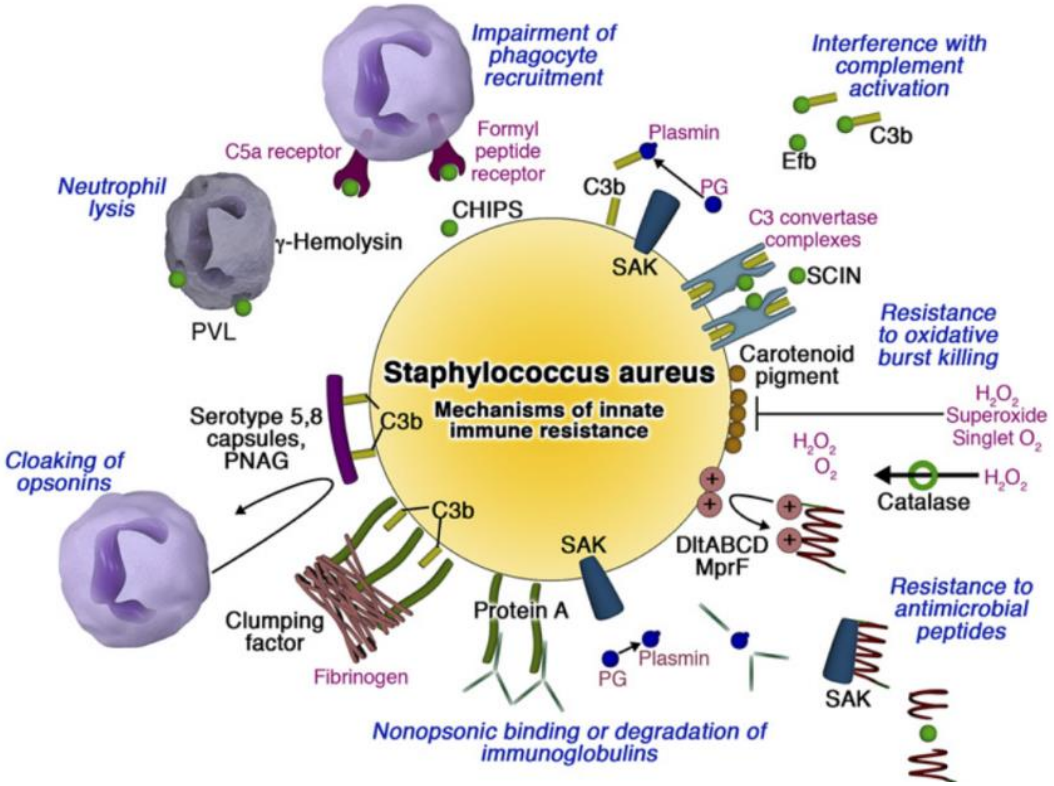


Figure 8: Mechanisms by which *S. aureus* subverts host innate immune defense. Phagocyte recruitment is limited by binding of CHIPS (Chemotaxis inhibitory protein of *Staphylococcus aureus*) to chemokine receptors. Complement activation is blocked by protein Efb binding of soluble C3 and inhibition of the both the classic/lectin and alternative C3 convertases by SCIN (Staphylococcal complement inhibitor). Golden carotenoid pigment provides an antioxidant shield whereas catalase detoxifies hydrogen peroxide. Resistance to cationic antimicrobial peptides is afforded by positive charge modifications of the cell wall, aureolysin-mediated proteolysis, and binding/inactivation by staphylokinase. Protein A binds Fc domains of Igs in a nonopsonic manner, whereas fibrinogen binding clumping factor and the surface polysaccharide capsule and poly-N-acetylglucosamine (PNAG) act to cloak surface bound opsonins from phagocyte recognition. The heptameric pore-forming toxins γ-hemolysin and Panton-Valentine leukocidin (PVL) preferentially target leukocyte membranes. The plasminogen (PG) binding protein staphylokinase (SAK) activates the zymogen to the active protease plasmin, which can degrade complement opsonin C3b and the immunoglobulin Fc domain [From (Nizet 2007)].

III. Overview of bacterial competitive fitness

The fitness of a bacterial strain could be explained from a Darwinian point of view. From his theory, “survival of the fittest” is the main concept of natural selection that is mechanism by which species adapt and evolve. It could be defined as an increase of

frequency or its probability of survival in competition with others. The individuals having variants that best fit to the environment (fittest) have better potential for survival, reproduction and passing their desirable variations to their offsprings.

Based on this concept, one way to define the bacterial fitness in the laboratory conditions is a competitive fitness assay by growing two or more strains together that allow them to compete and evaluate their ratio at different times (Figure 9). In addition, growth in the presence of other strains reflects a more "real" situation, as the fitness of one strain may be affected by the genetically different surrounding strains. Therefore, these experiments could reveal patterns of interaction or epistasis among different strains and possibly whether particular combinations of strains interact synergistically or antagonistically (Zhan & McDonald 2013). Interestingly, variants leading to improved fitness in one growth condition can lead to altered fitness in another condition, as the result is often a compromise (Mariam et al. 2004) (MacLean & Vogwill 2014).

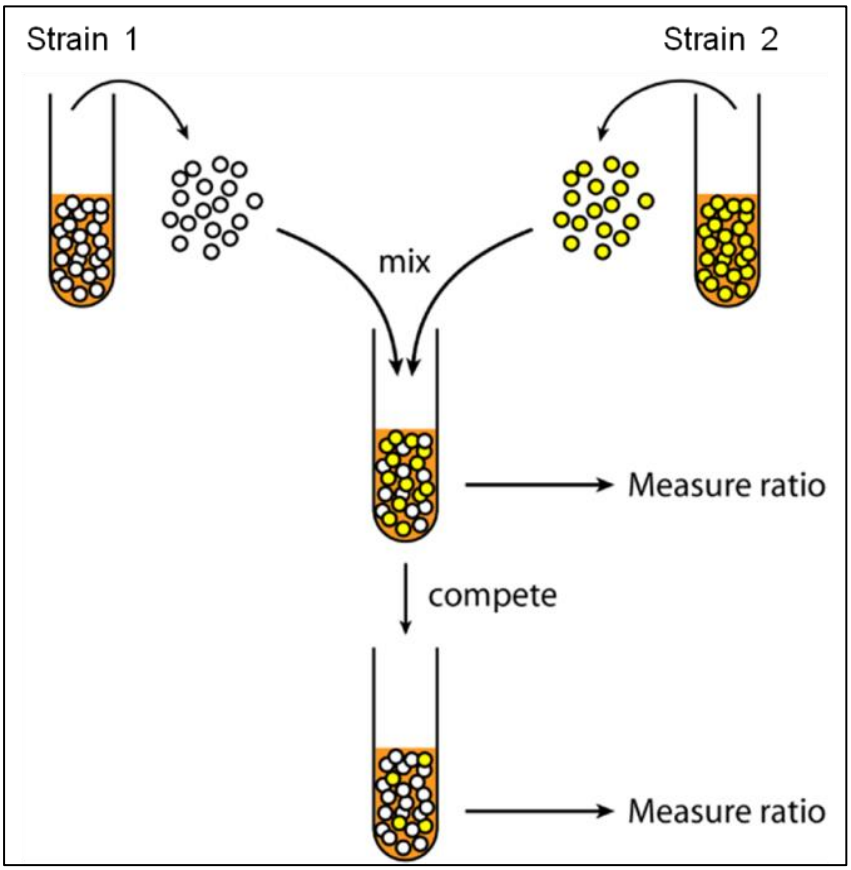


Figure 9: Scheme of a competitive fitness assay. The strain to be assayed is mixed with a reference strain, and the ratio of the two is measured before and after growth. [From (Desai 2013)].

The classical way to carry out fitness assays is to label the strains with different antibiotic resistance or fluorescent markers. However, one impediment is the limited number of available markers; hence fitness assays are difficult to perform on a large scale. As a result, one possibility to easily follow many strains in the same culture is to introduce specific DNA sequences for each constructed strains. These sequences, called DNA barcodes, can then be quantitatively detected within a mix culture. A DNA barcode acts as a specific "marker" that represents the relative presence of a strain in the population.

The first application of DNA barcodes was in *Salmonella typhimurium* to identify genes involved in pathogenesis using a murine model of typhoid fever (Hensel et al. 1995). Briefly, the DNA barcodes contained 40 random nucleotides flanked by common priming regions on each side. The DNA barcodes were ligated with transposons and used to mutagenize *S. typhimurium* genome. A bank of 1152 transposon-tagged mutants was obtained and arrayed in twelve 96-well microtiter plates. DNA colony blots were made from microtiter dishes by replica plating them on a membrane. The mutants of each microtiter plate were pooled together and used to infect mice. After 3 days of infection, spleens were recovered, homogenized and plated to recover the infecting mutants. Approximately 10,000 obtained colonies were pooled together. Chromosomal DNAs were extracted and DNA barcodes were amplified (using conserved priming regions for all tags), radiolabeled and hybridized with DNA colony blots (Figure 4). Virulence genes were found by identifying DNA barcodes that were present in the control samples but not in the infected ones. These DNA barcodes corresponded to insertions leading to attenuated virulence (Figure 10).

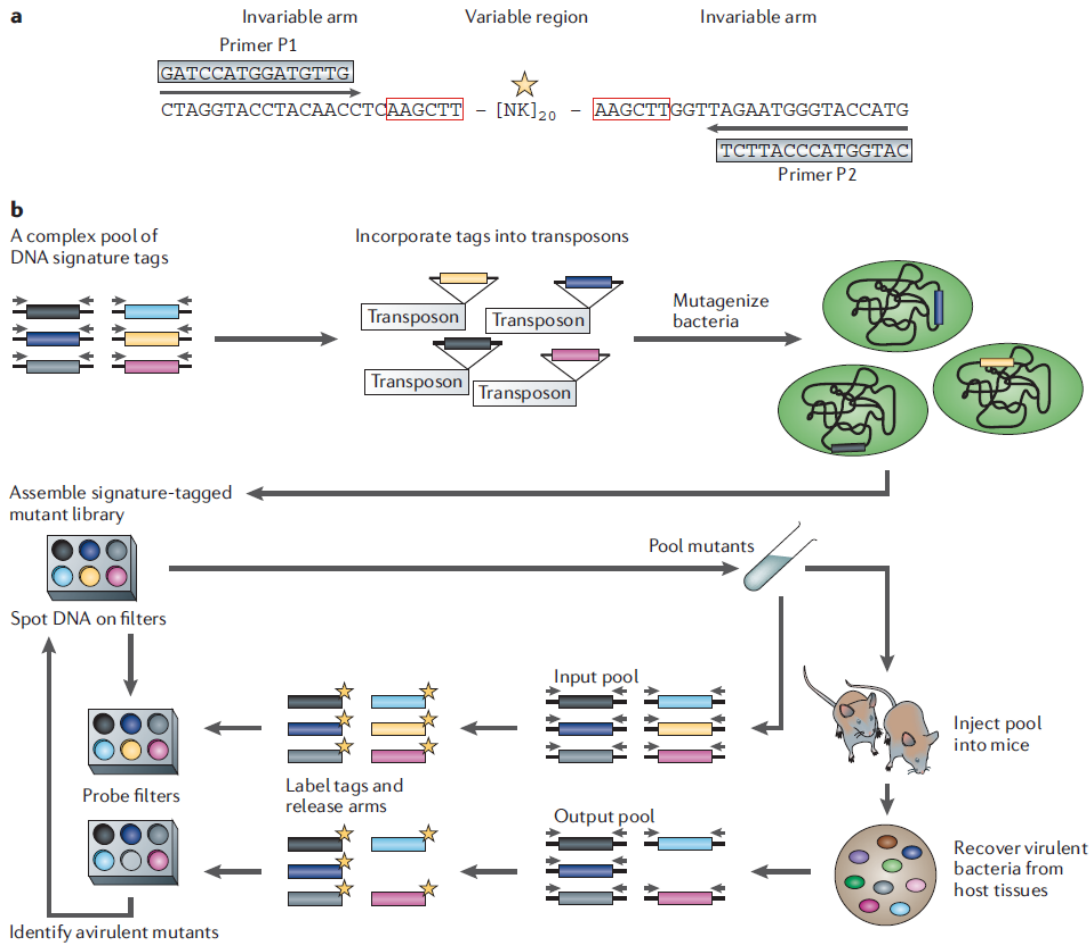


Figure 10: Signature-tagged mutagenesis in *Salmonella*. A) Design of a signature tag. Each tag has a unique central sequence of 40 bp ([NK] 20; N = A, C, G, or T; K = G or T), flanked by invariable arms of 20 bp, which are common to all the tags. B) Signature-tagged mutagenesis screening in mice. A complex pool of tags (shown as colored rectangles) is ligated to transposons. The tagged transposons are then used to mutagenize bacteria, which are subsequently assembled into a library. Only bacteria with tags that are efficiently amplified by PCR and are not cross reactive with other tags in hybridization experiments are selected for inclusion in the pool that is used to infect the mice. [From (Mazurkiewicz et al. 2006)].

This technique was subsequently developed in yeast (Shoemaker et al. 1996; Pierce et al. 2007; Smith et al. 2009; Han et al. 2010; Chen et al. 2012), and used with other bacteria (Rooney et al. 2008; Hobbs et al. 2010).

In many published fitness protocols, tags were analyzed by hybridizing labeled PCR products on dedicated DNA arrays (Hensel et al. 1995; Shoemaker et al. 1996; Pierce et al. 2007; Rooney et al. 2008; Hobbs et al. 2010). These experiments are rather heavy and expensive as each tested condition required at least one array. The protocol was adapted to deep sequencing technology to improve its sensitivity and its ease-of-use (Smith et al. 2009; Han et al. 2010) (chapter A).

IV. Overview of regulatory RNAs in *S. aureus*

Besides the central roles of mRNA, rRNA and tRNA in translation, the regulatory role of RNAs in prokaryote gene expression is nowadays well established. They can act either in *trans*, targeting RNAs and proteins, or in *cis* by affecting adjacent or associated sequences. Through sophisticated mechanisms, these regulatory RNAs fine-tune genetic expression to allow bacterial fitness and adaptation to varied environments including those within their dedicated hosts. They usually exert their functions at the levels of transcription and/or translation of their mRNA targets (Storz et al. 2011; Guillet et al. 2013).

1. Identification of regulatory RNAs by various approaches

The first regulatory RNAs in *S. aureus* was discovered in 1993 and named RNAIII (see chapter IV.3 for more details). Later, several studies contributed to identify numerous *S. aureus* regulatory RNAs based on computational prediction (Geissmann et al. 2009; Marchais et al. 2009; Pichon & Felden 2005), Affymetrix microarrays (Anderson et al. 2010; Roberts et al. 2006; Anderson et al. 2006), sequence cDNA libraries (Abu-Qatouseh et al. 2010) and high throughput sequencing (Bohn et al. 2010; Beaume et al. 2010; Lasa et al. 2011; Lioliou et al. 2012; Howden et al. 2013). Several targets from these identified sRNAs were experimentally validated (see Table 1). So far, the transcription of approximately 250 staphylococcal regulatory RNAs was experimentally confirmed [review from (Felden et al. 2011)].

Table 1: Summary of experimentally validated regulatory RNAs in *S. aureus* [a] (Tomasini et al. 2014)

Study	**Strain used**	**sRNA discovery methodology (number of *in silico* predicted sRNAs)**	**Experimentally validated sRNAs** [e]	**Experimental validation method**	**Target and mechanism**	**Comment**
(Roberts et al. 2006)	UAMS-1	Gene chip analysis	**SSR42** (srn_4470, RsaX28, Teg27, sRNA363)	NB, RT-PCR	Spa, hla, hglC, lukF Unknown	
(Pichon & Felden 2005) and (Sayed et al. 2011)	Mu50 (clonal Complex 5)	Bioinformatic predictions	**SprA** **SprA2** (srn_4550, WAN014FZW, IGR2049, IGR8bis, Teg26as, sRNA371), **SprA3** (IGR2125), **SprB** (srn_3600, Teg9, IGR18), **SprC** (srn_3610, Teg10), **SprD** (srn_3800, Teg14_sRNA300), **SprE**, **SprF**, **SprG**, **SprFG2**, **SprFG3** **4.5S RNA** (ffs, Teg42, IGRLF1, WAN01CBPQ, sRNA98), **6S RNA** (Teg97, ssrS, ssr80, IGR2, WAN01CC8T, sRNA256), **RNAIII** (srn_3910, sRNA317), **tmRNA** (tmR, WAN014GIY, Teg150, ssrA, sRNA166), **RNase P** (RseP, rnp, Teg65, IGR1215, sRNA240)	NB [b] NB [b] NB [b]	ABC transporter (SA2216), possible antisense sRNA Unknown, SprA2 encodes a cytolytic peptide Housekeeping ncRNAs	
(Geissmann et al. 2009)	RN6390, COL, Newman, HG001	Bioinformatic predictions and experimental validation	**RsaE** (srn_2130, RsaON, Sau20, Teg92, IGR6, sRNA183), **RsaA** (srn_1510, rsaOJ, Teg88, sau64, IGR1, IGR14, sRNA132), **RsaB** (srn_3410), **RsaC** (srn_1590, Teg90, sRNA135), **RsaD** (srn_1640, Teg91, sRNA138), **RsaF, RsaG** (srn_0510, Teg93, sRNA31), **RsaH** (srn_1910, rsaOK, Teg94, sau6059, IGR7, sRNA162), **RsaI** (srn_4390, rsaOG, Teg24, sRNA356), **RsaJ** (srn_4530, sprAs2prime, Teg96, sau5837, sRNA369), **RsaK** (srn_0440, Teg38, sRNA27)	NB, PE, RACE NB, PE, RACE	Masking of ribosomal binding site for oppB, sucD, SA0873 Unknown	Genetic manipulation demonstrated a role for RsaE in controlling metabolic pathways

Study	Strain used	sRNA discovery methodology (number of *in silico* predicted sRNAs)	Experimentally validated sRNAs [e]	Experimental validation method	Target and mechanism	Comment
(Marchais et al. 2009)	N315 (clonal Complex 5)	Bioinformatic (NAPP) (189) and Northern analysis	**RsaOA**, **RsaOB** (srn_0860,Teg40, sRNA61), **RsaOC** (srn_1770, RsaX08, Teg50), **RsaOD** (srn_3160, Teg67_sRNA250), **RsaOE** (srn_3490, Teg73_sRNA276), **RsaOF** (srn_1930.3, Teg12), **RsaOG**[b] (srn_4390, RsaI, Teg24, sRNA356)	NB	Unknown	
(Jesper S Nielsen et al. 2011)	N315 (clonal complex 5)	Bioinformatic search for intergenic σ^B consensus sites and experimental validation	**SbrA** (srn_2290, RsaOO, Teg54, sbrA, sRZN), **SbrB** (srn_2830, Teg111), **SbrC** (srn_1580)	NB	σ^B regulated. SbrC interacts with SA0587 (mntC)	*sbrA* and *sbrB* potential CDS
(Abu-Qatouseh et al. 2010)	-	Cloning and sequencing of cDNAs	**Ssr-72**, **Ssr-80** (6S, Teg97, ssrS, IGR2, WAN01CC8T, sRNA256), **Ssr-87**, **Sau-02** (Teg19a, Teg102, sRZN, sRNA190), **Sau-13** (srn_5000), **Sau-19** (srn_4680, Teg131, RsaX21, sRNA382), **Sau-24** (srn_2610, Teg81), **Sau-27** (srn_2690), **Sau-30** (srn_4260, SSR154, sRZI), **Sau-31** (srn_4250), **Sau-41** (srn_1070), **Sau-50** (srn_3040), **Sau-53** (srn_0430), **Sau-59** (srn_2340), **Sau-63** (srn_0950, Teg146, sRNA83), **Sau-64**, **Sau-66** (srn_1780), **Sau-5949** (srn_3460, Teg120, sRNA272), **Sau-5971** (srn_0880), **Sau-6053** (srn_2200, sRNA189, Teg78), **Sau-6072** (srn_4830, sRNA389)	NB [c] NB	Unknown Unknown. Sau-66, putative posttranslational control of antisense gene SA0671	142 candidate sRNA identified

Study	Strain used	sRNA discovery methodology (number of in silico predicted sRNAs)	Experimentally validated sRNAs [e]	Experimental validation method	Target and mechanism	Comment
(Bohn et al. 2010)	N315 (clonal complex 5)	454 pyrosequencing followed by experimental validation	**RsaON** (srn_2130, rsaE, Sau20, Teg92, IGR6, sRNA183)[d] **RsaOH**, **RsaOI** (srn_1490, SSR156, Teg47, Sau6477, sRZR, sRNA131), **RsaOL** (srn_1960, Teg100, Sau07, IGR14, sRNA168), **RsaOM** (srn_2030, Teg52_IGR20_sRNA172), **RsaOO** (srn_2290, Teg54, sbrA, sRZN), **RsaOP** (srn_2350), **RsaOQ** (srn_2880, Teg82, IGR23, sRNA230), **RsaOR** (srn_3820.1, SprX2, ssr6, teg15, IGR12), **RsaOT** (srn_4670, ssr43, RsaON, Teg29, sRNA381), **RsaOU** (srn_4800, sRZG), **RsaOV** (srn_4840, Sau40, sRZV) **RsaOW**, **RsaOX**	NB NB	Binds opp3A mRNA ribosome binding site. Overexpression of RsaE reduces central metabolic pathways and increases amino acid pool	30 sRNAs identified, 14 new
(Beaume et al. 2010)	N315 (clonal complex 5)	Illumina sequencing	**Teg1** (srn_0050, sRNA3), **Teg4**, **Teg17** (srn_0360, sRNA21), **Teg18** (srn_0770, sRNA53), **Teg19b, Teg21** (srn_4130), **Teg24** (srn_4390, rsaOG, RsaI, sRNA356), **Teg26** (srn_4460, sRNA362), **Teg28** (srn_4590, sRNA375), **Teg35** (srn_0030, sRNA2), **Teg38** (srn_0440, rsaK, sRNA27), **Teg42** (4.5S, ffs, IGRLF1, WAN01CBPQ, sRNA98), **Teg45** (srn_1350, sRNA120), **Teg47** (srn_1490, RsaOI, SSR156, Sau6477, sRZR, sRNA131), **Teg55** (srn_2370, sRNA198), **Teg56** (srn_2440, sRNA203), **Teg57** (srn_2450, Sau6229, sRNA204), **Teg60** (srn_2520, sRNA208), **Teg61** (srn_2620, sRNA216), **Teg69** (srn_3260, sRNA257), **Teg70** (srn_3300, sRNA262), **Teg72**	RT-PCR	Unknown	195 sRNAs predicted by HTS

			(srn_3340), **Teg73** (srn_3490, RsaOE, sRNA276), **Teg76** (srn_0930, sRNA74), **Teg91** (srn_1640, rsaD, sRNA138), **Teg2pl**			

[a] Validated by Northern blot, RNA extremity mapping, or RT-qPCR.
[b] Also experimentally validated using Northern blot in the study by (Abu-Qatouseh et al. 2010).
[c] Originally described by (Anderson et al. 2006).
[d] Previously described and validated. NB, Northern blot; PE, primer extension; RACE, random amplification of cDNA ends; RT-PCR, real-time PCR. Several long and stable
RNAs (SSR) expressed under particular conditions of growth have been assigned by microarrays (Roberts et al. 2006).
[e] A number of different names have been assigned to many of these sRNAs. In this table, the initial name from the relevant study has been used. A full list of alternate names can be found in the supplementary table from (Felden et al. 2011). Recently, a uniform nomenclature for *S. aureus* regulatory RNAs was proposed (Sassi et al. 2015).

2. Classification

Regulatory RNAs exert usually their activity by base-pairing with target mRNAs or by binding to proteins. Some regulatory RNAs have a *cis*-regulatory activity on associated RNA sequences. Most regulatory RNAs are active under specific conditions and influence gene expression in response to environmental changes. They can bind mRNA ribosome-binding site (RBS), inhibit and stimulate translation or RNA degradation. According to their predicted mode of action, regulatory RNAs can be categorized as follows.

2.1 Regulatory small RNAs targeting mRNAs

2.1.1 *Cis*-encoded antisense RNAs

Cis-encoded antisense RNAs (asRNAs) are expressed from DNA strands opposite to genes. The predicted putative target of asRNAs is the mRNA expressed from the opposite strand. asRNAs share extended regions of complete complementarity with their target (often 75 nucleotides or more) with which they fully or partially overlap to activate or inhibit mRNA functions (Waters & Storz 2009; Georg & Hess 2011) (Figure 11). Most studied asRNAs are from bacteriophages, plasmids, and transposons and control phage development, plasmid replication and copy-number control of mobile elements, respectively.

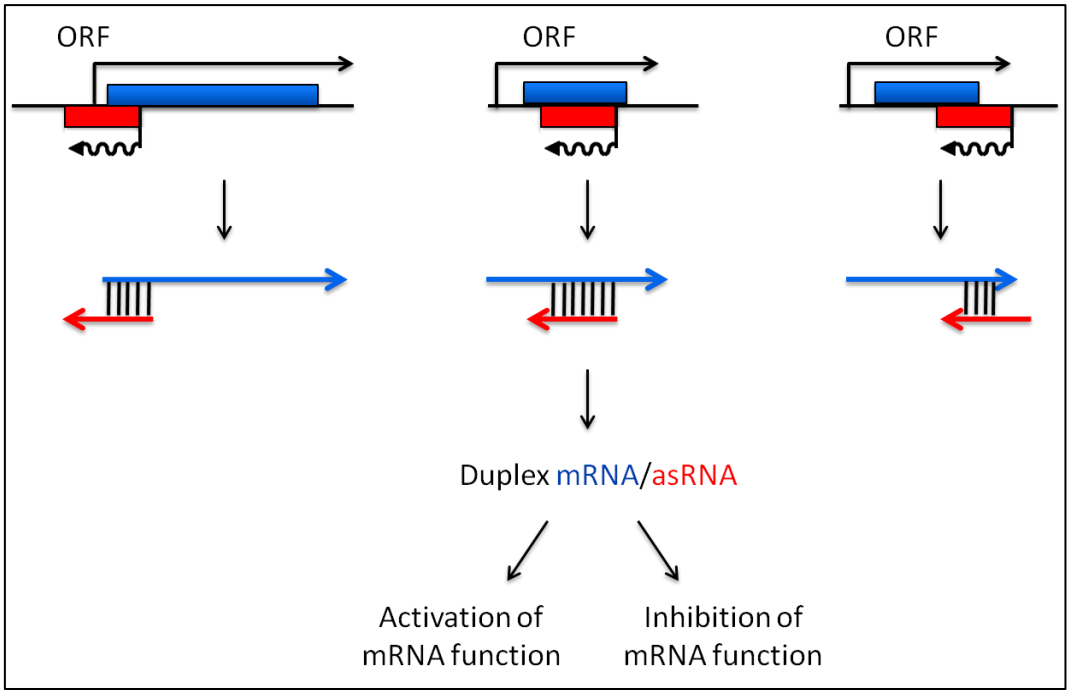


Figure 11: Example of *cis*-encoded antisense RNAs (asRNAs). mRNAs in blue, asRNAs in red.

The first asRNA reported in *S. aureus* was RNAI from plasmid pT181 (Novick et al. 1989). RNAI associates by base-pairing with *repC* mRNA and consequently inhibits the expression of RepC, a replication initiation protein. A second asRNA, RNAII, transcribed from the same promoter as RNAI, but longer than RNAI is also involved in pT181 replication as show on figure 12.

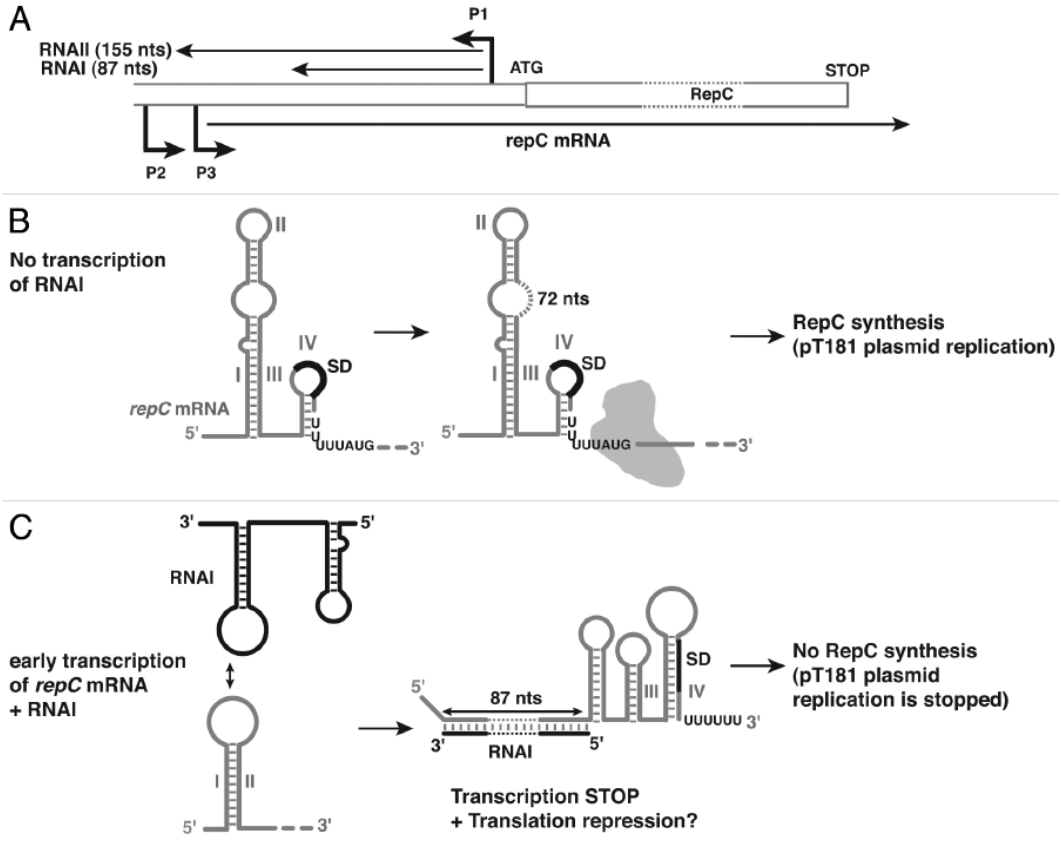


Figure 12: Antisense regulation of plasmid pT181 replication. (A) Genetic organization of pT181 plasmid and its control region. RNAI and RNAII are asRNAs. (B) Schematic secondary structure model of *repC* mRNA leader region as proposed by (Novick et al. 1989). The formation of a large helical domain formed by helices I and III favors the formation of an anti-terminator hairpin that stimulates the transcription of the complete mRNA. In this structure, the Shine and Dalgarno sequence (SD) and the initiation codon are available for translation. (C) The antisense RNAI traps a transient hairpin structure of *repC* mRNA during transcription, and the formation of the RNAI-mRNA duplex stabilizes a Rho-independent terminator to arrest transcription. RepC synthesis is thus prevented. [From (Romilly et al. 2012)].

RsaOW is an example of an asRNA expressed from mobile elements, which pairs with the 5'UTR of the transposase gene of IS1181. RsaOW has eight copies from *rsaOW1* to *rsaOW8* in N315 genome and is expressed constitutively likely to block expression of the IS1181 transposase (Bohn et al. 2010).

asRNAs were also reported from *S. aureus* core genome. The first high-throughput sequencing of N315 transcriptome revealed that at least 1.3% of mRNAs were covered by asRNAs (Beaume et al. 2010). However, this percentage is likely much higher. In this recent study, long (>75 nt) and short (<50 nt) transcripts of strain NCTC8325 were analyzed by RNA deep sequencing. The authors concluded that 49% of the ORFs are covered on at least 50% of their length by long asRNAs and up to 75% of ORF in case of short transcripts (Lasa et al. 2011).

2.1.2 *Trans*-encoded RNAs (sRNAs)

In contrast to *cis*-encoded asRNAs, *trans*-encoded RNAs (often referred to as sRNAs), are expressed from DNA regions with no RNA expressed on their opposite strand. sRNAs are usually small, typically between 50 and 500 nucleotides in length, and non-coding (Gottesman and Storz 2011). Their RNA targets are usually located at different positions on the chromosome. Hence, the base pairing length between a sRNA and its target is often limited, typically about 10-25 nucleotides (Waters & Storz 2009). Their mode of action is quite depicted in figure 13.

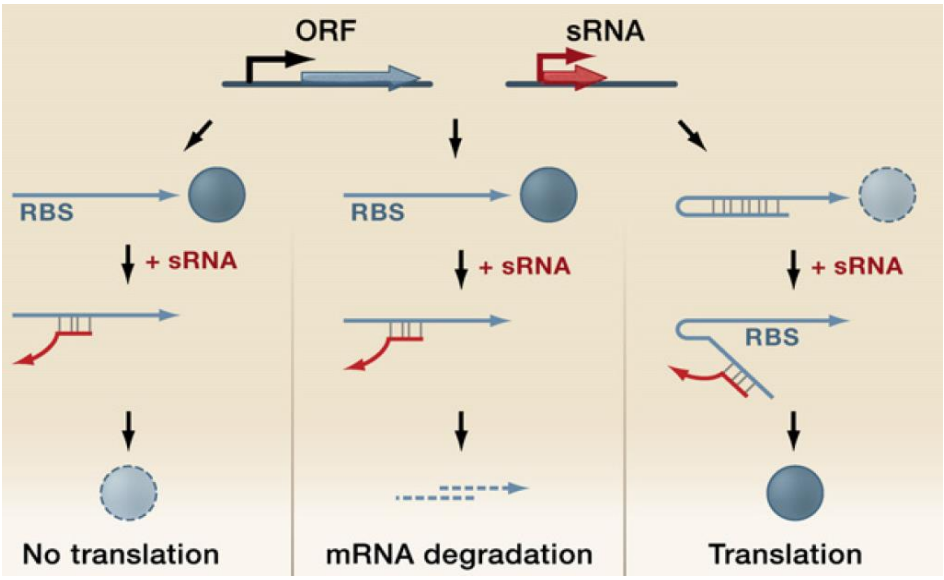
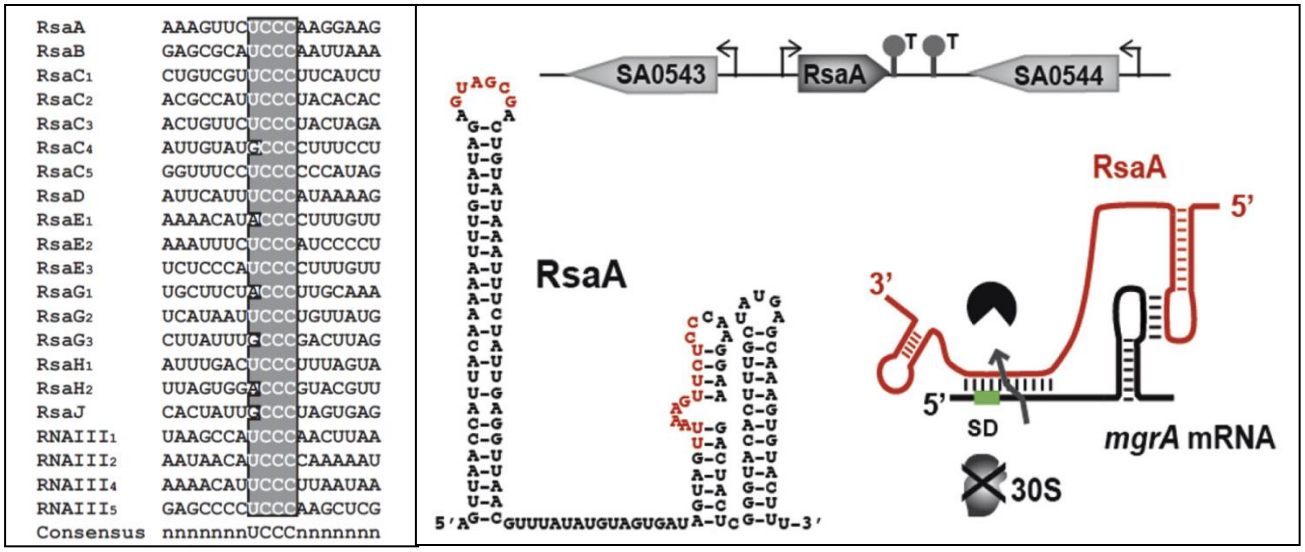


Figure 13: Gene arrangement and regulatory functions of sRNAs. Genes encoding *trans*-encoded sRNAs (red) are located separately from the genes encoding their target RNAs (blue) sRNAs have a limited base-pair complementarity with their targets. (Left panel) sRNAs can base pairing to 5'UTRs and block ribosome-binding site (RBS) and/or (Middle panel) target the mRNAs to degradation by ribonuclease. (Right

panel) sRNAs can also prevent the formation of an inhibitory structure which sequester RBSs. [From (Waters & Storz 2009)].

In *S. aureus*, the sRNA/RNA interaction seems to require a longer pairing than in many other organisms, possibly because *S. aureus* has a low GC content genome therefore generating weaker RNA-RNA interactions. Most of sRNAs have a conserved "seed" motif UCCC to initiate the pairing with RNA targets (Figure 14) (Geissmann et al. 2009).



A B

Figure 14: C-rich box conserved motif in *S. aureus* sRNAs and an example of example sRNA/mRNA base pairing. (A) Alignment of the C-rich sequence motifs of *S. aureus* sRNAs [From (Geissmann et al. 2009)]. (B) Repression of *mgrA* mRNA translation by the sRNA RsaA. Two regions of RsaA base pair i) with the *mgrA* mRNA RBS and ii) with the coding region via a loop-loop interaction. The two regions are essential to repress translation and to enhance the *mgrA* mRNA degradation [From (Fechter et al. 2014)].

2.2 *Cis*-regulatory elements

In bacteria, *cis*-regulatory RNAs may be located in the 5' or 3' untranslated regions (UTR) of mRNA and hence act in *cis*. Under specific condition, *cis*-regulatory RNAs can sense environmental signals such as metabolites (riboswitches), uncharged tRNAs (T-boxes), metal ions, pH and temperature (thermoswitches) and then adopt different conformations thus regulating the expression of downstream genes.

The most widespread examples of *cis*-regulatory sRNAs in bacteria are riboswitches that play a major role in regulating genes involved in metabolic pathways. Riboswitches

sense metabolites such as cofactors, vitamins, amino acids, nucleotides, the second messenger cyclic di-GMP, metal ions. Generally, riboswitches are composed of two parts: i) the aptamer region which binds ligands (metabolites) and ii) the expression platform which changes its structure in response to the ligand binding. mRNA modifications usually involve alternative hairpin structures creating transcription terminators and antiterminators or structures occluding and exposing RBSs, thus controlling translation. In most cases, the presence of ligands inhibit transcription or translation (Figure 15).

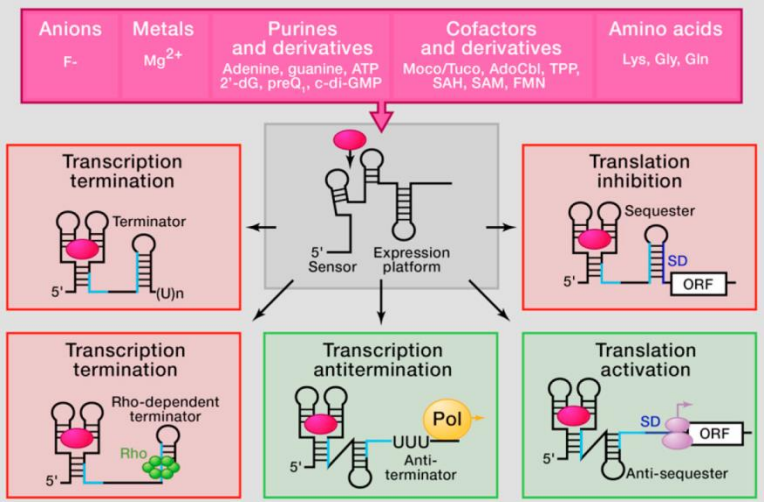


Figure 15: Diversity of riboswitches and mechanisms of gene control in bacteria. Mechanisms of modulation of gene expression are highly divergent in prokaryotes and involve control of transcription, translation and mRNA stability. SD (Shine-Dalgarno), Pol (RNA Polymerase), ORF (Open Reading Frame) [From (Serganov & Nudler 2013)].

Many *S. aureus* riboswitches were discovered by biocomputing analysis of genome sequences to identify conserved metabolite-binding domains (Barrick & Breaker 2007) (Yao et al. 2007); among them riboswitches sensing S-adenosylmethionine (SAM), thiamine pyrophosphate (TPP), flavin mononucleotide (FMN), lysine, glycine, guanine, 7-aminomethyl-7-deazaguanine (preQ1) and glucosamine-6-phosphate (Glc-6P) (Geissmann et al. 2009; Marchais et al. 2009; Abu-Qatouseh et al. 2010; Beaume et al. 2010; Bohn et al. 2010; Ten Broeke-Smits et al. 2010).

Antibiotic binding to riboswitches can be used to develop new treatments for multiple drug resistant strains. Some riboswitches are known as antimicrobial targets such as TPP, lysine, FMN and guanine riboswitches [review in (Mulhbacher, St-Pierre, et al. 2010)]. Recently, novel putative ligands that can bind to guanine riboswitches were selected based

on a model of the crystal structures. Two pyrimidine-based molecules named pyrimidine compound 1 (PC1) and pyrimidine compound 2 (PC2) were identified. PC1 showed antibacterial activity *in vitro* against *S. aureus* and *Clostridium difficile.* PC1 was also tested in a mouse model and shown to decrease *S. aureus* growth in the mammary gland (Mulhbacher, Brouillette, et al. 2010).

Another example of riboswitch is associated with the tightly control of the methionine biosynthesis operon in *S. aureus*. This regulation is a complex combination of the action of stringent-mediated repressor CodY, T-box riboswitch and methionine *metICFE-mdh* operon as explained in figure 16 (Schoenfelder et al. 2013).

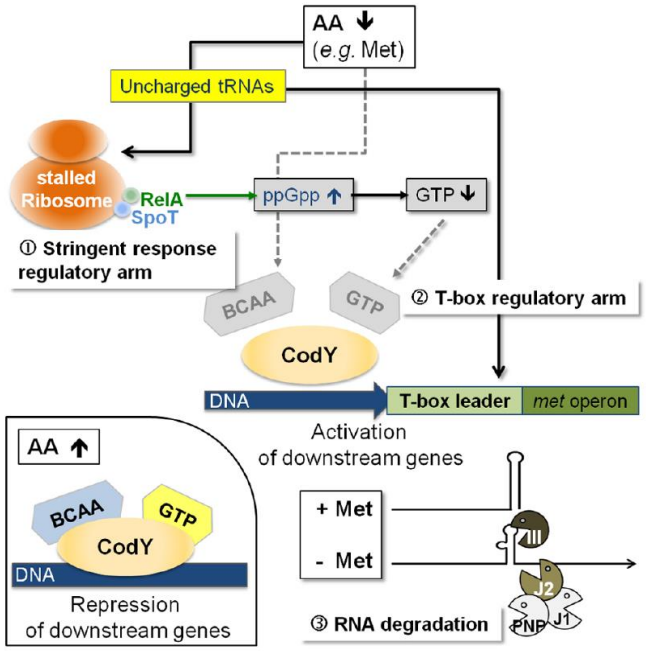


Figure 16: Model of a regulatory cascade for methionine biosynthesis operon control. (i) At high amino acid concentration, branched-chain amino acids (BCAA) and GTP are bound to the CodY repressor, increasing its affinity for target DNA binding; downstream genes are repressed (small picture, bottom left). (ii) Low amino acid levels will trigger the stringent response due to stalled ribosomes, which leads to an increase in RelA-mediated ppGpp alarmone synthesis resulting in less GTP. Subsequently, CodY dissociates from the DNA activating downstream transcription of the T-box leader RNA. The T-box acts as the crucial check-point sensing uncharged $tRNA_i^{fMet}$ levels and determines transcription of the met biosynthesis genes in a highly methionine-dependent manner. Rapid degradation of the met mRNA by the RNA degradosome is an additional mechanism to limit unnecessary translation of methionine biosynthesis mRNA. [From (Schoenfelder et al. 2013)].

Many *cis*-regulatory elements are present in *S. aureus*; however, so far, most of them are not characterized.

2.3 Protein-targeting RNAs

Protein-targeting RNAs are regulatory RNAs that directly interact with proteins to modulate their activity. Some of these proteins belong to ribonucleoprotein complexes and contribute to the function of housekeeping complexes such as M1 [the RNA component of Ribonuclease P, RNase P (Esakova & Krasilnikov 2010)], 4.5S RNA [component of the signal recognition particles (Ribes et al. 1990)] and tmRNA [tRNA-like mRNA-like dual functions (Keiler et al. 1996)]. Protein-targeting sRNAs can regulate protein activity by mimicking their substrate. In *E.* coli, some well-known examples are 6S sRNA that interacts with σ^{70} RNA polymerase (Wassarman 2007), CrsB sRNA that interacts with CsrA protein (carbon storage regulator) (Babitzke & Romeo 2007) and GlmY sRNA that interacts with YhbJ protein (Görke & Vogel 2008). In *S. aureus*, tmRNA, RNase P RNA, 4.5S and 6S were detected by comparative genomics and experimentally validated (Pichon & Felden 2005). However, this bacterium does not have the CsrB/CsrA and GlmY/YhbJ systems.

6S sRNA is a global regulator conserved in bacteria (Barrick et al. 2005; Trotochaud & Wassarman 2005). In *E.coli*, 6S sRNA sequestrates σ^{70}-core RNA polymerase by mimicking a promoter sequence (Figure 17). This interaction represses the expression of many σ^{70}-dependent transcription in stationary phase due to the abundance of 6S in this phase (Wassarman & Storz 2000). Moreover, 6S sRNA also upregulates the general stress σ^{S}-dependent transcription *in vivo* (Trotochaud & Wassarman 2005; Cavanagh & Wassarman 2014). 6S sRNA was found to be highly expressed in stationary phase of four *S. aureus* pathogenic strains (Pichon & Felden 2005). However, there is no homolog of σ^{S} in *S. aureus*. Instead, σ^{B} is the general stress sigma factor involved in environmental response and virulence factor expression (Gertz et al. 2000). Therefore, staphylococcal 6S RNA is possibly associated with different regulations than in *E. coli*.

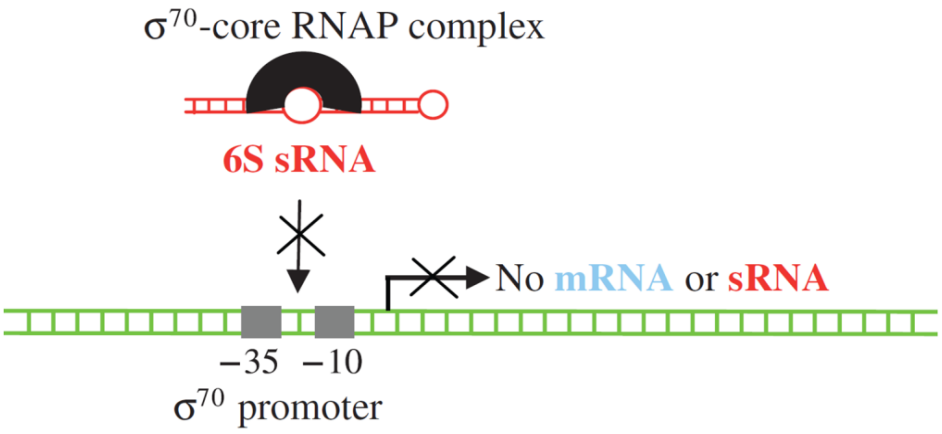


Figure 17: RNA polymerase interacts with 6S sRNA in *E.coli*. RNA synthesis by the "σ^{70}–core RNA polymerase" complex (black). 6S sRNA (red) sequesters the polymerase complex during nutrient limitation (stationary phase of growth), and restrain gene expression to the ones controlled by an alternative σ factor [From (Pichon & Felden 2007)].

3. Role of regulatory RNAs in *S. aureus*

Regulatory RNAs play multiple roles in *S. aureus.* Their expression was observed in several stress conditions including starvation, antibiotic treatment and host infection (Anderson et al. 2006; Geissmann et al. 2009; Beaume et al. 2010; Bohn et al. 2010; Anderson et al. 2010; Howden et al. 2013). Several examples of *S. aureus* regulatory RNAs involved in metabolism, stress response, environmental adaptation and virulence will be presented below.

RNAIII is the most well-known Staphylococcal sRNA. It is the intracellular effector of the *agr* system (Novick et al. 1993) that controls the expression of many virulence genes by a two-component signaling module. The *agr* locus encodes a quorum sensing system (agrBDCA) and RNAIII driven by the P2 and P3 promoters, respectively (Figure 18). AgrD encodes an autoinducing peptide (AIP) while AgrB is a transmembrane protein that processes and secretes this peptide. At the high cell densities, the secreted AIP molecules accumulate and reach a threshold level that can bind and activate the receptor histidine kinase AgrC. Then, the phosphorylated AgrC can activate the sensor regulator AgrA by transferring its phosphate group. As a consequence, the phosphorylated AgrA binds to the P2 and P3 promoters to induce RNAII and RNAIII transcripts. Therefore, RNAIII accumulates and its maximal expression is in late-exponential and stationary growth phases.

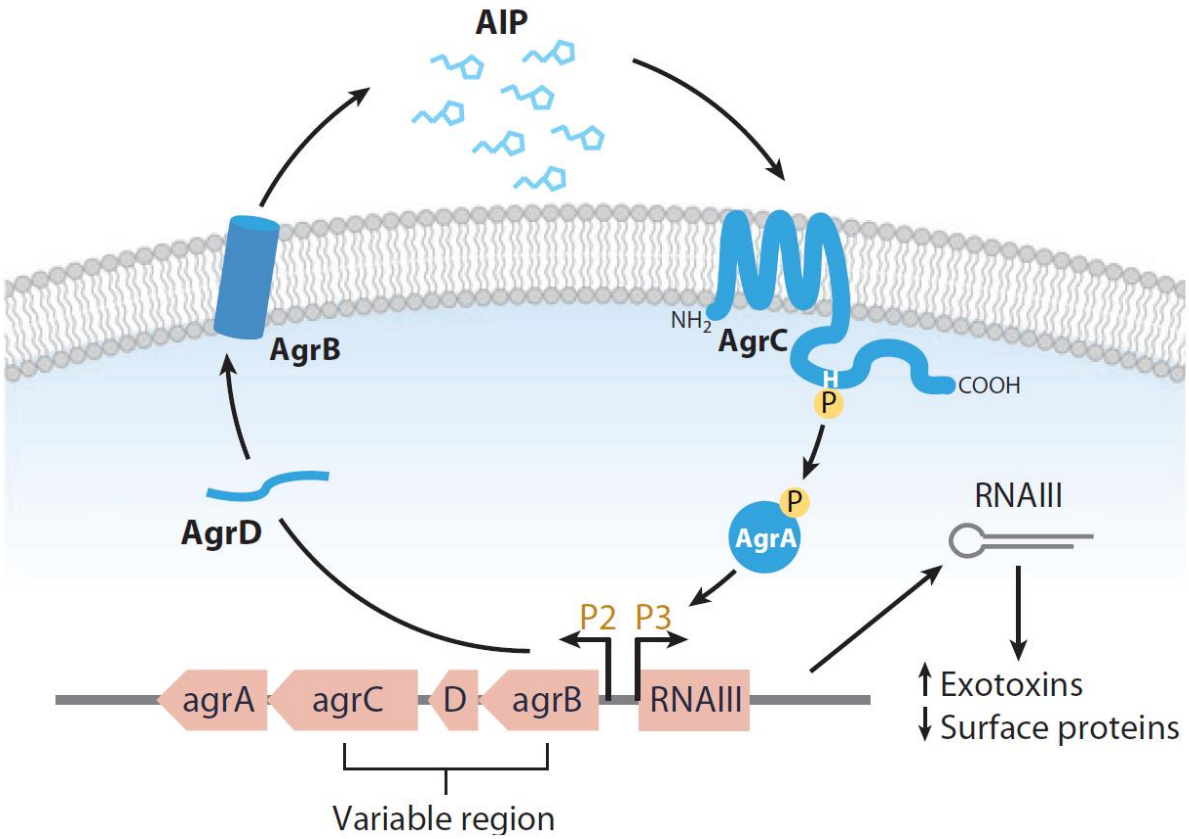


Figure 18: The accessory gene regulator (agr) quorum sensing system of *S. aureus.* [From (Novick & Geisinger 2008)].

RNAIII regulates important processes such as biofilm formation, peptidoglycan and amino acid metabolism, and transport pathways (Novick and Geisinger 2008). RNAIII upregulates genes involved in toxin secretion and enzyme production whereas it affects many downstream genes that encode cell wall–associated proteins. Thus, RNAIII plays a role in the switch of gene expression occurring when *S. aureus* reaches stationary phase. RNAIII is a unique example of one RNA molecule containing activities such as mRNA, an activator RNA, and an inhibitory RNA (Figure 19): i) RNAIII is a messenger RNA containing a small ORF encoding the δ-hemolysin. ii) the secondary structure near the 5' end of RNAIII acts as an antisense RNA. It base-pairs to a 5' segment of *hla* (hemolysin α) mRNA and sequesters the anti-Shine and Dalgarno sequence thus activating *hla* translation. iii) RNAIII directly base-pairs with *rot* (repressor of toxins), *spa* (protein A), *lytM* (autolysin) and *sbi* mRNA and represses their translations (Novick et al. 1993; Geisinger et al. 2006; Chunhua et al. 2012;

Chabelskaya et al. 2014). In addition, RNAIII also was shown to negatively regulate the transcriptional regulator *sarT* mRNA (Boisset et al. 2007).

In addition to RNAIII, a second sRNA named ArtR (**A**grA-**r**epressed, **t**oxin-regulating s**R**NA) is regulated by the *agr* system (Xue et al. 2013). ArtR directly binds to *sarT* mRNA (encoding a repressor of α-hemolysin) and promotes the duplex degradation by RNase III. Through SarT, ArtR indirectly activates *hla* expression (Figure 19).

The *psm-mec* RNA transcribed from the *SCCmec* mobile element (which contributes to *S. aureus* antibiotic methicillin resistance) is another example of sRNA with a dual-function. First, *psm-mec* contains a small ORF that encodes a 22 amino-acid cytolysin, phenol-soluble modulin (PSMα). Second, *psm-mec* RNA inhibits translation of *agrA* through direct interaction, leading to decreased extracellular toxin production, hence reduce virulence (Kaito et al. 2013).

Other known sRNAs important for virulence of *S. aureus* are $SprA1_{AS}$, SSR42 and Teg49. Firstly, $SprA1_{AS}$ is an antisense of SprA1, a sRNA encoding a human cytolytic peptide. Surprisingly, the pairing region between $SprA1_{AS}$ and SprA1 is not in the 35-nucleotide perfectly complementary region. Instead, $SprA1_{AS}$ asRNA interacts with the 5'end of *SprA1* to sequester the *SprA1* SD sequence and prevents translation initiation (Sayed et al. 2011). Secondly, SSR42 is a 891-nucleotide long RNA that belongs to the small stable RNAs (SSRs) group. SSR42 is induced in stationary phase and controls a large number of genes including virulence ones; it is required for pathogenicity in a murine model of *S. aureus* skin and soft tissue infection (Morrison et al. 2012). Thirdly, Teg49 RNA is located upstream of *sarA,* which encodes a transcriptional regulator. Teg49 was shown to modulate *sarA* expression which is involved in *S. aureus* pathogenicity (Kim et al. 2014).

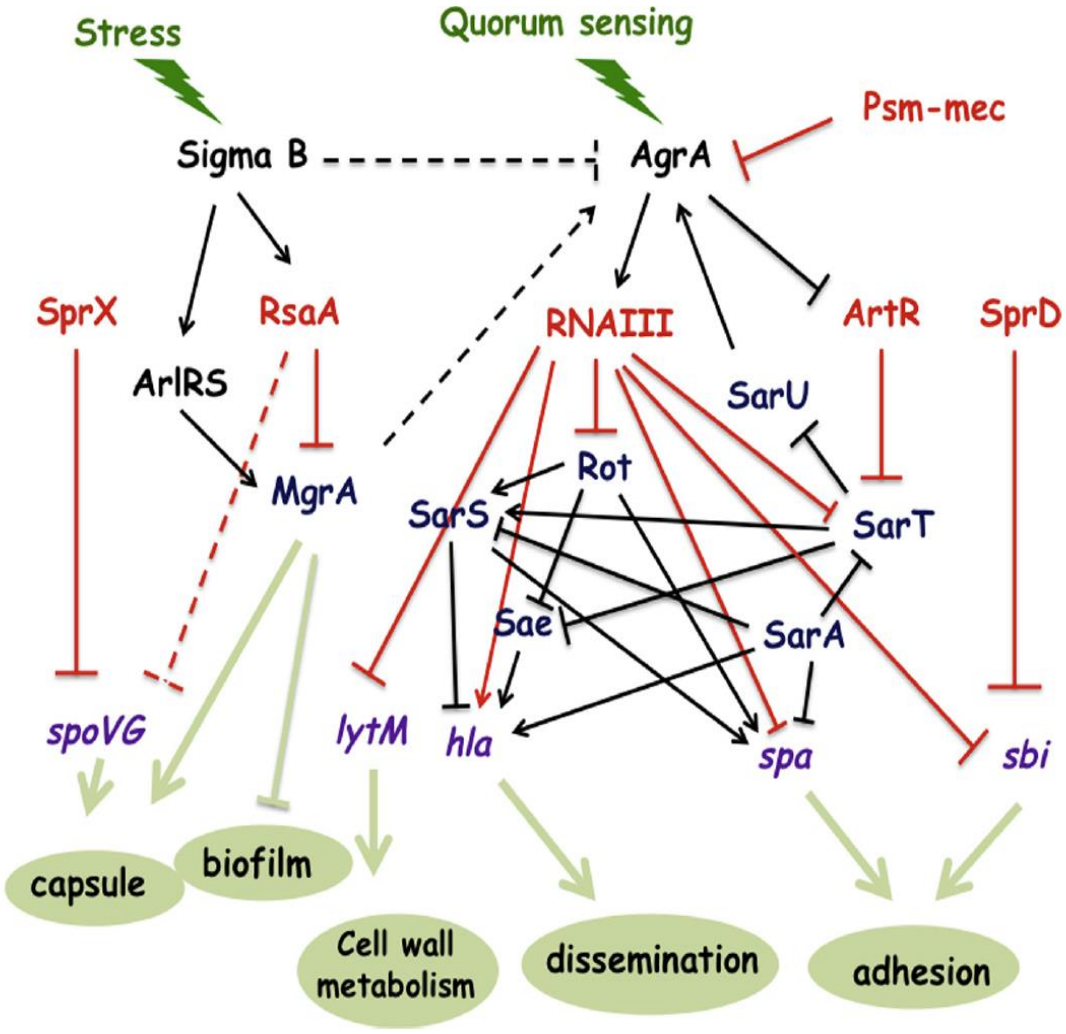


Figure 19: Regulatory circuits involved in virulence gene expression. The networks are based on the knowledge acquired from the literature (see the accompanying text). The transcriptional regulatory proteins are in black, the regulatory RNAs are in red and the target proteins are in purple. The transcriptional regulation is shown by black lines while post-transcriptional regulation is shown by red lines. Arrows corresponded to activation while bars corresponded to repression. Indirect regulation is given by dotted lines. The functional consequences of the regulation are also given. [From (Fechter et al. 2014)].

One interesting example is RsaE, a 93-nt long unique sRNA conserved in *Staphylococcus*, *Macrococcus* and *Bacillus* genera (Geissmann et al. 2009; Bohn et al. 2010). The transcription of RsaE is strongly enhanced in stationary phase in strain RN6390 (Geissmann et al. 2009) but in many clinical strains, RsaE accumulates in pre-stationary phase and is repressed in stationary phase (Bohn et al. 2010; Song et al. 2012). The *rsaE* transcription is up-regulated by the *agr* system and down-regulated in σ^{B+} strain (Geissmann et al. 2009).

RsaE plays an important role in *S. aureus* metabolism (Figure 20). It downregulates various enzymes involved in the Krebs cycle and the folate metabolic pathway. Specifically, RsaE directly represses the expression of two enzymes of the TCA cycle (SucC/SucD) and two oligopeptide transporters (Opp3A/B) by pairing to SD sequences of the targeted mRNAs hence preventing translation initiation (Geissmann et al. 2009; Bohn et al. 2010). In addition, RsaE upregulates genes encoding membrane proteins (*opp4A*/o*pp4D*) and several operons such as valine, leucine and isoleucine biosynthetic operons.

So far no phenotype has been associated with the *rsaE* deletion in *S. aureus* (Geissmann et al. 2009; Bohn et al. 2010). Recently it was reported the *rsaE* orthologue gene in *B. subtilis* was induced by nitric oxide (Durand et al. 2015). Many genes involved in the oxidative stress response were upregulated in the *rsaE* mutant as compared to the wild-type strain. Thus, the *rsaE* gene was renamed *roxS* (Related to oxidative stress). RoxS was shown to affect the *ppnKB* mRNA (encoding an NAD+/NADH kinase) stability but also to prevent its translation.

Due to the interplay between RsaE, *agr* system and σ^B, it was suggested that RsaE could be an example of sRNA at the crossroad between metabolism and virulence (Tomasini et al. 2014).

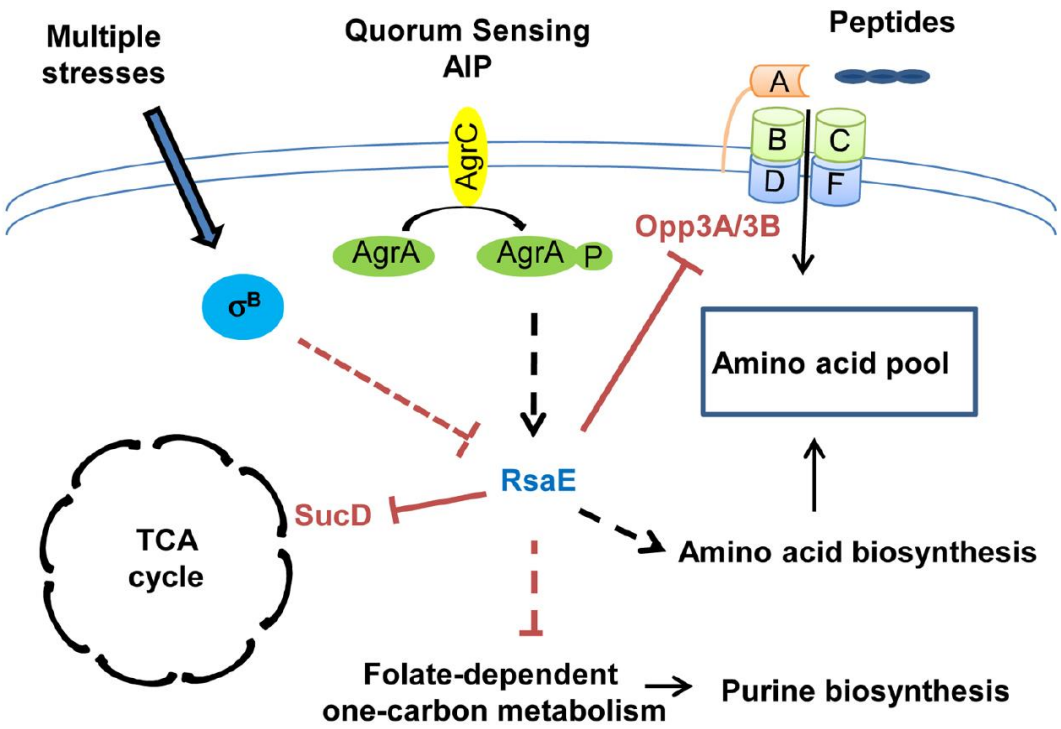


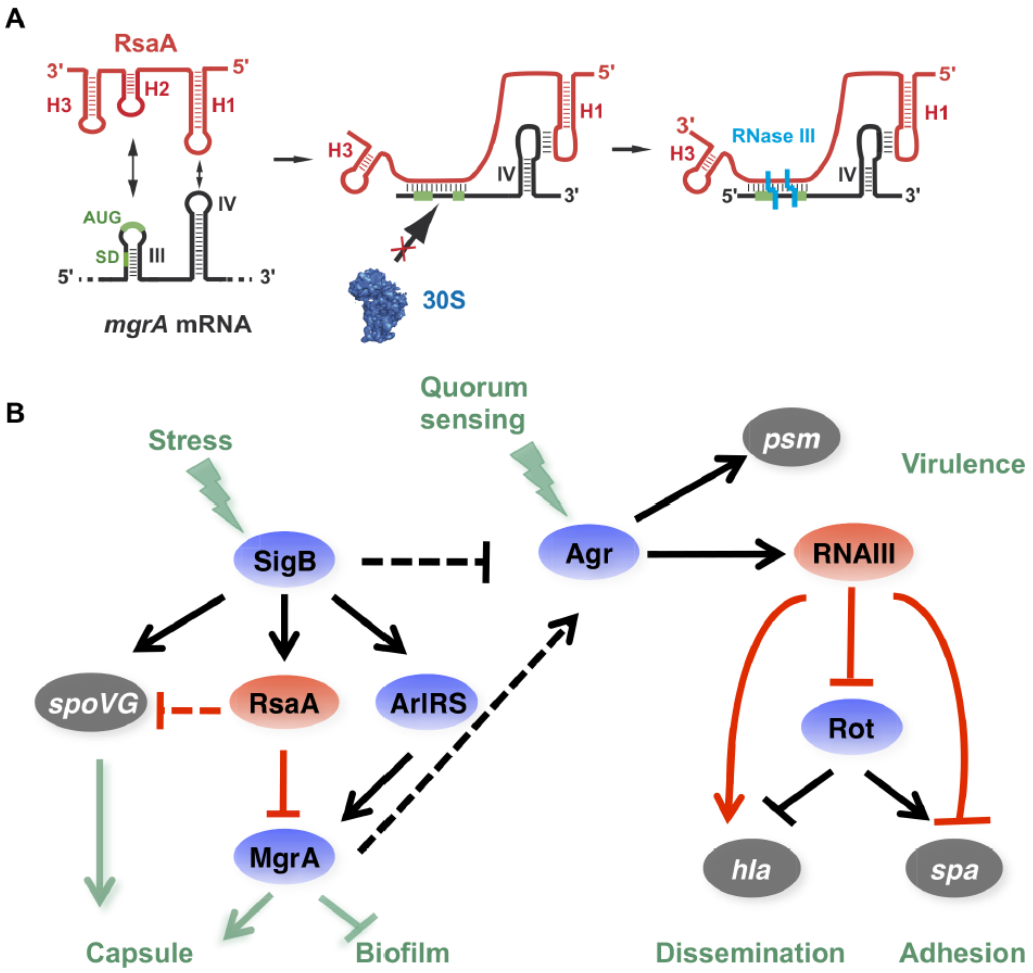
Figure 20: RsaE controls metabolic pathways. Autoinducing peptide (AIP), tricarboxylic acid cycle (TCA). The plain and dashed lines indicate the direct and indirect gene regulations, respectively (red bars: inhibitions, black arrows: stimulations). [From (Guillet et al. 2013)].

Another sRNA in *S. aureus* revealing links between adaptation and virulence is RsaA. It is a typical σ^B-dependent sRNA, hence strongly expressed in σ^{B+} strains (COL and Newman strains) (Geissmann et al. 2009). RsaA is also induced in small colony variants (SCV), a slow-growing subpopulation in *S. aureus* (Abu-Qatouseh et al. 2010). Moreover, RsaA was found to be expressed in all *S. aureus* clinical isolates (Romilly et al. 2014).

Recently, the global transcriptional regulator MgrA involved in biofilm formation and capsule synthesis was found to be negatively regulated by RsaA (Romilly et al. 2014) (and chapter III.2.1.2). RsaA base-pairs with *mgrA* mRNA in two different regions; a conserved C-rich motif of RsaA pairs with the SD sequence of *mgrA* mRNA and another loop-loop interaction in the coding sequence of *mgrA* that recruit RNase III to degrade the duplex, and hence inhibits translation initiation (Romilly et al. 2014) (see also chapter III.2.1.2). Through MgrA, RsaA activates the production of biofilm and represses the capsule synthesis (Figure 21). At the same time, MgrA is positively controlled by two component system ArlRS which is also activated by σ^B (Luong et al. 2006). Altogether, *mgrA* expression is controlled by a circle loop including both positive and negative regulation mediated by ArlRS and RsaA, respectively.

In addition, the expression of SpoVG (a predicted target of RsaA by computational approach) involved in capsule synthesis was significantly increased in a *rsaA* mutant (Romilly et al. 2014). Importantly, RsaA was shown to affect *S. aureus* virulence in two mouse infection models by reducing bacterial invasiveness and enhancing local colonization. RsaA is the first characterized conserved sRNA in *S. aureus* that diminishes the severity of acute infection and favors chronic infection (Romilly et al. 2014).

Figure 21: RsaA and its regulatory circuits. (A) Schematic drawing summarizing the regulatory



mechanism. RsaA binds to *mgrA* mRNA and inhibits translation by preventing the 30S subunit binding, and recruits RNase III to induce the simultaneous degradation of both RNAs. (B) RsaA is activated by σ^B and in turn represses *mgrA* mRNA translation. RsaA is thus indirectly linked to RNAIII regulatory networks because MgrA activates *agrACDB* expression while σ^B represses it. Arrows are for activation, bars for repression. In blue are the transcriptional protein regulators, in red the regulatory RNAs and in grey the virulence factors. Red lines corresponded to post-transcriptional regulation and black lines to transcriptional regulation. The regulatory events for with no direct regulation demonstrated yet are shown by dotted lines. [From (Romilly et al. 2014)].

Several regulatory RNAs expressed from genomic pathogenicity islands (containing virulence and antibiotic resistance genes) in *S. aureus* were named **Spr** for "**S**mall **p**athogenicity island **R**NAs" (Pichon & Felden 2005). One of them expressed from the Pathogenicity Island ϕ (PI), SprD, contributes significantly to disease in a mouse infection model. It prevents the translation initiation of the *sbi* mRNA (encoding immune evasion protein). SprD base-pairs with the *sbi* mRNA 5'-end to prevent ribosome loading by occluding the SD sequence and the initiation codon (Chabelskaya *et al.*, 2010) (Figure 22). Sbi is also repressed by RNAIII by a similar mechanism (Chabelskaya et al. 2014) (see also chapter III.3.1). The regulation of *sbi* specifically depends on the expression of these 2 sRNAs which are expressed in exponential phase and stationary phase for SprD and RNAIII, respectively.

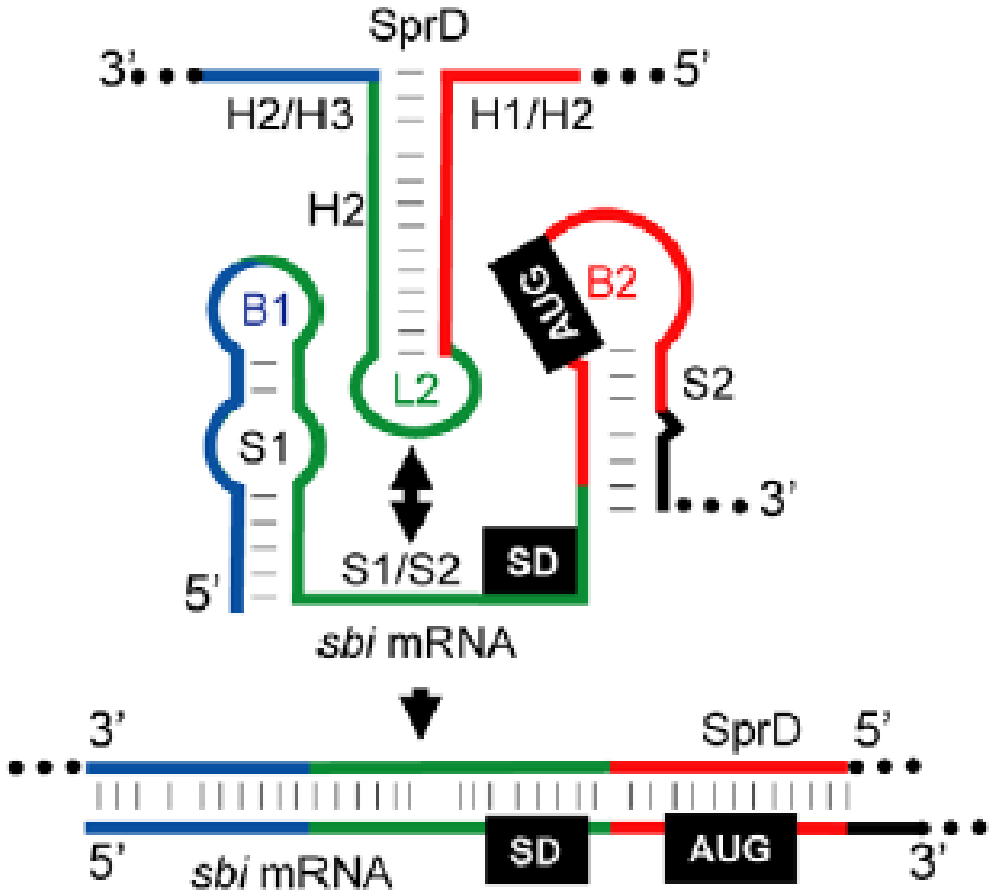


Figure 22: Base-pair association of SprD with *sbi* mRNA. SprD recognizes its target mRNA via a "loop–single strand" interaction (green) that extends further upstream and downstream. [From (Chabelskaya *et al.*, 2010)].

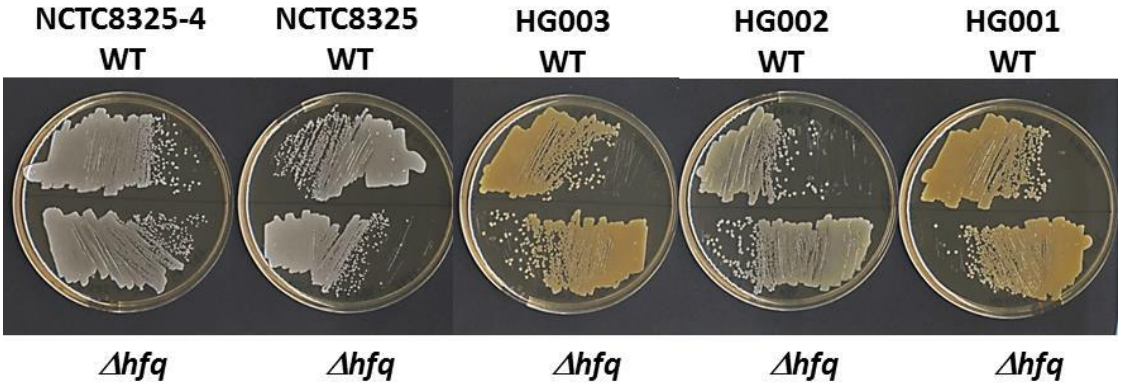
4. Proteins involved in *S. aureus* sRNA functions

4.1 RNA chaperone Hfq: a controversial factor

Hfq (aka HF-I, host factor-I) encoded by the *hfq* gene, is a RNA-binding protein first discovered in *E. coli* as a essential host factor for bacteriophage Qβ replication (Franze de Fernandez et al. 1968; Franze de Fernandez et al. 1972). Hfq facilitates the imperfect complementary pairings of sRNAs and their targets by stabilizing the interaction between the two RNA molecules and/or by increasing their local concentration. In many bacteria, including *E. coli*, Hfq is required for sRNA activities and contributes to the recruitment of the endoribonuclease RNase E (Morita et al. 2005; Urban & Vogel 2007; Bandyra et al. 2012).

The *hfq* gene is present, so far, in all sequences of staphylococcal strains and the Hfq crystal structure of *S. aureus* (Hfq_{Sa}) was the first obtained (Schumacher et al. 2002). Unexpectedly, the deletion of hfq_{Sa} in strain RN6390 did not generate any detectable phenotype in ~ 2000 tested growth conditions (Bohn et al. 2007). On the other hand, an *hfq* deletion in *S. aureus* NCTC8325-4 was shown to increase carotenoid pigments and to confer higher resistance to oxidative stress and reduced virulence (Liu et al. 2010). These apparently contradictory results can be explained by a different expression of *hfq* between strains. hfq_{Sa} is detected in NCTC8325-4 but not in RN6390 (Liu et al. 2010). Intriguingly, in contrast to Liu et al results, hfq_{Sa} mutants in several backgrounds including NCTC8325-4 constructed in our laboratory did not stimulate the production of *S. aureus* pigmentation (Chantal Bohn, unpublished results and figure 23).

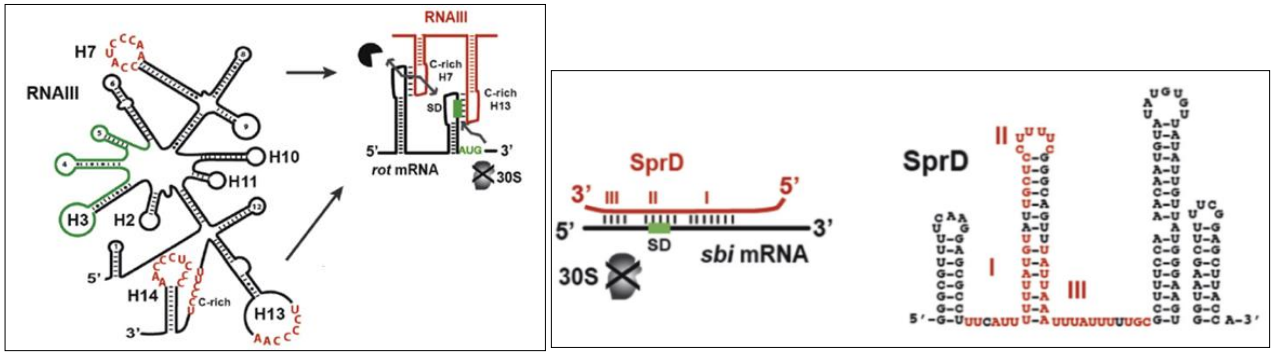
Figure 23: Effect of *hfq* deletion on pigmentation in different *S. aureus* strains.



Surprisingly, Hfq_{Sa} was not required for any sRNA activities tested, including interactions of the well-known regulatory RNA RNAIII with its targets (Geisinger et al. 2006; Boisset et al. 2007; Geissmann et al. 2009; Chabelskaya et al. 2010).

In *S. aureus*, long duplex pairings and formation of two-part binding sites between sRNAs and their mRNA targets are observed (*e.g.*, RNAIII-*rot* mRNA, SprD-*sbi* mRNA or RsaA-*mgrA* mRNA) (Figure 24 and Chapter IV.3). In addition, *S. aureus* sRNAs have often C-rich motifs within loops involved in pairing interactions [(Geissmann et al. 2009) and Chapter IV)]. Therefore, Hfq_{Sa} may be dispensable because of these features (Tomasini et al. 2014). In addition, Hfq_{Sa} contains a KANQ motif instead of an arginine rich motif RRER which is required for Hfq chaperone activity in *E. coli* (Panja et al. 2013); this difference may explain why hfq_{Sa} cannot complement neither *E. coli* nor *Salmonella typhimurium hfq*s (Večerek et al. 2008; Rochat et al. 2012). Similarly, the mutant *hfq* in *B. subtilis* did not have any phenotype under 2000 growth conditions except a growth defect in rich medium in stationary phase (Rochat et al. 2015). Moreover, *B. subtilis hfq* also cannot complement *S. typhimurium hfq*.

As Hfq_{Sa} seems not involved in sRNA-dependent regulations, other staphylococcal proteins may be required for sRNA activities. For example, YbeY, a *Sinorhizobium meliloti* protein sharing structural similarities with Argonaute (an RNA-binding protein involved in RNA silencing in eukaryote) was found to act as Hfq and indeed, the mutant *ybeY* has similar phenotypes as those of *hfq* mutant (Pandey et al. 2011).



(A) (B)

Figure 24: Examples of regulatory RNAs and their mechanism of action in *S. aureus.* (A) Inactivation of *rot* mRNAs by RNAIII. C-rich sequence motifs of RNAIII (in red) are seed sequences binding to SD sequences of mRNA targets. The formation of a RNAIII-*rot* mRNA duplex prevents the small ribosomal subunit (30S) binding and promotes RNase III specific cleavages (grey arrows). (B) Repression of *sbi* mRNA translation by SprD. Three different regions of SprD base-pair with *sbi* mRNA 5'-UTR and the beginning of its coding sequence. The duplex prevents the formation of an initiation ribosomal complex. [From (Fechter et al. 2014)].

4.2 Main RNases in *S. aureus*

Ribonucleases (RNases) contribute to RNA degradation and RNA processing. They can be divided into 2 groups: endoribonucleases (cleaving inside RNA molecules) and exoribonucleases (removing nucleotides from 5' or 3' ends of RNAs). RNases identified in *S. aureus* are listed in Table 2. Main RNases, *i.e.* RNase III, RNase Y, RNase J1/J2 and PNPase, are discussed below.

Table 2: Ribonucleases in *S. aureus*. [From (Bonnin & Bouloc 2015)].

Ribonuclease	Gene	Function [a]	Amino acid identity between NCTC8325 and *B. subtilis*168 orthologs[c]	Amino acid identity between NCTC8325 and *E. coli* MG1655 orthologs[c]	Nomenclature N315	Nomenclature NCTC8325	Essentiality [b]
RNase III	*rnc*	ds-RNA endonuclease *	0.49	0.34	SA1076	SAOUHSC_01203	N
Mini-III	*mrnC*	ds-RNA endonuclease ¤	0.56	None	SA0489	SAOUHSC_00512	N[b]
RNase Y	*rny/cvfA*	ss-RNA endonuclease *	0.69	None	SA1129	SAOUHSC_01263	N
RNase J1	*rnjA*	strong 5'-3' exonuclease activity * ss-RNA endonuclease	0.67	None	SA0940	SAOUHSC_01035	N**
RNase J2	*rnjB*	weak 5'-3' exonuclease activity * ss-RNA endonuclease?	0.50	None	SA1118	SAOUHSC_01252	N**
RNase P	*rnpA*	Endonucleolytic cleavage of RNA, removing 5'-extranucleotides from tRNA precursor with *rnpB* ribozyme *	0.49	0.24	SA2502	SAOUHSC_03054	Y
RNase Z	*Rnz*	Endonucleolytic cleavage of RNA involved in removing extra 3' nucleotides from the tRNA precursor ¤	0.45	0.41	SA1335	SAOUHSC_01598	Y

RNase M5	*rnmV*	ds-RNA endonuclease, maturation of 5S rRNA ¤	0.53	None	SA0450	SAOUHSC_00463	N
PNPase	*pnpA*	3’-5’ exonuclease *	0.68	0.50	SA1117	SAOUHSC_01251	N
RNase R	*Rnr*	3’-5’ exonuclease ¤	0.55	0.37	SA0735	SAOUHSC_00803	Y
YhaM	*yhaM*	3’-5’ exonuclease ¤	0.52	None	SA1660	SAOUHSC_01973	N
RNase HI	*ypqD/rnhA*	RNase HI-family protein of unknown function ¤	0.33	None	SA1266	SAOUHSC_01443	N
RNase HII	*rnhB*	Endonuclease, degradation of RNA/DNA duplexes ¤	0.47	0.44	SA1087	SAOUHSC_01215	N
RNase HIII	*rnhC*	Endonuclease, degradation of RNA/DNA duplexes ¤	0.46	None	SA0987	SAOUHSC_01095	N
nano-RNase A	*nrnA*	Oligoribonuclease, 3',5'-bisphosphate nucleotidase ¤	0.49	None	SA1526	SAOUHSC_01812	N

[a] Function: * demonstrated experimentally; ¤ function based on results of *B. subtilis* or *E. coli* studies.

[b] Essentiality: Y, demonstrated experimentally using transposon mutagenesis (Chaudhuri et al. 2009); N not essential demonstrated experimentally, N[b] not essential based on *B. subtilis* studies. ** RNase J1 and J2 are essential at 42°C but not at lower temperatures (Chaudhuri et al. 2009; Linder et al. 2014).

[c] Accession numbers: *B. subtilis* 168, NC_000964.3; *E. coli* MG1655; NC_000913.3.

4.2.1. RNase III: a major RNase involved in sRNA-dependent regulations

RNase III (encoded by *rnc*) is an endoribonuclease that belongs to a ubiquitous family of double-strand (ds)-RNA specific enzymes. In *E. coli,* RNase III is involved in various cell processes such as ribosomal RNA (rRNA) maturation (Srivastava et al. 1990; Deutscher 2009), mRNA processing and its autoregulation (Bardwell et al. 1989). In addition, RNase III degrades mRNA-sRNA duplexes (Stead et al. 2011).

RNase III is the best characterized RNase n *S. aureus*. This 243-amino-acid enzyme was first characterized via its function in the *agr* system. sRNA-mRNA duplexes such as RNAIII-*spa* mRNA, RNAIII-*rot* mRNA, RNAIII-*coa* mRNA are degraded by RNase III (Boisset et al. 2007; Huntzinger et al. 2005; Liu et al. 2011; Novick et al. 1993) (Figure 25).

Type I toxin-antitoxin (TA) systems encode a poison and an antidote which is an asRNA that associates with the mRNA encoding the toxin (Fozo et al. 2008; Brantl 2012). As toxin and antitoxin are expressed from opposite strands of a same DNA region, the two transcripts are complementary and form a duplex that can be targeted by RNase III. In *B. subtilis,* RNase III is essential because it prevents the expression of toxins from type I TA systems (Durand et al. 2012). In *S. aureus*, the essentiality of RNase III is observed in specific backgrounds containing phages phi 11, phi 12, phi 13; these prophages are carrying type I TA systems (see chapter C).

Three studies on the role of RNase III in *S.* aureus were recently published. The first one is a transcriptome comparative analysis between a wild-type strain and its corresponding isogenic *rnc* mutant (Lasa et al. 2011). The *rnc* depletion mutant revealed an increased antisense transcription (74.2% in *Δrnc* compared with 49.2% in wild-type) and a reduced number of short transcripts. This study demonstrates the role of RNase III in antisense-sense regulation and in reducing antisense transcription. The two other studies report coimmunoprecipitation assays of RNAs with RNase III and altered RNases III (Lioliou et al. 2012; Lioliou et al. 2013). Several RNAs were identified as RNase III substrates; its roles in rRNA and tRNA processings, and autoregulation of its own synthesis were confirmed. Moreover, RNase III was shown to be involved in mRNA turnover and

mRNA-sRNA duplexes like in *E. coli*. Interestingly, RNase III was found to cleave the *cspA* (cold shock induced protein) 5'UTR (Figure 25).

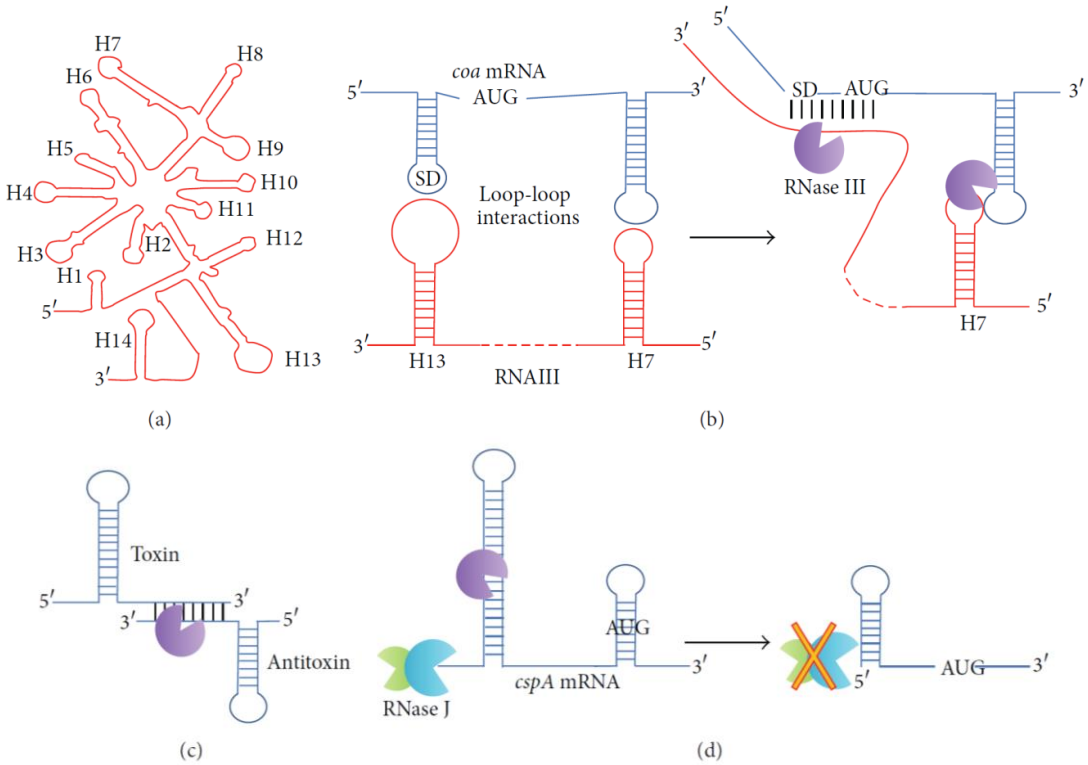


Figure 25: Examples of RNase III functions. (a) Schematic view of *S. aureus* RNAIII structure. RNAIII is involved in the regulation of virulence genes by base-pairing with specific mRNAs (b) The region around the SD sequence of *coa* mRNA (encoding coagulase) base-pairs with the RNAIII helix H13 and is stabilized by a second interaction involving the RNAIII helixH7. RNase III degrades the *coa* mRNA-RNAIII duplex, both in the SD region and within the loop-loop interaction region. (c) RNase III degrades ds-RNAs including sense antisense RNA duplexes as exemplified by type I toxin-antitoxin systems. (d) Cleavage inside a stem-loop can give rise to a more stable mRNA, as demonstrated for the cold shock protein A *cspA* mRNA. Cleavage of the stem-loop releases the translation start codon and a new stem-loop protects the 5' end from RNase J-mediated degradation. [From (Bonnin & Bouloc 2015)].

4.2.2. RNase Y, RNase J1 and J2

In Gram-positive bacteria such as *B. subtilis* or *S. aureus*, no single-strand specific-RNA endonuclease RNase E is present. However, single-strand endonuclease RNase Y and

two paralogs RNases named RNase J1 (formerly YkqC) and RNase J2 (formerly YmfA) were discovered and are functionally equivalent to RNase E in *E. coli*.

In *B. subtilis*, the depletion of RNase Y (encoded by *rny*) generates pleiotropic effects: reduced biofilm formation, altered the expression of many genes involved in folate, amino acid biosynthesis and extracellular polysaccharide synthesis (Lehnik-Habrink et al. 2011).

In *S. aureus*, the *rny* ortholog (formerly *cvfA*) was first identified in a screen for new virulence factors in silkworm and mouse infection models (Kaito et al. 2005). *rny* mutant showed a decrease of hemolysin production, and reduced virulence. Later, it was shown that CvfA protein has two domains: an RNA binding domain (KH domain), and a metal-dependent phosphohydrolase domain (HD domain). The HD domain was required for the virulence phenotype (Nagata et al. 2008). Moreover, RNase Y was also shown to control the expression of SaeS/SaeR TCS, a global regulator of virulence. The *sae* operon has 4 overlapping transcripts from T1 to T4. RNase Y processes the T1 transcript leading to the stabilization of a T2 transcript (Marincola et al. 2012) (Figure 26).

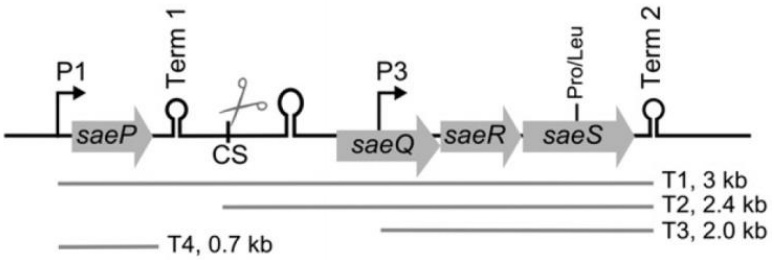


Figure 26: Schematic representation of the *saePQRS* operon with primary and mature transcripts (T1–T4), the promoters (P1 and P3), the terminators (Term1 and Term2) and the cutting site (CS). Adapted from (Marincola et al. 2012).

RNase J1 and RNase J2 (encoded by *rnjA* and *rnjB*, respectively) are 5' to 3' exonucleases and single-strand endonucleases (Even et al. 2005; Britton et al. 2007). RNases J1 and J2 are 5'-monophosphate group specific and are inhibited by RNAs with a 5'-triphosphate group (Deikus et al. 2008) (Li de la Sierra-Gallay et al. 2008). The 5'-3' exonuclease activity of RNase J2 was weaker than the activities of RNase J1 or of the RNase J1/J2 complex. In *B. subtilis*, *rnjA* and *rnjB* are not essential (Figaro et al. 2013). The

rnjA mutant had longer doubling time (~76 min) compared to the wild-type strain (~26 min) but no change was observed in mutant *rnjB*. In addition, *rnjA* depletion affected sporulation, competence and cell morphology.

In contrast to *B. subtilis*, a study of essential genes by transposon mutagenesis concluded that *rnjA and rnjB* were essential in *S. aureus* (Chaudhuri et al. 2009). However, single mutants *rnjA, rnjB* and double mutant *rnjA/rnjB* were obtained by allelic exchange in a more recent study (Redder & Linder 2012; Linder et al. 2014). These mutants cannot grow at 42^{o}C; it explained why these mutants were not obtained by transposon mutagenesis since a step was carried out at 44^{o}C. RNases J1 and J2 are essential under specific condition. *rnjB* leads to a stronger growth defect than *rnjA*. Interestingly, a mutation affecting RNase J1 active site leads to a phenotype equivalent to the *rnjA* deletion whereas a mutation affecting RNase J2 active site had no effect suggesting that RNase J2 plays a structural role. RNase J1 was shown to play a major role in RNA decay with the help of RNase J2 while both RNase J1 and J2 are responsible for cleaving precursor to mature 16s rRNA and RNase P ribozyme (Linder et al. 2014).

4.2.3. PNPase

Polynucleotide phosphorylase (PNPase) is a bifunctional enzyme encoded by *pnpA* gene. PNPase is 3' to 5' exonuclease that uses inorganic phosphate P_i instead of H_20 to degrade RNA and thus generates diphosphate nucleosides. PNPase can also have a second function as polymerase when the concentration of P_i is lower than diphosphate nucleosides in the cell (Deutscher and Li 2001).

PNPase is not essential in both Gram-positive and Gram-negative bacteria. However, *pnpA* mutants have cold-sensitive phenotype in *E. coli* and *B. subtilis* (Wang & Bechhofer 1996).

In *S. aureus*, the *pnpA* mutant is also cold-sensitive confirming the important role of PNPase in cold shock adaptation (Anderson & Dunman 2009). In addition, PNPase is involved in bulk RNA turnover: in wild-type strain, about 51% of transcripts are entirely

degraded after 5 min of posttranscriptional arrest while only 17% are degraded in the *pnpA* mutant (Anderson & Dunman 2009).

Interestingly, it was shown that PNPase and RNase Y regulates the turnover of mRNAs involved in virulence in opposite manner (Numata et al. 2014). The disruption of *pnpA* can suppress *rny* phenotypes (decreased hemolysin production and *agr* expression). A model was proposed in which specific 3'OH RNAs involved in hemolysin production were first cleaved by RNase Y producing 2',3'-cyclic RNAs. Then, 2',3'-cyclic RNAs are converted to 3'phosphorylated RNAs through hydrolysis of RNase Y. In this process, specific 3'OH RNAs and 2',3'-cyclic RNAs were sensitive to PNPase degradation while 3'phosphorylated RNAs were resistant to PNPase (Figure 27).

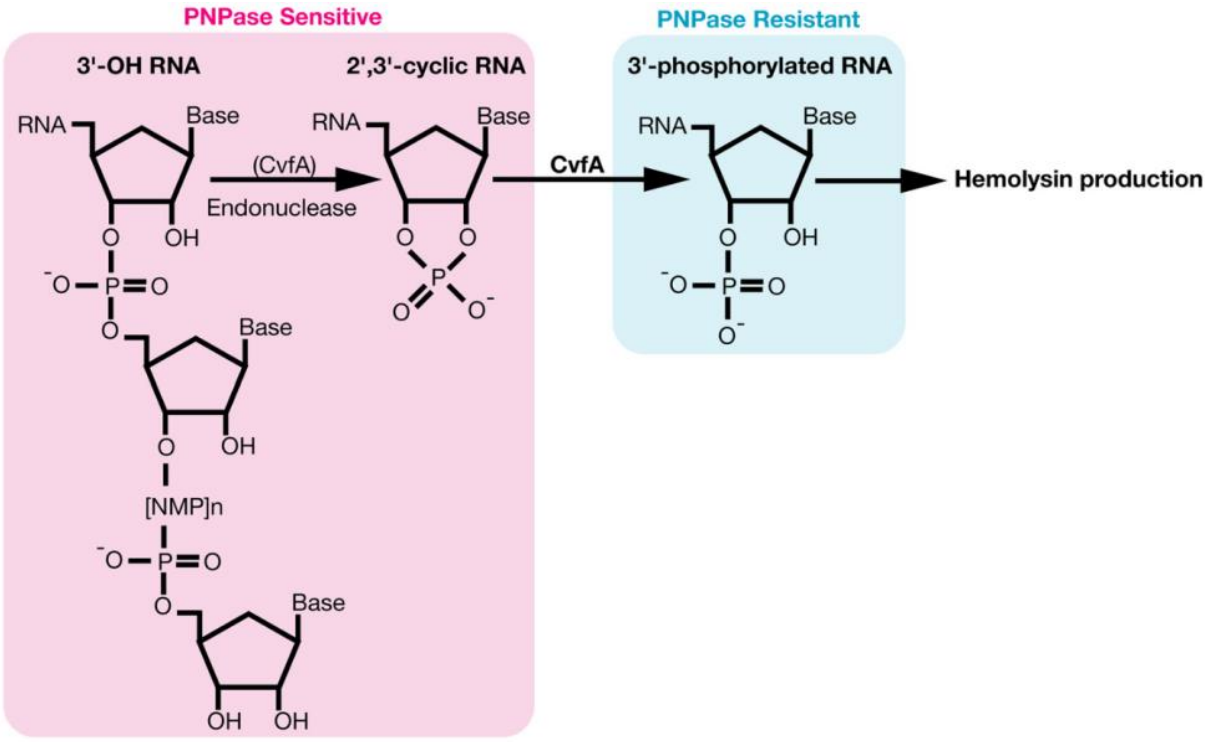


Figure 27: *S. aureus* hemolysin production via RNA stability control by RNase Y (CvfA) and PNPase. A specific RNA (3'-OH RNA) required for hemolysin production cleaved by RNase Y or other endonucleases results in the production of 2',3'-cyclic RNA. Next, the 2',3'-cyclic RNA is converted to 3'-phosphorylated RNA by RNase Y. 3'-OH RNA and 2',3'-cyclic RNA are degraded by PNPase, whereas 3'-phosphorylated RNA is resistant to PNPase degradation. [From (Numata et al. 2014)].

V. Selected samples of regulatory RNAs in some Firmicutes species

1. sRNA in the pathogenesis of *Streptococcus*

Most studies on Streptococci sRNAs concern three human pathogen species. Until now, 75 sRNAs were discovered in *S. pyogenes* (Perez et al. 2009; Patenge et al. 2012; Tesorero et al. 2013) and 179 sRNAs in *S. pneumonia*, respectively (Tsui et al. 2010; Kumar et al. 2010; Acebo et al. 2012; Mann et al. 2012). In addition, 197 regulatory RNAs were predicted *in silico*, of which 26 were valided whereas 39 riboswitches and cis-regulatory regions, 39 asRNAs and 47 sRNAs were found by using single nucleotide resolution RNA-seq in the opportunistic pathogen *S. agalactiae*, or Group B Streptococcus (Pichon et al. 2012; Rosinski-Chupin et al. 2015).

In *S. pyogenes*, Pel, FasX and RivX are three characterized sRNAs involved in virulence. *fasX* (fibronectin/fibrinogen-binding/hemolytic-activity/streptokinase-regulator-X) is the last gene of the *fasBCAX* operon and it regulates its own operon. In addition, FasX negatively regulates the expression of *fbp54* (fibronectin binding protein), and *mrp* (fibrogen binding protein) (Kreikemeyer et al. 2001). It also interacts with the 5'-end of *ska* mRNA (secreted plasminogen activator streptokinase) to stabilize the transcript and stimulate its translation (Ramirez-Peña et al. 2010) (Figure 28).

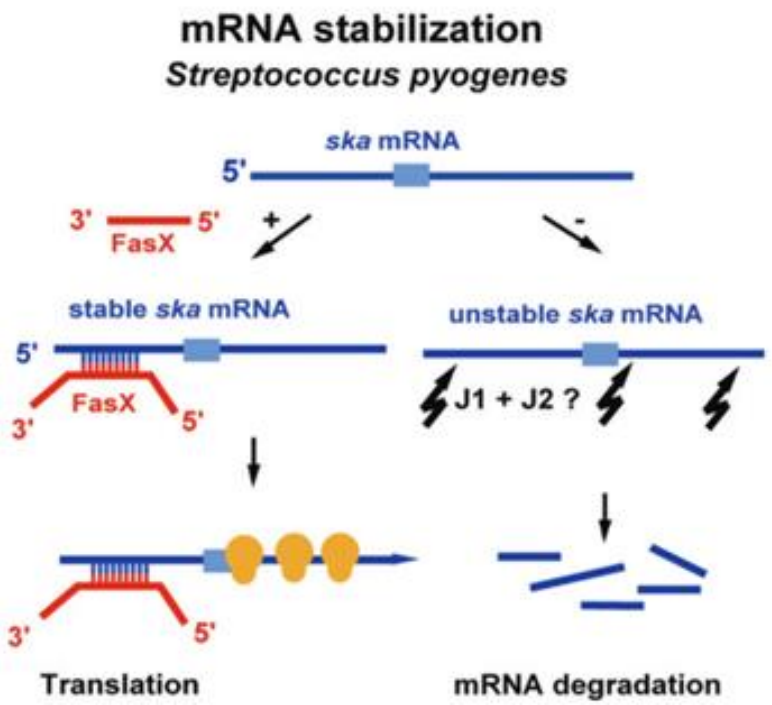


Figure 28: Regulatory mechanism of FasX with the *ska* mRNA. sRNAs are drawn in red, mRNAs in blue. Light blue, ribosome binding sites (RBS). Yellow symbols indicate ribosomes. Black arrows indicate unknown RNase action. [From (Brantl & Bruckner 2014)].

2. sRNA in the pathogenesis of *Clostridium*

Clostridium is a large genus containing around 100 species. This genus not only includes human and animal pathogens but also many strains involved in cellulose degradation, the carbon cycle, bioremediation and biotechnology. sRNAs were identified in three *Clostridium* species: 251 sRNAs (94 trans-encoded, 91 cis-encoded sRNA and 66 riboswitches) in the human pathogen *Clostridium difficile* (Soutourina et al. 2013), 159 sRNAs in *Clostridium acetobutylicum* (Venkataramanan et al. 2013) and 36 sRNAs in *Clostridium ljungdahlii* (Tan et al. 2013).

An interesting example is the regulation of the *ubiGmccBA* operon in *C. acetobutylicum*. This operon is involved in the conversion of methionine and cysteine. Four antisense RNAs with their length varying from 264 nt to 1000 nt are transcribed from the downstream region of the *ubiGmccBA* operon. Their expression is controlled by a S-box riboswitch; these different antisenses interfere with *ubiGmccBA* mRNA by base-pairing (André et al. 2008) (Figure 29).

Another example in the food poisoning pathogen *Clostridium perfringens*, is VR-RNA (VirR-regulated RNA), a sRNA positively regulated by the VirR/VirS TCS (Shimizu et al. 2002; Ohtani et al. 2003). VR-RNA activates many genes such as *pfoA* (theta toxin), *plc* (alpha-toxin), *cpd* (2',3'-cyclic nucleotide phosphodi-esterase), *ptp* (protein tyrosine phosphatase) and *colA* (kappa-toxin or collagenase) and represses the operon *ycgJ-metB-cysK-luxS* which includes *ycgJ* (encoding a hypothetical protein), *metB* (encoding cystathionine gamma-lyase), *cysK* (encoding cysteine synthase), and *luxS* (encoding the autoinducer 2 synthase) (Shimizu et al. 2002). Interestingly, VR-RNA directly binds to the 5'UTR of *colA* mRNA and induces the cleavage of this mRNA. The cleaved mRNA is stable and hence proficient for translation (Obana et al. 2010) (Figure 29).

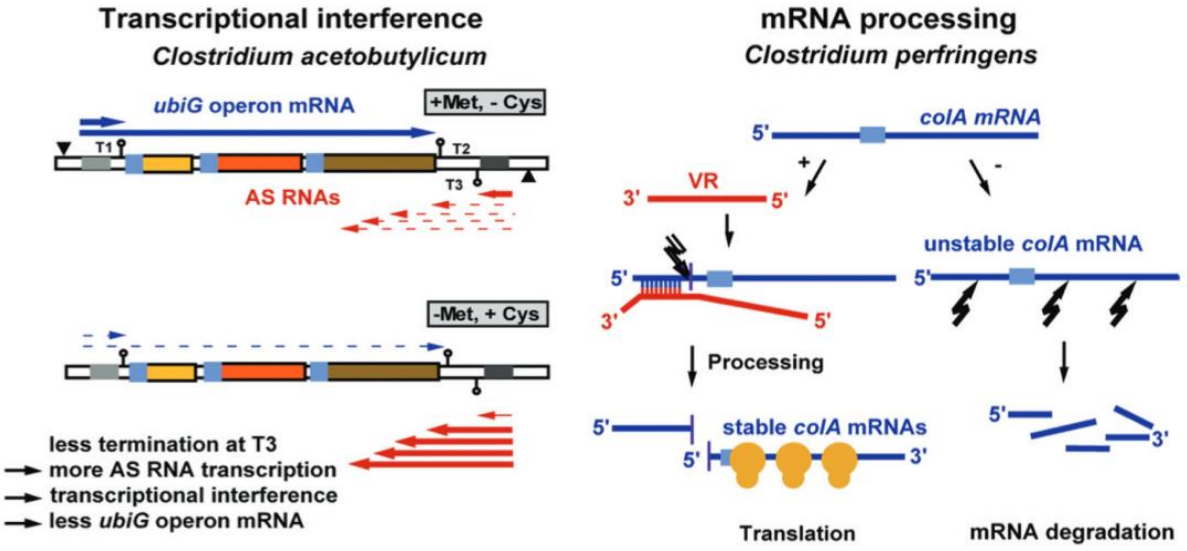


Figure 29: Examples of regulatory RNAs in *Clostridium*. sRNAs are drawn in red, mRNAs in blue. Light blue, ribosome binding sites (RBS). Yellow symbols indicate ribosomes. Black arrows indicate unknown RNase action. [From (Brantl & Bruckner 2014)].

3. sRNA in the pathogenesis of *Listeria monocytogenes*

In *L. monocytogenes,* 113 sRNAs and 70 asRNAs were discovered by performing comparative transcriptomes with its relative non-pathogenic *L. innocua* (Wurtzel et al. 2012). Combine with previous study (Mraheil et al. 2011; Oliver et al. 2009; Toledo-Arana et al. 2009), total 134 sRNAs and 86 asRNAs were found in this bacterium. In Gram-

positive, the interaction of sRNAs and their target seems not to require the RNA chaperone Hfq, but there is an exception: LhrA (Nielsen et al. 2009).

The chitinase ChiA is an enzyme that catalyzes chitin hydrolysis and is also involved in *L. monocytogenes* pathogenesis in a mice model (Chaudhuri et al. 2010). The liver and spleen colonization was significantly reduced with *chiA* mutant as compared to parental strain. LhrA sRNA regulates *chiA* mRNA and two hypothetical mRNAs (*lmo0850* and *lmo0302*) (Nielsen et al. 2009) (Jesper S. Nielsen et al. 2011). LhrA sequester the RBS of *chiA* mRNA and inhibit its translation initiation (Figure 30).

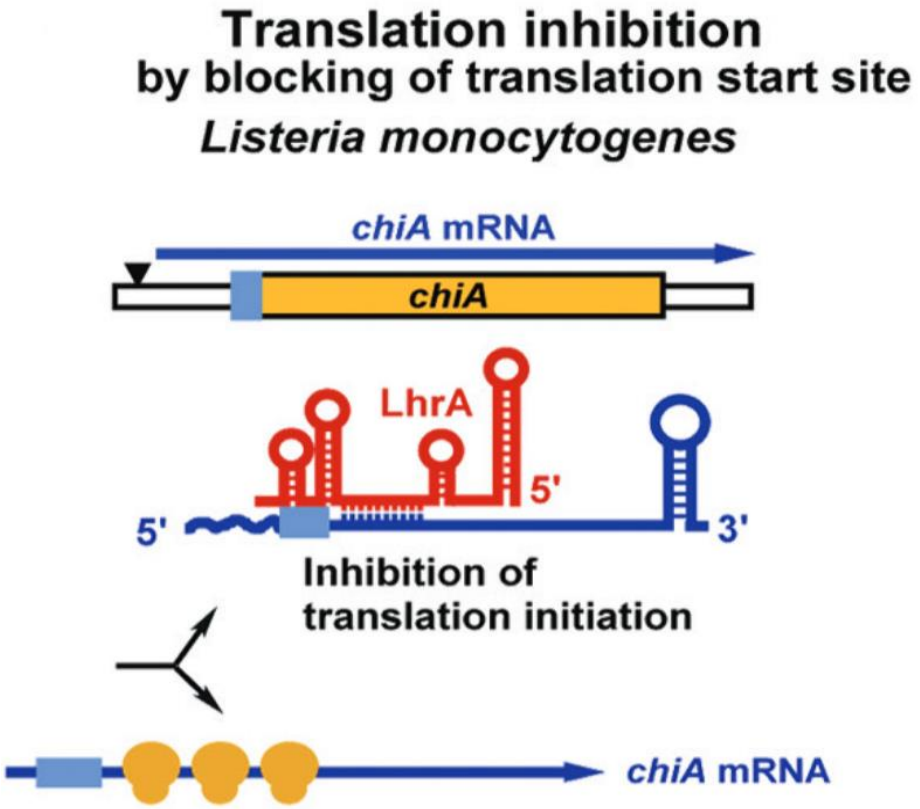


Figure 30: Regulatory mechanism of LhrA and *chiA* mRNA. sRNAs are drawn in red, mRNAs in blue. Light blue, ribosome binding sites (RBS). Yellow symbols indicate ribosomes. Black arrows indicate unknown RNase action. [From (Brantl & Bruckner 2014)].

PrfA is a *L. monocytogenes* regulator of virulence active at 37^{o}C (host human-body temperature) but not at 30^{o}C (Johansson et al. 2002). Its expression is controlled by a RNA thermoswitch located in the 5'UTR of *prfA* mRNA. Moreover, the *prfA* thermoswitch is also linked to an S-adenosyl methionine (SAM) riboswitch element A (SreA) (Loh et al. 2009). When the level of SAM is high (as inside the host), it binds to SreA, resulting in a conformational change of the RNA structure. SreA then acts in *trans*, binding to *prfA* thermoswitch and repressing its translation. Therefore, the expression of PrfA is tightly controlled during infection process due the combined activities of *prfA* thermoswitch and SAM-metabolite riboswitch integrating the temperature and the presence of SAM, respectively (Figure 31).

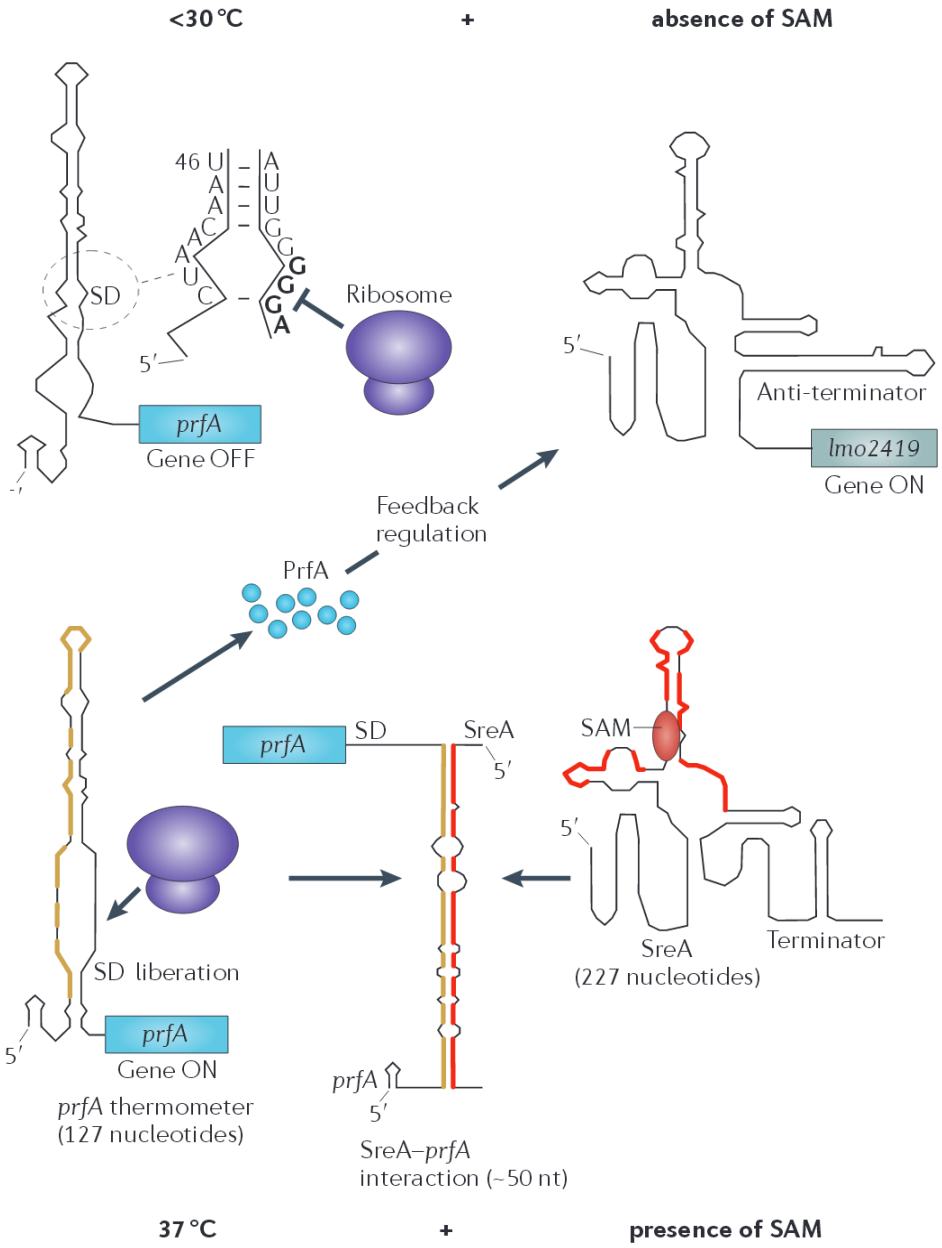


Figure 31: Interplay between a metabolite-sensing riboswitch and a temperature-sensing RNA thermometer. Translation initiation of PrfA-encoding mRNA of *Listeria monocyogenes* at low temperatures (<30°C) is hindered by a secondary structure that masks the Shine–Dalgarno (SD) site (bold). At host body temperature (37°C), the secondary structure is partially disrupted, enabling docking of the ribosome and translation initiation. After translation of the *prfA* mRNA, PrfA activates the transcription of virulence genes that are important for host infection. The truncated SAM riboswitch element A (SreA), which is encoded upstream of *lmo2419* and expressed only when levels of *S*-adenosyl methionine (SAM) are high (such as when the bacterium is inside the host), can function in *trans* by binding to the *prfA* RNA thermometer. The interaction between SreA and the *prfA* mRNA leads to diminished expression of PrfA. The exact mechanism by which this interaction hampers ribosome binding remains to be elucidated. SreA and *prfA* interaction sites are depicted in red and yellow. [From (Kortmann & Narberhaus 2012)].

4. sRNA in *Bacillus subtilis*

FsrA was the first characterized sRNA in *B. subtilis* (Gaballa et al. 2008). FsrA is homologous to RyhB in *E. coli* which is involved in iron metabolism and storage. FsrA is a global regulator controlling genes related to iron such as *sdhCAB* (succinate dehydrogenase) and *citB* (aconitase). However, unlike RyhB in *E. coli*, FsrA does not require the RNA chaperone Hfq. On the other hand, FsrA requires the Fur-regulated small proteins FbpA, FbpB and FbpC. At low iron concentration, FsrA represses the expression of *lutABC* operon (encoding iron sulfur cluster-containing enzymes) by binding to *lutA* mRNA translation initiation region. The interaction FsrA/lutABC is facilitated by FbpB. FbpB not only facilitates the interaction but also recruits the RNA degradosome.

B. subtilis has also a dual-function sRNA named SR1. SR1 is only expressed in gluconeogenic conditions. In glycolytic conditions, SR1 is repressed by a catabolite control protein A (CcpA) and a control catabolite protein of gluconeogenic genes (CcpN). The first function of SR1 is to base-pair with the transcriptional activator *ahrC* mRNA. The interaction involves 7 complementary regions (~100 nt downstream from RBS of *ahrC* mRNA), hence remodeling the mRNA to inhibit the translation initiation. AhrC is the transcriptional activator of two arginine catabolism operons RocABC and RocDEF. Consequently, SR1 indirectly controls these two operons. The second function of SR1 is associated with a 39 amino-acid peptide, called SR1P, encoded by SR1, that interacts with *gapA* mRNA to stabilize it by an unknown mechanism (Figure 32).

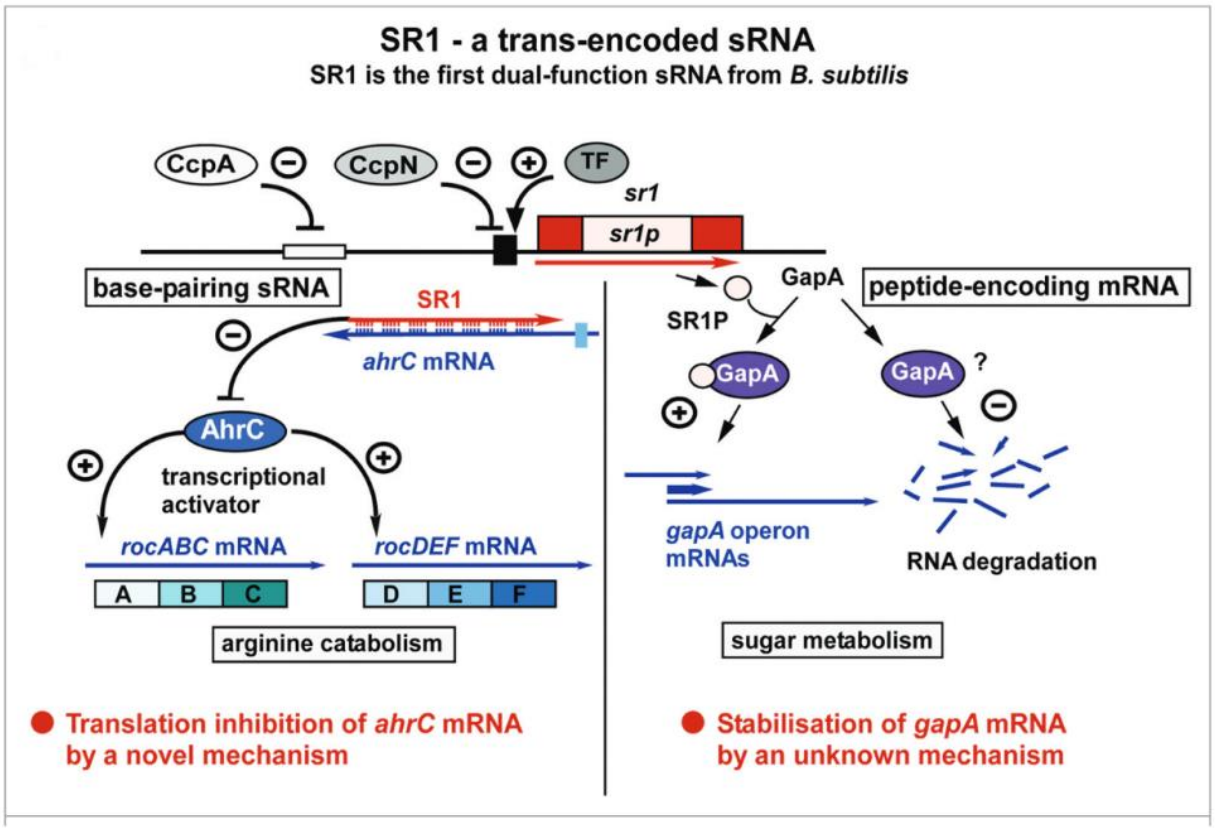


Figure 32: SR1, a *B. subtilis* trans-encoded sRNA with dual-function. +, activation; -, repression. CcpA and CcpN repress *sr1* transcription under glycolytic conditions. TF is a novel transcription factor that activates *sr1* transcription at cold-shock. The antisense RNAs are indicated in red, the sense RNAs in blue, RBS in light blue. [From (Brantl & Brückner 2014)].

5. sRNAs in the pathogenesis of *Enterococcus faecalis*

E. faecalis is an opportunistic human pathogen that belongs to the *Enterococci* family. This bacterium is hospital-acquired and multi-drug resistant. More than 100 sRNAs were identified in *E. faecalis* through various approaches such as bioinformatic prediction, 5' and 3' RACE mapping, microarray and northern blot (Livny et al. 2008; Fouquier D'Hérouel et al. 2011; Shioya et al. 2011; Innocenti et al. 2015). Among them, *ef0408-0409* sRNA is homologous to RNAII which is a component of a type I component TA system; its deletion increased virulence, and organ colonization in a mouse model, and survived better in macrophage. In addition, mutant *ef0408-0409* grew better in oxidative and osmotic stress conditions and was more resistant to acid. In contrast, three other mutants

(*ef0605-00606*, *ef1368-1369* and *ef3314-3315*) were less virulent than the wild-type strain (Michaux et al. 2014). This study showed a connection between stress resistance and pathogenicity.

Recently, a new model came out revealing a riboswitch-regulated sRNA that controls gene expression by sequestering a response regulator in *E. faecalis* (DebRoy et al. 2014) (Figure 33). This original regulation was also observed in *L. monocytogenes* (Mellin et al. 2014). Briefly, this system regulates ethanolamine (EA) metabolism as a source of carbon and nitrogen. Many genes involved in ethanolamine utilization (*eut*) are located on the same locus and expressed only when both ethanolamine and vitamin B12 are present.

E. faecalis responds to the presence of EA via the EutW/EutV TCS. In the presence of EA, the response regulator EutV is phosphorylated by the EutW histidine kinase (Baker & Perego 2011). This active EutV forms dimers and binds RNA hairpins expressed from the *eut* locus. Active EutV acts as an "anti-terminator". However, the transcription of the *eut* operon is still turned off when vitamin B12 is not present because EutV is sequestered by a riboswitch containing a noncoding RNA. When vitamin B12 is present, it binds to the riboswitch and changes its structure hence forming a terminator. Thus, the transcription of the noncoding RNA stops freeing the protein EutV. Therefore, EutV promotes the expression of the *eut* operon only when both ethanolamine and vitamin B12 are present (Figure 33).

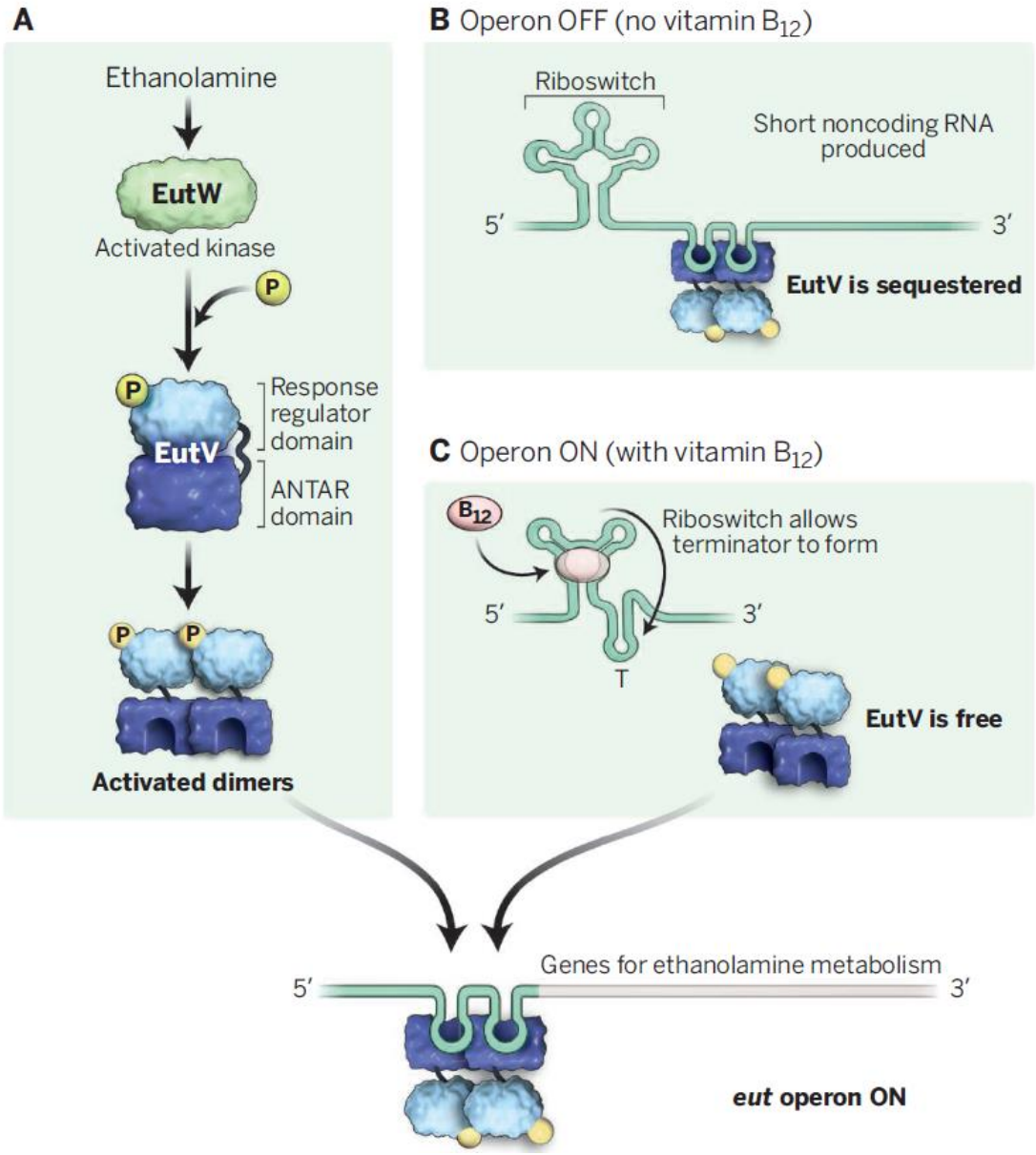


Figure 33: Riboswitch-based regulation. The *eut* operon contains genes involved in ethanolamine metabolism in bacteria. (A) EutV is phosphorylated and activated by EutW in response to ethanolamine. Active EutV forms a dimer that binds to adjacent RNA hairpins in the target transcript. Binding at these sites leads to antitermination; thus, transcription of the operon is turned ON, but this can only happen in the presence of vitamin B12. (B) In the absence of vitamin B12, a noncoding RNA is generated that sequesters EutV; thus, transcription of the operon is turned OFF. (C) In the presence of vitamin B12, the riboswitch blocks transcription of the noncoding RNA through a structural change that produces a terminating hairpin RNA (T). Active EutV protein is then free to bind to the target transcript and promote expression of ethanolamine metabolic genes. [From (Chen & Gottesman 2014)].

AIM OF THE THESIS

In *S. aureus*, about 200 regulatory RNAs (sRNAs) were identified but up to date, their functions in most cases are unknown.

1) The main goal to this thesis was to setup a methodology to determine phenotypes associated with *S. aureus* sRNAs genes on a large scale. A strategy was developed to evaluate the adaptative ability of a collection of sRNA gene mutants to various tested environmental conditions by performing competitive fitness experiments. 14 mutants with a chromosomal tagged deletion among 39 tested were either accumulating or disappearing in the 13 tested conditions. The observed phenotypes in these mutants are indications to help to determine the functions associated with these sRNAs (Chapter A).

2) An important step to determine the sRNA functions is the identification of sRNA targets. sRNAs usually associate by base-pairing with targeted mRNAs to affect their expression. Several computational methods propose lists of putative sRNA targets based on the identification of sRNA/RNA pairing regions. However, the pairing rules are not fully understood and these methods generate numerous false positive candidates and sometimes do not retain true positive targets. Therefore, we developed a robust procedure to identify reliably sRNA targets based on synthetic sRNAs that were used *in vitro* as bait to trap their corresponding targets which were subsequently identified by deep sequencing. The second chapter of this thesis reported the method and its application to four staphylococcal sRNAs RsaA, RsaE, RsaH and RNAIII (Chapter B).

The binding of sRNAs to their targets mRNAs usually affect their stability by recruiting RNase(s). In *S. aureus,* RNase III encoded by *rnc* gene is a major RNase involved in the degradation of sRNA-mRNA duplexes. RNase III was reported as nonessential in *S. aureus.* In chapter C, we report that the *rnc* gene is essential in strains containing prophages carrying type I toxin/antitoxin systems.

CHAPTER A1

Competition experiments with regulatory RNA gene mutants in *Staphylococcus aureus*: identification of a 3'UTR contributing to optimal growth at low-temperatures

Competition experiments with regulatory RNA gene mutants in *Staphylococcus aureus*: identification of a 3'UTR contributing to optimal growth at low-temperatures

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Keywords: *Staphylococcus aureus*, fitness, sRNA, phenotype

Running title: sRNA phenotypes in *S. aureus*

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Abstract

Bacterial gene expression is widely linked and controlled by growth conditions. Its tight regulation contributes to optimize bacterial fitness to environment. Many factors contribute to growth adaptions via transcriptional and post-transcriptional regulations and sigma factors, regulatory proteins and regulatory RNAs are key players. The identification of phenotypes associated with gene deletions is a classical method to find gene functions but may require testing many conditions for each studied mutant. Regulatory RNAs often contribute to fine-tune gene expression and phenotypes associated with their inactivation are often weak and difficult to detect. Nevertheless, minor phenotypes conferring modest advantages, may emerge as dominant traits after a few generations under selective pressure. Gene replacements with DNA barcode sequences allow monitoring many mutants simultaneously and detect weak phenotypes via fitness experiments. We adapted this strategy to deep sequencing and apply it to study regulatory RNAs in *Staphylococcus aureus*, a harmful animal, including human, pathogen. We constructed 39 Staphylococcal tagged-deletion mutants and tested their accumulation in competition experiments at different temperatures. Three and five tagged-deletion mutants were significantly underrepresented at 42°C and 20°C, respectively. One of the mutations, *rsaOV*, generated a strong growth defect at 20°C but not at 37°C or 42°C. Complementation and transcriptome studies indicate that *rsaOV* is within the *cwrA* 3'UTR and that the cold sensibility is directly associated to *cwrA* expression.

Introduction

Staphylococcus aureus is a major human and animal opportunistic pathogen. It causes syndromes ranging in severity from minor skin infections to life threatening diseases such as infective endocarditis and necrotizing pneumonia (Lowy 1998). The bacterium proliferation and pathogenicity are due to rapid adaptations to environmental conditions and controlled expression of virulence factors (Arvidson and Tegmark 2001; Bronner et al. 2004; Cheung et al. 2004). Numerous elements orchestrate the adaptive regulatory networks. Among them, sigma factors, regulatory proteins are contributing to transcriptional regulations, and a second line of control is posttranscriptional in which regulatory RNAs are essential contributors (Felden et al. 2011; Rochat et al. 2013; Bonnin and Bouloc 2015).

Bacterial regulatory RNAs are divided in two categories, cis- and trans-acting: the first one exerts its regulatory activity on associated or interdependent adjacent RNA sequences, while the second one base-pairs with independent RNAs or bind to proteins. Trans-acting RNAs targeting RNAs i) that are expressed from a complementary strand of another RNA (usually an mRNA) are called asRNAs (for antisense RNAs) (Georg and Hess 2011) and ii) those expressed from DNA sequence with no transcript on the complementary strand are usually referred to as sRNAs (for small RNAs) (Storz et al. 2011; Guillet et al. 2013). Bacterial sRNAs are often between 50 to 300 nucleotides, non-coding, conditionally expressed (*e.g.*, upon specific growth stresses or growth phases) and their association by base-pairing to mRNA targets affects the stability and/or translation of the target.

In *S. aureus*, RNAIII is the paradigm for a growing number of sRNAs associated with virulence (Novick and Geisinger 2008). Induced at high cell density by a quorum sensing regulation, RNAIII modulates the expression of numerous genes contributing to

staphylococcal virulence. However, *S. aureus* has hundreds of regulatory RNAs for which the function and mechanism is mostly unknown (Felden et al. 2011). As sRNAs often contribute to the "fine-tuning" of gene expression, their associated phenotypes are difficult to determine. For example, no obvious growth phenotype was found for the absence of RsaE in *S. aureus*, a widely conserved sRNA in bacteria which down-regulates the Krebs cycle and folate metabolism (Geissmann et al. 2009; Bohn et al. 2010). However, minor sRNA-mediated phenotypes conferring modest advantages may nevertheless affect bacterial fitness and emerge as dominant traits after a few generations under selective pressure.

Finding phenotypes associated with sRNA gene deletions usually require testing of many conditions for each mutant, with no assurance of success. To tackle this problem for *S. aureus* sRNAs, we used an alternative method based on the detection of barcoded deletions which was developed in yeast (Shoemaker et al. 1996) and also apply to bacteria (Mazurkiewicz et al. 2006; Hobbs et al. 2010; Hobbs and Storz 2012). We adapted the protocol to deep sequencing technology. We tested three growth conditions in triplicate with 39 tagged *S. aureus* sRNA mutants. We identified nine sRNA deletion mutants that resulted in the accumulation or disappearance of strains carrying them in at least one of the tested conditions. The strategy develop will be instrumental to identify sRNA-dependent phenotype and to find their functions.

Results and Discussion

Identification of mutants within a population having altered growths: general principal.

The goal is to the follow the amount of each mutant within a mutant collection growing under different conditions. Comparison to a reference condition allows to identify mutations conferring selective advantages or disadvantages with respect to the tested growth conditions. Each mutant is recognized and counted thanks to specific DNA tag sequences. The first step for this method was to obtain a large collection of tagged mutants in the background of interest. Then, mutant sets were grown in different conditions, the genomic DNA from these mixed populations were extracted, tags were PCR-amplified and the proportion of each specific tag was determined (Figure 1).

Construction of a set of tagged sRNA-deletion genes in *S. aureus*.

Tagged deletions were constructed in HG003, a NCTC8325 derivative in which *rsbU* and *tcaR* mutations were repaired and that is used as a model strain for staphylococcal regulation studies (Herbert et al. 2010). Loci replacements were performed by two-step homologous recombinations with integration and excision at targeted loci of a conditionally replicative plasmid containing the sequence the desired sequence. We used pMAD2 (Bonnin et al., unpublished results), a replication thermo-sensitive plasmid derived from pMAD (Arnaud et al. 2004). In order to follow all mutated strains within a population of different mutants, each deleted region was replaced by a tag sequence containing a specific 40-mer DNA sequence of each deletion (Shoemaker et al. 1996). The 40-mer, which is a DNA barcode identifier, is flanked on both sides by 26-mer sequences that are identical for all tags (Figure 1 and S4). The 26-mer sequences allow PCR amplifications of each barcode sequences with the same two primers. We generated the DNA barcode

sequences as followed. A DNA oligonucleotide made of the forward priming 26-mer region, a 40-mer random sequence and a reverse priming 26-mer region was PCR amplified, cloned into a plasmid and transformed into E. coli. The inserted sequences of about hundred transformants were characterized by DNA sequencing and those with adequate inserts were used to mark the deletions (Table S4). Efficient recombination of pMAD2 derivatives in *S. aureus* requires homologous fragment of about 1 kb. We therefore cloned into pMAD2, i) DNA tags (generated as described above) flanked on both sides ii) by about 1kb sequences of the surrounding deleted regions. The pMAD2 derivatives with confirmed expected inserts (by DNA sequencing) were transformed into RN4220, extracted and transformed into HG003 to perform allelic replacements as described (Arnaud et al. 2004). All gene substitutions constructed were with non-antibiotic marker remaining (Material and Methods).

sRNA genes selected for disruption were chosen based on data available when the project started (*e.g.* (Felden et al. 2011)). We retained those corresponding to apparent *bona fide* sRNAs. Deleted regions comprised, when information were available, promoter regions and full-length of sRNA genes. As i) the limits of many sRNA genes were unknown, ii) constructed deletions depends on the cloning of flanking regions and iii) *S. aureus* is 32% GC with long AT rich regions, some constructed deletions were either longer or shorter than the sRNA genes (Table S3). Recent deep sequencing data indicate that some RNA sequences retained for our study and initially considered as sRNA are 5' or 3' UTR of mRNAs. Altogether, 39 strains, each one containing a different tagged deletion, were constructed (Table S1)

Competition experiments

Each mutant strain was grown individually in a rich medium and assembled together in the same amount (normalized to OD_{600}) to generate a starting culture of a tag deletion set. The

procedure was repeated three times to generate three independent sets. Competition experiments were performed by diluting 1000 times the three tag deletion sets into a fresh culture medium and growing them in the tested conditions. A first sampling was performed during exponential phase when cultures reached OD_{600} 0.6 to 0.7 (Sampling 1). The remaining cultures left to grow and the day after, the overnight cultures were diluted 1000 times into a fresh culture medium and grown again in the same tested conditions. A second sampling was performed when the cultures reached OD_{600} 0.6 to 0.7 (Sampling 2). As many sRNA genes are expressed during stationary phases, Sampling 2 should allow the detection of phenotypes with sRNAs expressed preferentially at high density. In addition, phenotypes detected in Sampling 1 may be more pronounced in Sampling 2. In order to evaluate the proportion of each mutant within a mix population, the proportion of each DNA barcode were evaluated. 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 (Mazurkiewicz et al. 2006). These experiments are tedious and expensive, as each tested 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 banks as tested conditions increasing significantly the cost of the method. We therefore adapted the protocol as follow. PCR products of each experiment were obtained with two primers having 5'-extensions of 5 nucleotides; these "experiment identifiers" were specific of each sample counted (Table S5). The same quantity of PCR products from different conditions were mixed together. In a pilot experiment, a DNA-seq bank 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 each experiment differ only by their 5 terminal nucleotides, the denaturation steps and PCR-amplification during DNA-seq bank constructions lead to illegitimate pairing of identical barcodes coming from different experiments and artefactual results (data not shown). We solved this technical issue by removing the amplification step from the standard DNA-seq bank construction protocol. The resulting protocol adapted to deep sequencing technology is time saving, increases the response linearity, and cheaper if several conditions are pooled, as compared to the array technology.

As prove of principle, two growth conditions were tested, 42°C and 20°C. Barcode sequences from Sampling 1 and 2 in both conditions were identified and counted for the three assemble sets of sRNA-tagged deletions. Barcode sequences were also counted for samples grown at 37°C. The frequency of each mutant within the remaining population of Samplings at 42°C and 20°C were determined and normalize to the 37°C conditions. Consequently the data at high and low temperature are relative to 37°C. A standard deviation for each barcode tag frequency was determined from the results of triplicates. Mutants were considered either accumulate or disappear in the tested condition when a significant five-fold difference was observed with the reference condition.

At 42°C, in Sampling 1, Δ*sau30*, Δ*sau6836* and Δ*rsaH* were significantly underrepresented (Figure 2A) while in Sampling 2, only Δ*sau6428* was found significantly underrepresented (Figure 2B). In Sampling 2, Δ*sau30*, Δ*sau6836* and Δ*rsaH* mutants are still strongly underrepresented when the results from the 3 sets are average, however, with an unacceptable standard deviation.

At 20°C in Sampling 1, Δ*rsaOV* mutant was the most underrepresented followed by Δ*sau60* (Figure 3A). In Sampling 2, five mutants were found significantly

underrepresented, Δ*rsaOV*, Δ*rsaD*, Δ*teg49*, Δ*sau60* and Δ*sau6528*, with Δ*rsaOV* still the most underrepresented (Figure 3B).

Mutants with significant growth disadvantage at 42°C or 20°C revealed by these competition experiments were grown individually either at low or high temperature and compared to the parental strain.

In individual culture, the *sau30* deletion led to a growth defect at 42°C (Figure 4A) which was not present at 37°C (Figure 4B). The Sau30 sRNA (alias SSR154) was reported in two studies (Anderson et al. 2006; Abu-Qatouseh et al. 2010). The gene is located between SAOUHSC_02483 (*cbiO* encoding the subunit of a putative cobalt transporter) and SAOUHSC_02484 (*rplQ* encoding the 50S ribosomal protein L17). However, deep-sequencing transcriptome data indicates that Sau30 is possibly a 3'UTR part of *rplQ* mRNA rather that a sRNA *per se* (Figure S1). The *sau30* deletion constructed encompasses a large region that could affect *cbiO* gene. In individual cultures, no growth difference were observed between Δ*sau6836* Δ*rsaH* and the parental strain at 42°C, possibly because i) the growth differences are small and cannot be revealed by simple growth curves or ii) the growth defect may depends on the presence of other strains.

The Δ*rsaOV*, Δ*rsaD*, Δ*teg49*, Δ*sau60* and Δ*sau6528* mutants had significant slower growth rate than the parental strain in individual culture at low temperature (Figure 5A) in contrast to 37°C (Figure 5B). As the growth defect was much more pronounced for the *rsa*OV, this mutant was retained for further studies.

The 3'UTR of *cwrA* is required for optimal growth at low temperature

The RsaOV sRNA (alias Sau40) was reported in two studies (Abu-Qatouseh et al. 2010; Bohn et al. 2010). Its corresponding gene is located between SAOUHSC_02872 (*cwrA*) and SAOUHSC_02873 (encoding the subunit of a putative copper transporter).

We first try to complement unsuccessfully the *rsaOV* growth defect by a plasmid carrying the putative *rsaOV* gene (Figure 6). As for Sau30, deep-sequencing transcriptome data indicates that RsaOV may not be a sRNA *per* se, but more likely the 3'UTR end of the *cwrA* mRNA (Figure S2). These observations raised the possibility that the *cwrA* 3'UTR would be required for an efficient *cwrA* expression. Several plasmids were constructed to identify the required sequence to compensate the Δ*rsaOV* cold deficiency (Figure 7). Plasmids carrying the *cwrA* promoter i) and the *cwrA* open reading frame (without *rsaOV*), or ii) rsaOV (without *cwrA*) did not rescue the Δ*rsaOV* mutation. However, the plasmid carrying the complete region (*cwrA* promoter + *cwrA* open reading frame + *rsaOV*) complemented the Δ*rsaOV* growth defect a low temperature (Figure 6). To support the hypothesis that the deletion was rescued by the expression of *cwrA*, the *cwrA* initiation codon (ATG) of the complementing plasmid was mutated to a stop codon (TAA) (Figure 7); the resulting plasmid lost its ability to complement the Δ*rsaOV* cold deficiency (Figure 6). We concluded that *rsaOV* may be required to prevent the *cwrA* mRNA degradation and/or to stimulate its translation.

The *cwrA* gene is so far poorly characterized. It encodes a putative 63 amino-acid protein of unknown function. The combined analysis of several transcriptome studies of *S. aureus* treated with antibiotics targeting the cell wall revealed 15 genes always upregulated and 2 downregulated (Kuroda et al. 2003; Utaida et al. 2003; McAleese et al. 2006; McCallum et al. 2006) belonging the cell wall stimulon (Utaida et al. 2003). Among them, *cwrA* is

strongly upregulated. Its expression was studied with transcriptional gene fusions between *cwrA* and a bacterial *lux* cassette (Balibar et al. 2010). These experiments showed that *cwrA* expression was induced by cell wall-targeting antibiotics (*e.g.* vancomycin, oxacillin, penicillin) but not by antibiotics with other targets. However, because these results are obtained with gene fusions, they cannot take in account a possible regulatory role of the 3' UTR.

Material and methods

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Tables S1 and S2, respectively.

Allelic replacements of *S. aureus* genes were performed using pMAD2 derivatives. pMAD2 (GenBank accession number: KT323982) is a shuttle vector derived from pMAD (Arnaud et al. 2004) with a thermosensitive replication origin in *S. aureus*. pMAD2 is available for scientific community as Addgene plasmid N° 67682 (https://www.addgene.org/). Complementation studies were performed using derivatives from pCN38, a cloning shuttle vector (Charpentier et al. 2004). The tag library use to mark the sRNA gene deletions was cloned into pJET (GenBank/EMBL Accession number EF694056).

Staphylococcus aureus strains RN4220 and HG003 (Herbert et al. 2010) were routinely grown with aeration at 28°C, 37°C and 42°C in BHI broth or BHI agar. *Escherichia coli* DH5αZ1 was grown in LB broth or LB agar. When appropriate, the following antibiotics were used: erythromycin (0.5µg/ml), chloramphenicol (5µg/ml) for *S. aureus* strains and ampicillin (100µg/ml), chloramphenicol (20µg/ml) for *E. coli* DH5α.

DNA manipulation

Plasmids were extracted using NucleoSpin Plasmid Quick Pure kit (Macherey-Nagel, Düren, Germany), following manufacturer protocol with an additional step for *S. aureus*: cells were incubated for 1 h at 37°C with the lysis solution containing lysostaphin (10 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA). PCR amplifications were performed using high fidelity Phusion DNA Polymerase (Thermo Scientific Finnzymes) for cloning or Taq Polymerase (Fermentas) for verification following supplier's recommendations. DNA

concentration was measured using Nanodrop Spectrophotometer ND-1000 (Peqlab, Erlangen, Germany). For transformation, we use chemically-competent cells for *E. coli,* and electro-competent cells for *S. aureus*. As pMAD2 derivatives carry the *bgal* gene encoding ß-galactosidase, their presence in *S. aureus* was visualized by formation blue colonies on BHI plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Generation of DNA Tag (barcodes) library

To mark specifically each constructed gene deletion, a library of DNA tags was generated. The oligonucleotide "tag_random" containing a 40-mer random sequence between two non-random 26 nucleotide long regions was PCR-amplified using primers tag_F and tag_R (See Table S3, Supplementary data). PCR products were cloned into pJET plasmid using CloneJET PCR Cloning Kit following manufacturer instructions (Thermo Scientific). Ligation products were transformed into DH5αZ1, plasmids were extracted from about 100 clones and the inserts were identified by DNA sequencing of the inserts. The sequence of the retained tags is listed in Table S4.

Construction of tagged sRNA deletion in S. aureus HG003

Locus replacements in HG003 were performed by a two-step homologous recombination with integration and excision of conditionally replicative pMAD2 derivatives at targeted loci. pMAD2 derivatives contained sequences (about 1 kb) of upstream and downstream flanking regions of the deleted locus and in between these two regions, a given tag sequence.

HG003 *rsaA* tagged deletion was constructed as followed. The upstream and downstream rsaA sequences were PCR-amplified from HG003 chromosomal DNA using primers RsaA-

UpF/RsaA-UpR and RsaA-DwF/ RsaA-DwR, respectively (for primers, see Table S3). The tag1 sequence was PCR-amplified from pJET-tag1 (for tag sequences, see Table S4). pMAD2 was PCR-amplified using pMAD-F/pMAD-R primers. pDErsaA::tag1 (for DEletion of *rsaA* substituted by tag1) was obtained by assembling the four PCR fragments as described (Gibson et al. 2009). The assembled mix was used to transform *E. coli* DHαZ1. Usually, two plasmids isolated from DH5αZ1 were verified by DNA sequencing of inserts. As HG003 is not permissive for plasmids originating from *E. coli*, a confirmed plasmid was used to transform RN4220, extracted from RN4220 and transferred into HG003. Allelic chromosome/plasmid exchanges leading to chromosomal loci substitution by tag sequences were performed in HG003 as described (Arnaud et al. 2004) and verified by PCR tests. All forty tagged deletion mutants (Table S1) were constructed in the same way using the appropriate pMAD2 derivatives (for primers use to plasmid constructions, see Table S3).

Fitness assays/Competition assays/Bacterial competition assays

Each sRNA tag-mutant was inoculated separately in BHI broth and grown for 16h at 37^{o}C with aeration. Overnight cultures were diluted 1:1000 in fresh BHI broth and grown to OD_{600} ~ 0.6 -0.7. The forty cultures of mutated strains were pooled together with the same quantity for each one and stored in aliquot at -80^{o}C. Three sets of aliquots were assembled, with each sRNA tag-mutant culture originating from three independent clones. The three sets were used to perform each experiment in triplicate.

The tagged mutant sets were used to test the fitness of each mutation in various conditions as follow. For a given set, one aliquot was keep (time 0), one was grown in standard condition (BHI broth, 37^{o}C with aeration) and the others aliquots were grown in various stress conditions. Except time 0, aliquots for competition assays were diluted

1:1000. Sixteen competition conditions were tested and analyzed. Each experimental condition was performed in triplicate using the three independent sets. To identify sRNAs that would affect *S. aureus* growth in cold or warm conditions, each triplicate sets were grown at 20°C or 42°C under aeration. Bacteria cultures were sampled for each competition assay at OD~0.6. The remaining cultures were grown overnight, diluted 1:1000 the day after in the same medium, grown in the same condition and samples were harvested at OD~0.6.

DNA-seq library, high-throughput sequencing and data analysis

Chromosomal DNA was extracted from each culture as described above and tagged sequences were amplified. To construct only one deep sequencing Illumina bank with many samples and to be able to discriminate each experiment, we use specific primers for each experiment differing in the sequence of their last five 5' nucleotides (Table S5). The primer remaining part contained the conserved region for amplification of all tags.

Sequencing bank on PCR amplified DNA fragments for Illumina sequencing were constructed following manufacturer instruction. They were paired-end sequenced (2x 50 nt) by the CNRS IMAGIF platform (Gif-sur-Yvette, France).

Sequencing data was analyzed by using tools given by bioinformatics plateform MIGALE (Jouy-en-Josas, France). Briefly, two paired-end libraries were combined and each experiment/competition assay was separated by splitting 5-nucleotide-experiment-specific barcodes/primers at both ends. In each sample, the numbers of Tag sequences were verified corresponding to all mutant strains. We performed normalization by comparing with control samples and finally did statistical analysis for 3 biological replicates.

Growth curves

Growth curves were done in triplicate in 96-Well multiwell plates covered with a semipermeable film (4titude, Bagneux France) under constant vigorous shaking using the microplate reader CLARIOstar (BMG Labtech, Ortenberg, Germany). Overnight culture of *S. aureus* strains were diluted at 1:1000 in 200 µl of BHI medium with antibiotic when necessary. The growth curve was measured by OD_{600nm} every 15 minutes for 18 hours at either 25°C, 37°C or 42°C.

Disclosure of Potential Conflicts of Interest

None to declare.

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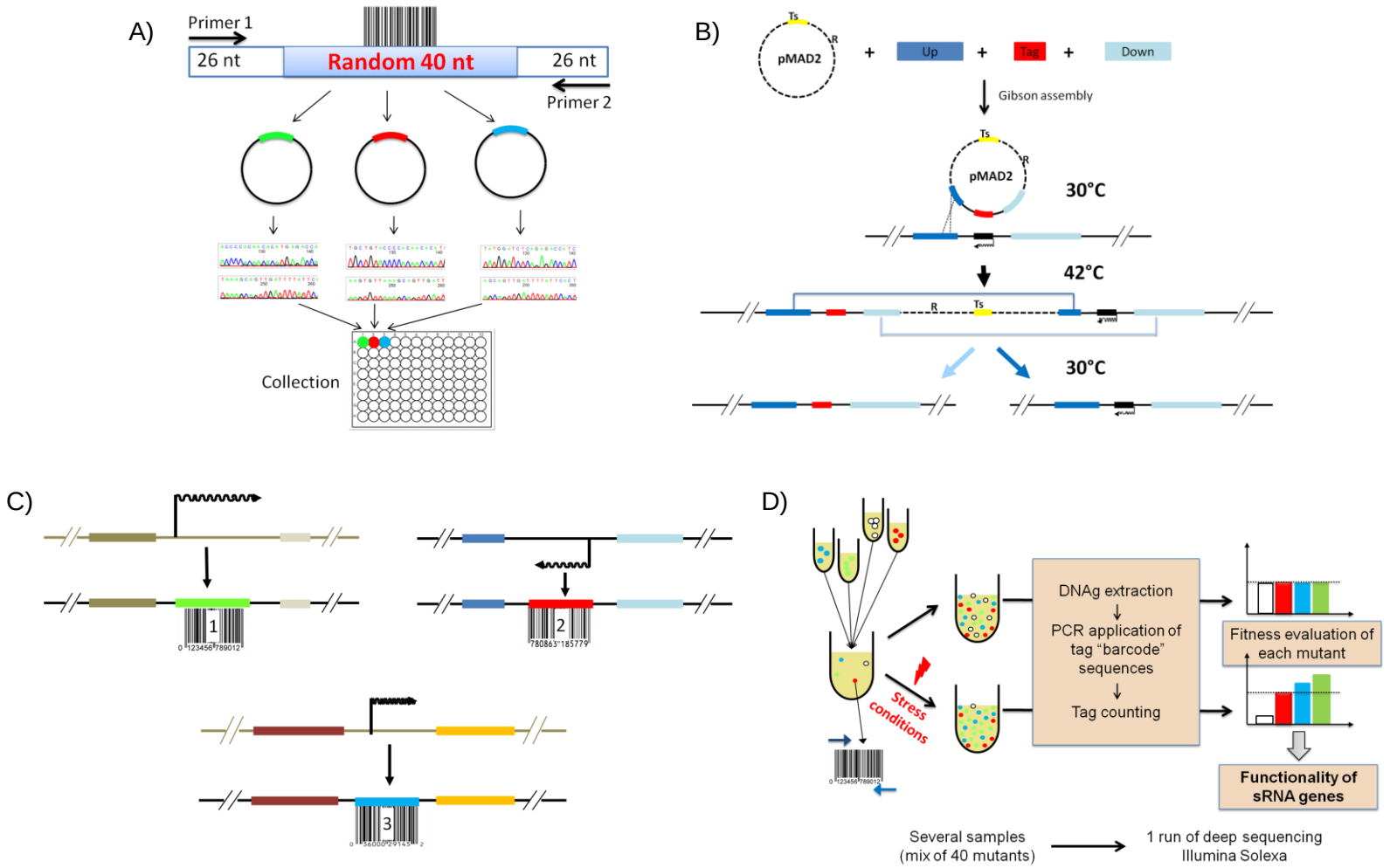
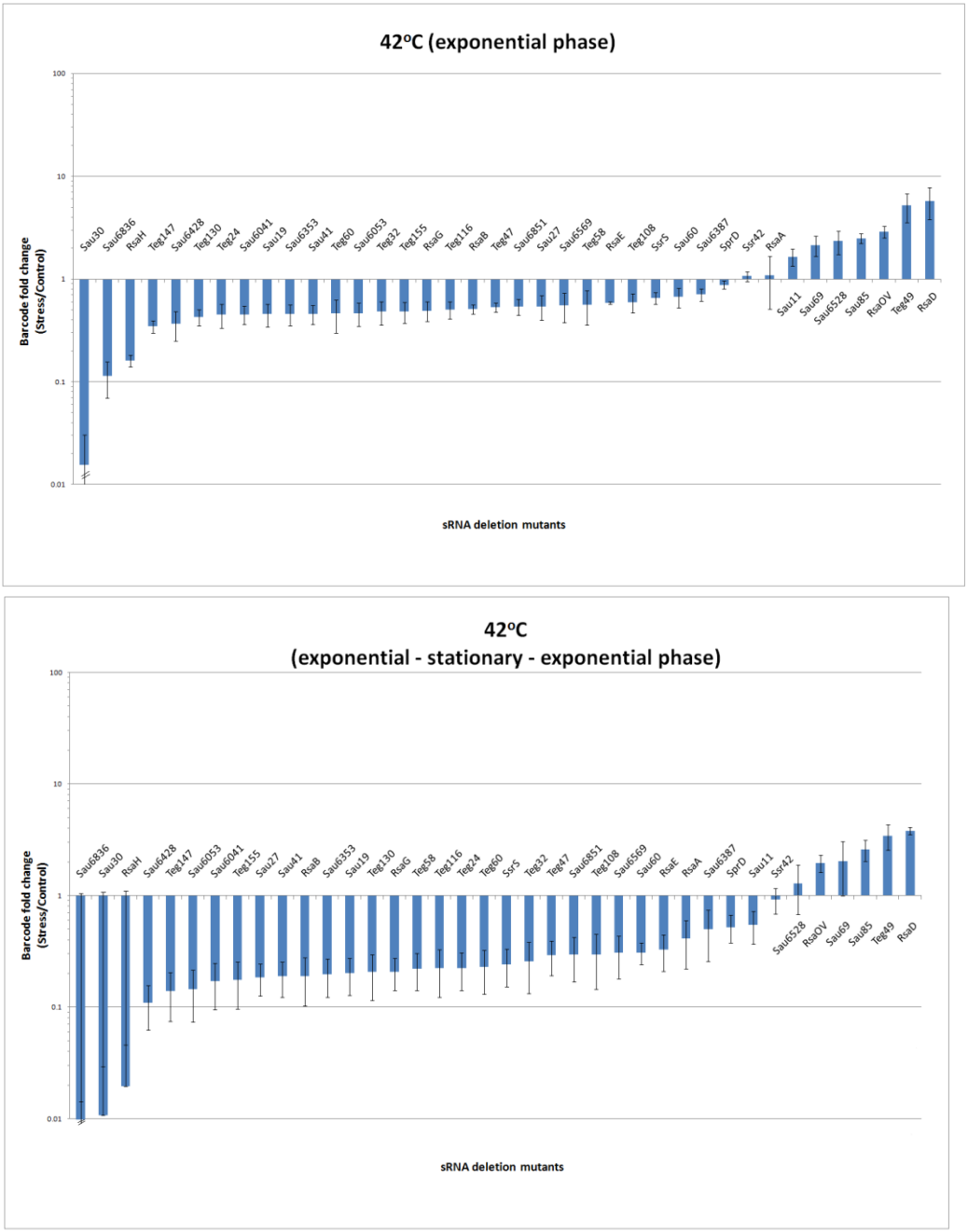
Figure 1



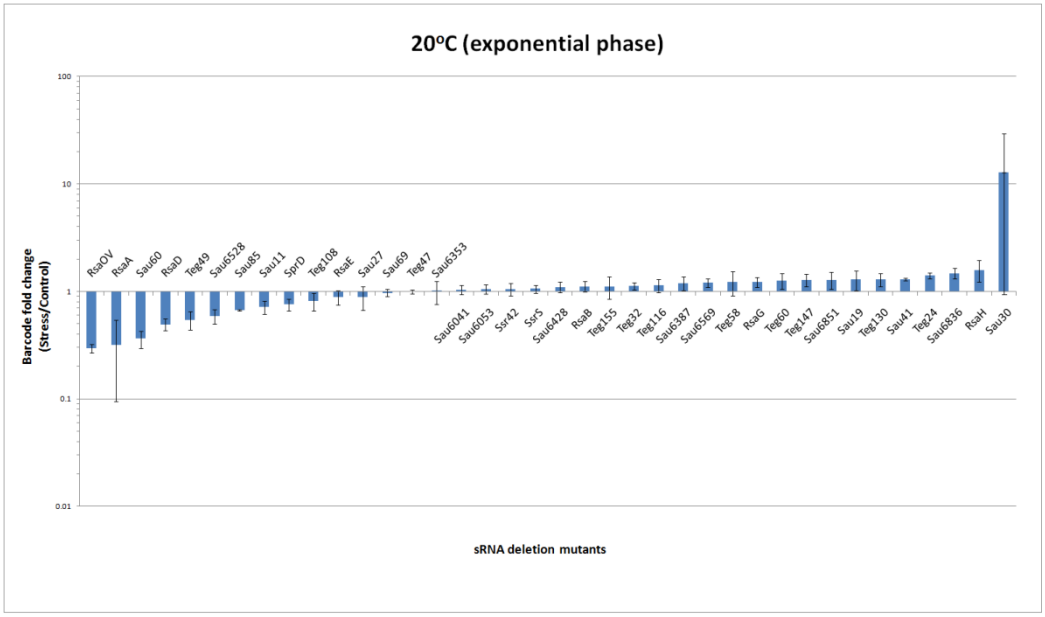
Fitness experiments with barcoded deletion mutants in *S. aureus*. A) Construction of a DNA Tag barcode library. B) Schematic view of gene inactivation by using pMAD2. C) Representation of the mix of sRNA-deleted mutant strains. Each mutant carries a specific Tag barcode. D) Protocol of fitness experiments.

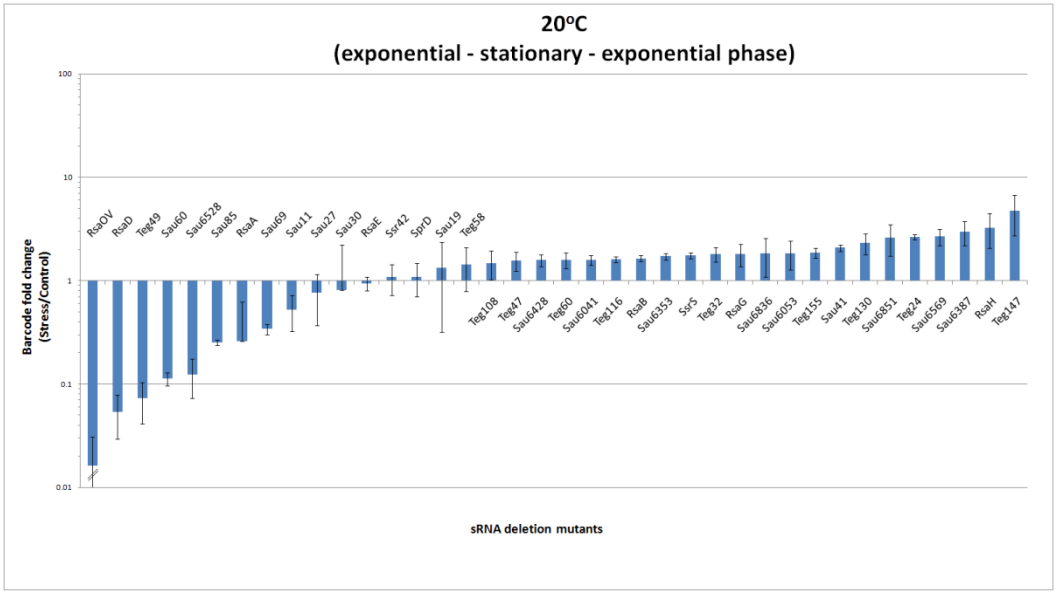
Figure 2



Competition assay at 42°C. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) at 42°C compared to a reference at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.

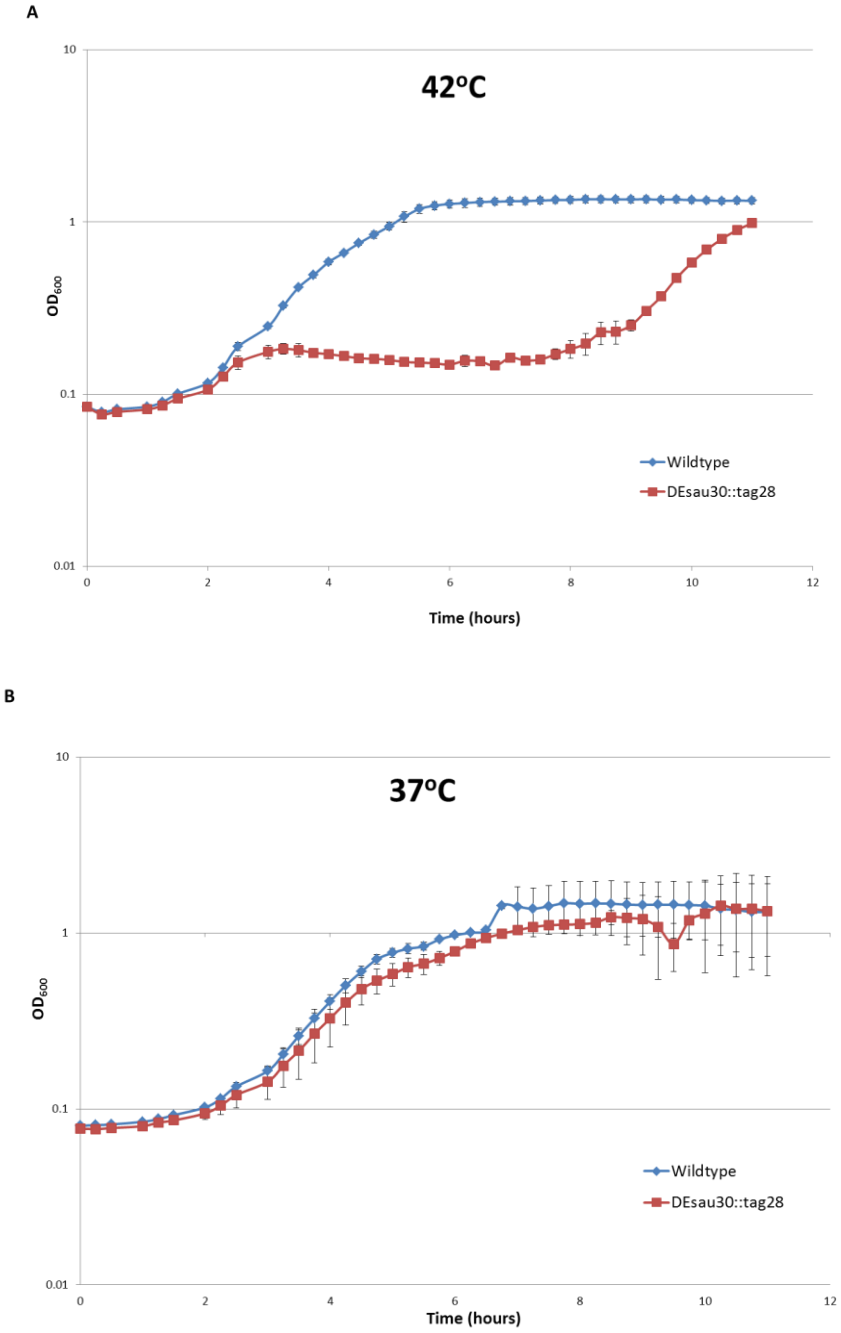
Figure 3





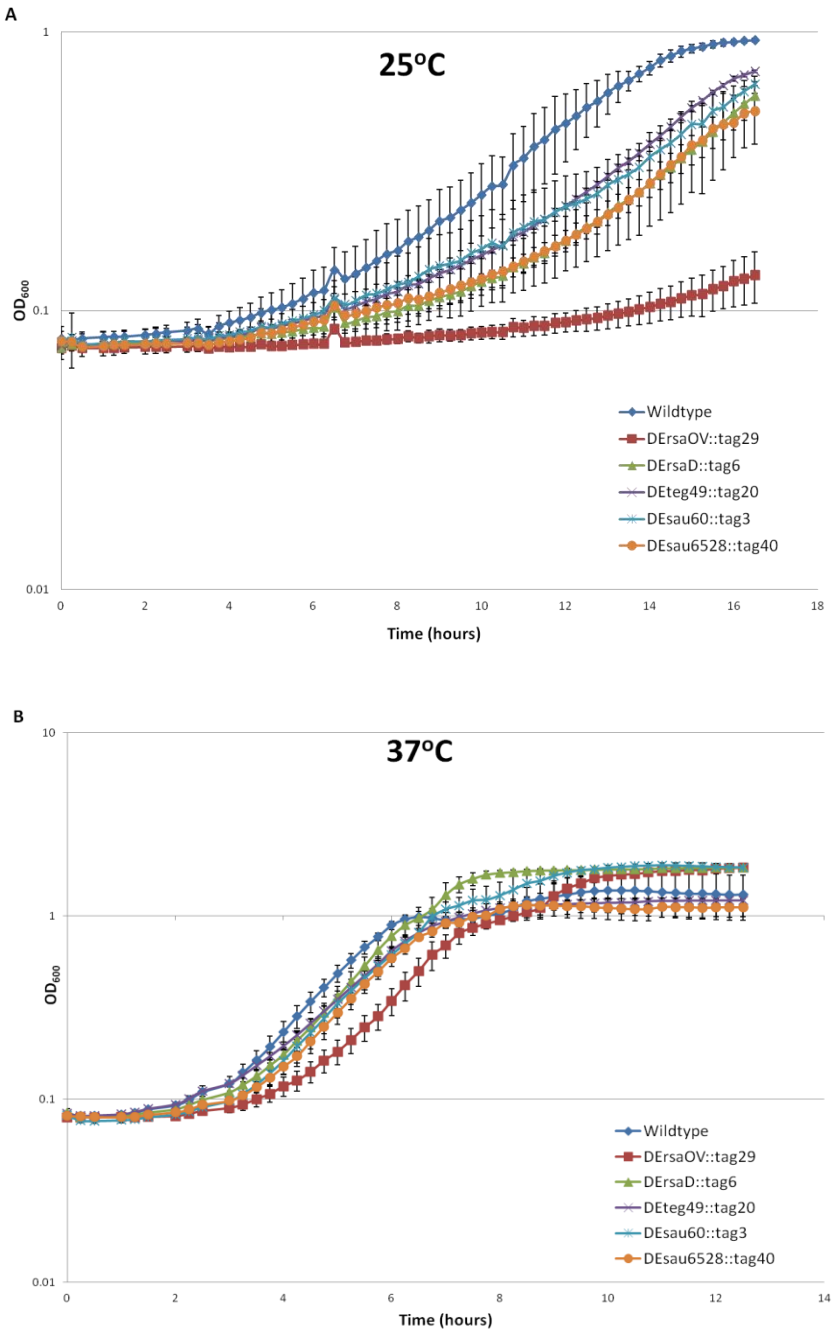
Competition assay at 20°C. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) at 20°C compared to a reference at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.

Figure 4



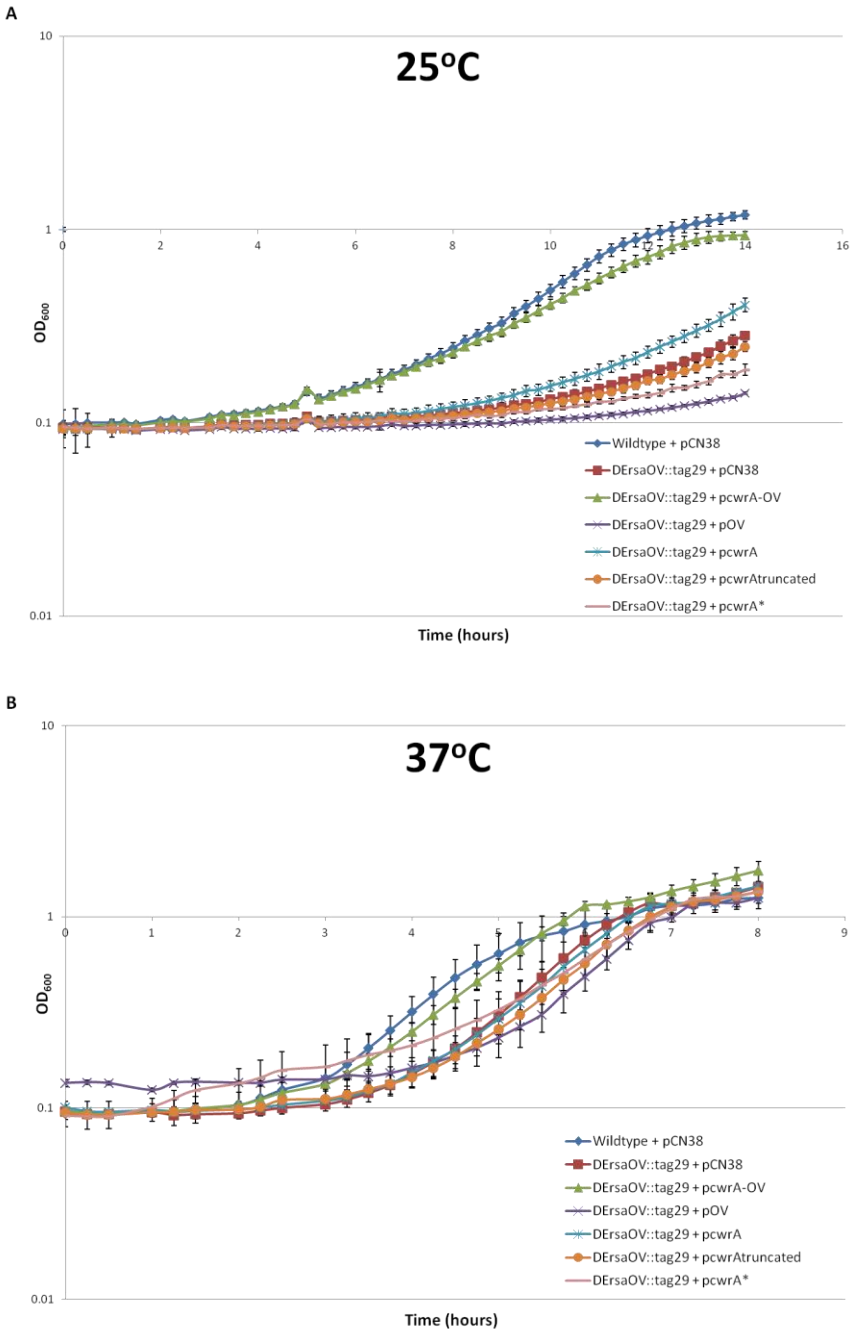
Growth defect of *sau30* mutant at 42°C. Growth curves of HG003 (blue) and *sau30* (red). Overnight cultures were diluted 200-fold in (A) BHI medium at 42°C and (B) at 37°C. Cultures were grown in microtiter plates under a vigorous agitation. *OD_{600} was measured* periodically.

Figure 5



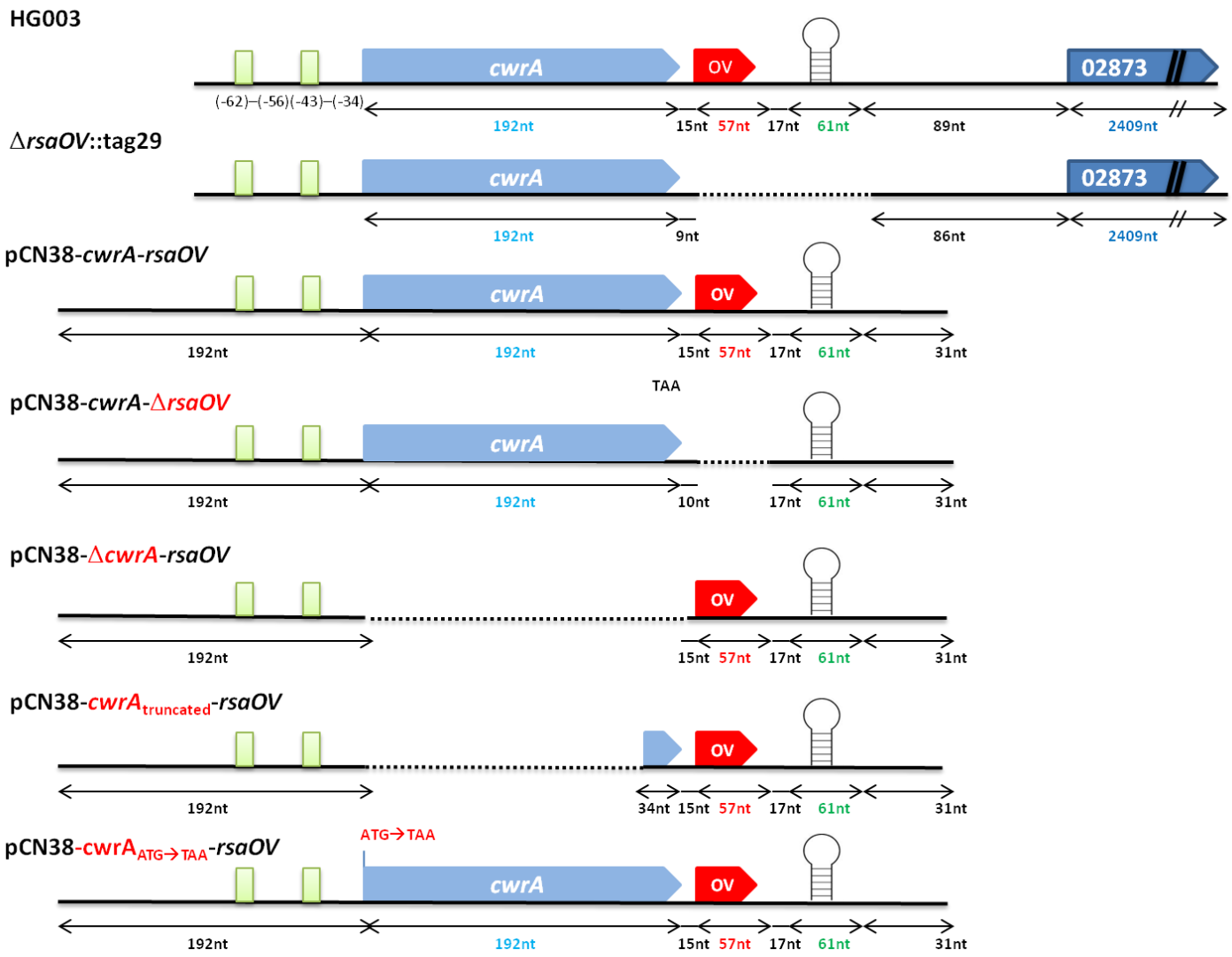
Growth defect of deletion mutants at 25°C. Growth curves of HG003 (blue) and indicated mutants. Overnight cultures were diluted 200-fold in (A) BHI medium at 25°C and (B) at 37°C. Cultures were grown in microtiter plates under a vigorous agitation. OD_{600} was measured periodically.

Figure 6



Complementation test of *rsaOV* growth defect at 25°C. Growth curves of HG003 (blue) and the indicated strains. Overnight cultures were diluted 200-fold in (A) BHI medium at 25°C and (B) at 37°C. Cultures were grown in microtiter plates under a vigorous agitation. OD_{600} was measured periodically.

Figure 7



Plasmids used for Δ*rsaOV* complementation studies. Schematic representation of the *cwrA*/*rsaOV* loci in HG003, Δ*rsaOV*::*tag29* mutant and carried on pCN38 derivatives as indicated on the figure. (green boxes) *cwrA* promoters; (light blue plain arrow) *cwrA* gene; (red plain arrow) *rsaOV*; (dashed line) absent sequences. Transcriptional terminator, promoter positions, ATG to TAA site directed mutation and nucleotide sequence lengths are indicated.

Supplementary data

Table S1: Bacterial strains

Strains	Relevant genotype	Reference
E. coli		
DH5αZ1	F^- (ϕ80d*lacZ*ΔM15) Δ(*lacZYA-argF*)*U169 deoR recA1 hsdR17*(r_k^- m_k^-) *endA1 supE44* λ^-*thi-1 gyrA*(Nal^r) *relA1 tetR*$^+$ *lacR*$^+$ Sp^r	(Lutz & Bujard, 1997)
S. aureus		
RN4220	Restriction-defective derivative of 8325-4	(Kreiswirth et al, 1983)
HG003	NCTC8325 derivative	(Herbert et al, 2010)
	rsbU and *tcaR* repaired, *agr*+	
SAPhB194	As HG003 *ΔrsaA*::*tag1*	This study
SAPhB343	As HG003 Δsau60::*tag3*	This study
SAPhB345	As HG003 Δ*rsaD*::*tag6*	This study
SAPhB347	As HG003 Δ*teg24*::*tag9*	This study
SAPhB349	As HG003 Δ*rsaG*::*tag11*	This study
SAPhB353	As HG003 Δ*ssrS*::*tag12*	This study
SAPhB360	As HG003 Δ*sau6041*::*tag14*	This study
SAPhB363	As HG003 Δ*sau41*::*tag15*	This study
SAPhB365	As HG003 Δ*sau6428*::*tag16*	This study
SAPhB366	As HG003 Δ*sau6836*::*tag17*	This study
SAPhB368	As HG003 Δ*teg147*::*tag18*	This study
SAPhB370	As HG003 Δ*teg47*::*tag19*	This study
SAPhB372	As HG003 Δ*teg49*::*tag20*	This study
SAPhB374	As HG003 Δ*sau6053*::*tag21*	This study
SAPhB376	As HG003 Δ*teg58*::*tag22*	This study
SAPhB378	As HG003 Δ*teg60*::*tag23*	This study
SAPhB380	As HG003 Δ*rsaB*::*tag25*	This study
SAPhB382	As HG003 Δ*sau69*::*tag27*	This study
SAPhB383	As HG003 Δ*sau30*::*tag28*	This study
SAPhB384	As HG003 Δ*sau40*::*tag29*	This study
SAPhB386	As HG003 Δ*teg116*::*tag30*	This study
SAPhB388	As HG003 Δ*sau6569*::*tag31*	This study

SAPhB390	As HG003 Δ*sau6851*::*tag32*	This study
SAPhB392	As HG003 Δ*sau11*::*tag33*	This study
SAPhB393	As HG003 Δ*sau27*::*tag36*	This study
SAPhB395	As HG003 Δ*teg108*::*tag37*	This study
SAPhB397	As HG003 Δ*sau85*::*tag38*	This study
SAPhB400	As HG003 Δ*sau6528*::*tag40*	This study
SAPhB401	As HG003 Δ*sau6387*::*tag41*	This study
SAPhB402	As HG003 Δ*sau6353*::*tag42*	This study
SAPhB404	As HG003 Δ*rsaE*::*tag45*	This study
SAPhB406	As HG003 Δ*teg130*::*tag46*	This study
SAPhB302	As HG003 Δ*RNAIII*::*tag47*	This study
SAPhB407	As HG003 Δ*sau19*::*tag48*	This study
SAPhB409	As HG003 Δ*rsaH*::*tag49*	This study
SAPhB412	As HG003 Δ*ssr42*::*tag50*	This study
SAPhB415	As HG003 Δ*teg155*::*tag53*	This study
SAPhB416	As HG003 Δ*teg32*::*tag54*	This study
SAPhB417	As HG003 Δ*sprD*::*tag57*	This study

Table S2: Plasmids

Name	Relevant genotype	Reference
pJET	rep (pMB1), *bla* (Ap^R), *eco47IR,* P_{lacUV5}, T7 promoter	Fermentas
pMAD	rep $pE194^{ts}$, rep pBR322, *pclpB* promoter, *bgaB, bla* (Ap^R), *ermC* (Ery^R)	(Arnaud et al, 2004)
pMAD2	rep $pE194^{ts}$, rep pBR322, *pclpB* promoter, *bgaB, bla* (Ap^R), *ermC* (Ery^R)	Bonnin et al. unpublished results
pDErsaA::tag1	pMAD2 derivative for chromosomal substitution of rsaAlocus with tag1 sequence	This study
pDEsau60::tag3	pMAD2 derivative for chromosomal substitution of sau60 locus with tag3 sequence	This study
pDErsaD::tag6	pMAD2 derivative for chromosomal substitution of rsaD locus with tag6 sequence	This study
pDEteg24::tag9	pMAD2 derivative for chromosomal substitution of teg24 locus with tag9 sequence	This study
pDErsaG::tag11	pMAD2 derivative for chromosomal substitution of rsaG locus with tag11 sequence	This study
pDEssrS::tag12	pMAD2 derivative for chromosomal substitution of ssrS locus with tag12 sequence	This study
pDEsau6041::tag14	pMAD2 derivative for chromosomal substitution of sau6041 locus with tag14 sequence	This study
pDEsau41::tag15	pMAD2 derivative for chromosomal substitution of sau41 locus with tag15 sequence	This study
pDEsau6428::tag16	pMAD2 derivative for chromosomal substitution of sau6428 locus with tag16 sequence	This study
pDEsau6836::tag17	pMAD2 derivative for chromosomal substitution of sau6836 locus with tag17 sequence	This study
pDEteg147::tag18	pMAD2 derivative for chromosomal substitution of teg147 locus with tag18 sequence	This study
pDEteg47::tag19	pMAD2 derivative for chromosomal substitution of teg47 locus with tag19 sequence	This study
pDEteg49::tag20	pMAD2 derivative for chromosomal substitution of teg49 locus with tag20 sequence	This study
pDEsau6053::tag21	pMAD2 derivative for chromosomal substitution of sau6053 locus with tag21 sequence	This study
pDEteg58::tag22	pMAD2 derivative for chromosomal substitution of teg58 locus with tag22 sequence	This study

pDEteg60::tag23	pMAD2 derivative for chromosomal substitution of teg60 locus with tag23 sequence	This study
pDErsaB::tag25	pMAD2 derivative for chromosomal substitution of rsaB locus with tag25 sequence	This study
pDEsau69::tag27	pMAD2 derivative for chromosomal substitution of sau69 locus with tag27 sequence	This study
pDEsau30::tag28	pMAD2 derivative for chromosomal substitution of sau30 locus with tag28 sequence	This study
pDEsau40::tag29	pMAD2 derivative for chromosomal substitution of sau40 locus with tag29 sequence	This study
pDEteg116::tag30	pMAD2 derivative for chromosomal substitution of teg116 locus with tag30 sequence	This study
pDEsau6569::tag31	pMAD2 derivative for chromosomal substitution of sau6569 locus with tag31 sequence	This study
pDEsau6851::tag32	pMAD2 derivative for chromosomal substitution of sau6851 locus with tag32 sequence	This study
pDEsau11::tag33	pMAD2 derivative for chromosomal substitution of sau11 locus with tag33 sequence	This study
pDEsau27::tag36	pMAD2 derivative for chromosomal substitution of sau27 locus with tag36 sequence	This study
pDEteg108::tag37	pMAD2 derivative for chromosomal substitution of teg108 locus with tag37 sequence	This study
pDE*sau85*::*tag38*	pMAD2 derivative for chromosomal substitution of *sau85* locus with *tag38* sequence	This study
pDE*sau6528*::*tag40*	pMAD2 derivative for chromosomal substitution of *sau6528* locus with *tag40* sequence	This study
pDE*sau6387*::*tag41*	pMAD2 derivative for chromosomal substitution of *sau6387* locus with *tag41* sequence	This study
pDE*sau6353*::*tag42*	pMAD2 derivative for chromosomal substitution of *sau6353* locus with *tag42* sequence	This study
pDE*rsaE*::*tag45*	pMAD2 derivative for chromosomal substitution of *rsaE* locus with *tag45* sequence	This study
pDE*teg130*::*tag46*	pMAD2 derivative for chromosomal substitution of *teg130* locus with *tag46* sequence	This study
pDE*RNAIII*::*tag47*	pMAD2 derivative for chromosomal substitution of *RNAIII* locus with *tag47* sequence	This study
pDE*sau19*::*tag48*	pMAD2 derivative for chromosomal substitution of *sau19* locus with *tag48* sequence	This study
pDE*rsaH*::*tag49*	pMAD2 derivative for chromosomal substitution of *rsaH* locus with *tag49* sequence	This study

pDE*ssr42*::*tag50*	pMAD2 derivative for chromosomal substitution of *ssr42* locus with *tag50* sequence	This study
pDE*teg155*::*tag53*	pMAD2 derivative for chromosomal substitution of *teg155* locus with *tag53* sequence	This study
pDE*teg32*::*tag54*	pMAD2 derivative for chromosomal substitution of *teg32* locus with *tag54* sequence	This study
pDE*sprD*::*tag57*	pMAD2 derivative for chromosomal substitution of *sprD* locus with *tag57* sequence	This study

Table S3: Primers to generate tagged deletion.

Name*	Sequence (5' to 3')
Tag_random	TTGTGGGGTACAGCAATGACAAGCTTN$_{40}$AAGCTTATTGCATAGCTGCGTATGGA
Tag_F	GGTCTCATGTGTTGTGGGGTACAGCAATGAC
Tag_R	GGTCTCTGAGATCCATACGCAGCTATGCAAT
RsaA UpF	ATATGGTCTCGAATTCCAAACGCAGTAACCAATGCT
RsaA UpR	AAGCTTGGTCTCACACACTTTTTATACTTCAAGAGAATTTTAAC
RsaA DwF	ATATGGTCTCGGATCTATGCAGTTGATTGGGCAT
RsaA DwR	AAGCTTGGTCTCATCTCAAATCACGTCTTATGTATACGG
Sau60 UpF	AAGCTTGGTCTCGAATTCCAGTAGCAGTAGCAGGTGCAG
Sau60 UpR	AAGCTTGGTCTCACACAATTATATAACTATAACAGAATATCCATTTC
Sau60 DwF	AAGCTTGGTCTCATCTCTGCCGTTTTCTTTTTGTCTTT
Sau60 DwR	AAGCTTGGTCTCGGATCCTCGCTGTTTGCATTTGATTC
RsaD UpF	GAATTCGAGACCGCTAGCGCTCATCGCATTTGTTATTAGTTTTG
RsaD UpR	GCGTATGGACCTAGGTATATTCATTTCCCATAAAAGCCAAG
RsaD DwF	ACCCCACAACCTAGGTATATTAAAGGGGATGGTTTCGTGA
RsaD DwR	GATATCGGATCCGAGACCCTCCTCTTCCAATTTGCTCGTC
New RsaD UpF	GAATTCGAGACCGCTAGCGCGGAGAACTGGTACTAACGGC
New RsaD UpR	GCGTATGGACCTAGGTATATGCTTCAATTTCGGTAACTTTAAA
New RsaD DwF	ACCCCACAACCTAGGTATATTGTGAGTGATATTTATTAGGGAAAGCT
RsaOG UpF	GAATTCGAGACCGCTAGCGCAATTGACCTTTTGCCACTCG
RsaOG UpR	GCGTATGGACCTAGGTATATGCATAAAATGAAGAAGTCTTCAGTTG
RsaOG DwF	ACCCCACAACCTAGGTATATCACAATCTTTTTTAAAATGTAAGCG
RsaOG DwR	GATATCGGATCCGAGACCCTCAATTTCGAGTTCGGCAGTT
RsaG UpF	GAATTCGAGACCGCTAGCGCAGGATGGAATCGTGCTGAAG
RsaG UpR	GCGTATGGACCTAGGTATATTTGCAATAGATTGGCGATACTTT

RsaG DwF	ACCCCACAACCTAGGTATATAGCGGTGTCAATATTGTAGGG
RsaG DwR	GATATCGGATCCGAGACCCTTTCAACAACATCAGCCAGAA
ssrS UpF	GAATTCGAGACCGCTAGCGCGCAGCACCCATACTGGAAAT
ssrS UpR	GCGTATGGACCTAGGTATATATGGGTTTTCTTGCAGCGTA
ssrS DwF	ACCCCACAACCTAGGTATATTTAAAATTTAGTGACGAATTCGCAAAG
ssrS DwR	GATATCGGATCCGAGACCCTGGTGCAGCTGAACAATATACTCG
Sau-6041 UpF	GAATTCGAGACCGCTAGCGCATTTATCGCAACCGGATCAT
Sau-6041 UpR	GCGTATGGACCTAGGTATATAAATTTGTGTACTATTCTTCGTCAAA
Sau-6041 DwF	ACCCCACAACCTAGGTATATAGTGCAAAAGTGCAAATTGA
Sau-6041 DwR	GATATCGGATCCGAGACCCTCTTTTGCTGTTTTATCAACTTTTTC
Sau-41 UpF	GAATTCGAGACCGCTAGCGCTCAAGATGTCGCAGCTGAAT
Sau-41 UpR	GCGTATGGACCTAGGTATATCGTTCTTAGTGGGACATACGG
Sau-41 DwF	ACCCCACAACCTAGGTATATGCCAGCGATGATACCCATTA
Sau-41 DwR	GATATCGGATCCGAGACCCTACCGAAAAAGCCAATGACTG
Sau-6428 UpF	GAATTCGAGACCGCTAGCGCATGATTTCGCCGAAGTGTTT
Sau-6428 UpR	GCGTATGGACCTAGGTATATACACATTATATTAATCATCATTTTGTTTC
Sau-6428 DwF	ACCCCACAACCTAGGTATATAAACGTTTGCTTTTTGTGTGA
Sau-6428 DwR	GATATCGGATCCGAGACCCTATCTCATCGCCGAAAAACTC
Sau-6836 UpF	GAATTCGAGACCGCTAGCGCTTGTTGGTGCTAACTGCTTTG
Sau-6836 UpR	GCGTATGGACCTAGGTATATTTTAATTAAGGTGAAGTGAATTAGCAA
Sau-6836 DwF	ACCCCACAACCTAGGTATATTGGGGCAACACTTTATTTGA
Sau-6836 DwR	GATATCGGATCCGAGACCCTAATTCAAGACGCTCTGTATTTGA
Teg147 UpF	GAATTCGAGACCGCTAGCGCTCTTGATGATTGAAGGGTCCA
Teg147 UpR	GCGTATGGACCTAGGTATATTTCGGTGTTGATTGGCATTA
Teg147 DwF	ACCCCACAACCTAGGTATATGGAAACAGAGGCAACGCTAC
Teg147 DwR	GATATCGGATCCGAGACCCTGAGGCATCAGGCACAGAAAT
RsaOI UpF	GAATTCGAGACCGCTAGCGCTGGTAACTGCATATTTACCAACC

RsaOI UpR	GCGTATGGACCTAGGTATATACGGCTAATTACAGTTCTCAATTT
RsaOI DwF	ACCCCACAACCTAGGTATATAGGCAGGTTTACCTGATAAAAA
RsaOI DwR	GATATCGGATCCGAGACCCTTGACATTGATTAAGTAACTTTTCAGGA
Teg49 UpF	GAATTCGAGACCGCTAGCGCACTGCTCGTTATGCGGCTAT
Teg49 UpR	GCGTATGGACCTAGGTATATTCACTGTGTCTAATGAATAATTTGTTT
Teg49 DwF	ACCCCACAACCTAGGTATATTTCCGATTGATAACGGGTAA
Teg49 DwR	GATATCGGATCCGAGACCCTACTCATTCACCAGCCTTTGC
Sau-6053 UpF	GAATTCGAGACCGCTAGCGCCGTTGAAGTAAGCCCGTTTG
Sau-6053 UpR	GCGTATGGACCTAGGTATATAATTCGATTATACAATTGAGCTGTT
Sau-6053 DwF	ACCCCACAACCTAGGTATATTTTATTTAGCATAGGTCTTTTTGTTTG
Sau-6053 DwR	GATATCGGATCCGAGACCCTGGGAAGTGCTCAGGCAATAC
Teg58 UpF	GAATTCGAGACCGCTAGCGCTTGTAATTTTGGAGAATGTGATTG
Teg58 UpR	GCGTATGGACCTAGGTATATTTGGATATAGCAAAAAGCCACA
Teg58 DwF	ACCCCACAACCTAGGTATATAAGCACGCCAATACGTTAGC
Teg58 DwR	GATATCGGATCCGAGACCCTTCCAACTTAGCAAACAAAATGTAGA
Teg60 UpF	GAATTCGAGACCGCTAGCGCCAATGCCTATCTTTGCACCA
Teg60 UpR	GCGTATGGACCTAGGTATATAATTAAACACCGTTATTTTTCCTTTG
Teg60 DwF	ACCCCACAACCTAGGTATATTCATATTAAATCAAAGAGGCATTG
Teg60 DwR	GATATCGGATCCGAGACCCTCTATTTGGATTTTATGCCTTGTGG
RsaB UpF	GAATTCGAGACCGCTAGCGCTTTGTTTCTTCTCCATCATCAA
RsaB UpR	GCGTATGGACCTAGGTATATGCGCTACAATTAACACTAATAATTG
RsaB DwF	ACCCCACAACCTAGGTATATATTCATTGCATCGCTTTCCT
RsaB DwR	GATATCGGATCCGAGACCCTTGATACCGATGCAGAAGTAGAA
Sau-69 UpF	GAATTCGAGACCGCTAGCGCTTGTTCTGCATTCTACTTCTACGC
Sau-69 UpR	GCGTATGGACCTAGGTATATCACCTTGCTATAATTATTTTGTTATAAATG
Sau-69 DwF	ACCCCACAACCTAGGTATATCATGGGTTATTGATTGGTGAT
Sau-69 DwR	GATATCGGATCCGAGACCCTCAACCTCTGATACTTCACCATCTT

Sau-30 UpF	GAATTCGAGACCGCTAGCGCGCTTCCATCGCCTCAGATAA
Sau-30 UpR	GCGTATGGACCTAGGTATATAACCCAGTCAATGTCATATACAGC
Sau-30 DwF	ACCCCACAACCTAGGTATATTCGTTGCACTCATTTGCTTT
Sau-30 DwR	GATATCGGATCCGAGACCCTTACTGATGAAGCGCAAAACG
New Sau-30 UpR	GCGTATGGACCTAGGTATATTGATGAAGCTAGTTTGATCAATTTCAC
RsaOV UpF	GAATTCGAGACCGCTAGCGCCGCCAATTGTAATCTGTCCA
RsaOV UpR	GCGTATGGACCTAGGTATATTTAGGTGACTTAAAAGAAATCAGATG
RsaOV DwF	ACCCCACAACCTAGGTATATTGGTAAAAGTAAAACGCAACGA
RsaOV DwR	GATATCGGATCCGAGACCCTGCGCCACCATTTCTTAAGTT
Teg116 UpF	GAATTCGAGACCGCTAGCGCAAATCACTGCGTCATTTCCA
Teg116 UpR	GCGTATGGACCTAGGTATATTCCTTGTCATTCGCTCATTT
Teg116 DwF	ACCCCACAACCTAGGTATATTGAAATTATATTTTACAATGCCCAAA
Teg116 DwR	GATATCGGATCCGAGACCCTAACGTTCACTTGGTACACCTACAA
Sau-6569 UpF	GAATTCGAGACCGCTAGCGCTTTTTCAATTTGGATGAACACA
Sau-6569 UpR	GCGTATGGACCTAGGTATATTCTTCGTACTTCGCCAGTGA
Sau-6569 DwF	ACCCCACAACCTAGGTATATGGTTCAAGCTACGCATTTTCA
Sau-6569 DwR	GATATCGGATCCGAGACCCTCAATACGGCATCTTCATTTCTG
Sau-6851 UpF	GAATTCGAGACCGCTAGCGCTTTCTTCAACAATCGTGACACC
Sau-6851 UpR	GCGTATGGACCTAGGTATATGCTTTATCCGAGTTTAAAATGTTG
Sau-6851 DwF	ACCCCACAACCTAGGTATATCCATTTCGATTTGTGCTATGA
Sau-6851 DwR	GATATCGGATCCGAGACCCTTTTTTCATTCTCCAATTATCTGTTT
Sau-11 UpF	GAATTCGAGACCGCTAGCGCAAATTTTACGTTGACCACTTGGA
Sau-11 UpR	GCGTATGGACCTAGGTATATATATTGTGAACGCATAACTTTCC
Sau-11 DwF	ACCCCACAACCTAGGTATATTTCATGAAATTTCGTTTAATTCG
Sau-11 DwR	GATATCGGATCCGAGACCCTAATGAGACCAGTGAAGAGTGAAA
Sau-27 UpF	GAATTCGAGACCGCTAGCGCGATGGACGTATTCATCCAGGT
Sau-27 UpR	GCGTATGGACCTAGGTATATGGGAGACAAAAATTATTTCGCATA
Sau-27 DwF	ACCCCACAACCTAGGTATATATAAAGATGATTGGTTTTCTATCCA

Sau-27 DwR	GATATCGGATCCGAGACCCTCGGAAAATTCGCTGGTCTTA
Teg108 UpF	GAATTCGAGACCGCTAGCGCGAAAATAGAATTTTTAATAGGGACGTT
Teg108 UpR	GCGTATGGACCTAGGTATATAATATCCATTCACCATATGATTTTT
Teg108 DwF	ACCCCACAACCTAGGTATATTCAGTCAGGAGGGACTTTCC
Teg108 DwR	GATATCGGATCCGAGACCCTAATATTTTTCCGTTGAGTGAATGA
Sau-85 UpF	GAATTCGAGACCGCTAGCGCTTTGCTGTTTATTCGTTTGATGA
Sau-85 UpR	GCGTATGGACCTAGGTATATTTGAGCTTAGGAAATCGATAGG
Sau-85 DwF	ACCCCACAACCTAGGTATATGATTTACCAGATGACATATAACAGCA
Sau-85 DwR	GATATCGGATCCGAGACCCTGGCGGTGCAATTGAATATAG
Sau-6528 UpF	GAATTCGAGACCGCTAGCGCGAAATCTGCATCTTTCGTTTCA
Sau-6528 UpR	GCGTATGGACCTAGGTATATTCAAAATCAACTGACCGATATTC
Sau-6528 DwF	ACCCCACAACCTAGGTATATTTTTGTGTTGTGGATTAAGATTCTA
Sau-6528 DwR	GATATCGGATCCGAGACCCTCACAACAAGCATCTGCAAAA
Sau-6387 UpF	GAATTCGAGACCGCTAGCGCCGTGACCTCGCTCTGCTAAT
Sau-6387 UpR	GCGTATGGACCTAGGTATATGATTGCACTAAACATGCATGAGA
Sau-6387 DwF	ACCCCACAACCTAGGTATATATTAATCACTTGAACGCGCAAT
Sau-6387 DwR	GATATCGGATCCGAGACCCTAAAAACGGCAAATGACAGTAAAA
Sau-6353 UpF	GAATTCGAGACCGCTAGCGCCATGGGATCCGAGTAAATCC
Sau-6353 UpR	GCGTATGGACCTAGGTATATTAGCGAATTGTACATAAACAACAGC
Sau-6353 DwF	ACCCCACAACCTAGGTATATAAGCAAACTTCTGCCACTTCA
Sau-6353 DwR	GATATCGGATCCGAGACCCTTGTTGAGACCATATTTAACATCTAACG
RsaE UpF	GAATTCGAGACCGCTAGCGCTCGTTGGGTCGATGTCTATG
RsaE UpR	GCGTATGGACCTAGGTATATCAATCTGTTCATAATGTAAGCGAATA
RsaE DwF	ACCCCACAACCTAGGTATATAAAAGACCTCGTTACATTTATGGTG
RsaE DwR	GATATCGGATCCGAGACCCTCGAAATTTATTCATTTTTCGATCC
Teg130 UpF	GAATTCGAGACCGCTAGCGCTATTTACCGCGTTCATGTGG
Teg130 UpR	GCGTATGGACCTAGGTATATCGAGCTAGGGATACTCGAAAA

Teg130 DwF	ACCCCACAACCTAGGTATATCACTCACTCCTTGTGTACATGC
Teg130 DwR	GATATCGGATCCGAGACCCTGGAAAGAGGTTATAAGTTATGCCAAA
RNAIII(ΔagrA) UpF	GAATTCGAGACCGCTAGCGCCCTGAAATGTGGAATAATGGCTA
RNAIII(ΔagrA) UpR	GCGTATGGACCTAGGTATATAGGGCGAAATGGGTTCTTAC
RNAIII(ΔagrA) DwF	ACCCCACAACCTAGGTATATTTAAGTATTTATTTCCTACAGTTAGGC
RNAIII(ΔagrA) DwR	GATATCGGATCCGAGACCCTTTTTGGTACTTCAACTTCATCCA
RNAIII UpF	GAATTCGAGACCGCTAGCGCCCTGAAATGTGGAATAATGGCTA
RNAIII UpR	GCGTATGGACCTAGGTATATATCATTATGAGACCCGCCGT
RNAIII DwF	ACCCCACAACCTAGGTATATCATGCTAAAAGCATTTATTTTCC
RNAIII DwR	GATATCGGATCCGAGACCCTTTTTGGTACTTCAACTTCATCCA
Sau-19 UpF	GAATTCGAGACCGCTAGCGCCCGCATTTGATTTTCGATTC
Sau-19 UpR	GCGTATGGACCTAGGTATATCACAAATCCCTTTATTTATTTGGAA
Sau-19 DwF	ACCCCACAACCTAGGTATATGCTATTAAACTTCCGTTCTTTGAA
Sau-19 DwR	GATATCGGATCCGAGACCCTGGGTGATAAAGGTACTTGGATAGTT
RsaH UpF	GAATTCGAGACCGCTAGCGCACGGACCACTAGCTGACTCG
RsaH UpR	GCGTATGGACCTAGGTATATTGTATAACCTTTGAACAACAATAATGA
RsaH DwF	ACCCCACAACCTAGGTATATAAATGAATCCGATTTACGAGTGA
RsaH DwR	GATATCGGATCCGAGACCCTCTTGTGGTTTTGCTTGCTGA
New RsaH UpR	GCGTATGGACCTAGGTATATCCTTTATTATAACTTATATCATTTTTATTA
RsaH DwF	ACCCCACAACCTAGGTATATCTTATTCCCATTATACATCAATTTAAAGCA
Ssr42 UpF	GAATTCGAGACCGCTAGCGCTTGTCCCCCAGTAGAAAACG
Ssr42 UpR	GCGTATGGACCTAGGTATATGTTTCAATCTATCTCTTTCTTTTTGTG
Ssr42 DwF	ACCCCACAACCTAGGTATATGCGCAATGCATAAAAACAAG
Ssr42 DwR	GATATCGGATCCGAGACCCTTTCATACTCAAATATCGAACAAAAAGA
Teg155 UpF	GAATTCGAGACCGCTAGCGCTTCTCACTCAAGAGTTAAAGCAACA
Teg155 UpR	GCGTATGGACCTAGGTATATTGATTGCTTATTTATTTTATCAAGAGG
Teg155 DwF	ACCCCACAACCTAGGTATATTCGTGTTCCAATTTTACTGAGTATC
Teg155 DwR	GATATCGGATCCGAGACCCTCGCGATTGAAGATCATTTTG

Teg32 UpF	GAATTCGAGACCGCTAGCGCTCTTCCGTTATAACCCCTCA
Teg32 UpR	GCGTATGGACCTAGGTATATGCAATTCGTATATTTTGCCAATG
Teg32 DwF	ACCCCACAACCTAGGTATATTTGGCATTTCCAAAATATCACTT
Teg32 DwR	GATATCGGATCCGAGACCCTTTTGATGATGATTCAAGATAGTATGG
sprD UpF	GAATTCGAGACCGCTAGCGCGGACGCCTATGACTACAGTTACG
sprD UpR	GCGTATGGACCTAGGTATATGCATTTCGGTGCTTACCTTT
sprD DwF	ACCCCACAACCTAGGTATATTGAAAATTTGAACACATTGCTG
sprD DwR	GATATCGGATCCGAGACCCTTTCATTAGTTTTACCAGGACCATT

* Up and Dw refer to upstream and downstream loci of the referred gene, respectively. F and R indicate that concerned primers are in the Forward or Reverse orientation with respect to the chromosome annotation, respectively.

Table S4: Tag sequences

Name	Sequence*
tag1	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCCTCACCACCTCCAACTATCC CGAGAACACTATCACTCTAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag2	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCACCACTACATCACTCTCAAA ACCCCGAGATAGCGCTCCAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag3	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCGATACCCCAAGCAATCACAAC ACCGCAACAGATCTAACAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag4	GGTCTCATGTGTTGTGGGGTACAGCAATGACAAGCTTGGTTTGCTCTGGCGATATTGGT CTATCGGTGGGTAGCTAGAAGCTTATTGCATAGCTGCGTATGGATCTCAGAGACC
tag5	GGTCTCATGTGTTGTGGGGTACAGCAATGACAAGCTTCGTTGTCGGTGGTTTGCGCGAG ATTTAGGGGGGTCGAGGGAAGCTTATTGCATAGCTGCGTATGGATCTCAGAGACC
tag6	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCTCTATCTAAAAACACCCCAAT AACCAACTAACTAACTCCAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag7	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTATCGAGAAATCCCTCGCAATAT CTATATCCATACCTCGCCAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag8	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCCTCCCAATATCTCACCAGAC CCCCCAACCTCGCACCCCAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag9	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTACAGCTAGCTCACTCCCACCAC CCACACAGCAAAAACTCGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag10	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCTCCCAAAACCTCTCCAGCCAA CTCAACAACTCGCTATCCAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag11	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTACATATCCCGCCAGCCCCAAAA AGCGCAAAACCCCGACCGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag12	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTACCCATAGACCTCCAACGAGCT AAATCACGCGACCCCTCTAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag13	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTACACCCCACGCGCAAACCCCAT AAACAGAACCCTCCACCAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag14	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAACGCTAACAACCTCCCGACAA AGACCTAGACACCAACAGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag15	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAAACCCCACCCCAGATACATCT CACGATCCACACCTACCAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag16	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCAATAGACATACCGAGATCTAA CAATAACACCCCCGAACTAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag17	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCTATACCCCGCGCCCTATCAAA ATCCCTCCCGCAAGCCCCAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag18	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCAGCCCCCACACGCGATAGAC AAATAGACCTAGCGACACAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag19 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCAAATCCCACTCCCCAGCCCG
AGAAAGCTAGACCCCGCTAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag20 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCATCCATAGCTCTATAGCCCG
CAACCAAAACCTCGATACAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag21 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCTCTCCACCGACACCCCCCACA
ATCCAGAAAACACCCTCGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag22 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCCGCGAAAGACACAGCTACCC
CCACAAAAATCCCCCACTAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag23 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAACTAGACCACCAGAGAAAGCT
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tag24 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAGAGCCCTCGAGAAACAAAGCA
AACCCGAGATACACATCAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag25 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAGACAGCTCCCCATCCACCCCA
ATCACGCGATCCCGCCAGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag26 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCGAACGAGCGAGACATAACGAT
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tag27 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCTCCCCCACGCAAGACCCAACC
ACACATCTACCCAGATAGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag28 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCGAGAACACCCCCGCTCGAACT
CTCTATCCCCCAACAACGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag29 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCGCTAGCTCTCCATCCCACAAG
AACGAACTACCCCCCAAAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag30 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCCTCCCAAAACCCAAACCTCG
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tag31 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTACCAACCGCGCCCCACCACCCC
CACAAACCAGCAACAAATAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag32 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCGAAATCTAACCCAATACCAAC
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tag33 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCGCGCGCGACCACCAGCCCCAT
ACCACTCACACCCTCCCAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag34 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTACCGATCACCATACCCCTCGCC
CCACAGCCCACAACATCAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag35 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCGACCAACATCTCACGCCCGCA
CTCTCTAGCGCCCGCGAGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag36 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAGAGATCGAGATAACCAACaac
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tag37 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCACCCAAGCGAGCCCCCCAACA
CGATCTATCCCACACTACAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag38 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTATCCCCAGCGAACTATAGATAA
CGATCGACACATCCACCAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag39 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCCACCCCCTCTAACACGAACT
CGACATCAAAATCACCCTAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag40 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAAAGACACACCAACATATAACC
CCAGAACCCTAGACCGACAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag41 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAGAGACACCCAACGACCTCGAG
ACCACCACAGACCTCACGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag42 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCCTCTACCACTAGCTCGATAT
ACCCCTACCACCAGCTCAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag43 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCCGCTATACCTCTCCCGCCCT
CAACCTAAACATCCAACAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag44 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAAATATATCCAACTACCGAGAC
CCATCCCGCAACACATATAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag45 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCGACCGCCCTACCGAACCATAC
CTCCCACGAAACCACACAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag46 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCTCACCCTCTCTCGCACCACCC
CTATCCCGAGAACCCAACAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag47 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCCACACAATAACTCAAGAAAT
AAAACCCCCGCGAGCTCTAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag48 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCTCTAGACATAACTCCCACCCC
AGACCCAAAAATCCCCCCAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag49 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCCCCTCTCAAGACAACGACCT
CAACCTACATCACAACCGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag50 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCAACGCGCCCACGATACATAC
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tag51 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTATCCCTACAAACAGACCGCACC
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tag52 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCAACGAGAAAGATCAAGACAG
ATAACTCCCGCCAGATCGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag53 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCTCTCGCTCCACAAACCCCACA
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tag54 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAAACCTCGCGCGAGAAAGAAAC
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tag56 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCACTAGAAAAACCAAAACAACG
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tag57 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAGCCAGCCAGCCCGATCAACAA
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tag58 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCACCTAAACACACCCCCCTAA
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tag59 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTATAAACAGCGCCCTACCCAACG
AAAGACAACACTCACGCTAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag60 GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTACAGAACACTCTCTACCTCTCT
AGCCACCACGAAAAATCAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

tag61	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCAATCAACATAGCCCGACACAC CACGACACCACTCCCGCTAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag62	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTAACCCCACACCTACCCCAATAA ATCTACACCACGCAAACGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag63	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCCTCACCCCCGATACCACACC CCCAACATCGATACCTCCAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag64	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCGCCATCACACGATCCCGCCCC AACCACAACTCAATACAAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag65	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCACGATACCCCTAGACACCCCG AGCCCGCCCAATCTCCAAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag66	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCGCGCTCCCTCTAACCAACCAC ATCGATAAAACGACATCAAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag67	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCTCGAAAAACACACATCCACAT CGAGCTCTCTACACAGAGAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag68	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTCCCACTAGATAAACAACGACAG ACCGCTCCCCATAGATCTAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC
tag69	GGTCTCTGAGATCCATACGCAGCTATGCAATAAGCTTACCTCCACAGACCTAGCTCGAC ATCCCACCATAAACAACCAAGCTTGTCATTGCTGTACCCCACAACACATGAGACC

* Grey highlighted sequences are tag specific.

Table S5: Primers with experiment specifiers used for deep sequencing

Name	Sequence
Primer 1–F	AAACAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 1-R	GCATTATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 2–F	AAGAGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 2-R	ATGTTATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 3–F	ATAGCATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 3-R	TACATATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 4–F	ATCACATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 4-R	TCAATATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 5–F	AGTCAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 5-R	GTCCTATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 6–F	AGCTTATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 6-R	TCGCTATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 7–F	ACACTATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 7-R	CGAGTATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 8–F	ACGCAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 8-R	CCGGTATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 9-F	TAAGAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 9–R	CTATAATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 10–F	TAGCTATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 10-R	CAGTAATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 11–F	TTACTATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 11-R	CCTAAATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 12–F	TGTTTATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 12-R	GGGAAATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 13–F	TGAAGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 13–R	TATCAATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 14-F	TGGATATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 14–R	CGACAATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 15–F	TCATTATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 15-R	GTCGAATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 16–F	TCGGAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 16-R	CTGGAATATACCTAGGTTGTGGGGTACAGCAATGAC

Primer 17–F	GAAGCATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 17-R	AAGTCATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 18–F	GAGCAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 18-R	GTATCATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 19–F	GGATGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 19-R	ACACCATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 20–F	GGCAGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 20–R	TTCCCATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 21–F	GTACCATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 21–R	CAGACATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 22–F	GTGAGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 22–R	TGAACATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 23–F	GCAAAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 23–R	ATGGCATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 24–F	GCGTTATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 24–R	GGAGCATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 25–F	CAAAAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 25–R	AATTGATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 26–F	CACCCATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 26–R	AGATGATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 27–F	CTAAGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 27–R	TCGAGATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 28–F	CTGCCATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 28–R	CCAAGATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 29–F	CGAATATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 29–R	GATCGATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 30–F	CGCACATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 30–R	AACCGATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 31–F	CGTTGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 31–R	TGGGGATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 32–F	CCCTTATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 32–R	CTCGGATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 33–F	AAATCATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 33–R	AACTTATATACCTAGGTTGTGGGGTACAGCAATGAC

Primer 34–F	AAGCCATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 34–R	CGTATATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 35–F	ATTCCATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 35–R	CCACTATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 36–F	ACTAAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 36–R	ACAGTATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 37–F	ACGTGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 37–R	GGGGTATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 38–F	TATCCATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 38–R	GGTCAATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 39–F	CAAGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 39–R	GCACAATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 40–F	TCGCGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 40–R	AGGGAATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 41–F	GATGAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 41–R	GTTCCATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 42–F	GTAGTATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 42–R	TATACATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 43–F	GTCAAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 43–R	AGGACATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 44–F	GTGGAATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 44–R	ATAACATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 45–F	GTTTCATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 45–R	TAGGCATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 46–F	GCTGGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 46–R	GAATGATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 47–F	CTCTCATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 47–R	ACCAGATATACCTAGGTTGTGGGGTACAGCAATGAC
Primer 48–F	CGAGGATATACCTAGGTCCATACGCAGCTATGCAAT
Primer 48–R	ACGCGATATACCTAGGTTGTGGGGTACAGCAATGAC

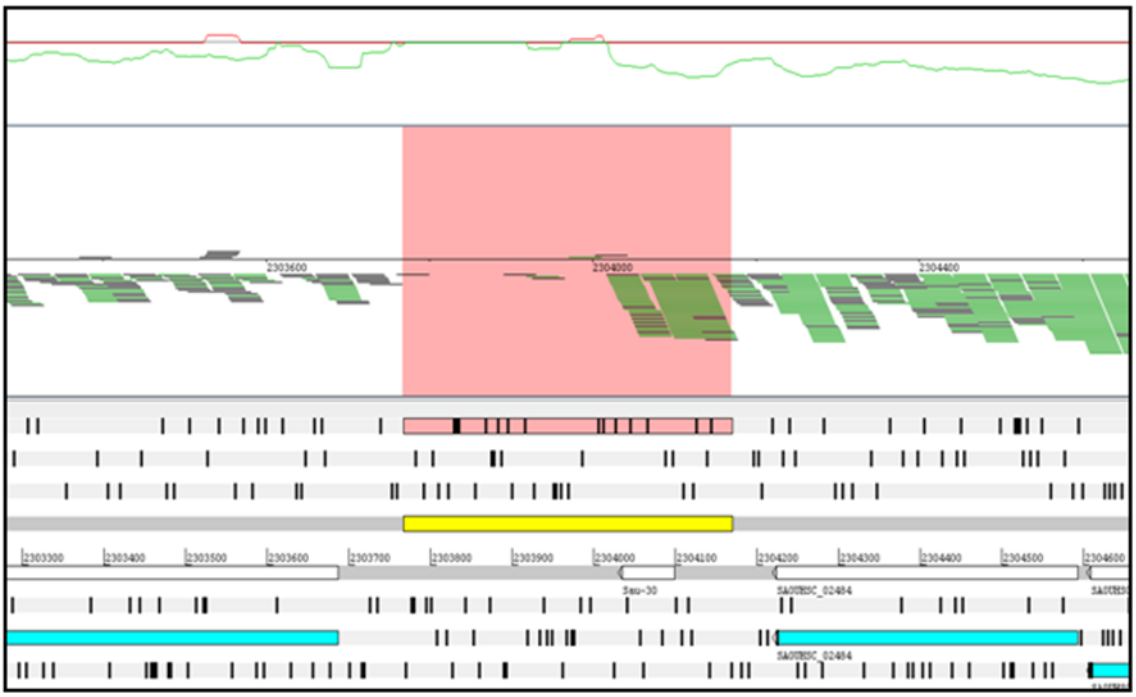
* Grey highlighted sequences are tag specific.

Table S6: Primers to generate *cwrA*/*rsaOV* complementing plasmids

Name*	Sequence
pcwrA-rsaOV-F	CTGCAGGTCGACTCTAGAGGTCAAATAAACCTTCGCCTATGC
pcwrA-rsaOV-R	CCATTCAGGCTGCGCAACTGAGTCAATCGTTGCGTTTTACTT
pcwrA-F	TTTCTTTTAAGTCACCTAAGACGACTTCTTTTATATAGATGCTAAGTAG
pcwrA-R	ATCTATATAAAAGAAGTCGTCTTAGGTGACTTAAAAGAAATCAGATGG
$pcwrA_{truncated}$-RsaOV-F	TATAAAGGAGTATGATAGCGTGCGAAAGAATTTAACCCATCTGA
$pcwrA_{truncated}$-RsaOV-R	ATGGGTTAAATTCTTTCGCACGCTATCATACTCCTTTATATTTCTCTT
prsaOV-F	TATAAAGGAGTATGATAGCGGTCACCTAAGAATTGCAAATCCAGA
prsaOV-R	ATTTGCAATTCTTAGGTGACCGCTATCATACTCCTTTATATTTCTCTT
$cwrA_{ATG\rightarrow TAA}$ UpF	GAATTCGAGACCGCTAGCGCACGAACGACTTTACAAGGGT
$cwrA_{ATG\rightarrow TAA}$ UpR	GTAATTAATATTCTTTACGCTATCATACTCC
$cwrA_{ATG\rightarrow TAA}$ DwF	GCGTAAAGAATATTAATTACAGGCACA
$cwrA_{ATG\rightarrow TAA}$ DwR	GATATCGGATCCGAGACCCTAGCTTTGCGTGACGTTTGAT

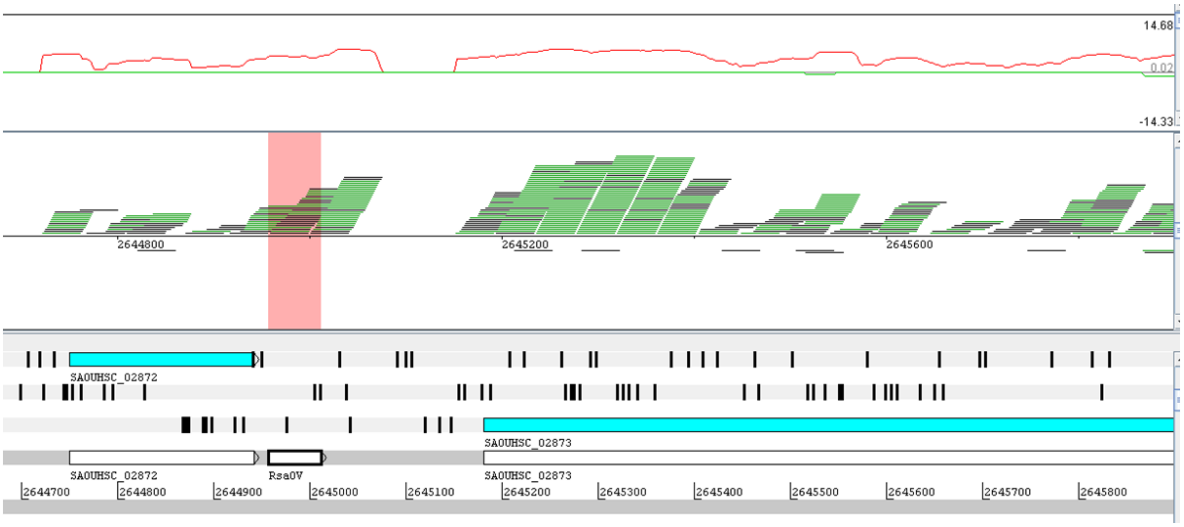
* Up and Dw refer to upstream and downstream loci of the referred gene, respectively. F and R indicate that concerned primers are in the Forward or Reverse orientation with respect to the chromosome annotation, respectively.

Figure S1.



Sau30 locus. RNA-seq results are visualized with Artemis sequence editor tool (Rutherford et al, 2000). (Top) Ln of read coverage track; forward and reverse sequences are in red and in green, respectively. (Middle) BamView of mapped reads; forward and reverse sequences are above and under the line, respectively. (Botton) HG003 Artemis representation using NCTC8325 nomenclature. (blue boxes) CDSs; (white boxes) CDSs and *sau30*; (yellow box) region covered by the *sau30* deletion.

Figure S2.



***cwrA rsaOV* locis**. RNA-seq results are visualized with Artemis sequence editor tool (Rutherford et al, 2000). (Top) Ln of read coverage track; forward and reverse sequences are in red and in green, respectively. (Middle) BamView of mapped reads; forward and reverse sequences are above and under the line, respectively. (Botton) HG003 Artemis representation using NCTC8325 nomenclature. (blue boxes) CDSs; (white boxes) CDSs and *rsaOV* locus.

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CHAPTER A2

Identification of sRNA phenotypes in *S. aureus* associated with growth adaptation and virulence

RESULTS

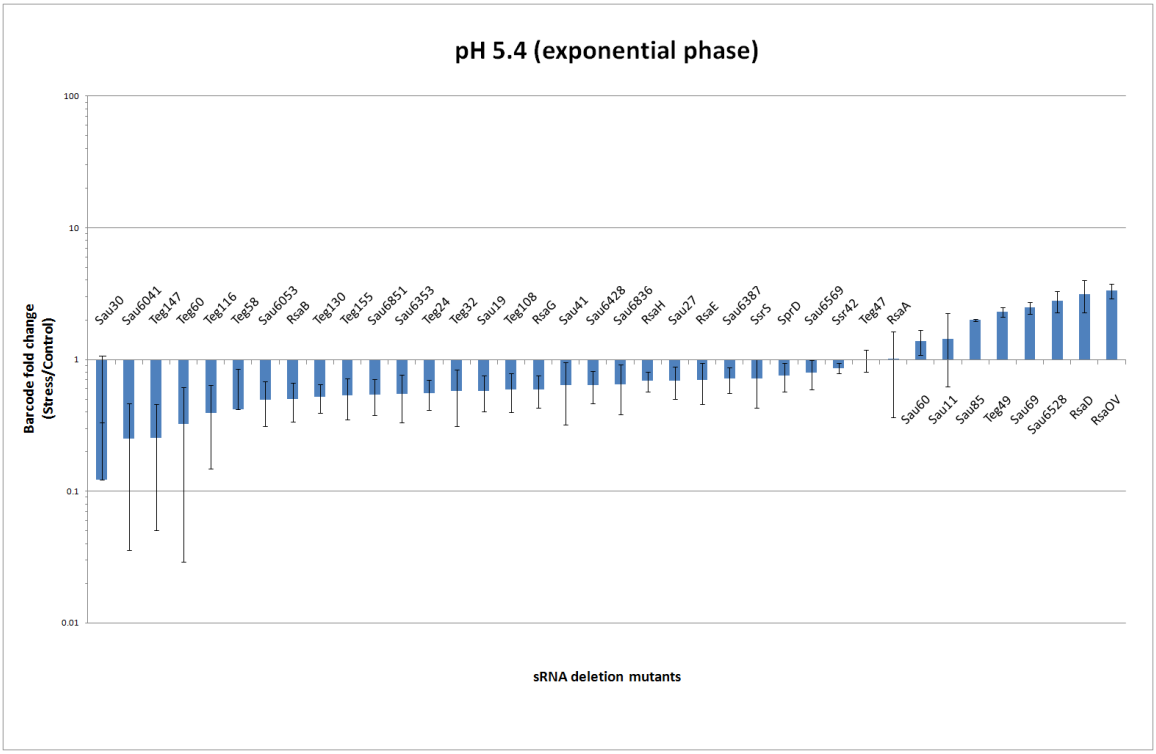
In addition to hot and cold temperatures, we tested the sRNA-deleted mutant set (in triplicate) previously described in competition assays with growth conditions mimicking situations associated with the infectious process. We also tested directly the fitness of mutants in a mouse model of infection. Experiments were performed as described in Chapter A2.

1. Adaptation to acidic and alkaline conditions

Acid and alkaline medium were obtained by adjusting the pH to 5.4 with 1M HCl or 8.68 with 1M NaOH, respectively. In acidic condition, no mutant had a significant phenotype in exponential phase (Sampling 1). Mutant *sau30* seemed underrepresented but the standard deviation was too important for the observation to validate the conclusion (Figure 1). However, no read corresponding to mutant *sau30* was detected when the mixed population went through stationary phase and underwent a second exponential growth phase (Sampling 2) at pH 5.4. The mutant *sau30* completely disappeared under this growth condition (Figure 1). It is the most drastic phenotype that we observed among all tested conditions. The mutant *sau6428* was also underrepresented in Sampling 2 (Figure 1).

The phenotype of mutant *sau30* was confirmed by comparing *sau30* and its parental strain growing individualy in a medium at pH 5.4 (Figure 2).

In alkaline growth medium, mutant *sau6836* had a significant growth defect in competition assays (Figure 3) and its phenotype was exacerbated by growing the mix population to stationary phase and diluting it again (Figure 3). The phenotype was confirmed by growing *sau6836* and its parental strain separately in an alkaline medium (Figure 4).



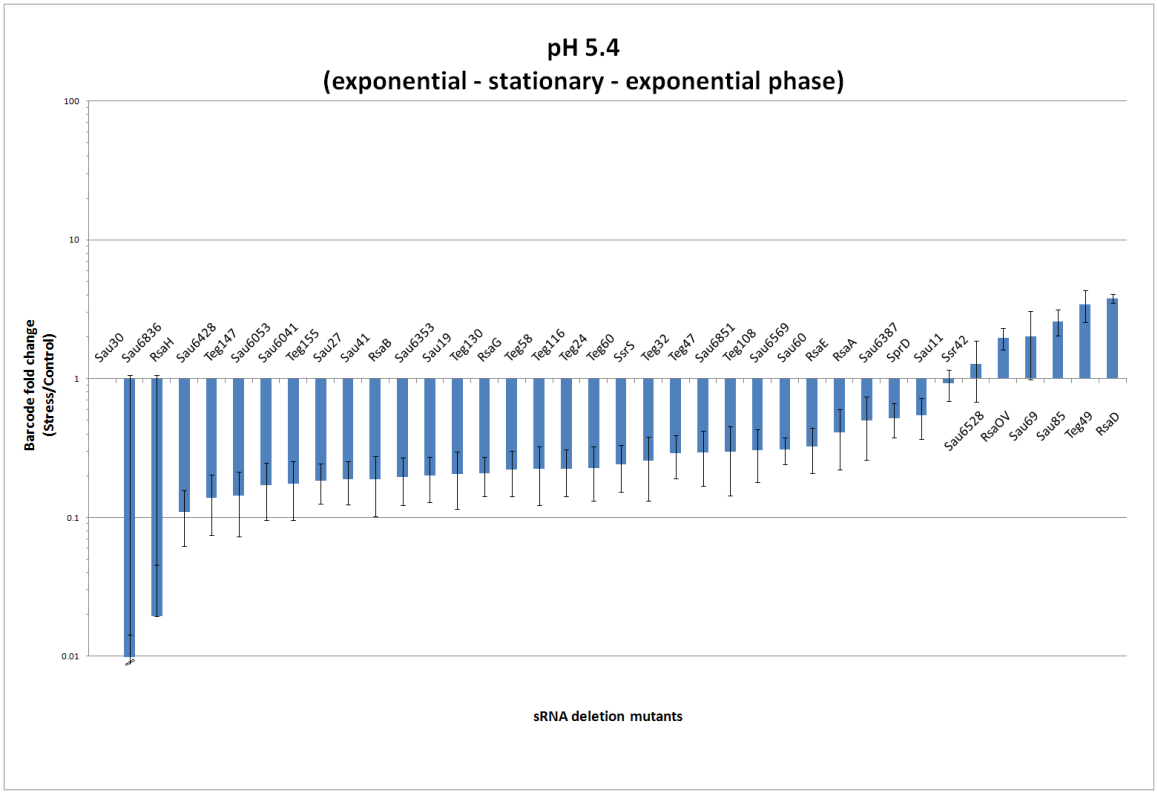
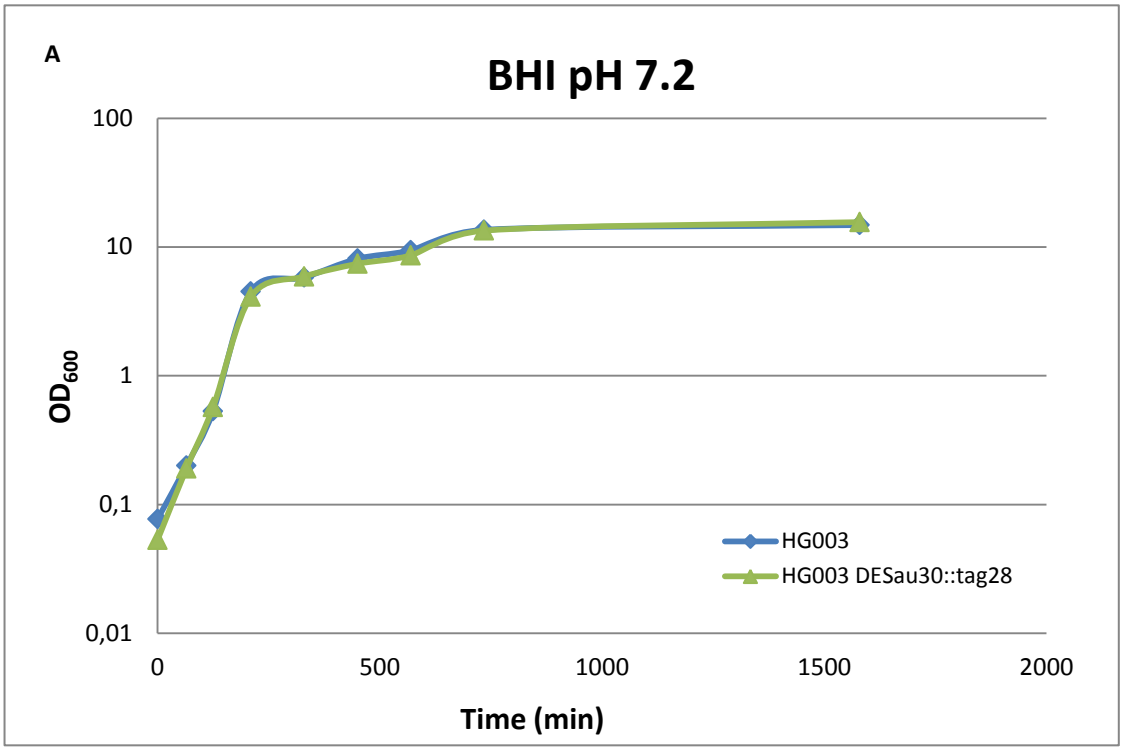


Figure 34: Competition assay at pH 5.4. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) at pH 5.4 compared to a reference at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.



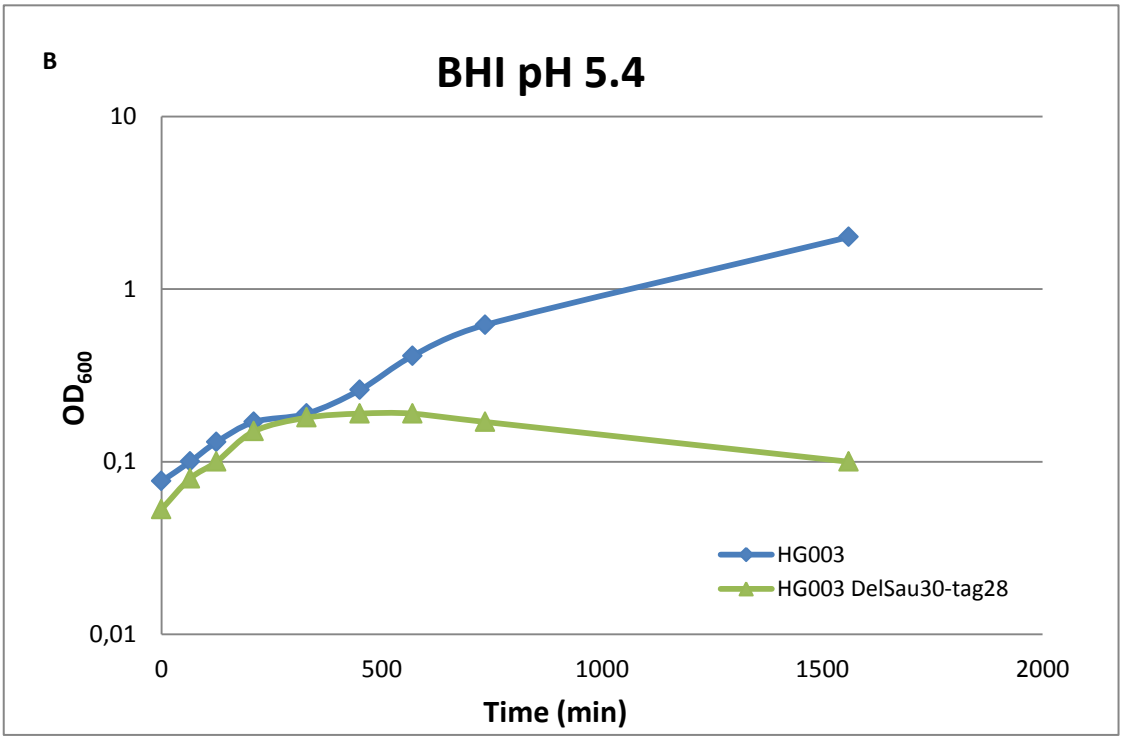


Figure 2: Growth defect of *sau30* mutant at pH 5.4. Growth curves of HG003 (blue) and *sau30* (green). Overnight cultures were diluted 200-fold in (A) BHI medium at pH 7 and (B) at pH 5.4. Cultures were grown under a vigorous agitation at 37°C.

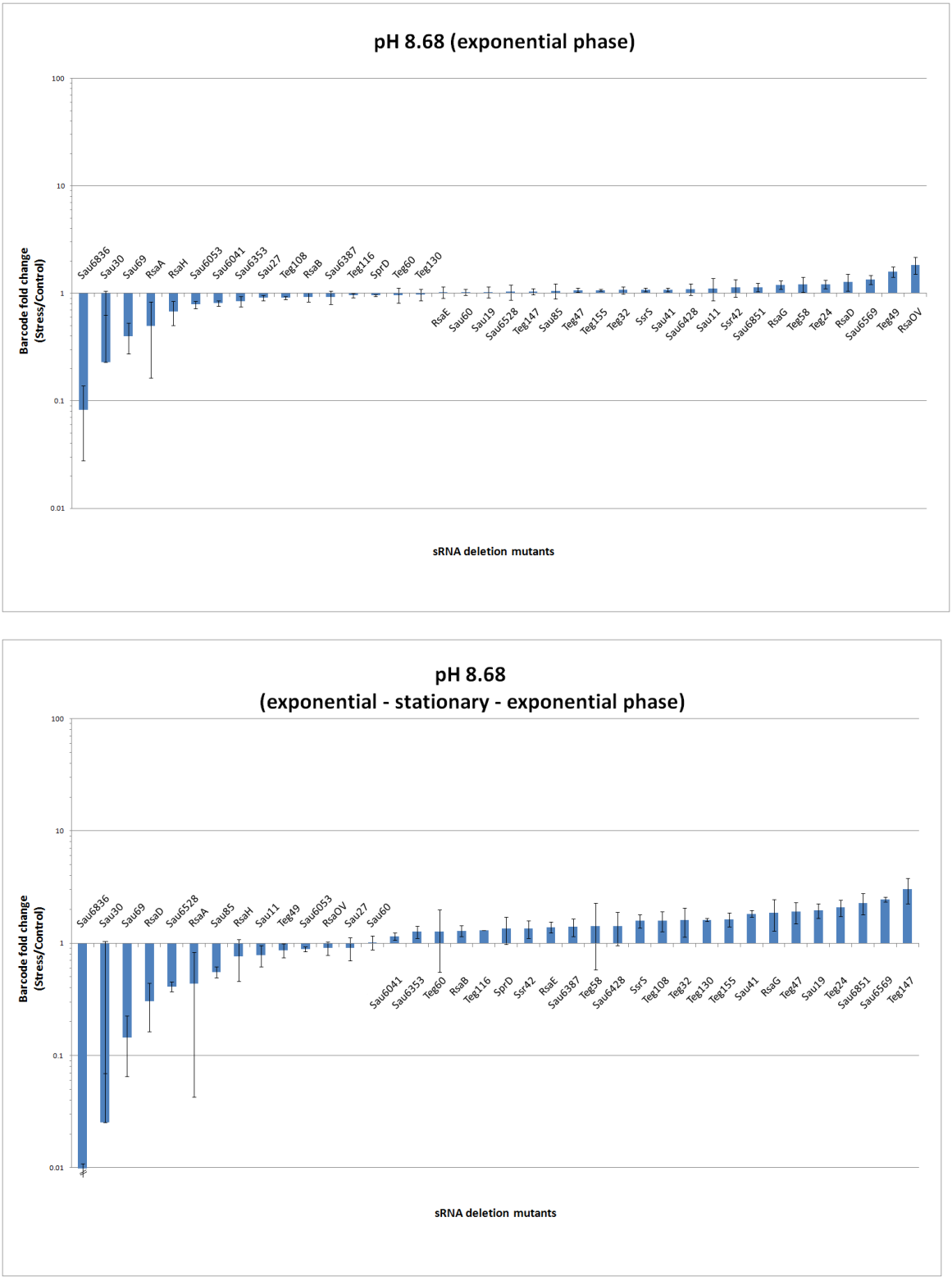


Figure 3: Competition assay at pH 8.68. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) at pH 8.68 compared to a reference at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.

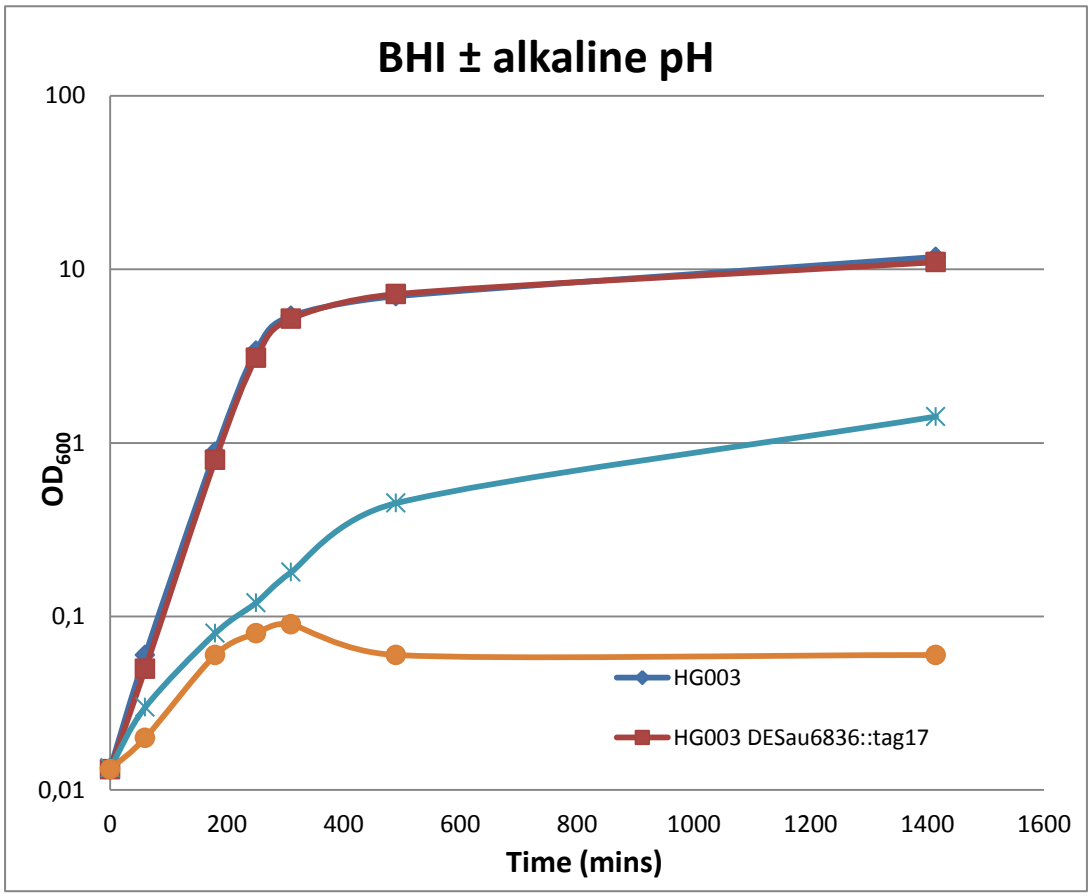
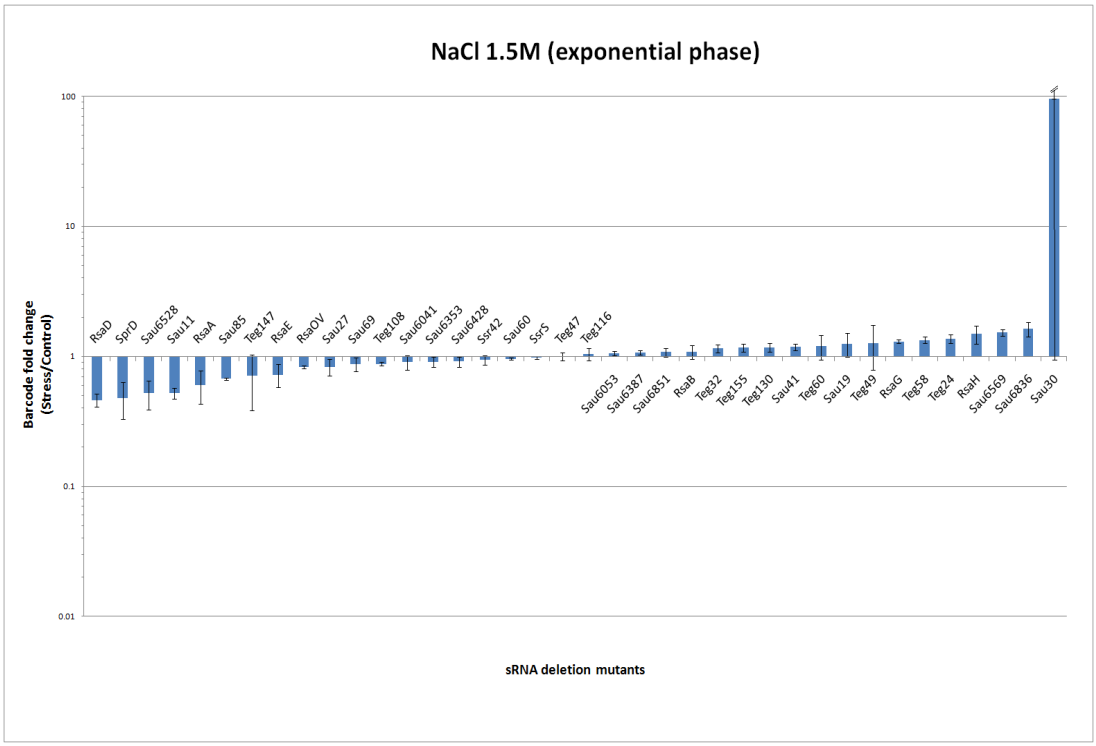


Figure 4: Growth defect of *sau6836* mutant at pH 8.68. Growth of HG003 (blue) and *sau6836* (red) in BHI medium; HG003 (light blue) and *sau6836* (orange) in BHI medium alkaline pH. Overnight cultures were diluted 200-fold in BHI medium at pH 7.2 (control) and at pH 8.6 (alkaline condition). These cultures were cultivated at 37°C under vigorous aeration.

2. Adaptation to high osmolarity and oxidative conditions

High osmolarity condition was set up by adding NaCl (1.5M) to BHI medium. None of the mutants from the tagged sRNA-deletion set was significantly affected when grown to exponential phase in high osmolarity medium (Figure 5). However, when the culture set was grown to stationary phase, diluted in the same medium (Sampling 2), mutants *teg147*, *rsaD* and *sau6528* were underrepresented and mutant sau6569 was accumulated (Figure 5).



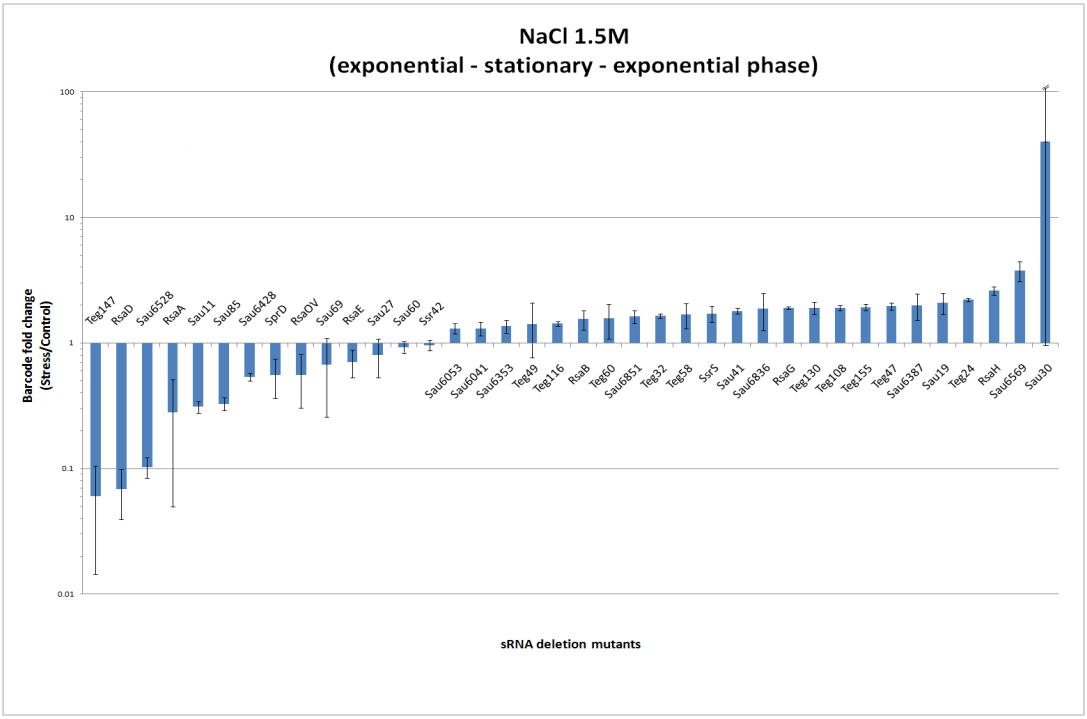
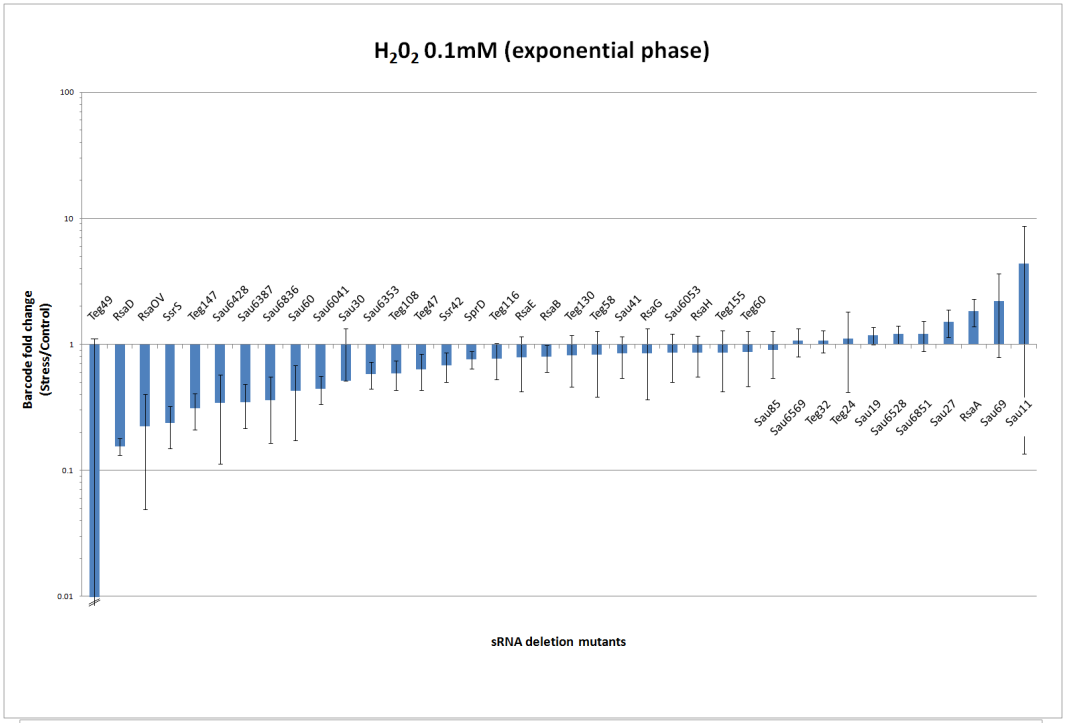


Figure 5: Competition assay at NaCl 1.5M. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) at NaCl 1.5M compared to a reference at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.

An oxidative condition was obtained by adding H_2O_2 (0.1mM) to the medium. Mutant *rsaD* was underrepresented in this condition (Figures 6). Two mutants *rsaOV* and *ssrS* were underrepresented, only when the population went through stationary phase (Figure 6).



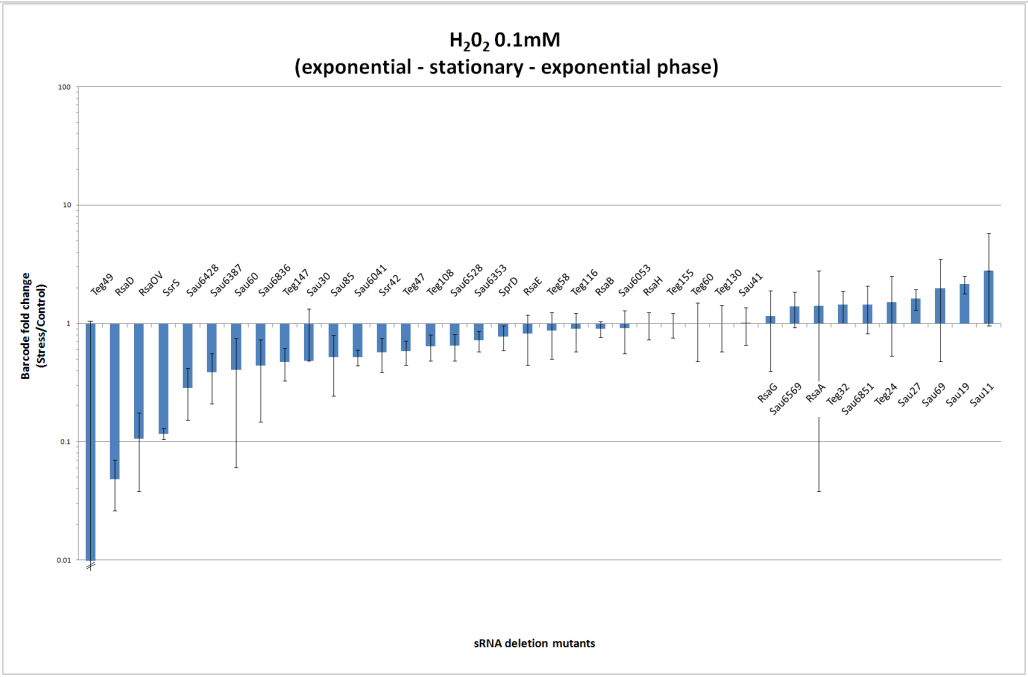


Figure 6: Competition assay at H_2O_2 0.1mM. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) at H_2O_2 0.1mM. compared to a reference at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.

3. Growth in RPMI and RPMI no-folate media

We investigated the effect of sRNA deletions on different culture media with our of tagged sRNA-deletion set. We used RPMI (Roswell Park Memorial Institute) medium which was originally used for human cell culture; it contains amino acids, vitamins, inorganic salts and glucose (Moore et al. 1967). This medium is also be used to culture bacteria under iron-limited condition. In addition, the RPMI-derivative named RPMI no-folate medium which does not contain folic acid was also used.

None of the mutants had significant phenotype in exponential phase when grown in these two media (Figures 7 & 8, upper histograms). While mutant *rsaH* grew badly in RPMI medium after the population went through stationary phase (Figure 7, lower histogram), mutant *sau6836* surprisingly completely disappeared in RPMI medium and also grew badly in the second phase in RPMI no-folate medium (Figures 7 & 8, lower histograms).

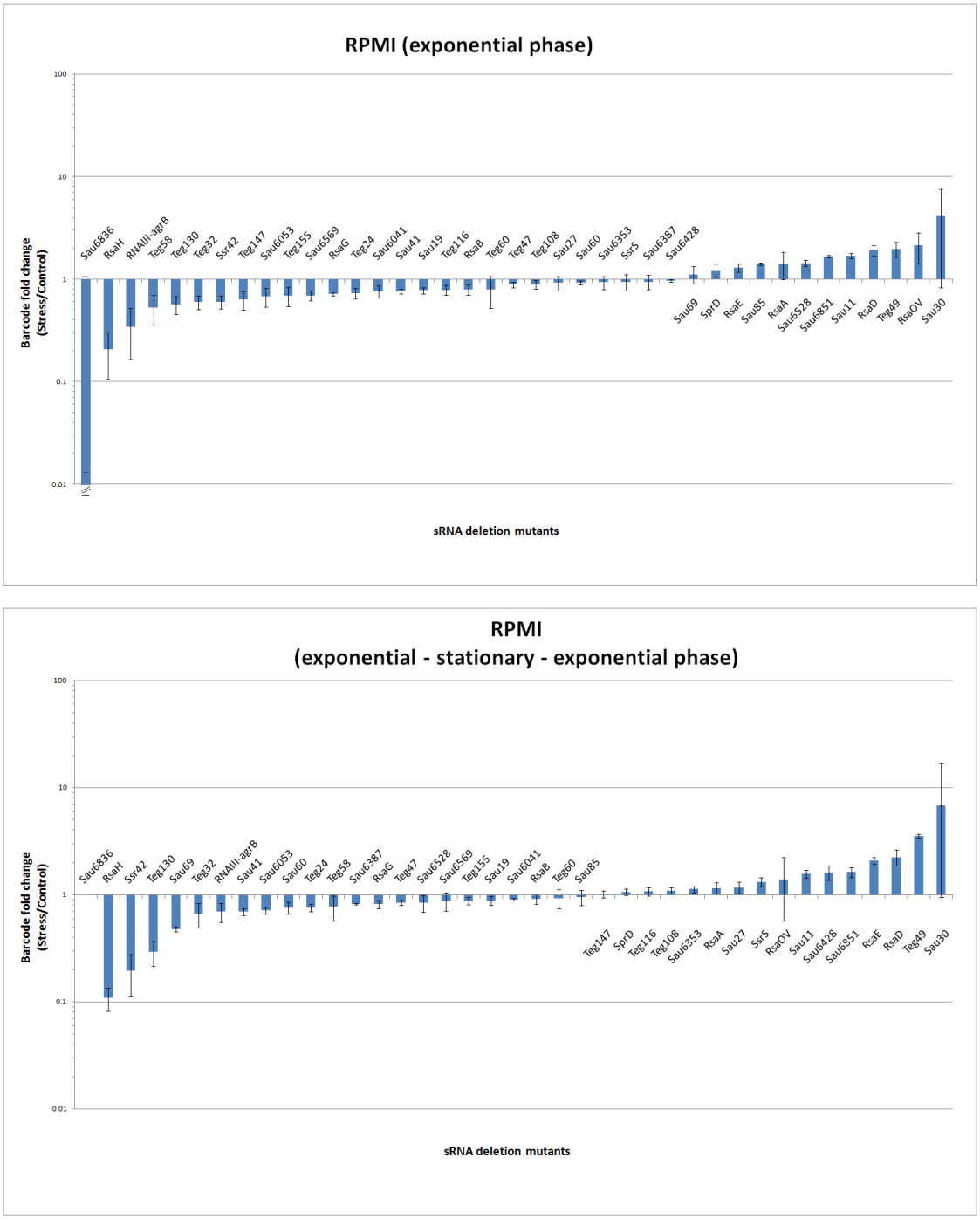


Figure 7: Competition assay in RPMI medium. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) in RPMI medium. compared to a reference in BHI medium at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.

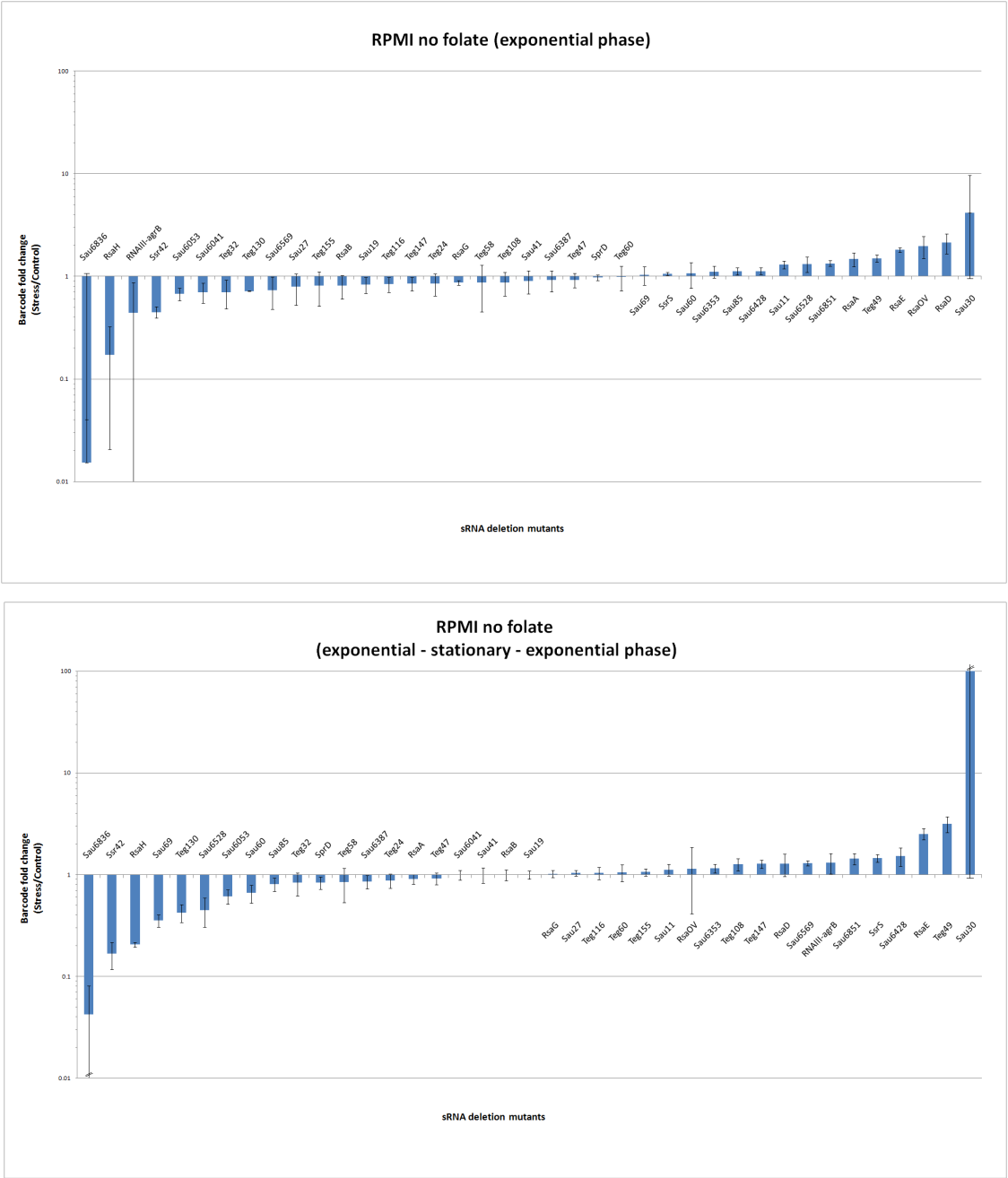
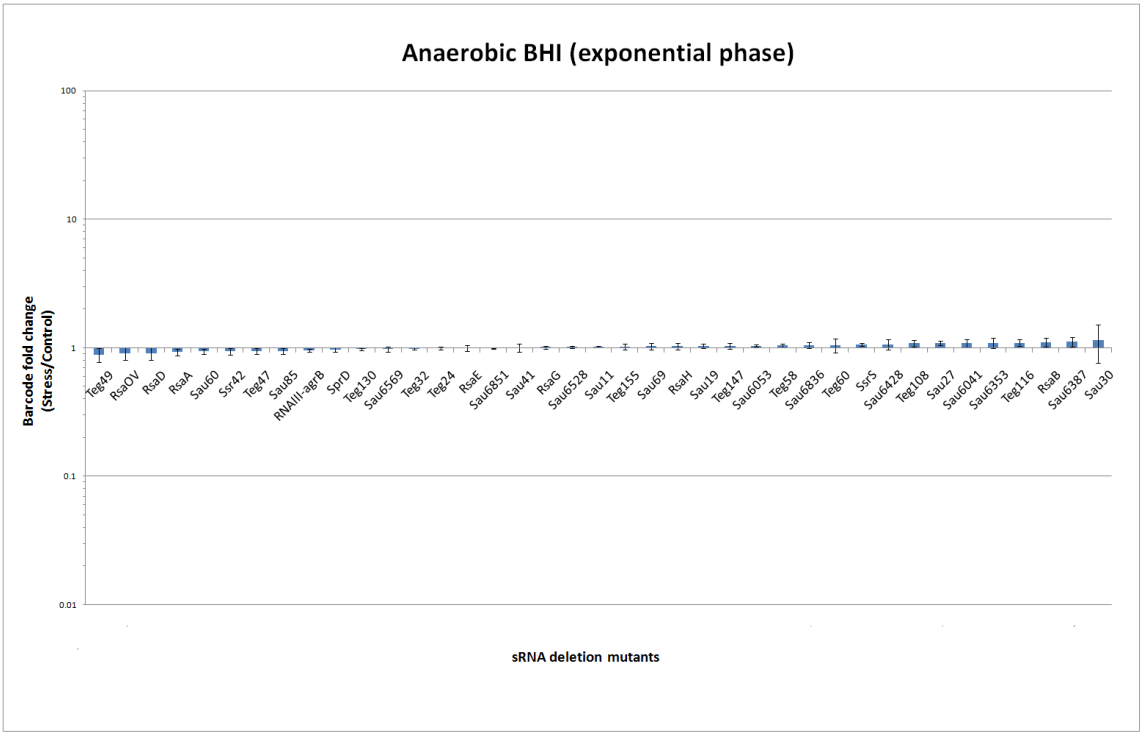


Figure 8: **Competition assay in RPMI no-folate medium.** Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) in RPMI medium. compared to a reference in BHI medium at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.

4. Anaerobic growth in BHI and RPMI media

S. aureus is a facultative anaerobe; hence we tested the effect of sRNAs under oxygen-limited conditions. These conditions were performed by growing bacteria in Falcon tube (50 mL) completely filled with BHI or RPMI medium.

No mutant had significant phenotype in exponential phase (Sampling 1) in anaerobic BHI and anaerobic RPMI media (Figures 9 & 10, upper histograms). However, when the population underwent stationary phase (Sampling 2), the quantity of mutant *sau30* varied either up (RMPI anaerobiosis) or down (BHI anaerobiosis) but with an unusual great variability making the results difficult to interpret (Figures 9 & 10, lower histograms). In contrast, mutant *teg49* accumulated in anaerobic RPMI medium (Figures 10).



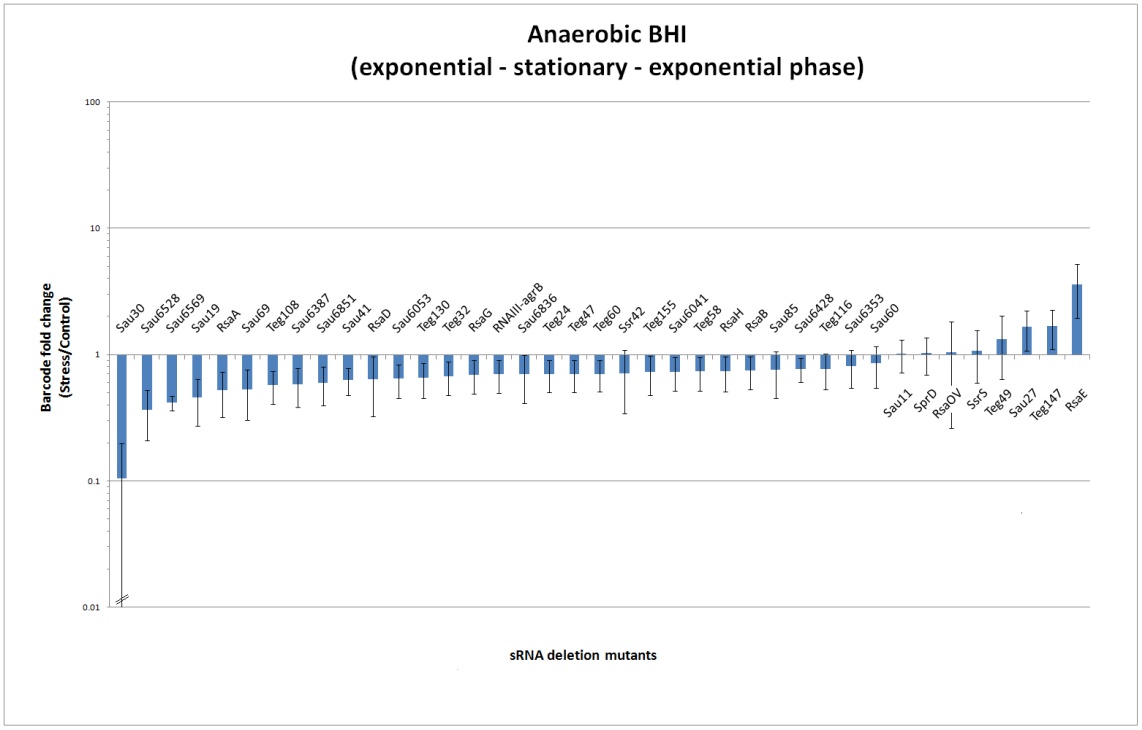
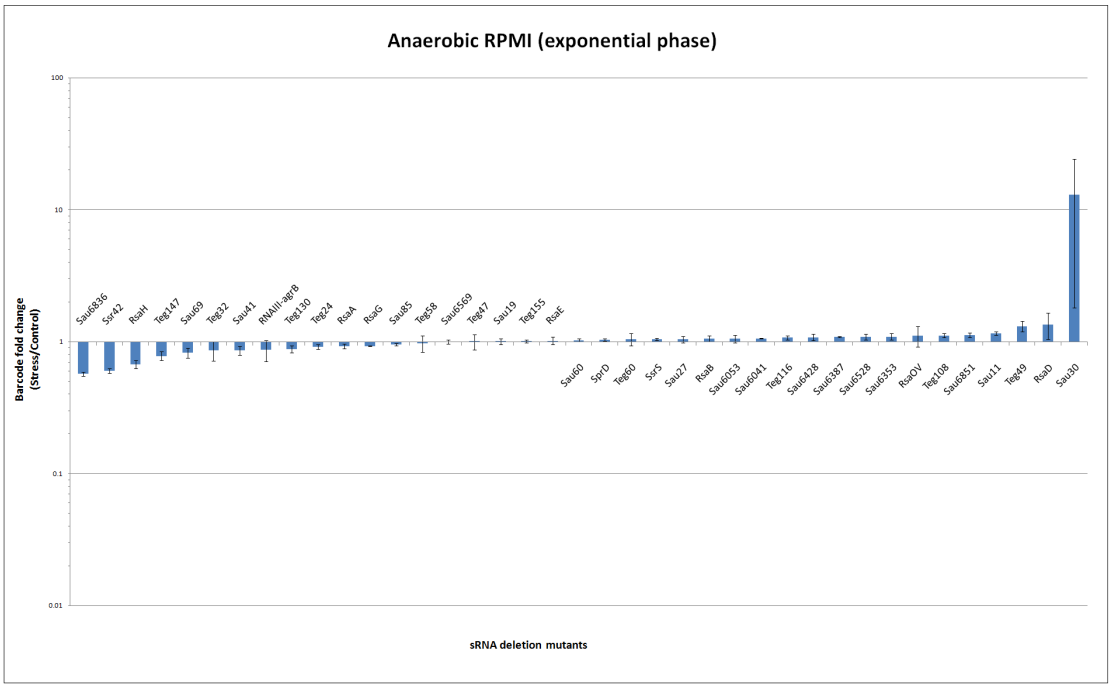


Figure 35: Competition assay in anaerobic BHI medium. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) in **anaerobic BHI** medium. compared to a reference in aerobic BHI medium at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.



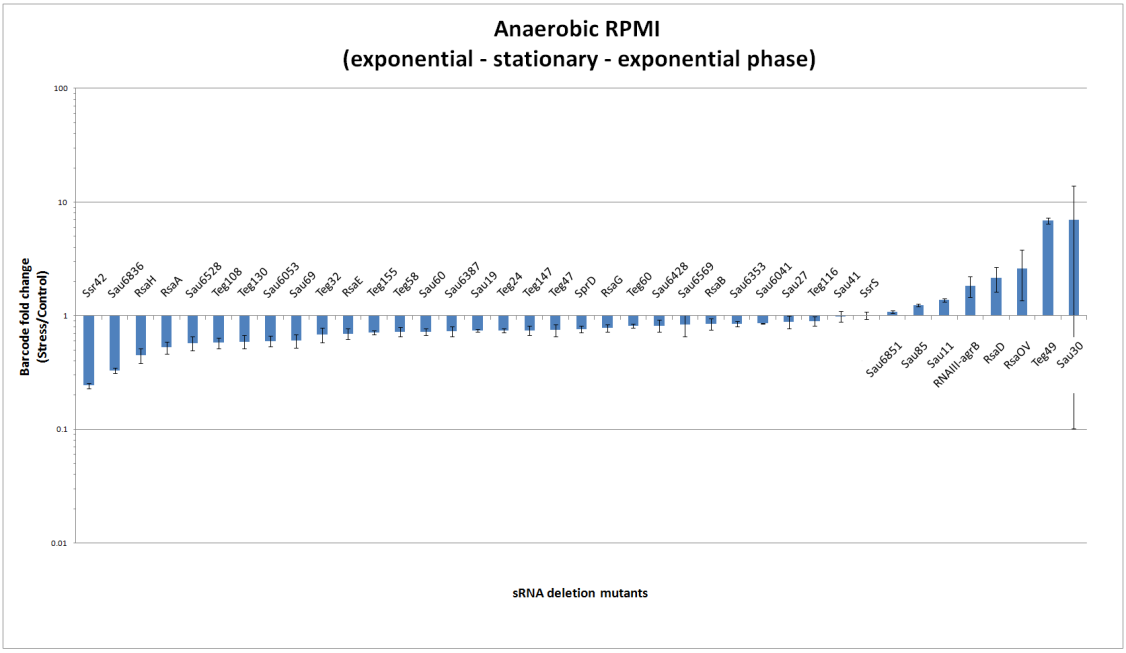
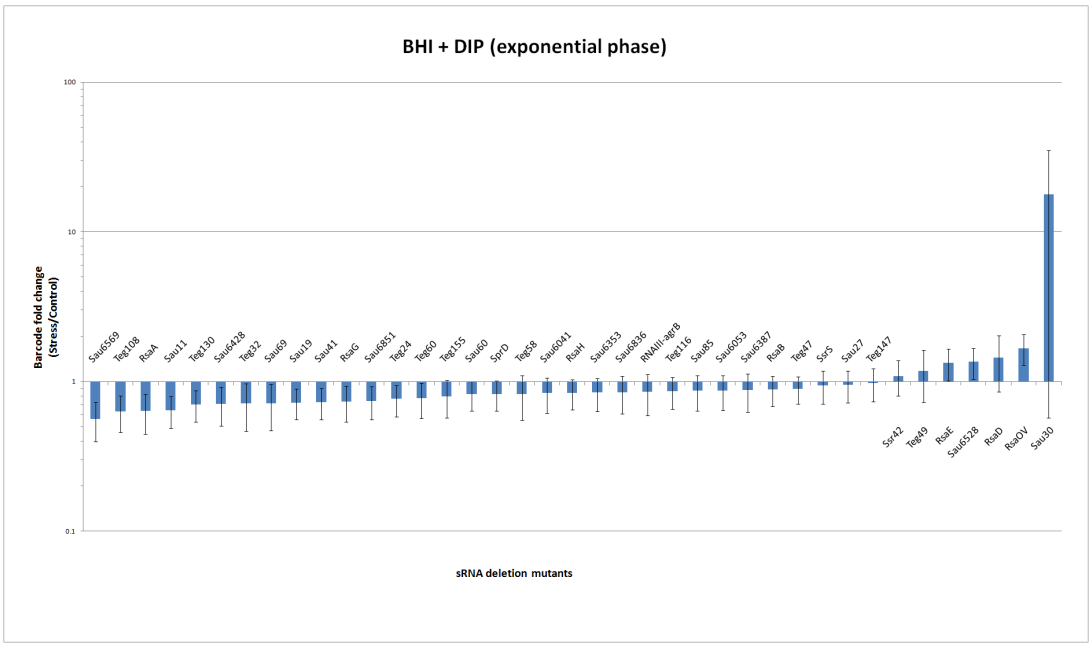


Figure 10: Competition assay in anaerobic RPMI medium. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) in anaerobic BHI medium. compared to a reference in aerobic BHI medium at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.

5. Adaptation to iron depletion and human serum

To investigate iron-*deficient* effects on sRNA gene deletions, DIP (2,2'-Bipyridyl) (1.4mM) was added to BHI medium to chelate its iron. To mimic the infection process, the mix of mutant strains was grown in BHI medium containing human serum (10%). In Sampling 1, no mutant had significant phenotype in both conditions (Figures 11 & 12).

However, when the population went through stationary phase (Sampling 2), mutants *teg49* and *rsaOV* were overrepresented in *iron-depleted medium* and in human serum, respectively (Figures 11 & 12, lower histograms).



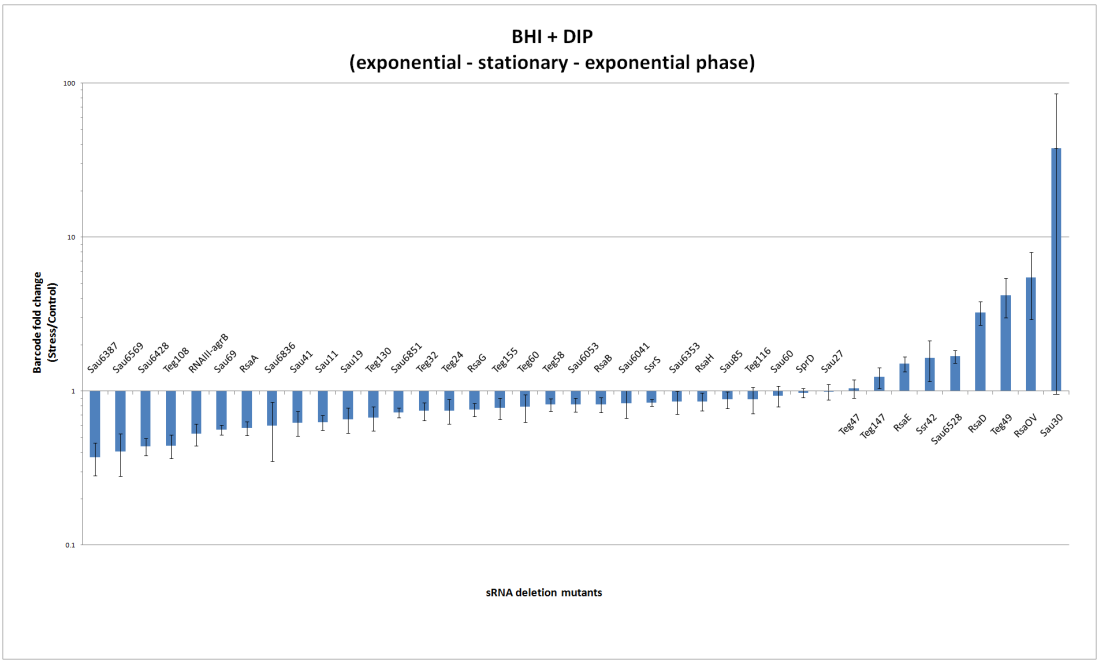
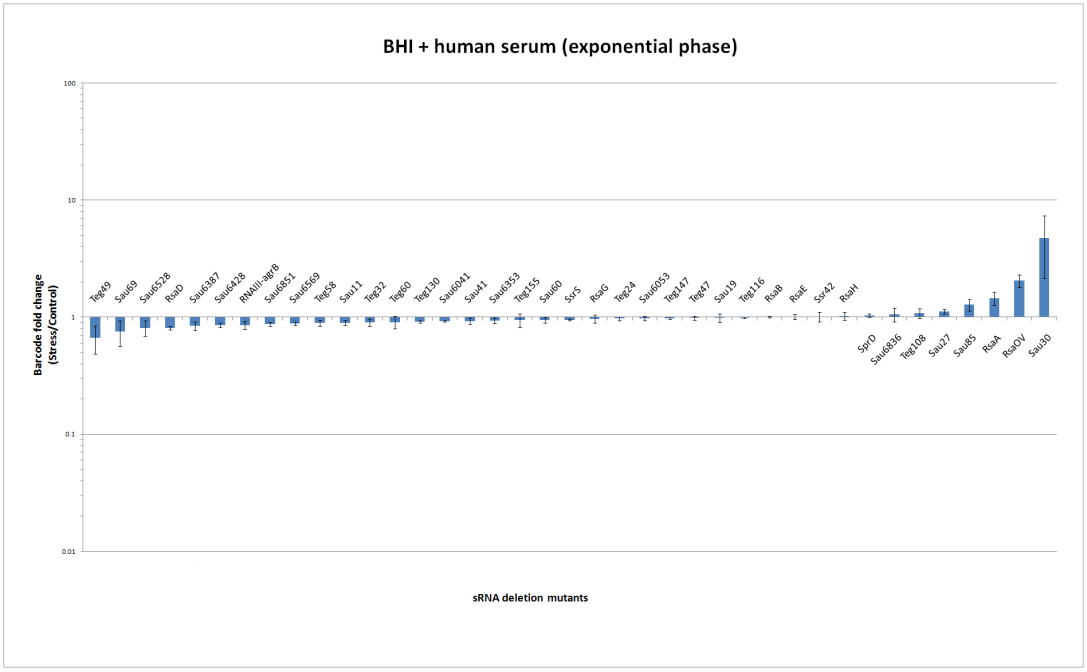


Figure 11: Competition assay in BHI medium containing DIP (1.4mM). Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) at DIP 1.4mM compared to a reference at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.



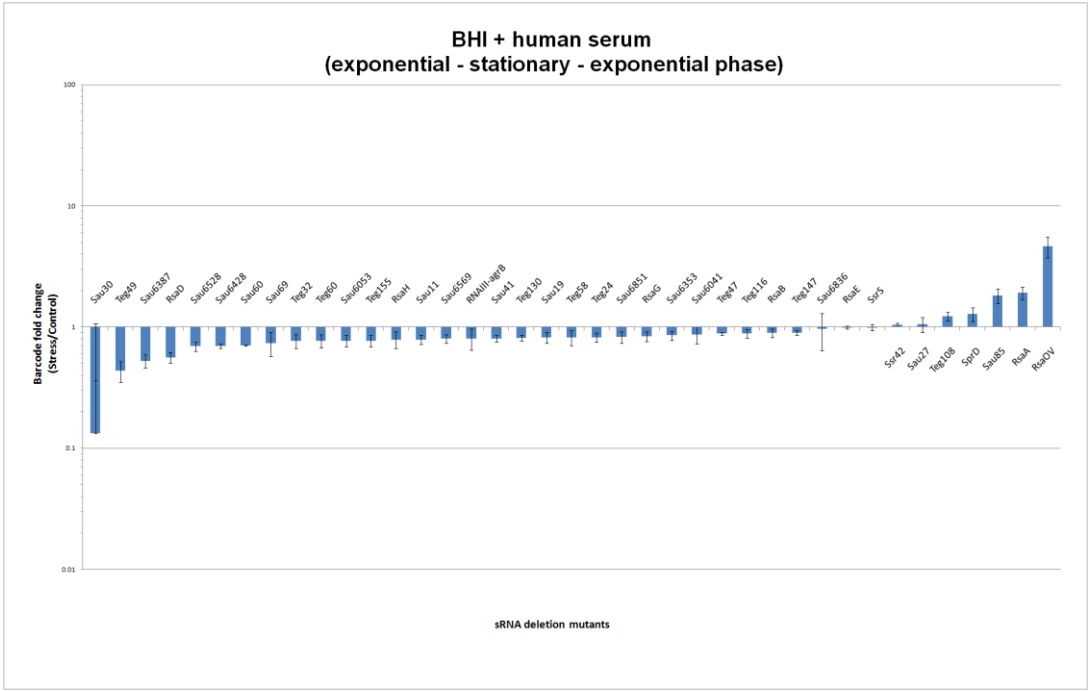


Figure 12: Competition assay in BHI medium containing human serum (10%). Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) in BHI medium containing human serum (10%) compared to a reference at 37°C. Upper and lower histograms correspond to Sampling 1 and 2, respectively. Data are shown as average values and the standard deviation of triplicate samples is indicated.

6. Growth competition experiments in mice

7-week-year-old BALB/c female mice were chosen for an animal model test. The mix of sRNA-deleted strains (6-7x10^7 CFU/500µl) was injected in 10 mice and performed in duplicate. Unexpectedly, only 3 in the total 20 mice survived just after 24-hours post infection. Blood, spleen and kidney samples were collected only from 3 surviving mice while liver samples were collected from total 20 mice. All mutants were evenly represented in blood, spleen and liver samples (Figures 13, 14, 16 & 17) while in kidney samples, mutant *rsaOV* grew badly (Figure 15). The validation of these observations will require repeating these experiments with inoculums containing fewer bacteria.

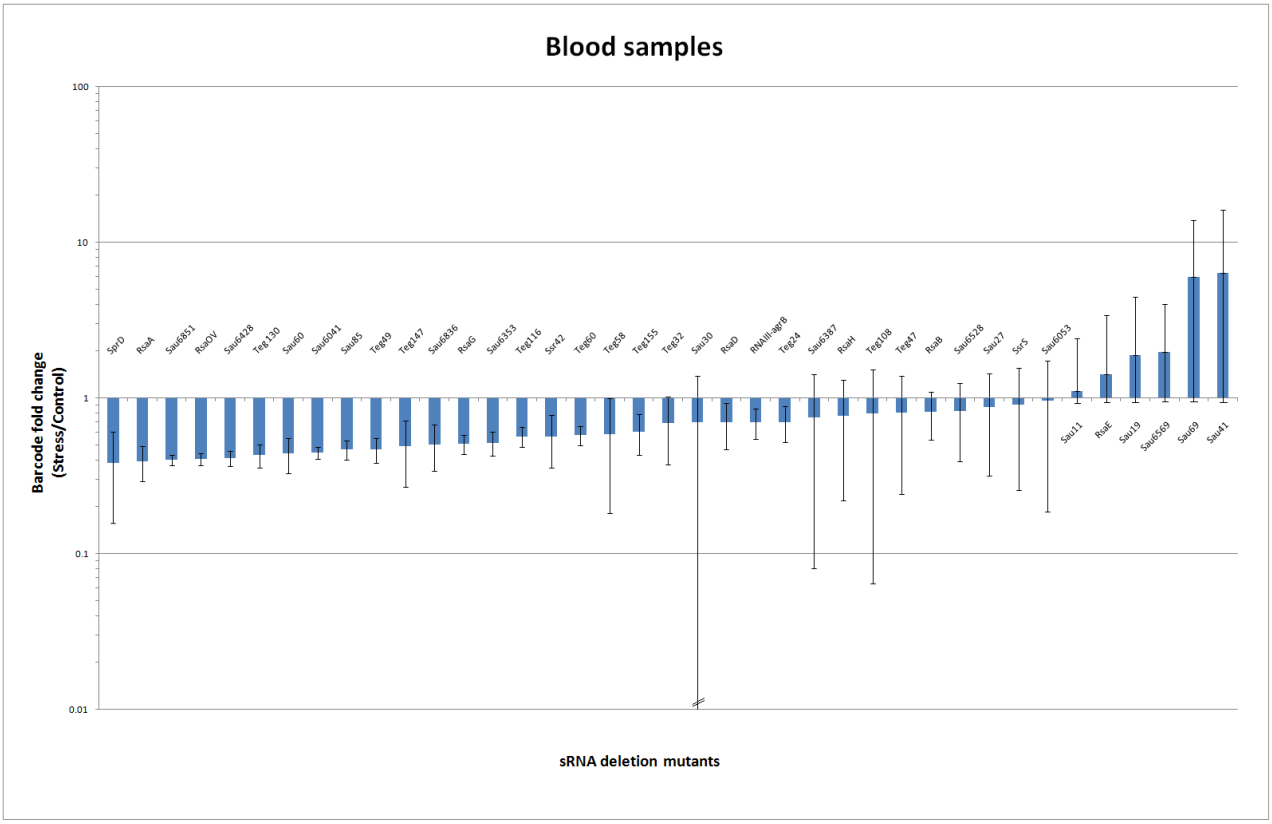


Figure 13: Competition assay from blood samples in mouse model. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) in blood samples compared to an uninfected reference sample. Data are shown as average values and the standard deviation of three samples is indicated.

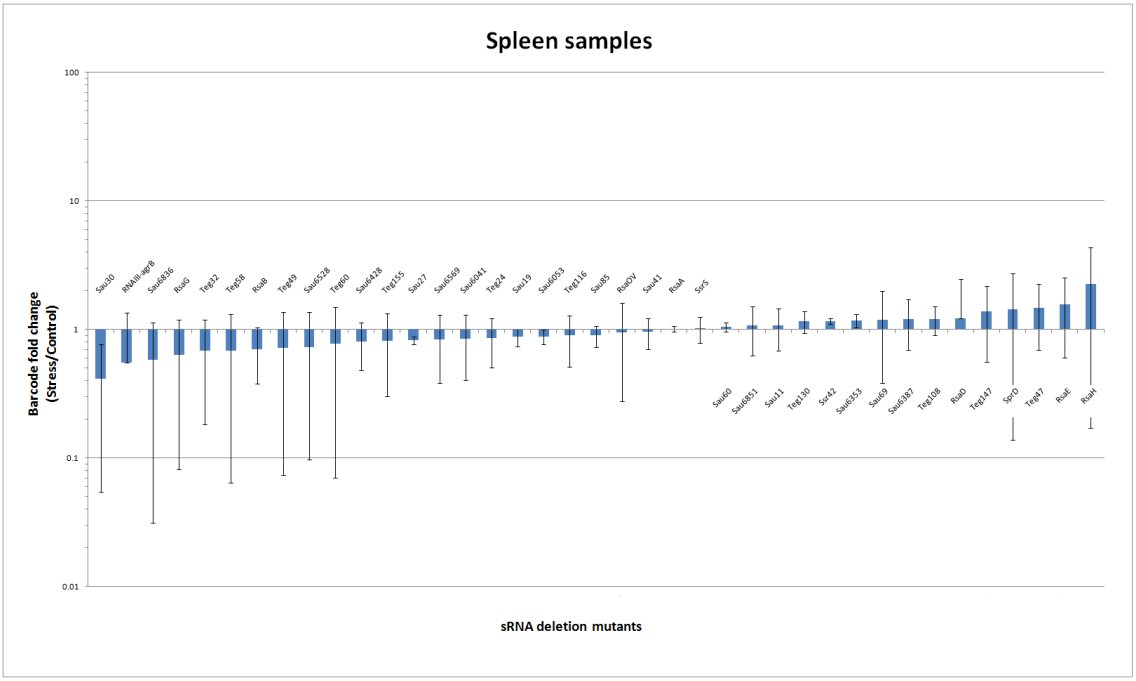


Figure 14: Competition assay from spleen samples in mouse model. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) in blood samples compared to an uninfected reference sample. Data are shown as average values and the standard deviation of three samples is indicated.

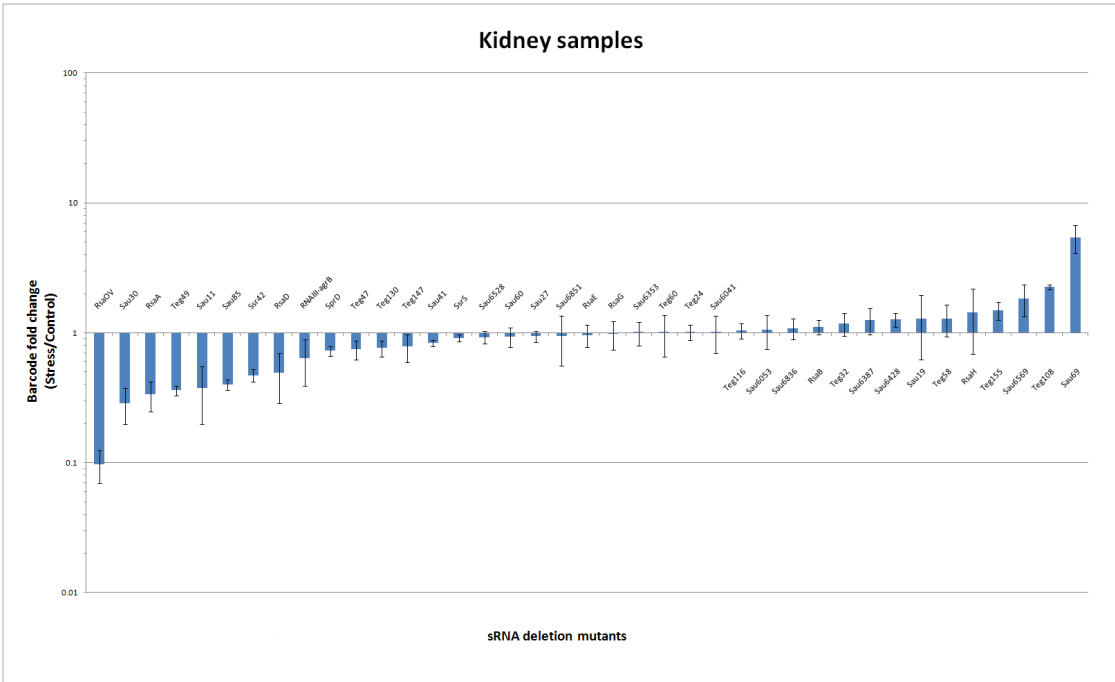


Figure 15: Competition assay from kidney samples in mouse model. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) in blood samples compared to an uninfected reference sample. Data are shown as average values and the standard deviation of three samples is indicated.

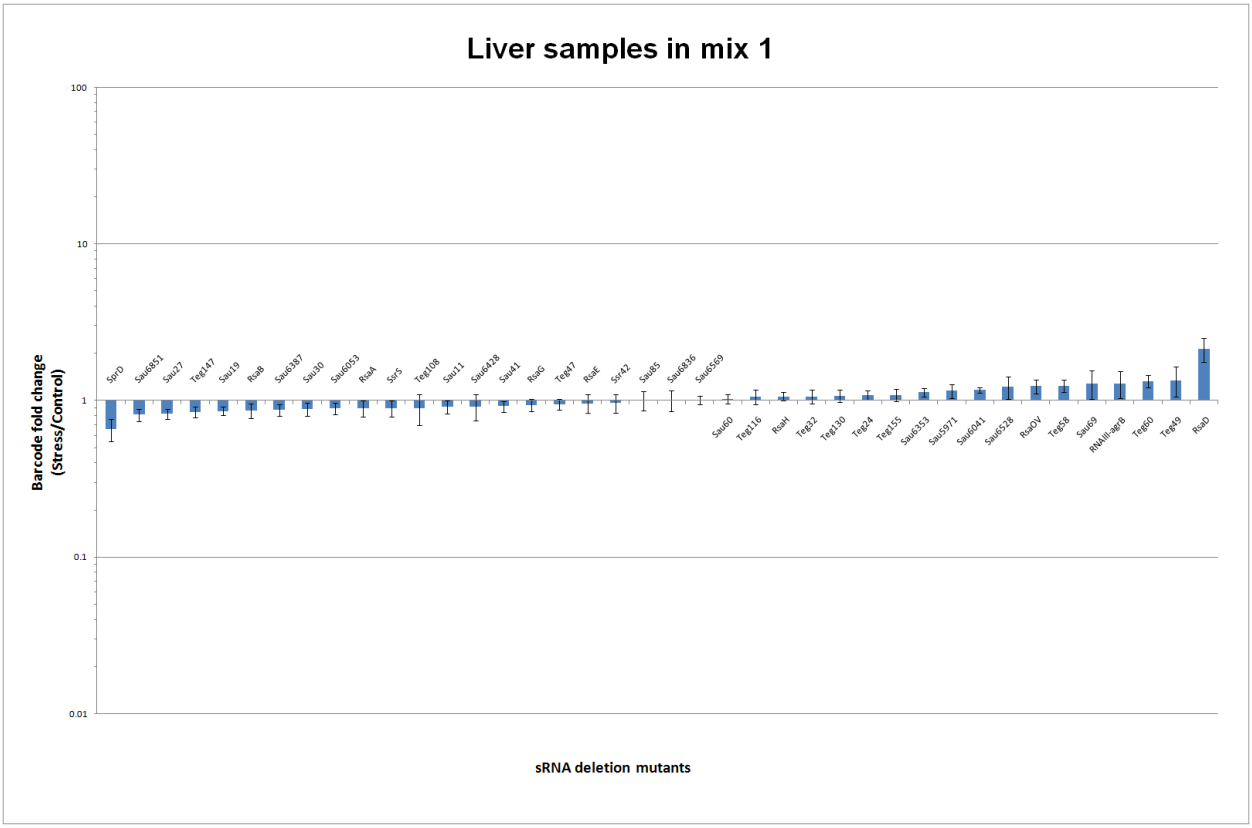


Figure 16: Competition assay from liver samples in mouse model. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) in blood samples compared to an uninfected reference sample. Data are shown as average values and the standard deviation of ten samples in mix 1 is indicated.

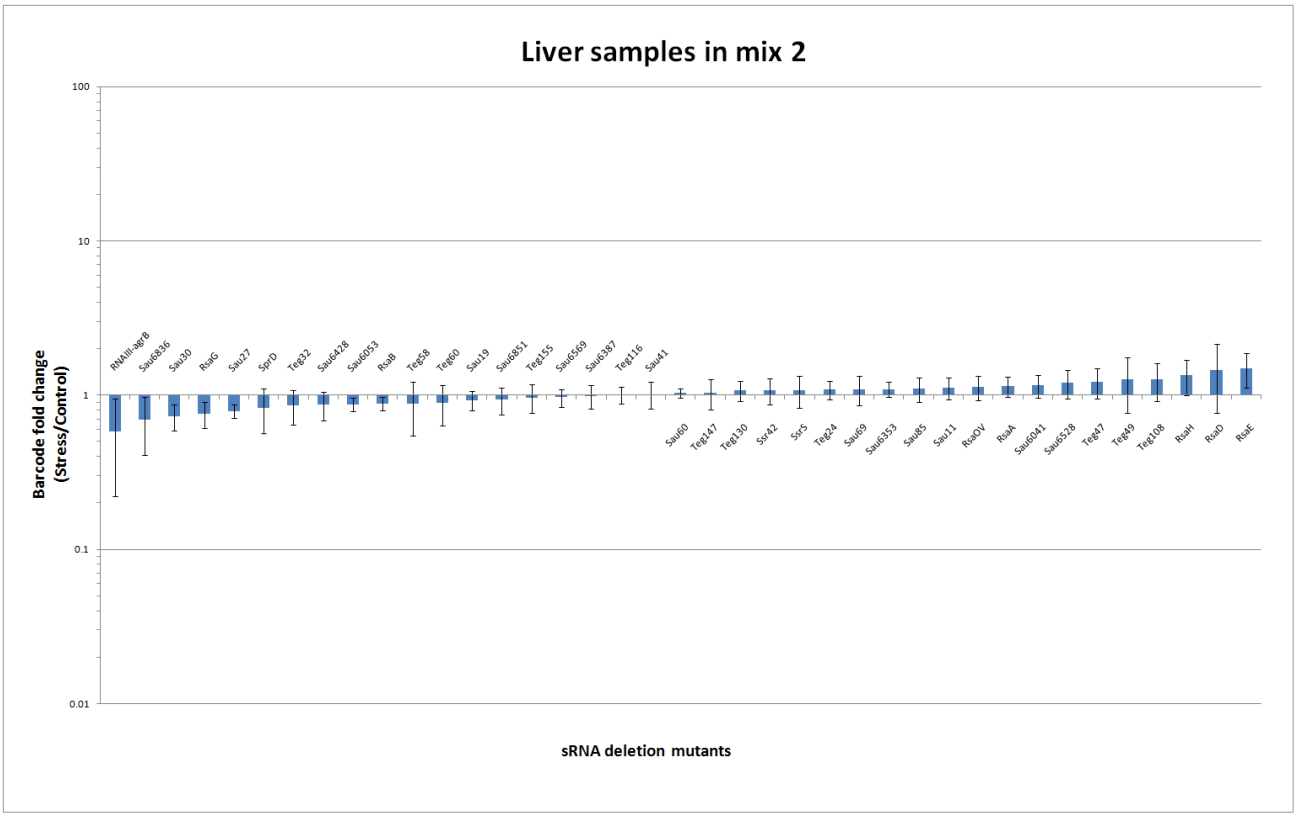


Figure 17: Competition assay from liver samples in mouse model. Histograms representing the disappearance (lower bars) or accumulation (upper bars) of indicated deletion mutants (x-axis) in blood samples compared to an uninfected reference sample. Data are shown as average values and the standard deviation of ten samples in mix 2 is indicated.

CHAPTER B

Trapping bacterial sRNA targets identifies sRNA-controlled pathways in *Staphylococcus aureus*

Trapping bacterial sRNA targets identifies sRNA-controlled pathways in *Staphylococcus aureus*

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Running title: Trapping sRNA-targets in *S. aureus*

Abstract

Bacterial regulatory RNAs (sRNA) generally act by base-pairing with target mRNAs. While identification of sRNA targets is the essential step in sRNA characterization, it remains a stumbling block in most studies. To study sRNA-regulated networks in the major human pathogen *Staphylococcus aureus*, we developed a robust procedure for identifying sRNA targets based on synthetic sRNAs that are used *in vitro* as bait to trap their corresponding targets. The key to target discovery lies in the differential analysis of RNA-seq data from captures with different sRNAs. This strategy was applied to study four staphylococcal sRNAs. Multiple putative targets per sRNA were identified and used to predict recurrent motifs that seed sRNA-target interactions. Confirmed targets demonstrate that RsaA, RsaE and RsaH sRNAs associated with mRNAs encoding autolysins, arginine and lactate metabolisms, respectively. RNAIII is the most extensively characterized *S. aureus* virulence regulator; we discovered new RNAIII targets that bring to light its control of mRNAs implicated in iron uptake.

Author summary

Bacterial small RNAs (sRNAs) are key regulators of homeostasis and adaptation responses that usually base-pair with their target RNAs. To decipher sRNA-dependent networks of *Staphylococcus aureus*, we developed a method to trap sRNA targets that combines RNA-seq, differential computational analysis and an *in vivo* validation step. We applied this strategy to investigate the function of four staphylococcal sRNAs; numerous targets were validated and specific roles for these sRNAs were assigned. One of them, RNAIII, is a paradigm for complex bacterial sRNAs and a virulence factor regulator that has been studied for over two decades. Here, we identified new RNAIII targets revealing that expression of virulence factors and iron import functions are coordinately controlled by a single sRNA.

Introduction

High throughput DNA sequencing methods to characterize bacterial transcriptomes have revealed an unexpectedly high number of small non-coding RNAs (sRNAs). So far, most characterized sRNAs exert regulatory activities via base-pairing with one or several RNAs, resulting in modulation of their translation and/or stability. The sRNAs seem to indiscriminately affect all cellular functions and contribute to bacterial homeostasis [1]. Remarkably however, sRNA gene deletions frequently have no detectable phenotype in laboratory conditions.

A major challenge in assessing the physiological roles of bacterial sRNAs is to find their targets [2]. Although widely used, computational target finding methods based on hybrid prediction algorithms do not yet reliably discriminate true targets from non-targets. A typical experimental approach relies on coprecipitation of RNA and RNA binding proteins such as Hfq, an RNA chaperone catalyzing intermolecular RNA pairing [3]. However, Hfq is not ubiquitous and in certain phyla (*e.g.*, Firmicutes) seems not to be required for general sRNA-based regulation [4]. We previously reported the identification of *Escherichia coli* sRNA targets using an sRNA as bait to capture its substrate within RNA extracts in the absence of RNA-binding proteins [5]. This method was accurate but limited by low sensitivity to the identification of abundant targets.

Staphylococcus aureus is a major opportunistic pathogen. Numerous trans-acting regulators, including regulatory RNAs, contribute to the coordinated expression of multiple virulence factors [6,7]. Although about 200 *S. aureus* sRNAs were identified, very few of their respective targets are known to date [4]. We present

here a target capture protocol, named "Hybrid-trap-seq" that exploits deep sequencing and bioinformatics to greatly improve sensitivity and specificity for the identification of biologically meaningful sRNA-target pairs. Applied to four staphylococcal sRNAs, Hybrid-trap-seq revealed numerous new sRNA-targets and sRNA-regulated pathways, and allowed us to connect sRNAs to specific biological functions.

Results and Discussion

***In vitro* sRNA target identification by Hybrid-trap-seq.** To identify sRNA targets, we assumed that biologically relevant sRNA-RNA intermolecular interactions could be isolated *in vitro*. As proof of concept, we chose four *S. aureus* regulatory RNAs: i) RNAIII as paradigm of staphylococcal regulatory RNAs [8] and ii) three sRNAs (RsaA, RsaE and RsaH) that were previously found to be expressed during specific growth phases [9,10].

Strain HG003 was used as a model for staphylococcal regulation studies [11]. Total RNAs were extracted from 16 different growth conditions, pooled together and sequenced by high-throughput technology (RNA-seq). The RNA pool covered most of the *S. aureus* transcriptome (Table S1). Synthetic RNAIII, RsaA, RsaE and RsaH sRNAs were produced, biotinylated, and fixed to streptavidin-associated magnetic beads; each streptavidin-biotinylated-sRNA complex was incubated with the pooled RNA mix following the same procedure that was used previously for the discovery of *omp* mRNAs-RseX sRNA interactions in *E. coli* [5]. After washing steps, RNAs bound to sRNAs were eluted (Figure 1). Recovered RNAs were converted to cDNAs and sequenced by RNA-seq. In each Hybrid-trap-seq dataset, analyses of mapped reads showed that 42 to 64% of all annotated genes were covered by at least ten reads (Table S1), indicating nonspecific binding to the sRNA-baits. To filter out background noise from putative sRNA targets, we performed a differential expression analysis between the four datasets using the DESeq software [12]. Transcription units accumulating significantly more reads in one dataset than in the others were classified in this way. This procedure identified

101, 23, 32 and 69 *in vitro* targets of RNAIII, RsaA, RsaE and RsaH, respectively (Figure 2 and Dataset S1). Among them were several known RNAIII and RsaE targets, indicating that the *in vitro* Hybrid-trap-seq procedure captures physiologically relevant RNA-RNA interactions.

sRNA-mRNA binding rules and seed motif prediction. We analyzed the potential interactions between sRNA-baits and their putative targets using IntaRNA [13]. RNAIII was not included in this analysis due to its complex structure comprising multiple RNA binding sites [14]. The 5' regions of putative targets showed significantly stronger base-pairing potential with their respective sRNA than 5' regions of random sets of *S. aureus* transcripts (Figure S1), indicating that Hybrid-trap-seq identifies a subset of RNAs with specific affinity to the bait sRNA.

Studies on Gram-negative bacteria suggested that sRNAs bind their target through a seed matching mechanism involving a short conserved sRNA region [15-18]. Similarly, in Gram-positive *S. aureus*, a UCCC sequence motif is present in the four sRNAs studied and is involved in target recognition in the case of RsaE [10]. The numerous target candidates produced by Hybrid-trap-seq provided the opportunity to assess seed binding potential on a quantitative basis. We found recurrent sequence motifs in each target set (Figure 3). The predominant motifs are akin to Shine Dalgarno (SD) sequences (Figure 3) and are indeed mainly located at the SD site. Strikingly, each motif had a complementary sequence in the corresponding sRNA that paired exactly to the most conserved positions of the motif (Figure 3). The anti-SD-like motifs in sRNAs are mostly single-stranded [10], hence accessible to base-pairing, and are located in evolutionary conserved regions (Figure S2). Altogether, four independent lines of evidence (motif

enrichment, complementarity with sRNA, accessibility and conservation) lead us to make it very likely that RsaA, RsaE, and RsaH operate through a seed binding mechanism targeting SD-like regions. The degeneracy (hence variability) of target motifs contrasts with the high conservation in cognate sRNA regions (Figure S2) consistent with previous phylogenetic studies that showed higher conservation on the sRNA side of sRNA-mRNA complexes [19,20].

A question raised by the use of an SD-like seed region is how specific recognition can be achieved. SD-like motifs in RsaA and RsaE targets (GGAGnnnUUU and AAGGGG) differ from the canonical *S. aureus* SD motif (AAGGAG, Figure S3) in notable ways and these differences are matched by specific complementary bases in the sRNA. RsaA may achieve further recognition specificity through two additional pairing sites (Figure 2A), potentially creating a double or triple anchored interaction, often spanning the mRNA start codon. RsaE has two CCCCTT repeats that match two AAGGGG repeats present in five potential targets (Figure 2B). Targets with such double seed contacts include mRNAs *rocD, rocF* and the sRNA RsaOG. RsaH targets a canonical SD consensus (UAAAGGAG); no further target motifs were detected that could improve binding specificity. However, frequent hybrid predictions outside the SD sequence (Figure 2C) suggest that a variety of additional anchor points can coexist with the major SD anchor. For the three RNAs analyzed, Hybrid-trap-seq thus enabled the identification of a major SD-like seed binding motif, which can be canonical or modified, and may be combined with additional binding sites.

***In vivo* assessment of putative sRNA-targets.** Hybrid-target-seq experiments are equivalent to genome-wide RNA-RNA retardation assays, with the same

caveat: Do putative targets uncovered *in vitro* correspond to real *in vivo* targets? As many regulatory RNAs that act by base-pairing affect the stability of their targets, we considered that putative targets would be validated if their abundance varied with sRNA expression levels. To test this, the relative amount of putative RNA targets was determined and compared by qRT-PCR upon induction of their corresponding regulatory RNAs or in the absence of the sRNA gene. A short induction time (5 min), was chosen to monitor the primary effect of sRNA induction on its targets.

To determine the accuracy of the method without bias, we systematically tested the most enriched RNAs with each of the sRNAs. In view of the large number of putative targets trapped (23, 32, 69, and 101 for RsaA, RsaE, RsaH and RNAIII, respectively), we chose for further study the 40% most enriched RNAs with RsaA or RsaE, and the 20% most enriched with RNAIII or RsaH.

Among the 54 tested candidates, a total of 11 were up-regulated and 7 down-regulated upon sRNA accumulation compared to the reference condition (Tables 1 and S2). An effect of sRNA gene deletions on putative targets was less frequently detectable; nevertheless four mRNAs were up-regulated and one down-regulated in sRNA gene deletion mutant strains as compared to the wild type strain (Table 1). Altogether, 21 out of 54 RNAs (39%) selected based on the unique criteria of *in vitro* enrichment were affected for their stability *in vivo*. Pulse-expression approaches are powerful methods for limiting the noise due to indirect effects. For example, after a short induction of *Salmonella enterica* GcvB or of *E. coli* RyhB sRNAs, only ~1% of the transcriptome was altered [21,22]. The high proportion of qRT-PCR-validated candidates identified after a similarly short induction time

indicates that Hybrid-target-seq is a powerful means of identifying biological sRNA targets. We also performed *in vivo* tests of additional putative identified targets of RNAIII and RsaH that are functionally related to already selected genes. Among the 14 RNAs tested, 6 were up regulated and 1 was down regulated upon sRNA induction (Tables 1 and S2). We also confirmed qRT-PCR results by Northern blot (Figure 4). As i) these RNAs were selected for their physical association with sRNAs and ii) their *in vivo* quantity was affected by the quantity of sRNAs, we conclude that they are direct sRNA targets. Taken together, 41% of sRNA targets predicted by Hybrid-trap-seq were confirmed *in vivo*.

mRNA down-regulation is expected to result in lower amounts of the corresponding protein. However, mRNA up-regulation by sRNAs should be interpreted cautiously, as pairing that prevents RNA degradation might inhibit translation by, for example, masking the ribosome binding site of targeted messengers. It is worth mentioning that for 10 out of 11 up-regulated mRNAs, an interaction site has been predicted by IntaRNA (Figure 3). Putative targets that remain constant in our test could also undergo sRNA-dependent translational controls; it is thus likely that among them, some are also true sRNA targets.

New targets for RNAIII including mRNAs involved in iron uptake. RNAIII is an atypical regulatory RNA because of its unusual long length (512-nts) and its expression of a small protein, the delta-hemolysin [23]. It contributes to the transition from host colonization to tissue destruction at high cell density by repressing early surface virulence factors and activating exotoxins [24]. RNAIII forms duplexes with *rot*, *spa*, *lytM*, *coa*, and *SAOUHSC_1110* mRNAs, encoding the regulator of toxin Rot, protein A, the peptidoglycan hydrolase LytM, a

coagulase and a fibrinogen binding protein, respectively [4]. As expected, known RNAIII targets, *i.e., spa*, *coa* and *SAOUHSC_1110* mRNAs, were significantly enriched when RNAIII was used as bait as compared to the other baits (Dataset S1). *rot* mRNA was highly enriched with RNAIII, but also retained by RsaH. The effect of RNAIII was tested *in vivo* on 24 putative targets and on *coa* and SAOUHSC_1110 mRNAs. Quantities of known RNAIII targets were reduced upon 5 min of RNAIII induction, thus validating our approach. Among the putative new targets, six mRNAs were down-regulated and three were up-regulated (Tables 1 and S2). Thus, in addition to four known RNAIII substrates, nine new targets were discovered.

Protein A (SpA) and Sbi prevent bacterial opsonophagocytic killing by lymphocytes and may act synergistically to promote *S. aureus* escape from immune responses during the host colonization phase [6]. *Spa* mRNA is down-regulated by RNAIII. *sbi* mRNA is controlled by SprD, a virulence related sRNA [25]. We show here that RNAIII also targets *sbi* mRNA revealing a RNAIII-dependent coordinated down-regulation of *sbi* and *spa*. While this work was in progress, an independent study reported that RNAIII targets *sbi* mRNA [26], thus providing independent validation of our Hybrid-trap-seq approach.

It was recently proposed that RpiRC, a sugar-responsive regulator indirectly down-regulates RNAIII [27]. However, our results identify *rpiRC* as an RNAIII target, as i) RNAIII interacts with *rpiRC* mRNA (Dataset S1), whose levels are increased *in vivo* by RNAIII overproduction (Table 1). One explanation that would reconcile the previous and present results is that RNAIII/*rpiRC* mRNA duplex would lead to RNAIII degradation.

Iron is a determining factor during the infection process and host iron sequestration inhibits bacterial growth [28,29]. In *S. aureus,* iron capture is mediated by staphyloferrin A and B siderophores [30], which are imported *via* the HtsABC and SirABC systems respectively. In addition, *S. aureus* imports xenosiderophores via FhuD1, FhuD2 and FhuBG. All three transport systems require the integrity of the FhuC ATPase. Another iron reservoir is heme which is imported by the Isd system [30]. Trapped RNAIII targets were strikingly enriched in mRNAs expressing proteins involved in iron homeostasis: *sirABC* operon, *fhuCB* operon, *fhuD2*, *htsA*, *fur* (encoding the intracellular Fe^{2+}-sensing regulator Fur), *hemH* (encoding a ferrochelatase), and two siderophore-related mRNAs, *iucB* and *sstD* (Dataset S1). Among those tested *in vivo*, we confirmed that mRNAs expressing SirA, FhuD2, and FhuC were down-regulated upon RNAIII induction (Table 1). *fur* mRNA levels did not vary when RNAIII was overproduced (Table S2). However RNAIII-*fur* pairing predictions involve the *fur* SD, leaving open the possibility that RNAIII acts at the translational level (Figure S4). During the host colonization phase, *S. aureus* does not produce hemolysins and the main pathways of iron acquisition likely utilize siderophores. In contrast, at higher cell densities, RNAIII expression is induced, and *S. aureus* enters into a tissue destruction phase characterized by erythrocyte lysis and the massive release of heme likely provides the major iron source [24,28,29]. RNAIII down-regulation of *S. aureus* iron acquisition systems may optimize bacterial fitness by limiting iron uptake and thus preventing toxicity when extracellular stocks are high, and switching between systems as a function of the growth phase and iron availability.

Host cells generate toxic reactive oxygen species as part of the primary response to bacterial infection. The Mn-containing superoxide dismutase SodA mediates elimination of superoxide radicals and contributes to reducing iron toxicity [31]. We observed that the trapped *sodA* mRNA was down-regulated *in vivo* upon RNAIII accumulation, possibly in parallel with reduced iron uptake. Interestingly, the response regulator AgrA is redox sensitive and consequently oxidative stress represses RNAIII expression [32], thereby stabilizing *sodA*.

RsaA is involved in autolysin regulation. RsaA, a 142-nt sRNA, is detected in all growth phases but its quantity strongly increases in stationary phase [9,10]. Trapping of RsaA targets identified seven mRNAs involved in cell envelope biosynthesis among a total of 21 enriched mRNAs (Dataset S1). This group included four mRNAs expressing CHAP-domain containing proteins; CHAP is mainly associated with peptidoglycan hydrolysis [33]. Each of the four identified mRNAs is transcribed from a distinct transcription unit. The probability of randomly finding four mRNAs expressing CHAP proteins (there are 14 such genes in the HG003 genome), among the selected candidates by RsaA trapping is 2.8×10^{-6}. This very low probability indicates a functional enrichment of RsaA targets. In addition, *mgrA* mRNA, which expresses a pleiotropic regulator affecting autolysis genes and repressing biofilm formation [34], was found in our screen.

Nine putative targets were tested for *in vivo* variation of abundance upon RsaA induction. *mgrA* and two mRNAs expressing CHAP proteins (SAOUHSC_02855 and SAOUHSC_2883) were significantly up-regulated upon RsaA accumulation. In addition, the amount of SAOUHSC_02576 autolysin mRNA was up-regulated when the *rsaA* gene was deleted (Table 1). These experiments confirm an RsaA-

dependent post-transcriptional regulation of genes involved in peptidoglycan homeostasis metabolism, which may impact biofilm- and stress- related functions. Measuring protein levels of the RsaA gene targets will be valuable for understanding the role of RsaA in bacterial physiology.

RsaE down-regulates arginine metabolism. RsaE is an astonishingly well conserved 93-nt sRNA found in the bacillales order. In *S. aureus*, it accumulates at the onset of stationary phase. RsaE affects expression of several genes involved in transport, folate metabolism and the Krebs cycle, and could contribute to *S. aureus* adaptation in stationary phase [9,10]. Among its previously reported targets, mRNAs corresponding to *opp3* and *opp4* operons were significantly enriched in the Hybrid-trap-seq experiment using RsaE as bait; others, however, expressing Krebs cycle and folate metabolism enzymes were not enriched. Twelve new RsaE putative targets were tested for their *in vivo* variation upon RsaE accumulation. Among them, two RNAs (RsaOG and SAOUHSC_02836) were up-regulated, and three mRNAs (*rocD*, *rocF* and SAOUHSC_01138) were down-regulated by RsaE (Table 1). The arginase RocF and ornithine aminotransferase RocD enzymes perform adjacent metabolic steps that mediate forward and reverse reactions to shift between proline and arginine pools via the urea cycle [35]. Strikingly, the *rocF* and *rocD* genes, which are genetically unlinked, are both negatively regulated at the post-transcription level by RsaE. The urea cycle is directly linked to the Krebs cycle via fumarate released by the formation of arginine from argininosuccinate. The newly identified trapped targets expand the metabolic pathway that is subject to coordinate negative regulation by RsaE.

RsaH contributes to lactate metabolism regulation. RsaH is a 128-nt sRNA that accumulates specifically in pre-stationary phase [9]. Putative RsaH targets found by Hybrid-trap-seq were associated with anaerobic and fermentation metabolism, virulence or oxidative stress (Dataset S1). Among the 21 putative targets tested *in vivo* for their variation upon RsaH induction, five were significantly up-regulated, expressing ferritin and proteins of unknown function, and three were down-regulated (Table 1). The two most down-regulated mRNAs correspond to genetically unlinked genes encoding L-lactate dehydrogenase (SAOUHSC_00206, *ldhE*) and lactate permease (SAOUHSC_02648, *lctP*). These two genes are also negatively controlled by the redox-sensing regulator of adaptation to anaerobic conditions Rex [36]. Our results indicate that RsaH affects a Rex-mediated regulation by directly down-regulating lactate metabolism and transport genes. The third down-regulated gene is *isaB*, which encodes a putative virulence factor implicated in immune evasion and is induced by acidity [37]. *rot* mRNA was highly enriched in the RsaH capture experiment (Dataset S1), while *in vivo rsaH* induction did not result in a significant change in its quantity (Table S2). Nevertheless, *in silico* analysis support specific highly stable interactions between RsaH and the 5'UTR of *rot* mRNA (Figure S4). This leads us to hypothesize that RsaH exerts translational control on Rot expression. Interestingly, the transition from aerobic to anaerobic growth reportedly induces expression of genes involved in lactate metabolism (*ldh*, *lctP*), and of *isaB* and *rot* [38]. Our results indicate that RsaH contributes to the coordinated regulation of these genes.

Conclusions. We developed an experimental strategy to identify RNAs selectively retained by regulatory RNAs. As the first step is based on the selection of RNA-

RNA interactions, the candidates should be considered as primary targets. Compared to previous methods, the use of deep sequencing greatly improved the sensitivity of target identification. The problem of inherent high background noise was solved by performing differential analyses of several trapping experiments in parallel. This approach increased the specificity of target identification and allowed detection of low abundance targets, a usual difficulty of affinity-based selections. Subsequent *in vivo* target validation gave supporting evidence that Hybrid-trap-seq efficiently identifies sRNA-mRNA partners.

The multiple targets identified for each sRNA provided valuable data for the identification of seed motifs. Computational analysis of mRNAs trapped by RsaA, RsaE and RsaH suggested a major binding mode based on multiple seed motifs, involving an SD or SD-like sequence plus other regions often spanning the start codon. Interestingly, the multiple seed binding mode evidenced for RsaA and RsaE may explain observed discrepancies between computational predictions and actual sRNA-target pairs.

Of the 68 putative mRNA targets we further analyzed, 41% had their amounts modulated *in vivo* in response to changes in their corresponding regulatory sRNA. This is a remarkably high success rate compared to other attempts to identify sRNA targets [2]. The fraction of valid targets is likely higher, as sRNAs can exert translational control without affecting mRNA stability. Strikingly, a substantial subset of targets for each given sRNA was functionally related. Identification of these new targets revealed that RNAIII, RsaA, RsaE and RsaH down-regulate iron transport, autolysins, arginine/proline pathway, and lactate metabolism, respectively (Figure 5). Altogether, the Hybrid-trap-seq experiments presented

here provided more staphylococcal sRNA targets than was obtained over many years of research. It should thus be a method of choice to decipher sRNA-controlled regulatory networks.

Materials and Methods

A detailed description of the experimental procedures can be found in Supporting Information.

Bacterial strains and plasmids. *In vivo* experiments were performed with *S. aureus* HG003 strain grown in BHI rich media [11]. For gene names, we used NCTC8325 nomenclature. The *rnaIII*, *rsaA*, *rsaE* and *rsaH* genes were inactivated by replacing the gene by a short tag sequence (Table S3). The *rnaIII*, *rsaA*, *rsaE* and *rsaH* genes were cloned into pRMC2, under the control of an anhydrotetracycline (aTc) inducible promoter [39].

Total RNA extractions in various biological conditions. Total RNA samples were extracted from HG003 grown in different conditions: i) eight samples in rich medium at OD_{600nm} 0.6, 1.8, 3.3, 4.5, 7.2, 9.8 and 12.8, and late stationary phase (24 hours), 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.

Hybrid-trap-seq. The procedure is summarized in Figure 1 and a complete explanation is provided in Supporting Information.

Computational analysis of RNA-RNA hybrids. Input sequences were the complete sRNA sequence and, for each putative target, the region from the transcriptional start site (TSS) to 100 nucleotides past the start codon, or the entire transcript when non-coding. TSSs were defined from sequencing of total pooled RNA as described [40]. Coordinates of coding and non-coding relevant transcripts are provided (Table S4). Target RNA-sRNA interactions were predicted using the

IntaRNA V.1.2.5 package [13] and consensus motifs in putative target sequences using the MEME suite V.4.9.0 [41]. We searched for the motif with highest raw counts using different combinations of MEME parameters as described in Supporting Information. The standard SD motif (Fig S2) was identified by MEME using as input the 1114 *S. aureus* sequences for which a TSS could be identified by the above protocol.

Quantitative reverse transcriptase PCR and northern blots. qRT–PCR experiments were performed on a subset of putative targets selected among the most enriched mRNAs of each Hybrid-trap-seq set (see Supporting Information for details). Experiments were performed on biological triplicates and data were analyzed as described [42]. Northern blots were performed as described [43].

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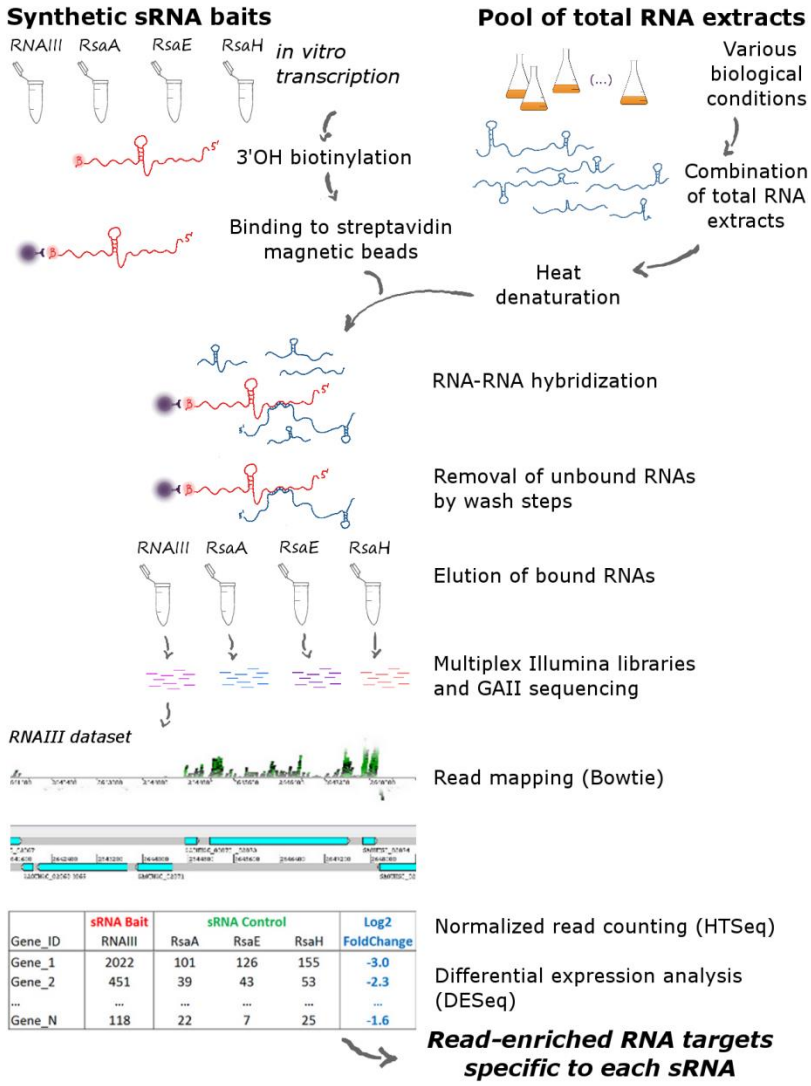

Figure 1. Overview of the Hybrid-trap-seq procedure. Synthetic sRNAs (RNAIII, RsaA, RsaE and RsaH) generated by *in vitro* transcription were biotinylated and incubated with magnetic streptavidin beads, to obtain sRNA-bound beads. Each of them was mixed with pooled total RNA samples of *S. aureus* grown in 16 different conditions. Unbound RNAs were removed by washing steps using magnetic separation. RNAs bound to sRNA-bound beads were then eluted and sequenced by RNA-seq technology. Reads were aligned to the chromosome sequence (NCTC8325) and visualized. Specifically retained RNAs corresponding to RNA targets of each sRNA-bait (an example with RNAIII is presented) were identified by differential expression analyses. The complete protocol is presented in Supporting information.

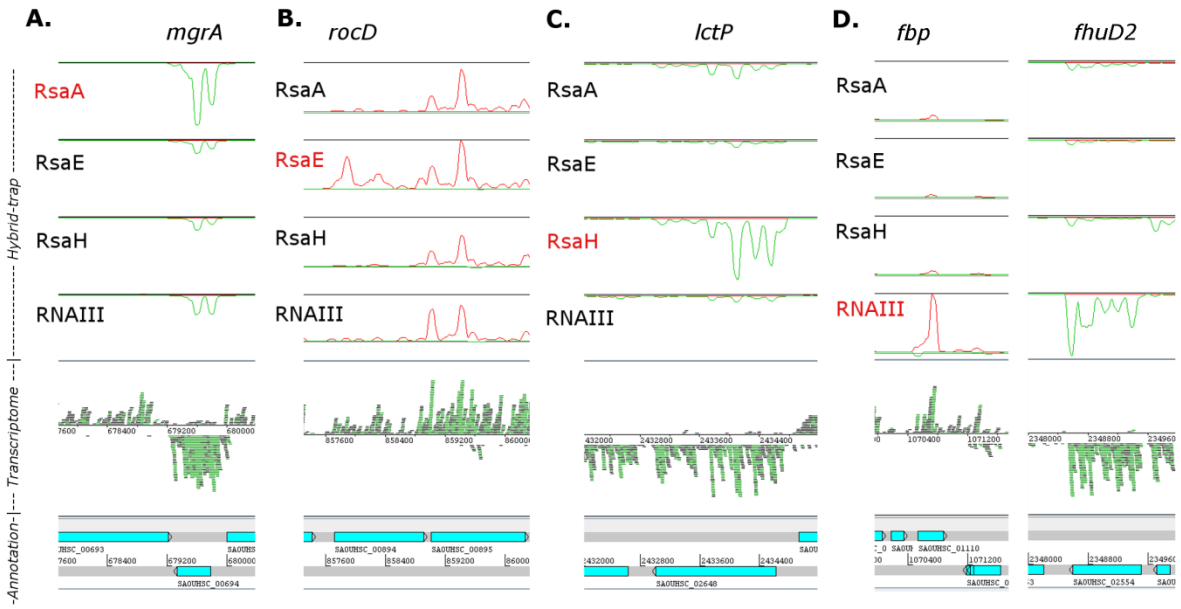

Figure 2. Examples of sRNA-dependent read enrichment regions. Selected Artemis genome viewer windows show i) read density profiles of RNAs trapped with sRNAs (Hybrid trap), ii) reads from RNA-seq of HG003 pooled total RNA extractions (Transcriptome), and iii) genome annotation with blue boxes indicating open reading frames (Annotation). For the complete set of putative and confirmed targets, see Dataset S1 and Table 1, respectively. Panels A, B, C, and D correspond to read density profiles enriched with RsaA, RsaE, RsaH, and RNAIII trapping experiments, respectively. Read density profiles of RNAs positioned above (in red) or below (in green) the horizontal line indicate reads mapping on the clockwise and counterclockwise genomic DNA strands, respectively; scales were normalized independently for each gene using the maximum and minimum density values among the four datasets.

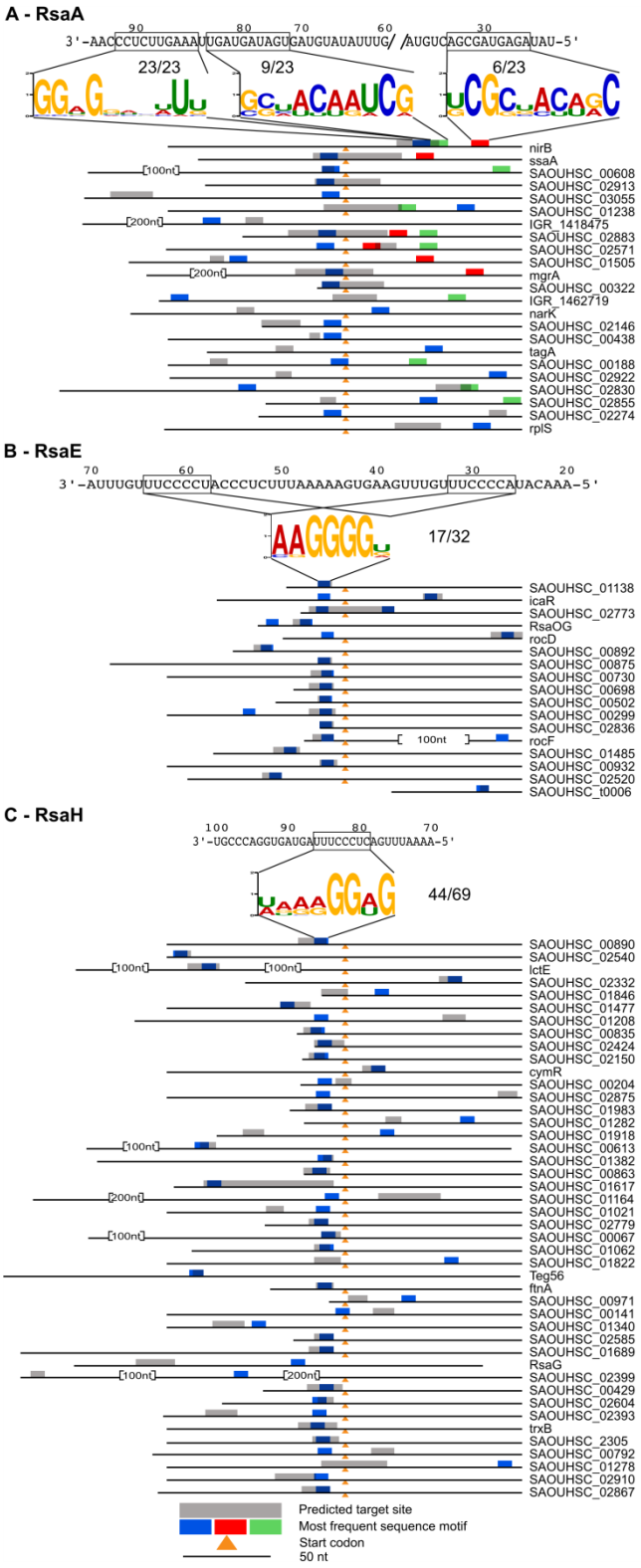

Figure 3. Analysis of the target-matching regions of RsaA, RsaE and RsaH.

A-C: Enriched MEME [41] sequence motifs found in each target set are shown as colored logos together with their total number of occurrences (coordinates of the consensus motif positions on the putative sRNA targets are presented in Table S4). x/y ratios represent the number of sequences featuring the motif / number of sequences in the target set. Each motif is connected to its locations in target RNAs (colored boxes) and to its best complementary site in the sRNA. Grey areas in targets correspond to regions of interaction with sRNA predicted by IntaRNA [13].

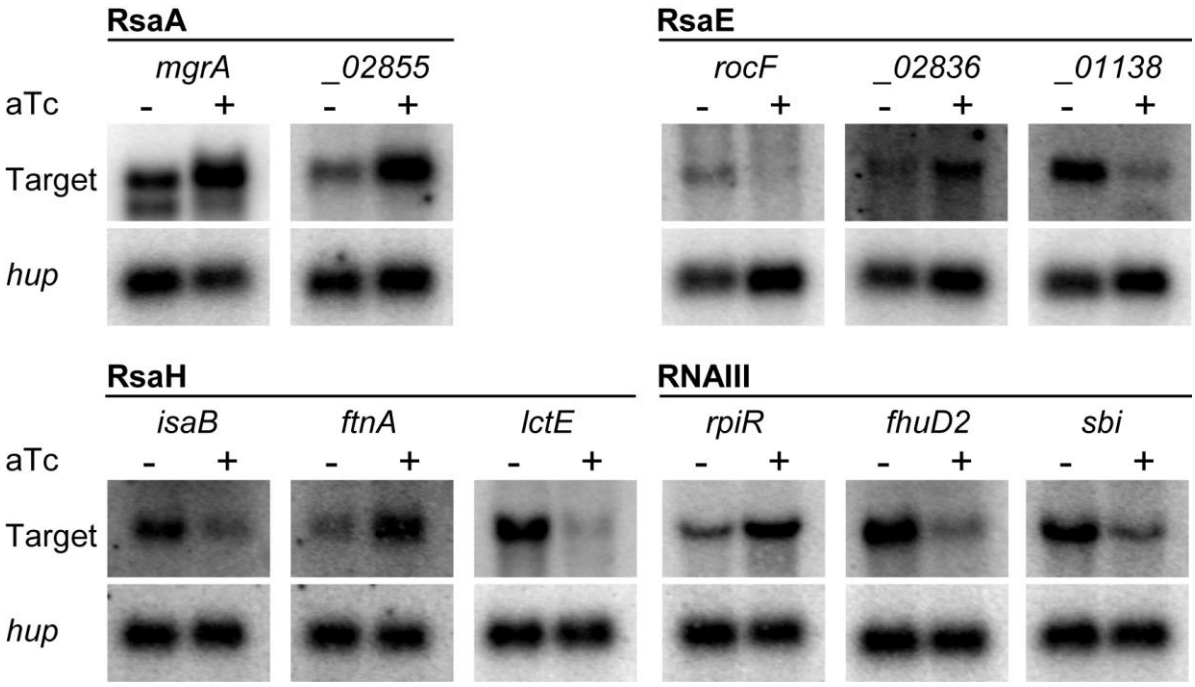

Figure 4. *In vivo* effect of sRNAs on their targets. Total RNAs were extracted from strains expressing conditionally the indicated sRNAs upon the addition of anhydrotetracycline (aTc, 1 mM) to the medium. (-), prior to aTc addition; (+), 5 min induction. The quantity of the indicated sRNA-targets was visualized by Northern blot experiments. *Hu* mRNA was used as a loading control for normalization. Numbers correspond to the SAOUHSC nomenclature.

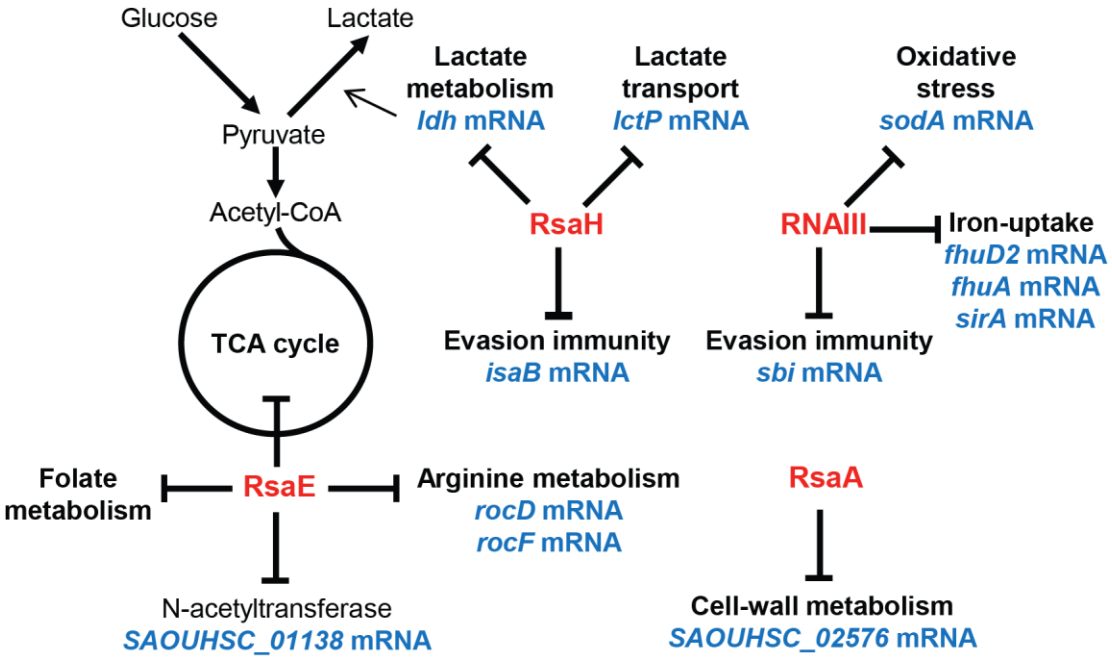

Figure 5. *Staphylococcus aureus* sRNA-controlled pathways identified by Hybrid-trap-seq.

sRNA, targeted mRNA and pathways are represented in red, blue, and bold black, respectively.

Table 1. Relative amounts of putative sRNA-targets in strains lacking or overexpressing sRNAs, determined by qRT-PCR.

	Δ*srna*/WT[1]	+aTc/-aTc[2]
RNAIII	0.00	358.7 ±25
SAOUHSC_00074 (*sirA*)	1.07 ± 0.63	**0.62** ± 0.05
SAOUHSC_00192 (*coa*)	1.14 ± 0.19	**0.23** ± 0.02
SAOUHSC_00652 (*fhuC*)	1.07 ± 0.12	**0.59** ± 0.01
SAOUHSC_00826	**1.94** ± 0.28	1.03 ± 0.05
SAOUHSC_01110 (*fbp*)	0.95 ± 0.04	**0.14** ± 0.01
SAOUHSC_01653 (*sodA*)	0.97 ± 0.02	**0.60** ± 0.05
SAOUHSC_02554 (*fhuD2*)	1.07 ± 0.10	**0.38** ± 0.01
SAOUHSC_02589 (*rpiR*)	1.04 ± 0.07	**2.47** ± 0.05
SAOUHSC_02670 (*hsp* gene)	0.99 ± 0.04	**1.98** ± 0.28
SAOUHSC_02706 (*sbi*)	1.25 ± 0.09	**0.47** ± 0.01
SAOUHSC_02872	**0.51** ± 0.04	1.42 ± 0.21
RsaA	0.00	155.9 ± 14.1
SAOUHSC_00694 (*mgrA*)	0.88 ± 0.08	**1.86** ± 0.26
SAOUHSC_02576 (*ssaA*)	**4.10** ± 1.99	1.17 ± 0.11
SAOUHSC_02855 (*ssaA*-like)	1.28 ± 1.01	**3.39** ±0.22
SAOUHSC_02883 (*ssaA*-like)	1.22 ± 0.84	**1.77** ±0.19
RsaE	0.00	359.8 ± 14.6
SAOUHSC_00894 (*rocD*)	2.42 ± 2.66	**0.46** ± 0.06

SAOUHSC_01138	**3.20** ± 1.61	**0.47** ± 0.08
SAOUHSC_02409 (*rocF*)	**3.84** ± 1.86	**0.31** ± 0.02
RsaOG sRNA	0.96 ± 1.08	**2.34** ± 0.24
SAOUHSC_02836	0.85 ± 0.22	**2.04** ± 0.02
RsaH	0.00	228.1 ± 21.6
SAOUHSC_00206 (*lctE*)	1.02 ± 0.45	**0.27** ± 0.14
SAOUHSC_02648 (*lctP*)	1.55 ± 0.27	**0.17** ± 0.08
SAOUHSC_02108 (*ftnA*)	1.07 ± 0.22	**2.09** ± 0.22
SAOUHSC_02972 (*isaB*)	1.07 ± 0.25	**0.54** ± 0.02
SAOUHSC_00465 (*veg*)	1.09 ± 0.20	**1.82** ± 0.14
SAOUHSC_00863	0.72 ± 0.00	**1.69** ± 0.16
SAOUHSC_01062	1.03 ± 0.32	**1.67** ± 0.10
SAOUHSC_02424	1.16 ± 0.13	**1.86** ± 0.27

[1]Relative amount of putative sRNA-targets in Δ*srna* to WT strains.

[2]Relative amount of putative sRNA-targets in Δ*srna* pRMC2-sRNA strains 5 min after aTc addition (induced state) to before aTc addition (non-induced state).

Studied sRNAs are either RNAIII, RsaA, RsaE or RsaH. Numbers in bold indicate that the target is significantly modulated by the absence (column Δ*srna*/WT) or accumulation (+aTc/-aTc) of the indicated sRNA. The indicated standard deviation is based on independent biological triplicates. Complete data are presented in Table S2.

SI Materials and Methods

Bacterial strains, plasmids and growth conditions. Experiments were performed using *S. aureus* RN4220 (for cloning purposes) and HG003 strains (1). For gene names, we used NCTC8325 nomenclature retrieved from Genbank file CP00025.1, which is the sequenced HG003 parental strain. Engineered plasmids were constructed in *E. coli* DH5α, transferred to RN4220 (a transformable strain with exogenous DNA) and subsequently to HG003. *S. aureus* strains were routinely grown in Brain Heart Infusion (BHI) broth at 37°C under vigorous agitation (180 rpm). DH5α was grown in Luria-Bertani (LB) broth at 37°C. Antibiotics were added to media as needed: ampicillin, chloramphenicol at 100 and 20 µg/ml for *E. coli*; chloramphenicol and erythromycine at 20 µg/ml and 5 µg/ml for RN4220; chloramphenicol and erythromycine at 20 µg/ml and 0.5 µg/ml for HG003, respectively.

HG003 derivatives that do not express RNAIII, RsaA, RsaE and RsaH were constructed by *rnaIII*, *rsaA*, *rsaE* and *rsaH* genes replacements with specific tag sequences using a replication thermosensitive plasmid pMAD (2) derivative (pMAD*), as described (3). In Brief, the pMAD* derivatives pMAD*rnaIII::tag47; pMAD*rsaA::tag1, pMAD*rsaE::tag45 and pMAD*rsaH::tag49, contained the tag sequences sandwiched between up- and downstream of sRNA gene sequences. pMAD* derivatives were constructed by Gibson assembly method (4) (primers in Table S4). The integrity of inserted DNA sequences was verified by DNA sequencing. The detailed strategy will be published elsewhere.

pRMC2 contains the *repA*, *repC*, *tetR* genes and the *xyl*/*tetO* promoter (5). The pRMC2 derivative containing *rnaIII*, *rsaA*, *rsaE*, *rsaH* (named pRMC2-RNAIII, pRMC2-RsaA, pRMC2-RsaE, pRMC2-RsaH, respectively) were constructed as follows: DNA sequences of interest were amplified from HG003 genomic DNA by PCR, using the primers indicated (Table S4). The resulting products were assembled into the pRMC2 PCR-amplified vector using the method developed by Gibson (4). The integrity of inserted DNA sequences was verified by DNA sequencing. Expression of inserted sequences upon addition of anhydrotetracycline (aTc) at 1 mM to the media was confirmed by qRT-PCR.

Total RNA extractions in various biological conditions. Total RNA was isolated as previously described (6) except for cells lysis. Briefly, cultures were centrifuged; the pellets were frozen in liquid nitrogen and stored at -80°C until RNA extraction. Cells pellets were resuspended into 400 µL of Lysis buffer (4 M guanidine thiocyanate, 25 mM sodium acetate pH 5.2, 5 g/L N-laurylsarcosinate), transferred into FastPrep tubes containing 0.6 g of glass beads (G4649, Sigma-Aldrich) and 400 µL of acid phenol:chloroform:IAA (25:24:1). Bacteria were mechanically lysed by using the Fastprep apparatus (MP Biomedicals) with 3 cycles of 45 s at speed 6.0 separated by incubation on ice during 5 min. After lysis, tubes were centrifuged 15 min at 17,900 g at 4 °C. The aqueous phase was acid phenol extracted, isopropanol precipitated and the pellet resuspended in RNase-free water. The RNA concentration of samples was measured using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Inc.). The quality of the RNA preparations was assessed by capillary electrophoresis using RNA Nano chips

with a Bioanalyzer Agilent 2100 (Agilent Technologies, Palo Alto, USA). Total RNA extracts were treated using the Turbo DNase I (Ambion, Foster City, CA, USA) before qRT-PCR.

Total RNA samples were extracted in 16 different biological conditions as described below. i) 8 samples harvested in the course of growth: Overnight cultures were diluted 2000 times in BHI medium and incubated at 37°C under shaking agitation (180 rpm). Cells were harvested in exponential phase (at OD_{600nm} 0.6, 1.8 and 3.3), transition phase (at OD_{600nm} 4.5 and 7.2), early stationary phase (at OD_{600nm} 9.8 and 12.8) and late stationary phase (24 hours). ii) 6 samples harvested under stress conditions: Exponential growing cells (at OD_{600nm} 0.6) were submitted before RNA extraction to cold shock (15 min at 10°C), heat shock (10 min at 48°C), oxygen limitation (the exponential growing culture was transferred into a 50 mL Falcon tube fully filled and was incubated during 30 min under static condition), alkaline stress (10 min after addition of KOH 30 mM), oxidative stress (10 min after addition of H_2O_2 10 mM) or disulfide stress (10 min after addition of diamide 1 mM). iii) 1 sample harvested under iron-depleted condition: Overnight culture was diluted in BHI supplemented with 2,2-dipyridyl at 1.4 mM and incubated for growth until OD_{600nm} reached 1. iv) 1 sample from cells grown on BHI-agar plates: Exponential growing cells were diluted at ~5.10^3 CFU/mL, 100 µl were spread on BHI-agar plates and incubated 16 h at 37°C. Colonies were resuspended in lysis buffer for RNA extraction.

Twenty µg of each of the 16 total RNA extracts were pooled to obtain the combined RNA extracts sample used as prey during the Hybrid-trap-seq procedure. In

parallel, 5 µg of this sample were processed using the MICROBExpress kit (Ambion, AM1905) as recommended by the suppliers, for large rRNAs removal and before Illumina high-throughput sequencing.

Hybrid-trap-seq procedure. Synthetic RNAs were generated using the T7 MEGAshortscript kit (Ambion) according to manufacturer's instructions. Briefly, the four sRNA genes under the control of the T7 RNA polymerase promoter were PCR amplified using specific oligonucleotides (Table S4). PCR-products were purified using clean-up gel extraction kit (Macherey-Nagel). *In vitro* transcription assays were performed at 37°C using 150 ng of DNA matrix. After 4 h of incubation, DNA was removed by treatment with 1 µl of TurboDNase I (Ambion) at 37°C during 15 min. Synthetic RNAs were phenol:chloroform extracted, isopropanol precipitated, the pellets were washed twice with 70 % ethanol then dried and resuspended in RNase-free water.

Synthetic RNAs were 3'-end biotinylated as previously described (7). Briefly, 50 µl of *in vitro* transcripts at ~5 µg/µl were mixed with 100 µl of potassium periodate 60 mM dissolved in NaAc 66 mM pH 4.8, then incubated 30 min at 20°C in the dark. The oxidation reactions were stopped by adding 150 µl ethylene glycol:water (50:50) during 5 min in the dark. 3'-OH oxidized RNAs were precipitated during 5 min on ice by adding 750 µl ethanol 100 %, then harvested by centrifugation at 13000 rpm during 20 min at 4°C. The pellets were resuspended in 100 µl of biotinamidocaproyl hydrazide 10 mM (Sigma-Aldrich, B3770) and incubated 2 h at 37°C. One hundred µl of $NaBH_4$ 200 mM and 200 µl Tris-HCl 1 M pH 8 were added to the samples. After 30 min at 4°C in the dark, the reduction reaction was stopped

by ethanol precipitation. RNA pellets were resuspended in RNase-free water. Full length RNAs were 5% urea PAGE purified as recommended in the T7 MEGAshortscript kit procedure.

For each Hybrid-trap-seq experiment, 25 µl of MasterBeads pre-coated with streptavidin (Ademtech, Pessac-France) were equilibrated in binding buffer (20 mM Tris-HCl, 0.5 M NaCl pH 8) then incubated 10 min at 20°C with 100 pmoles of biotinylated sRNA dissolved in binding buffer. Unbound sRNAs were removed by magnetic separation, and then sRNA-bound streptavidin beads were washed twice with 100 µl of binding buffer. For each Hybrid-trap-seq experiment, 50 µg of pooled total RNA sample were prepared in 20 mM Tris-HCl, 0.5 M NaCl pH 8, EDTA 1 mM, incubated at 55°C during 5 min, and then mixed with the sRNA-bound beads. After 15 min at 45°C, then 15 min at room temperature, the unbound RNAs were removed by magnetic beads separation. The RNA-bound beads were washed twice with 100 µl of wash buffer (7 mM Tris-HCl pH 8, NaCl 0.17 M). For RNA elution, beads were successively resuspended in 100 µl then 200 µl of RNase-free water separated by incubation during 2 min at 55°C. The eluted RNA fractions were ethanol precipitated and the pellets were resuspended in 19 µl of RNase-free water. RNA samples were analyzed by capillary electrophoresis using RNA Nano chips (Agilent) before Illumina high throughput sequencing.

Illumina high-throughput sequencing: The 4 Hybrid-trap eluted samples and the rRNA depleted pooled RNA sample were used to generate five oriented libraries as described (8). Libraries were sequenced using Illumina Genome Analyzer IIx to generate single-end 40-nt reads.

Read mapping on the genomic sequence: Reads passing the quality filter and trimmed of the sequencing adapters were aligned to *S. aureus* NCTC8325 chromosome sequence (CP000253.1) using bowtie VN:0.12.7 (9), converted to BAM files using SAMtools (10) and visualized in the Artemis viewer (11) as described (8).

Counting reads in each genomic region: The files of each four Hybrid-trap-seq experiments containing reads mapping at unique positions on the reference genome and deprived from the reads corresponding to bait-sRNAs were processed to identify putative targets as following. A genome annotation GFF file was built by combining the 2892 annotated genes retrieved from Genbank file CP00025.1 and the unannotated regions putatively expressed including antisense regions (specified as asSAOUHSC_XXXX for complementary strand of the annotated gene named SAOUHSC_XXXX) and intergenic regions (specified as IGRXXX-YYY for intergenic region between nucleotides XXX and YYY on the forward strand; ComplIGRXXX-YYY for intergenic region between nucleotides XXX to YYY on the backward strand). Read counts per gene were calculated for each dataset with HTSeq-count (http://www-huber.embl.de/users/anders/HTSeq) using the SAM file and this GFF file as the input.

Differential expression analysis: To identify specific RNA targets of each sRNA-bait, a differential expression analysis was performed using the DESeq software, an R Bioconductor package which was developed to test gene differential expressions between multiple experiments (12). For all expressed genes and each sRNA-bait, log2-fold changes were computed from normalized read number of

condition A (defined by the sRNA of interest dataset) to normalized read number of condition B (defined by the three other sRNAs datasets). Genes were considered as putative sRNA targets if with a log2-fold change ≤ -1.58 (3 fold change on the linear scale). This threshold was chosen in light of known RNAIII targets (*spa* mRNA had a log2-fold change of -1.5). Read-enriched transcription units were defined using side-by-side read-enriched regions. As one mRNA can putatively be targeted by several sRNAs, we specifically looked for RNAs enriched into two out of four sRNA datasets and we found that *rot* mRNA is enriched with RNAIII and RsaH.

Computational analysis of RNA-RNA hybrids. Target RNA-sRNA interactions were predicted using the IntaRNA package (13), the scoring of which is based on hybridization free energy and accessibility of the interaction sites in both RNA molecules. We used IntaRNA V.1.2.5 through the web interface at http://rna.informatik.uni-freiburg.de with parameters "Exact Number of Base Pairs in Seed" = 5 and "Max Number of Unpaired Bases" = 1. Input sequences were the complete sRNA sequence and, for each putative target, the region from the TSS to 100 nucleotides past the start codon. TSSs were defined from the above sequencing of total pooled RNA (62M 40 nt. reads) as described elsewhere (Toffano-Nioche et al. 2013). Briefly, reads were aligned onto the *S. aureus* genome using Bowtie V.1 [(9); options -v 2 -m 1] and the resulting BAM files were analyzed using the Det'rprok pipeline (Toffano-Nioche et al. 2013). For non-coding putative targets, the region analyzed was the entire transcript identified using the same protocol. Coordinates of coding and non-coding relevant transcripts are provided (Table S5). We sought consensus motifs in putative target sequences

using the MEME suite V.4.9.0 (14), run locally with options -dna -minw 4 -maxw 10. Input sequence fragments were the same as above, that is from TSS to ATG+100 or entire transcripts for non-coding RNAs. As we worked with small sequence datasets and degenerate motifs, we did not rely on the MEME motif E-values but instead sought the motifs with highest raw counts for each target family. To obtain the motif with highest counts, we compared MEME output obtained with parameters -mod anr or -mod zoops and retained any motif found in at least 25% of sequences. As no such motif was found in RsaH targets, we further used parameter -minsites 15, which yield a single motif present in 44 out of 69 sequences.

The standard SD motif (Fig. S4) was identified by MEME using as input the 1114 *S. aureus* sequences for which a TSS could be identified by the above protocol, again considering the sequence from TSS to 100 nucleotides past the start codon (first start codon in case of multicistronic loci).

Quantitative reverse transcriptase PCR (qRT-PCR) and northern blots. Overnight cultures grown in BHI supplemented with chloramphenicol were diluted 1000-fold in the same medium at 37°C. At OD600=0.6, aTc (1 mM) was added to the medium and cultures were sampled at 5 min. Each condition cultures were done in triplicate. RNAs were prepared as described (15). qRT–PCR experiments were performed on a subset of putative targets for each sRNA. For RsaA and RsaE, all of the 40 % most enriched targets were tested (log2-fold change ≤ -2.3 and -3, respectively). For RNAIII and RsaH the criteria applied for selection were: i) all of the 20 % most enriched targets (log2-fold change ≤ -3 and -3.8 respectively) and ii) manual analysis based on keeping genes that are functionally related to

already selected genes (for RNAIII: *fbp*, *sbi*, *fhuC*, *fhuD2*, *sarH1*, *saeR*; for RsaH: *isaB*, *ftnA*, *lctP*, *SAOUHSC_00681*, *rot*, *trxB*, *SAOUHSC_00067* and *pgsA*). Data were analyzed as described (16). The geometric mean of 4 genes (*recA*, *gyrA, glyA* and *ftsZ*) chosen among 13 was used to normalize the samples. Genes were considered as significantly affected by sRNA overexpression if the fold-change from + aTc to – aTc samples or WT to ΔsRNA samples were ≥ 1.5 or ≤ 0. 67.

Northern blots were performed as described (17-19). Samples were separated by either PAGE or agarose gels and probed with ^{32}P-labeled PCR probes using the Megaprime DNA labeling system (GE Healthcare) (for primer used, Table S3).

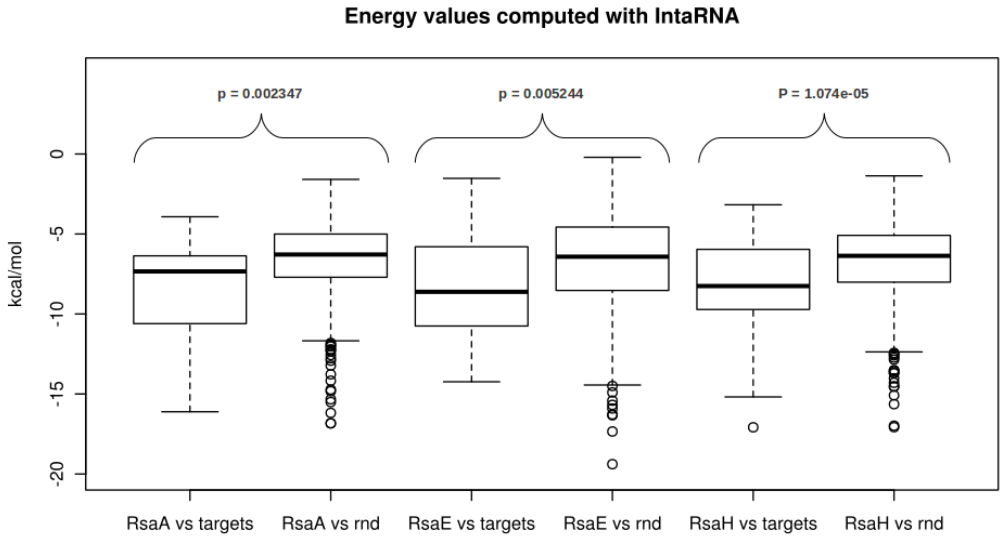

Fig. S1. Distribution of IntaRNA hybridization energy values obtained when matching each sRNA to its set of hybrid-trap-seq targets (RNA vs targets), compared to energy values obtained when matching the same sRNA to the same number of random genes (RNA vs rnd). P-values for hypothesis of identical distributions are computed using Wilcoxon's test.

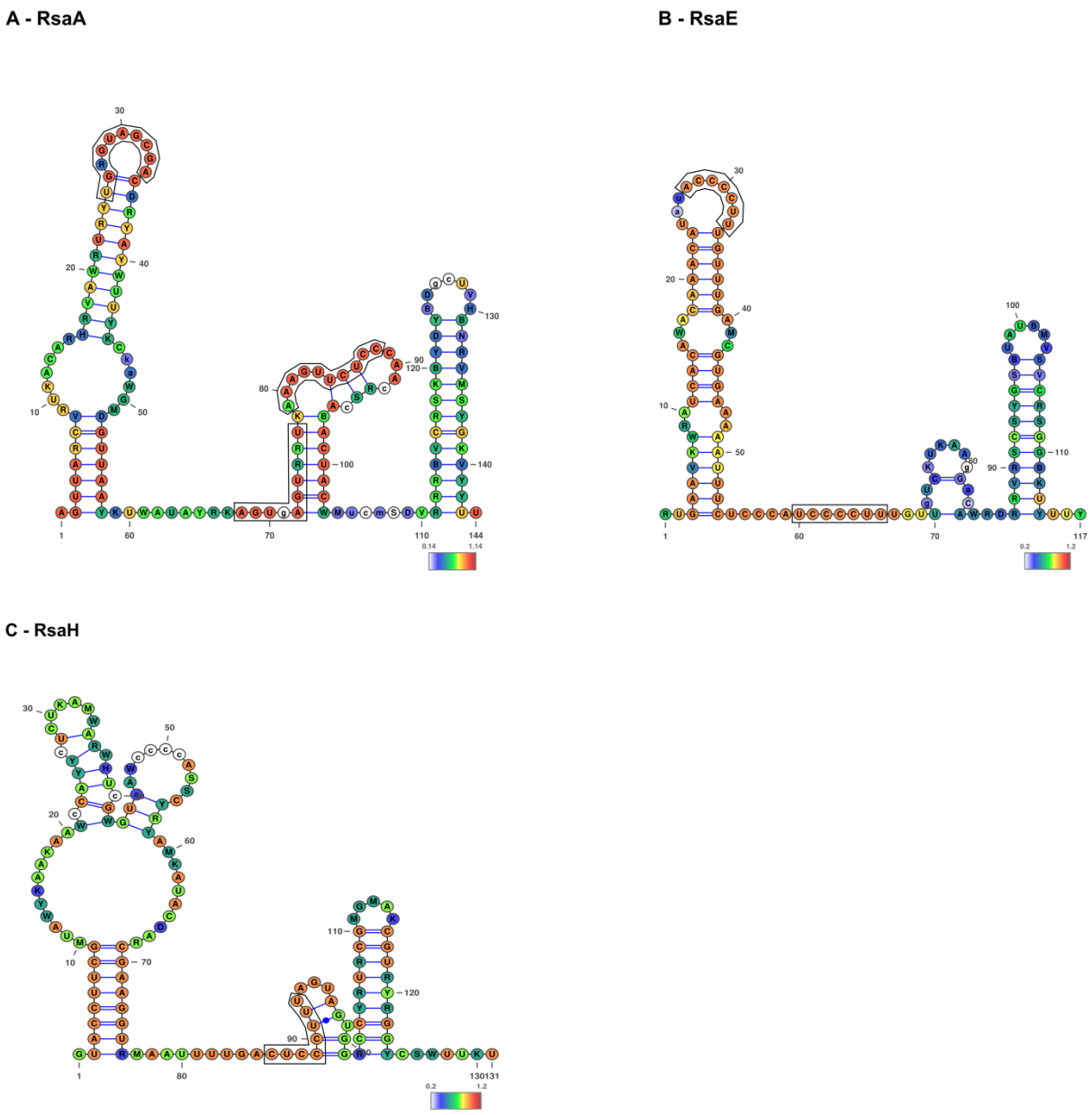

Fig. S2. Secondary structures of RsaA (A), RsaE (B) and RsaH (C) predicted from a structural alignment of homologous sequences, as provided by the RFAM database (20). Color scale indicates evolutionary conservation at each position (red: most conserved). Boxes around sequences indicate predicted seed matching regions as explained in Fig. 3. Structures are drawn using Varna (21). RFAM entries: RsaA: RF01816; RsaE: RF01820; RsaH: RF01821. [Experimental structures were also previously reported (22)]

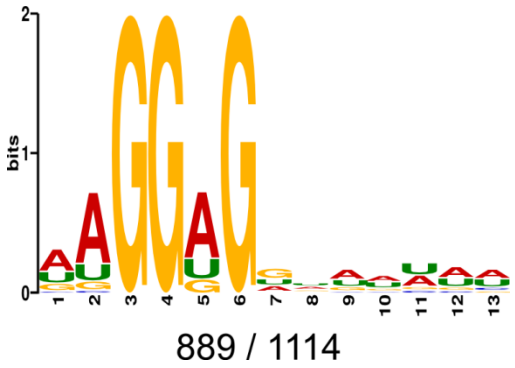

Fig. S3. Model for the standard *S. aureus* SD motif, identified by MEME (14) using all HG003 available 5' UTR regions (see Matérials and Method).

A

```
                    59               77
                    |                |
          5'-UUA...UUUAA      G      GACAU...GCU-3'    fur
                     UUAUUA UAGGGGAGUG
                     |||||| ||:||||:||
                     AAUAAU AUUCCCUUAC
          3'-CGG...GUAUG      A      AAAAU...AAU-5'    RNAIII
                    |                |
                    485              467
```

```
Energy:                   -13.0 kcal/mol          Position - mRNA:        60 -- 76
Hybridization Energy:     -19.2 kcal/mol          Position - ncRNA:      468 -- 484
Unfolding Energy - mRNA:    4.0 kcal/mol          Position Seed - mRNA:   72 -- 76
Unfolding Energy - ncRNA:   2.2 kcal/mol          Position Seed - ncRNA: 468 -- 472
```

```
>SAOUHSC_01592|fur
ttattgagaaaactgttagttttaattgtaaagtttgaaataatttgtaatgattttaattattagtaggggagtggacatcgTTGGAAGA
ACGATTAAATCGCGTTAAGCAACAATTACAACAATCATCATATAAGCTAACGCCACAACGCGAAGCTACTGTTAGAGTTCTAATTGAAAATG
AAAAAGATCATCTAAGTGCTGAAGACGTATATCTGAAAGTAAAAGATAAAGCGCCTGAAATTGGCTTGGCGACAGTATACAGAACGTTAGAG
TTGTTAGCTGAACTAAAAGTTGTCGACAAAATTAACTTTGGTGATGGCGTCGCTCGTTTTGATTTAAGAAAAGAAGGCGCAAAACATTTCCA
CCATCATTTAGTATGTATGGAATGTGGTCGTGTAGATGAAATCGATGAAGATTTGTTACCAGAAGTTGAAAATCGAGTTGAAAATGAGTTCA
ATTTTAAAATTTTAGATCATCGTTTAACTTTCCATGGTGTGTGTGAAACGTGCCAAGCTAAAGGTAAAGGATAGtaaattgcgtaggttaaa
ttaaccttcgct
```

B

```
             158                                                    213
             |                                                      |
5'-GUA...UUGUA  U       UAA   A         AAG  UU     U   GGGA       AUACU...UAA-3'   rot
              GU UUAUGCA   GUU  GCACAUAC   UU  GGGAU GUU    UGUUUGUUA
              || |:|||||   |||  :|||||||   ||  |:||| |||    |:||:||||
              CA AGUACGU   CAA  UGUGUAUG   AA  CUCUA CAA    AUAAGCAAU
3'-UUU...GCAAA  U       CG    AA        AA   UU                      AGCUUCCAUG-5'    RsaH
             |                                                      |
             59                                                     10
```

```
Energy:                   -14.9 kcal/mol          Position - mRNA:       159 -- 212
Hybridization Energy:     -33.9 kcal/mol          Position - ncRNA:       11 -- 58
Unfolding Energy - mRNA:    9.6 kcal/mol          Position Seed - mRNA:  208 -- 212
Unfolding Energy - ncRNA:   9.4 kcal/mol          Position Seed - ncRNA:  11 -- 15
```

```
>SAOUHSC_01879|rot, repressor of toxins
gtatataaattataaaattaatatgtaatagagtgatttgttttatgtactattatcttatttctaaatattaactctattgattattggtt
tttatacttatttaattttattcaactttgacaattgaatagaaagcaagtttatttacacttgtagtttatgcataagttagcacataca
agtttgggattgttgggatgtttgttaatacttgtatagtagctaaatatgtgattattaattgggagatgtttagcATGAAAAAAGTAA
ATAACGACACTGTATTTGGAATTTTGCAATTAGAAACACTTTTGGGTGACATTAACTCAATTTTCAGCGAGATTGAAAGCGAATACAAAATG
TCTAGAGAAGAAATTTTAATTTTACTAACTTTATGGCAAAAAGGTTCTATGACGCTTAAAGAAATGGACAGATTTGTTGAAGTTAAACCGTA
TAAGCGTACGAGAACGTATAATAATTTAGTTGAATTAGAATGGATTTACAAAGAGCGTCCTGTTGACGATGAAAGAACAGTTATTATTCATT
TCAATGAAAAGTTACAACAAGAGAAAGTAGAGTTGTTGAATTTCATCAGTGATGCGATTGCAAGTAGAGCAACAGCAATGCAAAATAGTTTA
AACGCAATTATTGCTGTGTAA
```

Figure S4. Predicted interaction between (A) RNAIII and *fur* mRNA and (B) RsaH and *rot* mRNA using the IntaRNA software. *fur* and rot mRNA sequences were retrieved from the reads profiles of HG003 transcriptome dataset. The start codons are boxed; untranslated regions are in lower cases and the coding DNA sequence in upper cases. The predicted pairing regions are highlighted in grey.

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Table S1. Read data summary

Read number	RNA3 trapping	RsaA trapping	RsaE trapping	RsaH trapping	HG003 transcriptome
Total	15211679 (100%)	12074287 (100%)	13679630 (100%)	11556376 (100%)	62293581 (100%)
Uniquely mapped	4102192 (27,0%)	2009060 (16,6%)	310720 (2,3%)	1441606 (12,5%)	9844630 (15,8%)
Unmapped	3693924 (24,3%)	3023715 (25,0%)	4101799 (30,0%)	2407864 (20,8%)	27107467 (43,5%)
Mapped in the sRNA gene	3739270 (24,6%)	1845837 (15,3%)	93005 (0,7%)	1182228 (10,2%)	-
Mapped more than once	7415563 (48,7%)	7041512 (58,3%)	9267111 (67,7%)	7706906 (66,7%)	25341484 (40,7%)
Used for DEseq analysis	362922 (2,4%)	163223 (1,4%)	217715 (1,6%)	259378 (2,2%)	-
Annotated CDSs					
Covered by at least 10 reads	1851 (64,0%)	1226 (42,4%)	1261 (43,6%)	1431 (49,5%)	2526 (87,3%)
Putative sRNA-targets					
Number of read-enriched TUs	101	23	32	69	-

Table S2. qRT-PCR results.

≥ 3.00												
≥ 1.50												
< 1.5 or > 0.67												
≤ 0.67												
≤ 0.33												
	1 - Variation of gene expression in *ΔsRNA* strains relative to the wild type strain (*wt*) using the four reference genes.						**2 - Variation of gene expression in *ΔsRNA pRMC2-sRNA* in the induced state (+aTc) relative to the same strains non-induced (-aTc) using the four reference genes.**					
RNAIII	**wt**			***ΔRNAIII***			**- aTc**			**+ aTc**		
4 references ftsZ/recA/gyrB/glyA	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
SAOUHSC_01150 (ftsZ)	0.92	0.98	1.10	0.98	1.00	0.90	1.01	0.98	1.01	0.98	1.01	1.00
SAOUHSC_01262 (recA)	1.05	1.01	0.94	0.99	1.02	1.12	0.98	1.04	0.98	1.07	1.03	1.04
SAOUHSC_00005 (gyrB)	1.13	0.99	0.90	0.93	0.89	0.94	0.98	1.00	1.03	0.94	0.94	0.98
SAOUHSC_02354 (glyA)	0.91	1.02	1.07	1.11	1.10	1.06	1.03	0.99	0.99	1.01	1.01	0.98
RNAIII	1.13	1.02	0.86	0.00	0.00	0.00	0.99	1.02	0.99	365.41	380.04	330.79
SAOUHSC_00070 (sarH1; Sar-like protein)	1.11	1.04	0.87	0.67	0.84	0.89	0.99	1.01	1.00	1.09	1.15	1.02
SAOUHSC_00074 (sirA, lipoprotein receptor)	1.52	1.04	0.63	0.66	0.76	1.80	0.88	1.31	0.86	0.60	0.68	0.60
SAOUHSC_00192 (coa, coagulase)	1.10	1.02	0.89	1.16	0.94	1.32	0.91	1.08	1.01	0.24	0.21	0.24
SAOUHSC_00257 (esxA, secreted antigen)	0.99	1.04	0.97	1.03	0.98	1.10	0.94	1.03	1.04	1.00	1.07	1.18
SAOUHSC_00362 (conserved protein)	1.13	1.00	0.88	0.82	0.80	0.85	1.01	0.99	1.01	1.02	1.08	1.07
SAOUHSC_00539 (conserved protein)	1.07	1.00	0.93	0.93	0.87	1.02	0.97	0.99	1.05	1.09	1.12	1.09
SAOUHSC_00652 (fhuC, iron transport)	1.02	1.03	0.96	1.00	1.00	1.20	1.02	1.02	0.96	0.59	0.58	0.58
SAOUHSC_00826 (conserved protein)	0.61	1.10	1.48	1.64	1.97	2.21	0.99	0.94	1.07	1.08	1.00	1.01
SAOUHSC_00715 (saeR, response regulator)	1.06	1.04	0.91	0.90	0.86	0.87	0.98	1.04	0.98	0.85	0.88	0.94
SAOUHSC_00935 (mecA, adapter protein)	0.99	1.02	0.99	1.02	0.94	1.03	0.92	1.01	1.08	0.95	0.97	1.08
SAOUHSC_01060 (conserved protein)	1.05	1.00	0.95	0.72	0.76	0.76	0.98	0.99	1.02	0.86	0.84	0.79

SAOUHSC_01081 (conserved protein)	0.92	1.03	1.06	0.88	0.85	0.85	1.04	0.97	1.00	1.21	1.14	1.09
SAOUHSC_01110 (fbp, fibrinogen-binding prot.)	0.92	1.00	1.09	0.99	0.92	0.95	0.97	1.03	1.00	0.15	0.14	0.14
SAOUHSC_01592 (fur, transcriptional regulator)	0.96	0.92	1.13	0.92	0.90	0.90	1.03	1.01	0.96	1.31	1.25	1.15
SAOUHSC_01653 (sodA, Mn-dependent SOD)	0.84	0.94	1.27	0.99	0.96	0.96	1.03	0.95	1.03	0.66	0.60	0.55
SAOUHSC_02019 (lytN, N-acetylmuramoyl-L-alanine amidase)	0.94	0.98	1.08	1.24	1.17	1.15	1.11	1.00	0.90	1.16	1.12	1.08
SAOUHSC_02419 (conserved protein)	0.91	1.03	1.06	0.89	0.90	0.94	1.04	1.03	0.93	1.46	1.39	1.24
SAOUHSC_02528 (conserved protein)	1.11	0.99	0.91	0.83	0.79	0.98	0.95	0.99	1.06	0.82	0.87	0.87
SAOUHSC_02554 (fhuD2, iron transport)	0.96	1.03	1.01	1.08	0.97	1.17	1.02	0.95	1.04	0.37	0.38	0.38
SAOUHSC_02568 (conserved protein)	0.81	0.91	1.36	0.92	0.88	0.86	0.97	1.02	1.01	0.92	0.87	0.77
SAOUHSC_02589 (rpiR, transcriptional regulator)	1.01	0.99	0.99	0.97	1.05	1.11	0.96	1.07	0.98	2.51	2.42	2.48
SAOUHSC_02660 (CorA-like transporter)	1.12	0.99	0.90	1.01	0.96	0.97	0.99	1.03	0.98	1.01	1.03	1.02
SAOUHSC_02670 (heat shock protein)	0.90	1.04	1.06	1.02	0.94	1.00	1.06	1.00	0.94	2.20	2.07	1.66
SAOUHSC_02706 (sbi, igG-binding protein)	1.03	1.01	0.96	1.35	1.17	1.23	1.04	1.02	0.94	0.46	0.47	0.48
SAOUHSC_02872 (conserved protein)	1.01	1.02	0.97	0.55	0.51	0.47	1.04	1.06	0.91	1.63	1.42	1.20
SAOUHSC_02931 (conserved protein)	0.81	0.99	1.25	1.26	1.50	2.27	0.95	1.03	1.03	1.17	1.16	1.10
RsaA	***wt***			***ΔrsaA***			**- aTc**			**+ aTc**		
4 references ftsZ/recA/gyrB/glyA	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
SAOUHSC_01150 (ftsZ)	0.92	1.02	1.07	1.09	0.91	0.99	0.87	0.94	1.21	1.11	0.88	1.07
SAOUHSC_01262 (recA)	1.12	0.80	1.12	0.90	0.87	1.00	1.03	0.98	0.99	1.21	1.24	1.17
SAOUHSC_00005 (gyrB)	1.01	1.06	0.94	0.96	1.16	1.02	1.03	1.06	0.92	0.84	0.96	0.85
SAOUHSC_02354 (glyA)	0.96	1.16	0.90	1.05	1.09	0.99	1.08	1.02	0.91	0.88	0.96	0.94
RsaA	0.58	3.03	0.57	0.00	0.00	0.00	1.06	0.95	1.00	162.17	139.79	165.94
SAOUHSC_00640 (tagA, teichoic acid bios.)	0.97	0.88	1.17	0.95	1.06	1.15	1.00	1.02	0.98	1.50	1.41	1.44
SAOUHSC_00694 (mgrA, norR, transcript. reg.)	0.92	1.04	1.05	0.97	0.86	0.82	0.92	0.84	1.30	2.13	1.61	1.85
SAOUHSC_01211 (rplS)	0.92	0.98	1.11	0.89	0.77	0.96	0.93	0.86	1.25	1.36	1.00	1.34
SAOUHSC_01238 (cdsA, lipid metabolism)	1.14	0.74	1.18	1.08	0.90	1.34	0.82	1.05	1.17	1.23	1.21	1.29
IGR-1418475	0.93	1.13	0.96	1.09	0.85	0.89	0.86	0.93	1.26	1.19	1.06	1.27

SAOUHSC_02571 (ssaA-like, CHAP domain)	0.81	1.24	1.00	0.66	0.69	0.98	0.81	0.93	1.33	0.82	0.74	0.83
SAOUHSC_02576 (ssaA, CHAP domain)	0.89	0.54	2.09	2.38	3.65	6.28	0.90	0.97	1.14	1.12	1.09	1.30
SAOUHSC_02855 (ssaA-like, CHAP domain)	0.88	0.79	1.44	0.63	0.76	2.44	0.98	0.84	1.21	3.55	3.14	3.48
SAOUHSC_02883 (ssaA-like, CHAP domain)	0.98	0.81	1.26	0.80	0.67	2.19	1.10	0.97	0.94	1.99	1.69	1.64
RsaE	***wt***			***ΔrsaE***			**- aTc**			**+ aTc**		
4 references ftsZ/recA/gyrB/glyA	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
SAOUHSC_01150 (ftsZ)	0.92	1.02	1.07	0.64	0.80	0.95	0.97	1.00	1.03	1.04	1.01	1.22
SAOUHSC_01262 (recA)	1.12	0.80	1.12	1.08	0.82	1.02	1.01	0.97	1.02	1.07	1.09	1.02
SAOUHSC_00005 (gyrB)	1.01	1.06	0.94	1.42	1.20	0.99	1.01	1.03	0.96	1.01	1.03	0.98
SAOUHSC_02354 (glyA)	0.96	1.16	0.90	1.02	1.27	1.04	1.01	1.00	0.99	0.89	0.88	0.82
RsaE	0.96	1.43	0.73	0.00	0.00	0.00	0.89	1.13	1.00	355.81	347.67	376.07
SAOUHSC_00819 (cspC, cold-shock protein)	1.40	0.72	0.99	0.97	0.47	0.76	0.98	1.05	0.97	1.51	1.70	2.30
SAOUHSC_00875 (pyridine oxidoreductase)	0.97	1.14	0.90	0.80	1.33	0.92	0.94	0.91	1.17	0.80	0.72	0.99
SAOUHSC_00894 (rocD, ornithine aminotransf.)	0.91	2.04	0.54	0.84	5.49	0.93	1.00	0.99	1.01	0.47	0.51	0.40
SAOUHSC_01016 (purN, purine and folate met.)	0.65	0.94	1.64	1.18	0.95	1.15	0.98	1.03	1.00	1.49	1.38	1.19
SAOUHSC_01138 (acetyltransferase)	0.83	1.39	0.86	2.48	5.04	2.06	0.97	1.03	1.00	0.46	0.56	0.39
SAOUHSC_01287 (glnA, glutamine synthetase)	0.87	0.94	1.23	0.56	0.80	1.31	1.06	1.02	0.92	1.13	1.17	0.96
SAOUHSC_01542 (SNF2-related protein)	0.92	1.29	0.85	1.38	1.21	0.85	0.99	1.13	0.89	1.27	1.46	1.19
SAOUHSC_02409 (rocF, arginase)	1.11	1.31	0.69	2.89	5.98	2.65	1.00	1.01	0.99	0.33	0.32	0.28
RsaOG (Teg24, RsaI) sRNA	1.15	2.62	0.33	0.47	2.20	0.20	0.91	1.08	1.02	2.07	2.55	2.40
SAOUHSC_02836 (acetyltransferase)	0.98	0.86	1.18	0.66	0.81	1.08	1.00	1.04	0.96	2.06	2.05	2.02
RsaX25/Teg141 sRNA	0.72	1.18	1.19	0.62	0.68	0.67	0.98	0.85	1.21	1.50	1.17	1.82
SAOUHSC_03001 (icaR, transcriptional regulator)	1.12	0.86	1.04	1.30	0.77	1.15	0.94	1.13	0.94	0.79	0.72	0.72
RsaH	***wt***			***ΔrsaH***			**- aTc**			**+ aTc**		
4 references ftsZ/recA/gyrB/glyA	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
SAOUHSC_01150 (ftsZ)	0.92	1.02	1.07	0.93	1.20	0.77	1.02	1.00	0.98	0.91	1.07	1.13
SAOUHSC_01262 (recA)	1.12	0.80	1.12	0.91	0.72	0.79	1.00	1.03	0.97	1.15	1.21	1.14

SAOUHSC_00005 (gyrB)	1.01	1.06	0.94	1.09	0.99	1.10	0.95	1.01	1.05	0.98	0.85	0.92
SAOUHSC_02354 (glyA)	0.96	1.16	0.90	1.07	1.16	1.49	1.03	0.96	1.01	0.98	0.91	0.85
RsaH	1.08	1.38	0.67	0.00	0.00	0.00	1.12	1.00	0.89	229.90	248.85	205.60
SAOUHSC_00067 (L-lactate permease)	0.83	1.14	1.05	0.96	1.78	1.96	1.04	1.12	0.86	1.27	1.55	1.34
SAOUHSC_00206 (lctE, L-lactate DH)	1.44	1.13	0.62	1.39	0.52	1.17	1.39	0.43	1.68	0.44	0.20	0.18
SAOUHSC_02648 (lctP, L-lactate permease)	1.31	1.41	0.54	1.62	1.24	1.77	1.63	0.38	1.60	0.27	0.12	0.13
SAOUHSC_01260 (pgsA)	0.88	1.08	1.05	0.83	1.20	0.75	1.07	1.10	0.85	0.94	0.99	0.88
SAOUHSC_00785 (trxB, thioredoxin reductase)	0.94	1.01	1.05	0.92	1.28	0.84	1.03	1.01	0.96	0.98	0.97	0.94
SAOUHSC_01732 (cymR, transcriptional factor)	1.32	0.72	1.05	1.56	0.67	0.84	1.05	0.97	0.98	0.90	0.80	0.83
SAOUHSC_01879 (rot, repressor of toxins)	0.87	1.25	0.93	0.78	1.48	0.99	1.13	0.87	1.01	0.67	0.81	0.67
SAOUHSC_02108 (ftnA, ferritin)	0.84	1.23	0.97	0.82	1.22	1.18	1.05	1.02	0.93	2.15	2.28	1.85
SAOUHSC_02972 (isaB, immunodominant ag. B)	1.01	1.32	0.75	0.77	1.21	1.21	1.04	0.92	1.04	0.53	0.57	0.54
SAOUHSC_00681 (MFS protein)	1.07	1.07	0.88	1.00	1.38	1.17	0.87	1.05	1.09	0.96	0.99	0.89
SAOUHSC_00465 (veg, conserved protein)	1.10	1.08	0.84	0.86	1.16	1.24	1.19	0.92	0.92	1.81	1.96	1.69
SAOUHSC_00863 (conserved protein)	0.98	0.62	1.64	0.72	0.72	0.72	1.05	1.07	0.88	1.69	1.85	1.54
SAOUHSC_01044 (conserved protein)	0.96	0.97	1.07	0.89	0.97	1.20	1.04	0.98	0.98	1.18	1.26	1.33
SAOUHSC_01062 (conserved protein)	0.87	1.21	0.95	0.92	1.39	0.78	1.15	0.97	0.90	1.68	1.78	1.57
SAOUHSC_01382 (conserved trans-menb. protein)	0.98	0.89	1.15	0.90	0.76	0.58	1.00	1.03	0.97	1.07	1.12	1.04
SAOUHSC_01721 (conserved protein)	0.95	1.12	0.94	1.00	1.07	0.92	0.96	1.04	1.00	1.17	1.11	1.06
SAOUHSC_01918 (conserved protein)	1.13	1.10	0.80	1.29	1.38	1.70	0.96	0.89	1.17	1.14	1.04	1.22
SAOUHSC_02303 (MazF-like protein)	0.97	1.16	0.90	0.91	1.01	0.84	1.07	0.97	0.97	1.43	1.26	1.25
SAOUHSC_02424 (conserved protein)	1.09	1.01	0.91	1.05	1.12	1.31	1.06	0.95	0.98	2.17	1.77	1.65
SAOUHSC_02555 (acyl-CoA DH-like protein)	1.13	1.37	0.65	1.17	1.14	0.73	1.00	1.00	1.01	1.35	1.36	1.37
SAOUHSC_02779 (conserved protein)	0.92	1.03	1.05	0.99	1.28	1.20	1.05	1.06	0.90	0.80	0.97	0.89

Variation of gene expression using the four reference genes (*ftsZ*, *recA, gyrB* and *glyA*): **1 -** in *ΔsRNA* strains relative to the wild type strain (*ΔsRNA* is either *ΔRNAIII*, *ΔrsaA*, *ΔrsaE* or *ΔrsaH*); **2 –** in *ΔsRNA* pRMC2-*sRNA* strain (*sRNA* is either *RNAIII*, *rsaA*, *rsaE* or *rsaH*) in the presence of aTc (induced state, column +aTc) relative to the same strains prior aTc addition (non-induced state, column -aTc). The values correspond to the three replicates from biological independent RNA samples.

Table S3. Oligonucleotides and strains.

Oligonucleotide Name	Description	Sequence (5' -> 3')
1) Synthetic RNAs production using T7 promoter:		
1166	T7-*rnaIII* forward	TAATACGACTCACTATAGGGTAACTAGATCACAGAGATGTGATGG
1167	*rnaIII* reverse	GCCGCGAGCTTGGGAGGG
SA79	T7-*rsaA* forward	TAATACGACTCACTATAGGGAGTTAACCATTACAAAAATTGTATAGAGTAGC
SA80	*rsaA* reverse	AACAAAGTACACTTTGCTCATAGCA
SA81	T7-rsaE forward	TAATACGACTCACTATAGGGATGAAATTAATCACATAACAAACATACCC
SA82	*rsaE* reverse	ATAAAAAAACGTCGTGTCTGAATACA
SA83	T7-*rsaH* forward	TAATACGACTCACTATAGGGTACCTTCGATAACGAATAAACATCTC
SA84	*rsaH* reverse	AAATAAAAACGACCCGCACG
2) Northern blots:		
RsaA_694_left	*mgrA* detection	CAATGCTCAAAGACAAGTTAATCG
RsaA_694_right	*mgrA* detection	GCTGAAGCGACTTTGTCAGA
RsaA_2855_left	*SAOUHSC_02855* detection	GTCGAACAAACGCATCAATC
RsaA_2855_right	*SAOUHSC_02855* detection	GTTACGTGCTGCCTTTTTGC
RsaE_2409_left	*rocF* detection	GCACCATCAACATTTGGACA
RsaE_2409_Right	*rocF* detection	GCGTTTCAAGCGGATCTAAA
RsaE_2836_left	*SAOUHSC_02809* detection	CCACAAACCATAGACGAACG
RsaE_2836_Right	SAOUHSC_02809 detection	GCAAACTTTTGATGCAACTGA
RsaE_1138_left	*SAOUHSC_01138* detection	TTTAGAGCGTTTGGCAACAA
RsaE_1138_Right	*SAOUHSC_01138* detection	AGCTTCCACATCTGTAAATCCA
RsaH_2972_left	*isaB* detection	TGGGCACACTGATTGGAGTA
RsaH_2972_Right	*isaB* detection	ACCGCTATCAGCTTCCTTTG
RsaH_2108_left	*ftnA* detection	CATATATGGCAATGGCAGCA
RsaH_2108_Right	*ftnA* detection	TACGAGCGCCAAGTTCTTTT
RsaH_206_left	*SAOUHSC_00206* detection	TGCCACACCATATTCTCCAA

RsaH_206_Right	*SAOUHSC_00206* detection	GCTAATCCCATTGCAACACC
RNAIII_2589_left	*rpiR* detection	CATCAGTCATTCGATTCAGCA
RNAIII_2589_Right	*rpiR* detection	TTGCTCCCATATGCATCTCA
RNAIII_2554_left	*fhuD2* detection	TTGGTGATGGCGATGTAGAA
RNAIII_2554_Right	*fhuD2* detection	GTTTCACTTCAGCCCAACCT
RNAIII_2706_left	*sbi* detection	ATTACGCGAACACCCAGAAC
RNAIII_2706_Right	*sbi* detection	CATCATGACGAACGATTGCT
HU-45_left	*hu* detection	AGATTTAATCAATGCAGTTGCAGA
HU_46_Right	*hu* detection	AATGCTTTACCAGCTTTGAATGCT

3) sRNA deletions:

Up-RsaE-F	*rsaE* upstream seq	GAATTCGAGACCGCTAGCGCTCGTTGGGTCGATGTCTATG
Up-RsaE-R	*rsaE* upstream seq	GCGTATGGACCTAGGTATATCAATCTGTTCATAATGTAAGCGAATA
Down-RsaE-F	*rsaE* downstream seq	ACCCCACAACCTAGGTATATAAAAGACCTCGTTACATTTATGGTG
Down-RsaE-R	*rsaE* downstream seq	GATATCGGATCCGAGACCCTCGAAATTTATTCATTTTTCGATCC
Up-RNAIII-F	*rnaIII* upstream seq	GAATTCGAGACCGCTAGCGCCCTGAAATGTGGAATAATGGCTA
Up-RNAIII-R	*rnaIII* upstream seq	GCGTATGGACCTAGGTATATAGGGCGAAATGGGTTCTTAC
Down-RNAIII-F	*rnaIII* downstream seq	ACCCCACAACCTAGGTATATTTAAGTATTTATTTCCTACAGTTAGGC
Down-RNAIII-R	*rnaIII* downstream seq	GATATCGGATCCGAGACCCTTTTTGGTACTTCAACTTCATCCA
Up-RsaH-F	*rsaH* upstream seq	GAATTCGAGACCGCTAGCGCACGGACCACTAGCTGACTCG
Up-RsaH-R	*rsaH* upstream seq	GCGTATGGACCTAGGTATATTGTATAACCTTTGAACAACAATAATGA
Down-RsaH-F	*rsaH* downstream seq	ACCCCACAACCTAGGTATATAAATGAATCCGATTTACGAGTGA
Down-RsaH-R	*rsaH* downstream seq	GATATCGGATCCGAGACCCTCTTGTGGTTTTGCTTGCTGA
pMADOF_R (733)	pMAD* amplification	GCGCTAGCGGTCTCGAAT
pMADOF_F (732)	pMAD* amplification	AGGGTCTCGGATCCGATATC
Tag primer2(736)	Tag amplification	ATATACCTAGGTCCATACGCAGCTATGCAAT
Tag primer1(735)	Tag amplification	ATATACCTAGGTTGTGGGGTACAGCAATGAC

4) pRMC2-sRNA constructions:

pRMC2_RsaA_F	*rsaA* ampl. for pRMC2 cloning	GATAGAGTATAATTAAAATAAGCAGTTAACCATTACAAAAATTGTATAGAG

pRMC2_RsaA_R	*rsaA* ampl. for pRMC2 cloning	TCAGATCTGTTAACGGTACCCCGTATACATAAGACGTGATTTGG
pRMC2_RsaH_F	*rsaH* ampl. for pRMC2 cloning	GATAGAGTATAATTAAAATAAGCGTACCTTCGATAACGAATAAACATCTC
pRMC2_RsaH_R	*rsaH* ampl. for pRMC2 cloning	TCAGATCTGTTAACGGTACCTGGGAATAAGAAATAAATAAAAACGA
pRMC2_RNAIII_F	*rnaIII* ampl. for pRMC2 cloning	GATAGAGTATAATTAAAATAAGCTAACTAGATCACAGAGATGTGATGG
pRMC2_RNAIII_R	*rnaIII* ampl. for pRMC2 cloning	TCAGATCTGTTAACGGTACCTATTTTAACGGCGGGTCTCA
pRMC2_F	pRMC2 amplification	
pRMC2_R	pRMC2 amplification	

5) qRT-PCR experiments:

0616-SAOUHSC01150-F1r	Quant. of the corresponding gene	ATCGTTATACCAAATGACCGTTTATTAG
0617-SAOUHSC01150-R1r	Quant. of the corresponding gene	GCGTAACACGTTGTCAGCTTCT
0622-SAOUHSC01262-F1r	Quant. of the corresponding gene	ATCGCAACCGGATCATGGT
0623-SAOUHSC01262-R1r	Quant. of the corresponding gene	AGCAGCAACTGAGTCTACAACTACAATA
0628-SAOUHSC00005-F1r	Quant. of the corresponding gene	GCACGTGTTGCTGCGAAA
0629-SAOUHSC00005-R1r	Quant. of the corresponding gene	CTTCAGGACTTTTACTAGAGCAATCG
0642-SAOUHSC02354-F1r	Quant. of the corresponding gene	TGTTTGGAGCTGAACATGTCAAT
0643-SAOUHSC02354-R1r	Quant. of the corresponding gene	TGTCGCCCATTTCTAATGCA
0438-SAO00640-F1	Quant. of the corresponding gene	TACGCGACGACACATCAAGC
0439-SAO00640-R1	Quant. of the corresponding gene	ACGATGCGCTAGAGGTTGCT
0440-SAO00694-F1	Quant. of the corresponding gene	GGGATGAATCTCCTGTAAACGTCA
0441-SAO00694-R1	Quant. of the corresponding gene	CGTTGATCGACTTCGGAACG
0442-SAO01211-F1	Quant. of the corresponding gene	CCAAGTTTCCGTCCTGGTGA
0443-SAO01211-R1	Quant. of the corresponding gene	GTTCCACGCCAACACCTGAT
0444-SAO01238-F1	Quant. of the corresponding gene	TGTGGCTTATGTAGGCATTGGTT
0445-SAO01238-R1	Quant. of the corresponding gene	CAAGCCACCTATGAATCCTTCG
0446-*1418475-F1	Quant. of the corresponding gene	AGGCATTAATTTGACGGCAATG
0447-*1418475-R1	Quant. of the corresponding gene	TTCGCGAAGTGTGTCTCGTTT
0450-SAO02571-F1	Quant. of the corresponding gene	GGCCGTTCAATCTCAAGTGGT
0451-SAO02571-R1	Quant. of the corresponding gene	TTGCATTGCCCCAAGTTGA

0452-SAO02576-F1	Quant. of the corresponding gene	TCATGCAGATGCTGCTGAAAAT
0453-SAO02576-R1	Quant. of the corresponding gene	AACCGATTTCTCCGCCAACT
0454-SAO02671-F1	Quant. of the corresponding gene	CCAAGCACAAACACCGGGTA
0455-SAO02671-R1	Quant. of the corresponding gene	CCGCTATCGGTGGTGCTAAA
0456-SAO02684-F1	Quant. of the corresponding gene	TCCAAAATGTCGACCAGCAA
0457-SAO02684-R1	Quant. of the corresponding gene	CTGTAACACCCCCACGCATT
0458-SAO02855-F1	Quant. of the corresponding gene	TGCAGTTGATTGCATGACAGC
0459-SAO02855-R1	Quant. of the corresponding gene	CATTTAAACGTCGCGCACAA
0460-SAO02883-F1	Quant. of the corresponding gene	CGCCATCTTCAAATGGTCGT
0461-SAO02883-R1	Quant. of the corresponding gene	CCAATTTTCCCACCAACACG
0462-RsaA-F1	Quant. of the corresponding gene	AGAGTAGCGACTGTATAATTTCTATTGAGG
0463-RsaA-R1	Quant. of the corresponding gene	AAAGTGTACCCGAGTAGTCTTCCTTG
0464-SAO00819-F1	Quant. of the corresponding gene	AGGTTTTGGTTTCATCGAAAGAGA
0465-SAO00819-R1	Quant. of the corresponding gene	CGATGTCGAATTCAACTTTTTGG
0466-SAO00875-F1	Quant. of the corresponding gene	TTGTTCGTAACTTGCCGATTGA
0467-SAO00875-R1	Quant. of the corresponding gene	TTGGCGCATGTGGTAAATCA
0468-SAO00894-F1	Quant. of the corresponding gene	TTGCTGACGAAATCCAAGCA
0469-SAO00894-R1	Quant. of the corresponding gene	TGAACCATGTGAGCCAGGTG
0470-SAO01016-F1	Quant. of the corresponding gene	GCTGGCTACATGCGTCTAATTG
0471-SAO01016-R1	Quant. of the corresponding gene	TTGGCCTATTGCGTCAATCC
0472-SAO01138-F1	Quant. of the corresponding gene	TGGCATATTTGTCGGAGATCAA
0473-SAO01138-R1	Quant. of the corresponding gene	TCTGAAGCGTACCCTCTGTTTTG
0474-SAO01287-F1	Quant. of the corresponding gene	TGGATGAAAAAGGGGAACCA
0475-SAO01287-R1	Quant. of the corresponding gene	GTTGACCAGGGGCAACTTCA
0476-SAO01542-F1	Quant. of the corresponding gene	GTAGCTCAGAATGGGGCATCA
0477-SAO01542-R1	Quant. of the corresponding gene	TTGGTTGGCCTTGAGACTCC
0478-SAO02409-F1	Quant. of the corresponding gene	TGCGGTAGGTTCAGTATCAGCA
0479-SAO02409-R1	Quant. of the corresponding gene	GACCTTCGCCTGTCAAAATCC
0480-*2377226-F1	Quant. of the corresponding gene	CAGGGGGAGCGATTAAACAA

0481-*2377226-R1	Quant. of the corresponding gene	GTCACGTGCTAGCCGACAAA
0482-SAO02836-F1	Quant. of the corresponding gene	TAGACGAACGTGTCGCATGG
0483-SAO02836-R1	Quant. of the corresponding gene	ATGAACCGAACGTCGCAAAC
0484-*2773602-F1	Quant. of the corresponding gene	TTCCCCTGAAAAGAATAAGTTGTCA
0485-*2773602-R1	Quant. of the corresponding gene	CCATCACATAGGCGCTTATCAA
0486-SAO03001-F1	Quant. of the corresponding gene	ATACGCCTGAGGAATTTTCTGGA
0487-SAO03001-R1	Quant. of the corresponding gene	TGCGAAAAGGATGCTTTCAAAT
0488-RsaE-F1	Quant. of the corresponding gene	AATCACATAACAAACATACCCCTTTGT
0489-RsaE-R1	Quant. of the corresponding gene	GTGTCTGAATACACGACGCTAAACA
0490-SAO00067-F1	Quant. of the corresponding gene	CTTTGCTTGGAGTCCGTTCG
0491-SAO00067-R1	Quant. of the corresponding gene	GCTGTCCCAGTTGCACCAAT
0492-SAO00206-F1	Quant. of the corresponding gene	AAGCGAAGCGTTCGATGTTG
0493-SAO00206-R1	Quant. of the corresponding gene	TTGCCCTCAGGACGTTGTTC
0494-SAO02648-F1	Quant. of the corresponding gene	GCATGGAGCCCATTCATTGT
0495-SAO02648-R1	Quant. of the corresponding gene	GCGTAACTTCGCTGATTGTTCC
0496-SAO01260-F1	Quant. of the corresponding gene	TTGCCAGAGAATTTGCCGTAA
0497-SAO01260-R1	Quant. of the corresponding gene	CAATGTTGCCAATGGATCACC
0498-SAO00785-F1	Quant. of the corresponding gene	GGTGTTCCGGGTGAACAAGA
0499-SAO00785-R1	Quant. of the corresponding gene	CTCATCACGACGGTGAACGA
0500-SAO01732-F1	Quant. of the corresponding gene	TCCTTTAAGAAATGCGGGGTTA
0501-SAO01732-R1	Quant. of the corresponding gene	TCCCCTGCTGAGATTTCTTCC
0502-SAO01879-F1	Quant. of the corresponding gene	AGAGCGTCCTGTTGACGATGA
0503-SAO01879-R1	Quant. of the corresponding gene	TTGCTCTACTTGCAATCGCATC
0504-SAO02108-F1	Quant. of the corresponding gene	GCACATGCAGAATTCAGAGCA
0505-SAO02108-R1	Quant. of the corresponding gene	TTTATCTTGACGAGCGATTTCAGA
0506-SAO02972-F1	Quant. of the corresponding gene	CAGGCAGCAATAACCCCATATT
0507-SAO02972-R1	Quant. of the corresponding gene	CGTTTCCTTTTGCAGAAACACC
0508-SAO00681-F1	Quant. of the corresponding gene	GCCCCAACTTTACGCATCTG
0509-SAO00681-R1	Quant. of the corresponding gene	GTTCCACCAAACAATCCAGCA

0510-SAO00465-F1	Quant. of the corresponding gene	CGTATTGTACTGAAAGCCAATGGA
0511-SAO00465-R1	Quant. of the corresponding gene	TCAACAATGAAAACTGACGGATATG
0512-SAO00863-F1	Quant. of the corresponding gene	TTGGTTTTGTTGGCGAGCAT
0513-SAO00863-R1	Quant. of the corresponding gene	CGCCTTCTTCAGCATTTACACC
0514-SAO01044-F1	Quant. of the corresponding gene	GAAGTATTATGAATCCGGCGTGAC
0515-SAO01044-R1	Quant. of the corresponding gene	TCCTCTGCTTTAGCAGGCACA
0516-SAO01062-F1	Quant. of the corresponding gene	CAATGAAAAATGCAGCTTTGAAAC
0517-SAO01062-R1	Quant. of the corresponding gene	GCCATCTTCGCGGTCAACTA
0518-SAO01382-F1	Quant. of the corresponding gene	CGCTGCATTTTTAGCGATTATG
0519-SAO01382-R1	Quant. of the corresponding gene	GTTCAGCGCCCTCATAGCC
0520-SAO01721-F1	Quant. of the corresponding gene	TCCAACGCAAGATGTAAGAGATGT
0521-SAO01721-R1	Quant. of the corresponding gene	GCTTCATTTTGGCGTGGAAT
0522-SAO01918-F1	Quant. of the corresponding gene	CATCATCAATCCACGCAACCT
0523-SAO01918-R1	Quant. of the corresponding gene	CTGGATGATCGGCAGTGACA
0524-SAO02303-F1	Quant. of the corresponding gene	ACAAGGGGGAGTCAGACCTGT
0525-SAO02303-R1	Quant. of the corresponding gene	ACATGTGTCGGTATTTTCGCTTT
0526-SAO02424-F1	Quant. of the corresponding gene	TTGGACATCGAACCTTTACGC
0527-SAO02424-R1	Quant. of the corresponding gene	TTCACGCCTTTTGGTGTTATCAT
0528-SAO02555-F1	Quant. of the corresponding gene	AGCGATGAGAATAGTTGGTGCTAAA
0529-SAO02555-R1	Quant. of the corresponding gene	GCATCTTCCATAGGTGGATTGTG
0530-SAO02779-F1	Quant. of the corresponding gene	AACCGCATTTGATTTTCGATTC
0531-SAO02779-R1	Quant. of the corresponding gene	ACTTGGGCGTCATGGACACT
0534-SAO00070-F1	Quant. of the corresponding gene	TCATCAGCAAGAAAACACACTTCC
0535-SAO00070-R1	Quant. of the corresponding gene	CACGTTCTGCAATTTTCTCTCG
0536-SAO00074-F1	Quant. of the corresponding gene	GTGCCACTGACGTCGCTGTA
0537-SAO00074-R1	Quant. of the corresponding gene	CGCGACAATTAAGTCCGGTTT
0538-SAO00192-F1	Quant. of the corresponding gene	TCCACAGGGCACAATTACAGG
0539-SAO00192-R1	Quant. of the corresponding gene	CTTAATGATGGGCCGCTTTG
0540-SAO00257-F1	Quant. of the corresponding gene	TACGGGCAAGGTTCAGACCA

0541-SAO00257-R1	Quant. of the corresponding gene	TCTTCGAAACGGCTGAAAGC
0542-SAO00362-F1	Quant. of the corresponding gene	TGGCATCCATGCACCTAAAA
0543-SAO00362-R1	Quant. of the corresponding gene	AGCTTGCTTCATCTCTGCATCA
0544-SAO00539-F1	Quant. of the corresponding gene	TGCCATGGTCATGACACCTTAT
0545-SAO00539-R1	Quant. of the corresponding gene	ATTTCGCGACCACGTTCAAT
0546-SAO00652-F1	Quant. of the corresponding gene	TAACGGCTGCGGGAAATCTA
0547-SAO00652-R1	Quant. of the corresponding gene	CCCAACAGTTAAGCCATCTGCT
0548-SAO00715-F1	Quant. of the corresponding gene	GTCCAAGGGAACTCGTTTTACG
0549-SAO00715-R1	Quant. of the corresponding gene	CGCATAGGGACTTCGTGACC
0550-SAO00826-F1	Quant. of the corresponding gene	CCACAAAATGTTTCACTTGACTGG
0551-SAO00826-R1	Quant. of the corresponding gene	GTGCATCCCAAATATCTTTTGTTTT
0552-SAO00935-F1	Quant. of the corresponding gene	AGGCCCGTGGATTTAGTCGT
0553-SAO00935-R1	Quant. of the corresponding gene	TTGTGACTTCGACACCTTTTTCAA
0554-SAO01060-F1	Quant. of the corresponding gene	GCGCACAACTGAGTCTAAACGA
0555-SAO01060-R1	Quant. of the corresponding gene	TTGCGCACGTTGTTTAGGTG
0556-SAO01110-F1	Quant. of the corresponding gene	CGTTTGCCGGTGAATCTCAT
0557-SAO01110-R1	Quant. of the corresponding gene	TTTGTGCTTTACGGTGTGTTGC
0558-SAO01592-F1	Quant. of the corresponding gene	GAAATTGGCTTGGCGACAGT
0559-SAO01592-R1	Quant. of the corresponding gene	TGGAAATGTTTTGCGCCTTC
0560-SAO01653-F1	Quant. of the corresponding gene	CACGCTTTGGTTCAGGTTGG
0561-SAO01653-R1	Quant. of the corresponding gene	GCCAATGTAGTCAGGGCGTTT
0562-SAO02019-F1	Quant. of the corresponding gene	GCTGGACTGACGGAATCGAA
0563-SAO02019-R1	Quant. of the corresponding gene	AACTGATCGTGGCGCTGTCT
0564-SAO02419-F1	Quant. of the corresponding gene	TTGCAGTCGAGCATTTAATGGA
0565-SAO02419-R1	Quant. of the corresponding gene	AACGTTGTTGCAACTGTGTAAGAAA
0566-SAO02528-F1	Quant. of the corresponding gene	TCAACCTCTCACCGTGAATTACTTT
0567-SAO02528-R1	Quant. of the corresponding gene	GAGCGAGAATGCCCATATGAAT
0568-SAO02554-F1	Quant. of the corresponding gene	TAACTGGGGTCGTGGTGGAG
0569-SAO02554-R1	Quant. of the corresponding gene	TCACTTCAGCCCAACCTGCT

0570-SAO02568-F1	Quant. of the corresponding gene	TTGAAGGGGCATTTATAGCAACA
0571-SAO02568-R1	Quant. of the corresponding gene	TCAACAAACATTTCAACGTTAGCAC
0572-SAO02589-F1	Quant. of the corresponding gene	ATGGCGGTTTTCACGACTTG
0573-SAO02589-R1	Quant. of the corresponding gene	GCATGATTAAGTGCGCGTGTAG
0574-SAO02660-F1	Quant. of the corresponding gene	GTGACCCTTTGGACTCAGAAGAAA
0575-SAO02660-R1	Quant. of the corresponding gene	CTGTTACGATAATACCGTTGCCAAT
0576-SAO02670-F1	Quant. of the corresponding gene	AGCGCAAAATACAAATCTGAACAA
0577-SAO02670-R1	Quant. of the corresponding gene	TGCTTGGTTTGATTTTAGGCAAG
0578-SAO02706-F1	Quant. of the corresponding gene	CGTCATGATGAGCGTGTGAAA
0579-SAO02706-R1	Quant. of the corresponding gene	TCAACTTTTGGCGCCACTTT
0580-SAO02872-F1	Quant. of the corresponding gene	TTACGAAATGACAAACGACACGA
0581-SAO02872-R1	Quant. of the corresponding gene	GATGGGTTAAATTCTTTCGCATGTA
0582-SAO02931-F1	Quant. of the corresponding gene	ATGATTCATTCCCAAAACAAGAGAA
0583-SAO02931-R1	Quant. of the corresponding gene	AATCACCGGCCTTTTATTTTAGC
0584-RNAIII-F1	Quant. of the corresponding gene	TGGATTATCGACACAGTGAACAAAT
0585-RNAIII-R1	Quant. of the corresponding gene	GCACTGAGTCCAAGGAAACTAACTC
0586-SAO01081-F1	Quant. of the corresponding gene	AACAACAAATGATGCGGATTT
0587-SAO01081-R1	Quant. of the corresponding gene	GGATAATCGACGTAAGAAGAATCAT
0592-RsaH-F3	Quant. of the corresponding gene	CCTTCGATAACGAATAAACATCTCT
0593-RsaH-R3	Quant. of the corresponding gene	GGGAGTCAAATTTTTACCTTCG

Strain Name	**Genotype**	**References/Construction**
RN4220	Mutant of strain 8325-4 (= 8325 N, UV-cured of phages Phi11, 12, and 13) that accepts foreign DNA	(Kreiswirth et al, 1983, Nature 305:709–712)
HG003	rsbU and tcaR repaired	(Herbert et al., 2010, Infect Immun. 78(6):2877-89)
SAPhB194	as HG003 DrsaA::tag1	HG003 + pMAD*rsaA::tag1
SAPhB194	as HG003 DrsaA::tag1	HG003 + pMAD*rsaA::tag1

SAPhB162	as HG003 DrsaE::tag45	HG003 + pMAD*rsaA::tag45
SAPhB163	as HG003 DrsaH::tag49	HG003 + pMAD*rsaA::tag49
SAPhB302	as HG003 DrnaIII::tag47	HG003 + pMAD*rsaA::tag47
SAPhB301	as HG003 DrsaA::tag1 pRMC2RsaA	SAPhB194 + pRMC2RsaA
SAPhB282	as HG003 DrsaE::tag45 pRMC2RsaE	SAPhB162 + pRMC2RsaE
SAPhB283	as HG003 DrsaH::tag49 pRMC2RsaH	SAPhB163 + pRMC2RsaH
SAPhB303	as HG003 DrnaIII::tag47 pRMC2RNAIII	SAPhB302 + pRMC2RNAIII

Table S4. Genome coordinates of putative targets, MEME motifs and IntaRNA predictions.

RsaA	**Size**	**UTR size**	**Genome pos.: start..end**	**Motif 1**	**Motif 2**	**Motif 3**	**IntaRNA**
SAOUHSC_00640 (*tagA*)	178	78	628836..629013	124			40 – 49
SAOUHSC_00694 (*mgrA*)	392	292	680066..679675	282		361	265 – 308
SAOUHSC_01211 (*rplS*)	202	102	1161439..1161640	175			131 – 156
SAOUHSC_01238 (*cdsA*)	200	100	1185133..1185332	164	131		89 – 132
0446-0447-*1418475	428	x	1419072..1418645	249			273 – 282
SAOUHSC_02571 (*ssaA-like*)	201	101	2361897..2362097	86	144	112	119 – 130
SAOUHSC_02576 (*ssaA*)	183	83	2366635..2366817	70		124	66 – 115
SAOUHSC_02671 (*narK*)	221	121	2456758..2456538	137			61 – 70
SAOUHSC_02684 (*nirB*)	200	100	2471681..2471481	139	149	172	130 – 153
SAOUHSC_02855 (*ssaA*-like)	145	45	2627965..2628109	88	135		32 – 40
SAOUHSC_02883 (*ssaA*-like)	158	58	2657294..2657137	44	101	84	27 – 82
SAOUHSC_02913	179	79	2680433..2680255	64			63 – 99
SAOUHSC_01505	222	122	1458752..1458531	58		163	47 – 54
IGR_1462719	205	x	1462936..1462739	8	164		99 – 123
SAOUHSC_02922	199	99	2687404..2687206	181			61 – 69
SAOUHSC_00188	200	100	208080..208279	93	137		25 – 34
SAOUHSC_00608	325	225	600043..600367	213	309		213 – 219
SAOUHSC_02830	232	132	2607971..2608202	102	227		213 – 232
SAOUHSC_02274	149	49	2104685..2104833	38			131 – 140
SAOUHSC_03055	247	147	2821294..2821048	135			16 – 39
SAOUHSC_00322	116	16	336664..336549	4			4 – 38
SAOUHSC_00438	200	100	441535..441734	89			81 – 86
SAOUHSC_02146	147	47	2019274..2019420	36			1 – 22
RsaE	**Size**	**UTR size**	**Genome pos.: start..end**	**Motif 1**	**Motif 2**	**Motif 3**	**IntaRNA**
SAOUHSC_00819 (*cspC*)	213	113	800964..801176				150 – 160
SAOUHSC_00875	232	132	840553..840322	118			118-125

SAOUHSC_00894 (*rocD*)	135	35	857683..857817	23.124	118-135
SAOUHSC_01016 (*purN*)	200	100	986097..986296		85-92
SAOUHSC_01138	133	33	1090169..1090037	19	19-26
SAOUHSC_01287 (*glnA*)	171	71	1242350..1242520		57 – 67
SAOUHSC_01542	200	100	1490508..1490309		92 – 105
SAOUHSC_02409 (*rocF*)	123	23	2237081..2236959	11	6 – 17
RsaOG (Teg24, RsaI) sRNA	149	x	2377463..2377315	6.25	21 – 30
SAOUHSC_02836	114	14	2612752..2612865	1	1 – 8
RsaX25/Teg141 sRNA	307	x	2774384..2774078		296 – 300
SAOUHSC_03001 (*icaR*)	172	72	2775082..2774911	58.118	117 – 127
SAOUHSC_00086	134	34	92424..92557		69 -- 84
SAOUHSC_00299	200	100	314008..313809	44.87	81 -- 95
SAOUHSC_00502	139	39	503130..503268	26	25 -- 31
SAOUHSC_00561	129	29	569598..569470		8 -- 18
SAOUHSC_00698	129	29	682514..682642	16	10 -- 23
SAOUHSC_00730	200	100	714159..714358	88	82 -- 93
SAOUHSC_00892	163	63	855514..855676	17	13 -- 22
SAOUHSC_00926	200	100	896996..897195		166 -- 178
SAOUHSC_00932	200	100	904525..904724	88	87 -- 96
SAOUHSC_T0006	166	x	1088090..1087966		61 -- 70
sRNA Teg103	144	x	1123950..1123807		124 -- 131
SAOUHSC_01320	163	63	1263356..1263518		47 -- 55
IGR_1247676	91	x	1248138..1248048		62 -- 66
IGR_1274679	118	x	1274715..1274832		10 -- 24
SAOUHSC_01485	174	74	1442876..1442703	41	35 -- 49
IGR_1821348	104	x	1821370..1821473		75 -- 80
SAOUHSC_T00055	76	x	492344..492419		40 -- 51
SAOUHSC_T00034	199	x	2128307..2128109		83 -- 110
SAOUHSC_02520	188	88	2323826..2324013	47	43 -- 53

SAOUHSC_02773	125	25	2550063..2550187	10.47			6 -- 53
RsaH	**Size**	**UTR size**	**Genome pos.: start..end**	**Motif 1**	**Motif 2**	**Motif 3**	**IntaRNA**
SAOUHSC_00067	324	224	71284..71607	212			212 – 222
SAOUHSC_00206 (*lctE*)	411	311	228171..228581	151			144 – 161
SAOUHSC_02648 (*lctP*)	224	124	2434732..2434509				93 -- 121
SAOUHSC_01260 (*pgsA*)/SAOUHSC_01257	200	100	1211921..1212120				108 -- 125
SAOUHSC_00785 (*trxB*)	200	100	766854..767053	82			75 – 96
SAOUHSC_01732 (*cymR*)	200	100	1636654..1636455	116			111 – 123
SAOUHSC_01879 (*rot*)	362	262	1795110..1794749				159 -- 212
SAOUHSC_02108 (*ftnA*)	142	42	1981577..1981718	28			27 – 36
SAOUHSC_02972 (*isaB*)	139	39	2735193..2735055				87 -- 101
SAOUHSC_00681/SAOUHSC_00679	200	100	667078..667277				90 -- 94
SAOUHSC_00465 (*veg*)	200	100	466101..466300				75 -- 88
SAOUHSC_00863	123	23	831415..831293	6			1 – 15
SAOUHSC_01044	200	100	1013361..1013560				141 -- 148
SAOUHSC_01062	186	86	1026355..1026540	73			70 – 78
SAOUHSC_01382	239	139	1325761..1325523	125			128 – 133
SAOUHSC_01721	200	100	1627989..1627790				113 -- 127
SAOUHSC_01918	172	72	1826595..1826766	93			16 – 27
SAOUHSC_02303/ SAOUHSC_02304	200	100	2135568..2135369				36 -- 45
SAOUHSC_02424	117	17	2252348..2252232	3			1 – 17
SAOUHSC_02555	170	70	2349961..2349792				2 -- 13
SAOUHSC_02779	145	45	2555347..2555491	29			26 – 36
SAOUHSC_00890	200	100	854571..854372	84			75 -- 90
SAOUHSC_02540	200	100	2338479..2338280	5			6 – 14
SAOUHSC_02332	156	56	2162523..2162368	115			110 – 122
SAOUHSC_01846	113	13	1754266..1754154	31			1 – 15
SAOUHSC_01477	200	100	1435623..1435424	65			65 – 81
SAOUHSC_01208	218	118	1159617..1159834	102			174 – 186

SAOUHSC_00835	127	27	809475..809601	9	5 – 14
SAOUHSC_02150	124	24	2022495..2022618	8	5 – 13
SAOUHSC_00204	125	25	227790..227666	11	21 – 29
SAOUHSC_02875	200	100	2649263..2649064	85	187 – 197
SAOUHSC_01983	131	31	1889320..1889190	17	10 – 24
SAOUHSC_01282	123	24	1239594..1239472	89	47 – 55
SAOUHSC_00613	319	219	604871..605189	142	145 – 153
SAOUHSC_01617	196	96	1541730..1541535	20	18 – 90
SAOUHSC_01164	455	355	1113245..1113701	345	375 – 409
SAOUHSC_01021	200	100	993120..992921	85	57 – 66
SAOUHSC_01822	200	100	1728492..1728293	157	84 – 98
Teg56	293	x	1051518..1051810	108	110 – 115
SAOUHSC_00971	109	9	946642..946534	42	12 – 22
SAOUHSC_00141	200	100	145305..145504	96	117 – 128
SAOUHSC_01340	200	100	1279179..1279378	49	27 – 44
SAOUHSC_02585	129	29	2377581..2377709	16	13 – 23
SAOUHSC_01689	282	182	1599924..1600205	169	163 – 176
RsaG	230	x	201741..201970	123	36 – 57
SAOUHSC_02399	542	442	2219308..2218767	201	7 – 14
SAOUHSC_02305	200	100	2136801..2136602	85	83 – 97
SAOUHSC_00792	208	108	?774234..774046	94	124 – 136
SAOUHSC_01278	200	100	1234835..1235034	187	88 – 124
SAOUHSC_02910	200	100	2677600..2677799	84	62 – 84
SAOUHSC_02867	205	105	2641169..2641373	90	82 – 97
SAOUHSC_00429	146	46	429170..429315	33	26 – 45
SAOUHSC_02604	169	69	2393726..2393558	52	55 – 63
SAOUHSC_02393	202	102	2214138..2213937	85	25 – 42
SAOUHSC_02848	231	131	2623111..2622881		92 -- 136
SAOUHSC_02794	156	56	2567336..2567491		124 -- 130

SAOUHSC_02351	200	100	2174538..2174339	56 -- 62
SAOUHSC_02317	200	100	2151662..2151463	32 -- 49
SAOUHSC_01181	198	98	1133513..1133316	159 -- 168
SAOUHSC_01131	180	80	1085535..1085714	152 -- 159
SAOUHSC_00942	200	100	914081..914280	19 -- 25
rsaD	177	x	639887..639711	3 -- 8
SAOUHSC_00642	281	181	630699..630979	127 -- 142
SAOUHSC_00501	154	54	503065..502912	35 -- 42
SAOUHSC_00229	116	16	250575..250690	43 -- 48
IGR_asSAOUHSC_A02856	200	x	2781897..2781698	29 -- 48
SAOUHSC_02625-AS02627	200	100	2413724..2413923	121 -- 133
SAOUHSC_00950	123	23	924110..924232	4 -- 31
SAOUHSC_00174	156	26	190474..190629	53 -- 58

CHAPTER C

Deciphering RNase III essentiality in *Staphylococcus aureus*

Deciphering RNase III essentiality in *Staphylococcus aureus*

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Abstract

RNase III is a conserved double-strand-specific endoribonuclease. In bacteria, it contributes to i) bulk RNA degradation, ii) reducing pervasive transcription and iii) maturing both rRNAs and mRNAs. In *Bacillus subtilis*, RNase III is essential, and ensures protection against Type I toxin-antitoxin (TA) systems by degrading antitoxin RNA-mRNA duplexes. Despite its involvement in essential functions, RNase III is dispensable in several bacterial species, presumably due to functional redundancy with other ribonucleases. Deletions in *rnc* (encoding RNase III) were previously constructed in *Staphylococcus aureus* and therefore RNase III was reported as non-essential in this human and animal opportunistic pathogen. Surprisingly, the *rnc* gene could not be deleted in HG003 *S. aureus* strain, which contains three prophages that are absent in strains in which *rnc* was dispensable. Phage-encoded RNA duplexes were thus candidate RNase III targets. A cloned Type I TA system, named here SapTA, encoded by one of the HG003 prophages, had no effect on the wild type strain, but was deleterious in the absence of RNase III due to the expression of the SapT toxin. Two phages produced clearer plaques when spotted on a *rnc* strain than on its corresponding wild type isogenic strain. These results show that RNase III provides a selective advantage in the presence of phages.

Introduction

RNA steady-state is maintained by a balance between RNA synthesis and RNA degradation. Degradation involves ribonucleases (RNases), which also contribute to RNA processing leading to active transcripts.[1-3] Ribonuclease III (RNase III) is an important ubiquitous endonuclease that cleaves double-stranded RNA.[4] It is involved in mRNA decay and mRNA processing. A major role is its contribution to ribosomal RNA (rRNA) maturation.[5] However, this function is also performed by other ribonucleases, and RNase III is dispensable in some bacteria.[4] In *Escherichia coli*, RNase III regulates its own expression by a feedback mechanism involving cleavage of a stem-loop within its own mRNA.[6] RNase III also contributes to numerous post-transcriptional regulatory systems, *via* the degradation of internal structures or RNA duplexes that involve mRNA/regulatory RNA interactions.[7,8]

Staphylococcus aureus is a Gram positive bacterium involved in hospital and community-acquired infections including endocarditis, pneumonia, osteomyelitis and skin infection.[9,10] In *S. aureus*, the *rnc* gene (encoding RNase III) was inactivated in several strains and reported not to affect growth in rich media.[11-13] However, the absence of RNase III affects expression of numerous virulence genes and strongly decreased *S. aureus* pathogenicity in a murine peritonitis model as compared to the parental strain.[13] RNAIII, a 514-nt regulatory RNA with 14 stem loop structures[14,15] associates by base-paring with targeted mRNAs related to virulence, thus affecting their translation and stability.[11,13,16] By degrading RNAIII targets, RNase III contributes to the irreversibility of regulatory processes.[11,16,17] The use of wild-type and mutated forms of RNase III to co-immunoprecipitate associated RNAs revealed numerous RNase III substrates and its global role in gene regulation.[18] RNase III was also recently shown to eliminate pervasive transcription that occurs in *S. aureus*.[12]

Despite its multiple roles, RNase III is not essential is some organisms, such as *E. coli*,[19] *Streptomyces coelicolor*,[20] and *Salmonella*.[21] In *Bacillus subtilis*, RNase III was

considered as essential;[22,23] recent experiments indicate that RNase III is required to eliminate toxins encoded by the type I toxin/antitoxin (TA) system.[22]

In *S. aureus*, RNase III was considered as non-essential. However, we were unable to delete *rnc* strain in some strains, leading us to revisit this view. Here, we demonstrate that the presence of lysogenic phages containing a Type I TA system renders *S. aureus* dependent on RNase III. In addition, Δ*rnc S. aureus* strains are more susceptible to phage infection, indicating that RNase III provides a selective advantage against deleterious effects of foreign DNA containing Type I TA systems.

Results and Discussion

RNase III may be essential for *S. aureus*.

NCTC8325 (aka RN1) is a clinical isolate from a corneal ulcer; this strain and its derivatives are often used for genetic studies.[24,25] However, the positive regulator of σ^B (*rsbU*) and a transcriptional activator of protein A (*tcaR*) are inactive, such that NCTC8325 is deficient for σ^B-dependent regulation and protein A expression. HG003 is a NCTC8325 derivative in which *rsbU* and *tcaR* were repaired, and is proposed as a model strain for staphylococcal regulation studies (Figure 1).[26] NCTC8325 was also cured of 3 resident phages (Φ11, Φ12 and Φ13) yielding NCTC8325-4 (named RN450),[24] and then subjected to chemical mutagenesis yielding RN4220.[27] The latter strain is used to uptake foreign DNA thanks to the inactivation of a restriction system.[28] Like its parental strain NCTC8325, RN4220 is *rsbU* and *tcaR*. Comparative sequence analysis between the two strains reveals four large-scale deletions and more than a hundred SNPs in RN4220.[29,30]

To characterize the role of RNase III in post-transcriptional regulation, we intended to construct *rnc* deletions in HG003 and RN4220. In *S. aureus*, locus replacements are classically performed by two-step homologous recombination with integration and excision of a conditionally replicative plasmid at targeted loci. This excision step usually results in ~50% clones having the expected mutations. We used a replication thermo-sensitive plasmid pMAD derivative to delete the *rnc* gene.[31] A PCR test following the excision step indicated that *rnc* deletions in RN4220 were obtained at the expected frequency. However, surprisingly none of the fifty tested colonies from a selection on HG003 had an *rnc* deletion indicating a strong selective advantage to remain *rnc*$^+$ in this strain, but not in RN4220 (Figure 1).

RNase III was considered as non-essential since the *rnc* gene was inactivated in several strains and reportedly did not affect growth in laboratory conditions.[32] A *S.*

aureus Δ*rnc* was constructed in RN6390.[11,16] This strain derives from NCTC8325-4 and has characteristics similar to those of RN4220. The above results suggested that an active σ^B regulation and/or the presence of prophages could prevent establishment of the *rnc* deletion in HG003.

The alternative σ^B is a *S. aureus* global regulator induced during stationary phase and upon various stress.[33] Because of the importance of σ^B regulation, we first tested the potential role of *rsbU* in RNase III essentiality. Two NCTC8325 derivatives deferring with their *rsbU* allele were tested: NCTC8325-4 (*rsbU*) and SH1000 (*rsbU*$^+$) (Figure 1). The *rnc* gene was successfully inactivated in both strains indicating that *rsbU* is not involved in RNase III essentiality. In agreement with this conclusion, a Δ*rnc* mutant was reported in *S. aureus* 15981 [12] and USA300;[34] these strains, not related to NCTC8325, are also *rsbU*$^+$ [35]. Successful inactivation of *rnc* in RN6390 and NCTC8325-4 also indicated that the nitrosoguanidine mutagenesis underwent by RN4220 was not a determining factor for RNase III essentiality.

NCTC8325-4 differs mainly from NCTC8325 by the absence of three prophages that were expelled after two UV treatments. In contrast to NCTC8325-4, we were unable to introduce Δ*rnc* in NCTC8325. This result indicates that prophages are likely associated with RNase III essentiality in *S. aureus*. In agreement with this proposal, neither NCTC8325 derivatives nor *S. aureus* 15981, in which the *rnc* gene was deleted, possessed Φ11, Φ12 or Φ13.

A phage encoded toxin is deleterious for *S. aureus* in the absence of RNase III.

TA systems encode poisons and their corresponding antidotes. In Type I TA systems, the antitoxin is an RNA that associates with the mRNA encoding a toxin to prevent its translation or to stimulate its degradation.[36,37] Toxin and antitoxin are often expressed from opposite strands of a same DNA region; in these cases, the two transcripts are complementary and may form duplexes targeted by RNase III. We considered that RNase III essentiality in HG003 could be due to the presence of Type I TA systems

carried by resident prophages. These putative systems were search by visual inspection of HG003 high-throughput RNA sequences aligned on a genome browser. Five loci with significant transcription on both strands were identified within the three prophage sequences (Figure S1). We hypothesized that these loci could generate toxicity in the absence of RNase III. Four loci, SAOUHSC_01580 within the prophage Φ12, SAOUHSC_02016 to SAOUHSC_02018 within prophage Φ11 and SAOUHSC_02176 and SAOUHSC_02236 to SAOUHSC_02238 within Φ13, were cloned into shuttle vector pCN38 (Table 1). No clone was obtained with the fifth locus (SAOUHSC_02076 to SAOUHSC_02078 sequence), maybe due to expression of the toxin in *E. coli*. pCN38 and its derivatives obtained in *E. coli* were recovered to transform RN4220 and RN4220 Δ*rnc*. After 12 hours, clones were obtained for all transformations except for pCN38 containing SAOUHSC_02018 (named pSAP-TA) into RN4220 Δ*rnc* (Table 2). However, this latter transformation gave rise to small size translucent colonies after 18 hours, which displayed altered growth in liquid rich medium (Figure 2). These results suggest that the SAOUHSC_02018 locus is deleterious for *S. aureus* in the absence of RNase III. As RNase Y is a major Staphylococcal RNase,[38] we also tested the transformation efficiency of pCN38 and its derivatives in its absence: no major difference was observed between RN4220 and its Δ*rny* derivative (Table 2).

To rule out that the above finding results from a polar effect associated with Δ*rnc* or mutations due to the inactivation procedure, we constructed RN4220 Δ*rnc* derivative in with the *rnc* gene was introduced at an ectopic position, yielding RN4220 Δ*rnc* ECTO::*rnc*$^{+}$ (cf. Material and Methods). The weak growth defect associated with Δ*rnc* was alleviated in the presence of the *rnc*$^{+}$ ectopic copy indicating its ability to complement Δ*rnc* (Figure 2). As expected, transformation of RN4220 Δ*rnc* ECTO::*rnc*$^{+}$ with pSAP-TA did not lead to slow growing translucent colonies; we therefore concluded that the pSAP-TA phenotypes in RN4220 Δ*rnc* were solely due to the absence of RNase III.

SAOUHSC_02018 encodes a putative small peptide of 50 amino acids with a possible small transmembrane domain. On the opposite strand an asRNA is transcribed, which likely prevents expression of SAOUHSC_02018 (Figure S1). This structure is reminiscent of Type I TA systems. SAOUHSC_2018 and its asRNA were accordingly renamed *sapT* and *sapA* for *S. aureus* phage encoded Toxin and Antitoxin, respectively (also see below). The *sapT* start codon of pSAP-TA was replaced by a stop codon yielding pSAP-T*A. This mutagenesis modified neither *sapT*/*sapA* RNA complementarity nor the transcriptional start sequence of *sapA*. In contrast to pSAP-TA, transformants of RN4220 and RN4220 Δ*rnc* with pSAP-T*A were observed after 12 hours. The presence of pSAP-T*A did not affect RN4220 Δ*rnc* growth (Figure 2). We concluded that the SAOUHSC_02018 locus toxicity in RN4220 Δ*rnc* was associated with the presence of SapT.

RN4220 Δ*rnc* pSAP-TA was grown in liquid with several rounds of dilution over about five days and plated on a rich medium. Fast-growing clones were observed and selected for further studies. Plasmids from four independently selected fast-growing clones were extracted and re-introduced into RN4220 Δ*rnc*. Three of them did not have any mutation in the *sapT/spaA* genes and led to initial phenotype (*i.e.*, slow growing translucent colonies); this result suggests that chromosomal mutations circumvent *sapT/spaA* associated toxicity. One extracted plasmid did not confer slow growth when re-introduced into RN4220 Δ*rnc*; the identified mutation changed TGG to TAG resulting in the replacement of SapT W28 by a stop codon. This result supports the conclusion that SapT is a toxic protein expressed in the absence of *rnc*.

RNase III interferes with the Type I TA *sapT*/*sapA* expression.

As sapT mRNA and SapA are expressed from complementary strands, they likely form duplexes targeted by RNase III. We therefore asked whether the Δ*rnc* deletion could affect the amounts and profiles of both RNAs. The putative *sapT* and *sapA* promoter sequences have canonical σ^{70} promoter sequences (TTTACT - n=17 - TATAAT for SapT and TTGAAA - n=17 - TATTAT for SapA) and are likely recognized by the *S.*

aureus housekeeping factor σ^A.[39] Indeed, Northern blotting experiments indicate that the antitoxin SapA is detected at all growth phases, and accumulates in stationary phase in both RN4220 and RN4220 Δ*rnc* strains (Figure 3). In RN4220, probing for *sapT* mRNA reveals a band of about 200 nucleotides, but not in stationary phase, suggesting that *sapT* mRNA may disappear as a result of SapA accumulation. Probing for *sapT* mRNA in RN4220 Δ*rnc* shows a drastically different profile as in RN4220: (i) several higher molecular weight bands were detected and (ii) the pattern remained similar in all growth phases (Figure 3). These experiments are interpreted as showing that the absence of RNase III results in an inability to degrade *sapT* mRNAs and therefore contributes to its toxicity.

RNase III contributes to *S. aureus* adaptation to phage infection.

The *sapT* gene is conserved in staphylococcal phages Φ11, SP6, SA12, SA13, Φ55, StauST398-5 and Φ80α. These phages belong to unrelated phage families from different serogroups and containing varied integrases.[40] SapT is also present within lysogenized phages of numerous *S. aureus* isolates including NCTC8325, Mu50 and USA300. The COL isolate has only one prophage devoid of *sapT* but the gene is present in the SaPI3 pathogenicity island, a mosaic sequence including phage DNA.[41] In some strains, the -35 box of *sapT* transcriptional promoter is TTTACT rather than TTGCTT, which may affect the level of the toxin. For all these *sapT*–containing strains, the amount of SapT has to remain low. Consequently, a main reason for RNase III maintenance might be to ensure protection against toxicity of incoming DNA carrying Type I TA systems. To test this hypothesis, two phages, Φ11 and Φ80α, which carry the *sapT* gene, and the COL phage, L54a, (devoid of *sapT*) were plated on different genetic backgrounds including HG003, RN4220 and RN4220 Δ*rnc*, RN4220 Δ*rny* and COL. The number of plaque forming units (PFUs) and plaque morphology were studied at 37°C. L54a did not form visible plaques when infecting COL and HG003. No significant difference was observed in terms of PFU for RN4220 and its mutants. For

Φ11 and Φ80α, turbid plaques were obtained in a parental strain whereas clear plaques were observed in absence of RNase III (Figure 4). L54a phage spotted on RN4220 and its Δ*rnc* derivative gave turbid plaques in both cases. These results show that the presence of RNase III contributes to improve *S. aureus* survival to phage infection in the case of phages containing type I TA systems.

Conclusion

Giving the role of RNase III in several crucial RNA processing, one could expect that *rnc* integrity would be required for cell survival. However, because other RNases can substitute for RNase III, Δ*rnc* mutants were obtained in several bacterial species. RNase III was considered essential in *B. subtilis*.[22,23] However, essentiality appears to be due to the presence of toxins that are controlled by antisense RNA mechanisms; RNase III was needed to silence such toxins.[22] In the absence of toxins, *B. subtilis rnc* becomes dispensable. In contrast, RNase III was considered dispensable in *S. aureus*. Here, we demonstrate that the absence of RNase III is deleterious in *S. aureus* strains carrying Type I TA systems. Our results allow the initial finding on the role of RNase III in *B subtilis* from Condon's lab to be generalized to other species. It was recently reported that *rnc* was inactivated by transposon mutagenesis in the HG003 genetic background; however, the strain used was a derivative in which the Φ11 prophage was specifically cured to prevent homologous recombination with the used transposon element.[42] Therefore, we can conclude that in the absence of Φ11, HG003 becomes permissive for *rnc* inactivation. We observed that a wild-type *S. aureus* strain was more resistant to phages containing Type I TA systems than its *rnc* isogenic derivative. Consequently, the essential role of RNase III would not be to process endogenous RNAs, but rather to ensure a defense system against incoming DNA, therefore providing a selective advantage against phage infections.

Material and methods

Bacterial strains, growth conditions and plasmids

Strains and plasmids used in this study are listed in Table 1. *S. aureus* and *E. coli* DH5α were grown at 37°C with aeration in BHI and LB media, respectively. When necessary, antibiotics were used as follows: chloramphenicol (5 µg/ml) and erythromycin (0.5 µg/ml) for *S. aureus*; chloramphenicol (10 µg/ml) and amipicillin (100 µg/ml) for *E. coli*.

Primers are listed in Table S1.

Expression of cloned genes in *S. aureus* was obtained using pCN38 derivatives.[43] The pSite1 plasmid was constructed as follows; Phage site1 (Figure S1) was PCR-amplified from HG003 chromosomal DNA using primers Site1_F/Site1_R, pCN38 was PCR-amplified using primers pCN38-lin_F/pCN38-lin_R. pSite1 was obtained by assembling the two PCR fragments as described.[44] pSAP-TA (pSite 2), pSite3 and pSite4 were constructed as per pSite1 except that *sapTA*, Site3 and Site4 were amplified using Site2_F/Site2_R, Site3_F/Site3_R, and Site4_F/Site4_R, respectively. pSAP-T*A is a plasmid in which the *sapT* start codon was replaced by a stop codon as follows. pSAP-TA was PCR-amplified using the mutagenic primers SapT-mutF/SapT-mutR, the PCR product was self-ligated by Gibson assembly.[44]

Chromosomal allelic exchanges were performed using pMAD2-based plasmids. pMAD2 is a pMAD [31] derivative that allows to use one-step cloning strategy with the Type IIS restriction enzyme BsaI.[45] pMAD2, designed for another purpose than the experiments described here, was constructed as follows: i) the unique pMAD BsaI site, within the *bla* gene, was removed and ii) two BsaI sites and additional unique restriction sites were introduced. pMAD2 was fully sequence (accession number pending). For construction and map, see Figure S2.

pMAD2-DErnc was constructed for the chromosomal deletion of *rnc* as follow. The upstream and downstream *rnc* sequences were PCR-amplified from HG003 chromosomal DNA using primers Rnc-upF/Rnc-upR and Rnc-dwF/ Rnc-dwR, respectively. pMAD2 was PCR-amplified using pMAD-F/pMAD-R primers. pMAD2-DErnc was obtained by assembling the three PCR fragments as described.[44]

pMAD2-DErny was constructed for the chromosomal deletion of *rny* as follow. The upstream and downstream *rny* sequences were PCR-amplified from HG003 chromosomal DNA using primers Rny-upF/Rny-upR and Rny-dwF/ Rny-dwR, respectively. pMAD2 was PCR-amplified using pMAD-F/pMAD-R primers. pMAD-DErny contains a specific tag sequence to mark the *rny* deletion which was designed for another purpose than the experiments described here. The tag sequence was obtained by a PCR-amplification on pTAG44 using Tag-F/Tag-R primers. pMAD2-DErny was obtained by assembling the four PCR fragments as described.[44]

We constructed pECTO, a plasmid derived from pMAD2 which can be used for any chromosomal gene ectopic complementation (Figure S3). This plasmid allows insertions between SAOUHSC_00278 and SAOUHSC_00279. This region was chosen because i) it is conserved across *S. aureus* species and ii) our deep-seq RNA data from samples grown in 16 conditions failed to detect any transcription (Figure S3). pECTO contains two fragments of ca. 800 bp corresponding to the region encompassing SAOUHSC_00278 (chromosomal coordinates 293951-294750) amplified using primers pECTO_A_F/pECTO_A_R, and SAOUHSC_00279 (chromosomal coordinates 294754-295555) amplified using pECTO_C_F/pECTO_C_R, and in between, a *E. coli rrn* strong transcription terminator, amplified using pECTO_B_F/pECTO_B_R, to avoid possible transcriptional interference associated with inserted. These three amplicons were assembled with the linear pMAD2 plasmid backbone as described. [44] The ectopic zone was deposited on genbank (accession number pending).

Plasmids pMAD2 and pECTO are available for scientific community as Addgene plasmids 67682 and 67683, respectively (https://www.addgene.org/).

The complementation of Δ*rnc* was performed by the chromosomal insertion of the *rnc* gene using pECTOrnc, constructed as followed. The *rnc* gene with its upstream regulatory region was PCR-amplified from HG003 chromosomal DNA using primers rnc -comp-F/rnc-comp-R. pECTO was PCR-amplified using primers pECTO-lin-F/pECTO-lin-R. pECTOrnc was obtained by assembling the two PCR fragments as described.[44]

DNA manipulation

DNA extraction, purification of PCR products and plasmid extraction were done according manufacturer's instructions (Macherey-Nagel, Hoerdt, France) with an additional step for *S. aureus*: cells were incubated for 1 h at 37°C with the lysis solution containing lysostaphin (10 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA). PCR amplifications were performed using Phusion DNA (for cloning) and DreamTaq (for construct verification) polymerases according supplier's recommendations (Life Technologies, Saint Aubin, France).

Gene replacement and functional complementation

The inactivation of *rnc* and *rny*, and the *rnc* ectopic chromosomal insertion were performed as described [31] using pMAD2-DErnc, pMAD2-DErny and pECTOrnc, respectively, with the following modification: plasmid integration steps were performed at 37°C instead of 42°C to avoid unwanted mutations that may arise at 42°C in the presence of erythromycin.[13]

RNA extraction & Northern blot analysis

Total RNAs were extracted as described.[46] Northern blots were performed as described.[47,48] Samples were separated by PAGE gels and probed with γ^{32}-P labeled oligonucleotides (Table S1). To decipher the role of RNase III in the regulation of SapA/*sapT* mRNA, samples were recovered at OD_{600} 0.4, 2, 4, 6 and in stationary phase.

Phage preparation and drop assay

The prophage L54a was induced by UV treatment and recovered from *S. aureus* COL as described.[49] Phages L54a, Φ11 and Φ80α were amplified on plates as follow. One ml of BHI [for Φ80α, $MgSO_4$ (12.5mM) was added to the medium] was mixed to 10 µl of an overnight culture of RN4220 and incubated 15 min at 37°C. Three ml of top agar were added and plated onto BHI plate. The day after, the top agar layer was recovered and 1ml of BHI broth and 100 of chloroform was added. The mixture was then centrifuged and 1 ml of supernatant was recovered. The titer of phage, expressed as PFU, was determined as previously described.[49] Lysis plaque turbidity was observed after 8 and 16 hours and PFU were counted after 16 hours.

Disclosure of Potential Conflicts of Interest

Authors declare no potential conflicts of interest.

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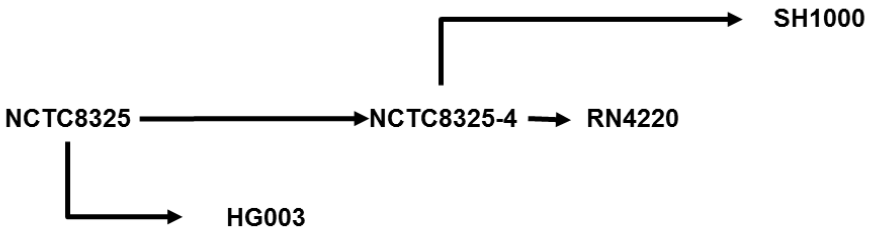

rsbU	-	+	-	-	+
Prophages	Φ11 Φ12 Φ13	Φ11 Φ12 Φ13	-	-	-
% of *rnc* disruption	< 2% [a]	< 2% [a]	40% [b]	45% [c]	50% [b]

Figure 1. *S. aureus* NCTC8325 prophages prevent the selection of *rnc* deletion. Upper part: simplified scheme of the NCTC8325 lineage. See ref. [26] for detailed NCTC8325 genealogy. Table, corresponding strain genotypes and frequency of *rnc* deletions obtained using pMAD2-DErnc (Material and Methods). a, b and c indicate that 50, 10 and 20 colonies were tested by PCR for the presence of *rnc* deletions, respectively.

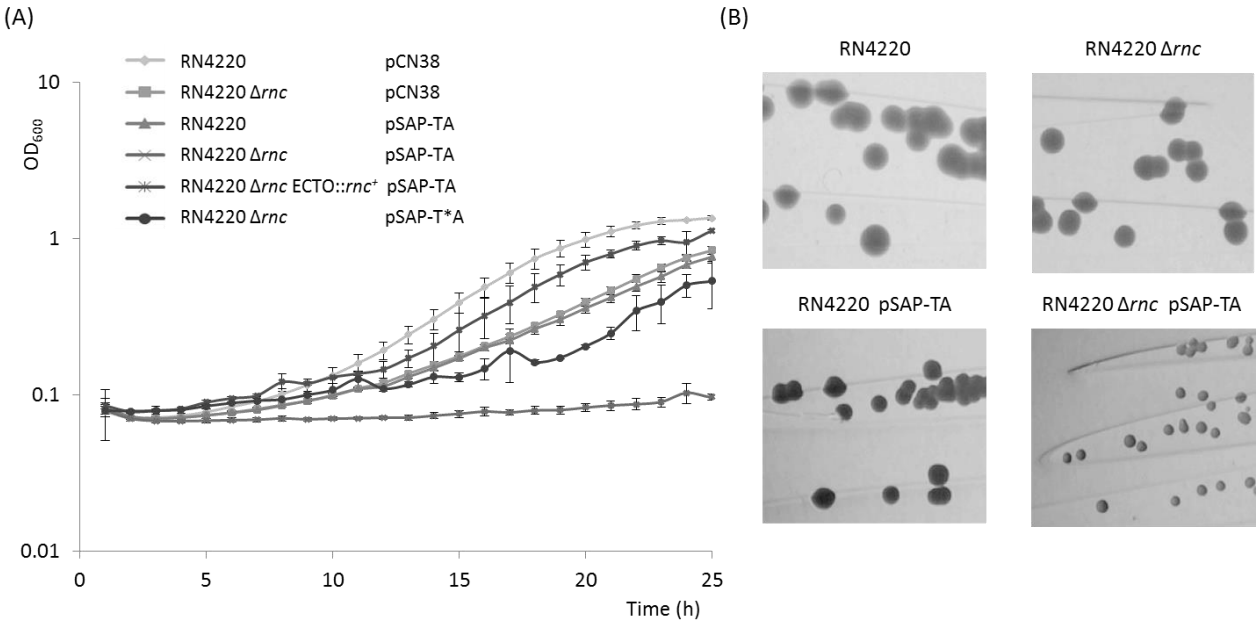

Figure 2. SapTA is deleterious in the absence of RNase III. (**A**) Growth curves of RN4220 and derivatives are presented as indicated. Growth curves were done in triplicate in 96-Well multiwell plates covered with a semipermeable film (4titude, Bagneux France) under constant vigorous shaking using the microplate reader CLARIOstar (BMG Labtech, Ortenberg, Germany). (**B**) Stereomicroscope (Motic, Wetzlar, Germany) photographs of the indicated strains grown on BHI plates after 18 hours of culture. Same settings were used for all photographs.

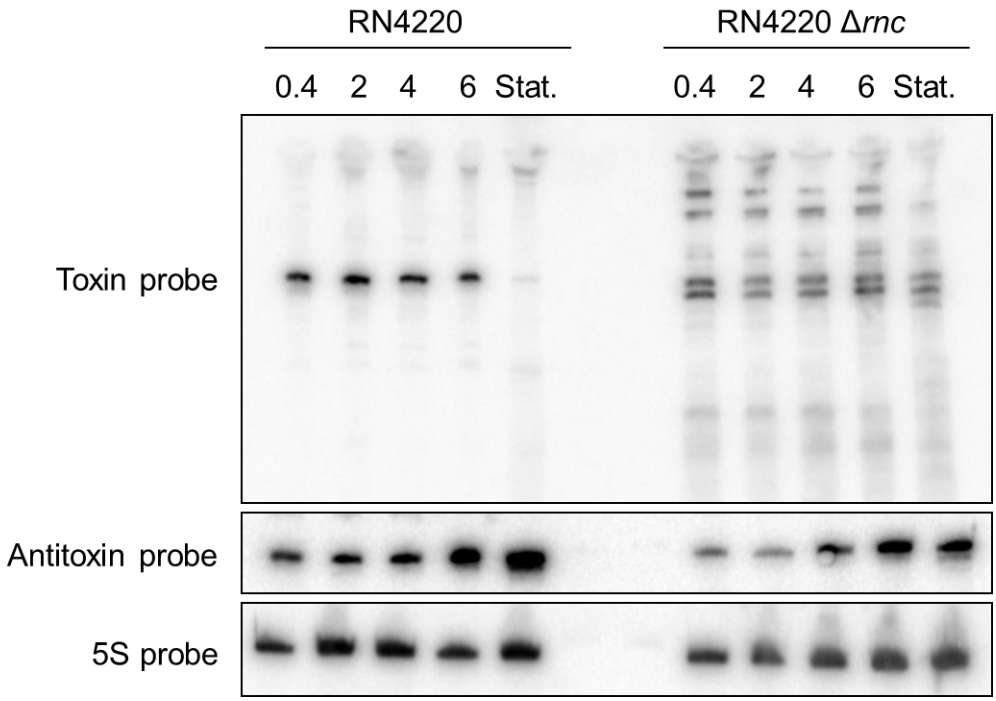

Figure 3. RNase III is involved in *sapTA* expression. Northern blot analysis of *sapT* mRNA and SapA in RN4220 and RN4220 Δ*rnc*. Total RNAs were prepared from cultures harvested at OD_{600} 0.4, 2, 4, 6 and in stationary phase as indicated. Probes against *sapT* mRNA, SapA and 5S were used as indicated (see Material and Methods).

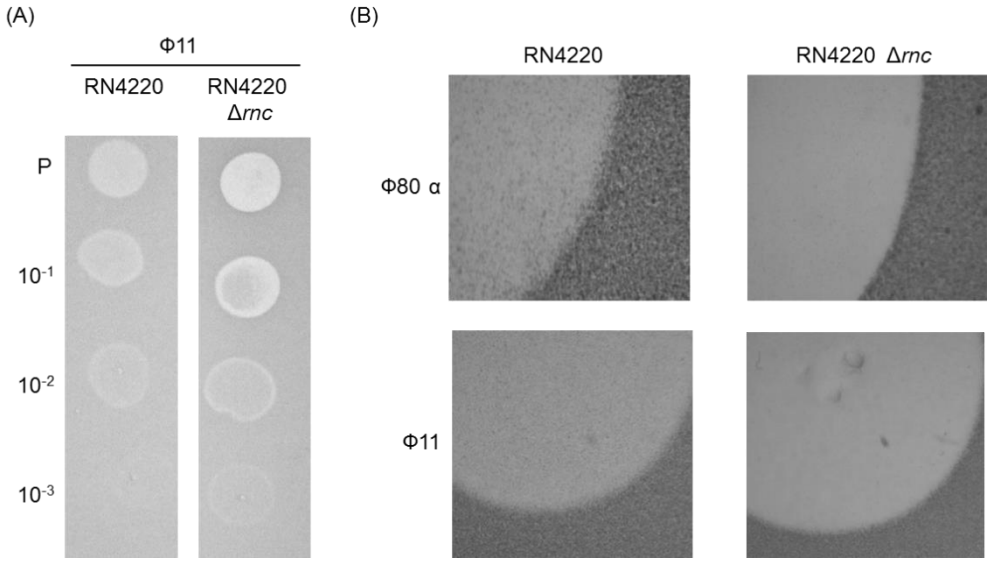

Figure 4. RNase III alters phage growth. (**A**) Phage spot dilution plating assay of Φ11 on RN4220 and RN4220 Δ*rnc*. (**B**) Stereomicroscope observation after 8h of Φ11 and Φ80α plaques spotted on RN4220 and RN4220 Δ*rnc*. Plaques are clearer in the absence of RNase III.

Table 1. Strains and plasmids used in this study

***S. aureus* strain**	**Relevant genotype**	**Reference**
RN4220	*rsbU tcaR*	Ref 30
NCTC8325 (RN1)	*rsbU tcaR* Φ11 Φ12 Φ13	Ref 24
HG003	Φ11 Φ12 Φ13	Ref 26
NCTC8325-4 (RN0450)	*rsbU tcaR*	Ref 24
SH1000	*tcaR*	Ref 50
COL	MRSA pT181	Ref 51
SAPhB245	As RN4220 Δ*rnc*	This study
SAPhB701	As RN4220 Δ*rnc* ECTO::*rnc*$^{+}$	This study
SAPhB809	As RN4220 Δ*rny*	This study

***E. coli* strain**	**Genotype**	**Reference**
DH5α	F– Φ80 *lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Laboratory collection

Plasmid	**Property/use**	**Reference**
pMAD	Allele exchange	Ref 31
pMAD2	Allele exchange with BsaI cloning	This study
pMAD2-DErnc	*rnc* gene deletion	This study
pMAD2-DErny	*rny* gene deletion	This study
pECTO	*S. aureus* chromosomal ectopic complementation	This study
pECTOrnc	*rnc* chromosomal ectopic complementation	This study
pCN38	Expression in *S. aureus*	Ref 43
pCN38-site1	pCN38 containing SAOUHSC_1580	This study
pSAP-TA	pCN38 containing *sapTA*	This study
pSAP-T*A	pSAP-TA derivative with the *sapT* gene inactivated (replacement of its start codon by a stop codon)	This study
pCN38-site3	pCN38 containing SAOUHSC_2176	This study
pCN38-site4	pCN38 containing SAOUHSC_2238	This study

Table 2. Transformation frequency test.[a]

	pCN38	pSite1	pSAP-TA	pSite3	pSite4
Δ*rnc*	1	0.5	< 0.003 0.3*	0.4	0.5
Δ*rnc* ECTO::*rnc*$^+$	1	1.2	0.9	0.2	1.2
Δ*rny*	1	1.3	0.9	0.2	1.1

[a] The number represented the mean ratio with the RN4220 of three independent experiments

* After 36 hours (instead of 16 hours). All clones are translucent.

Supplementary data

Deciphering RNase III essentiality in *Staphylococcus aureus*

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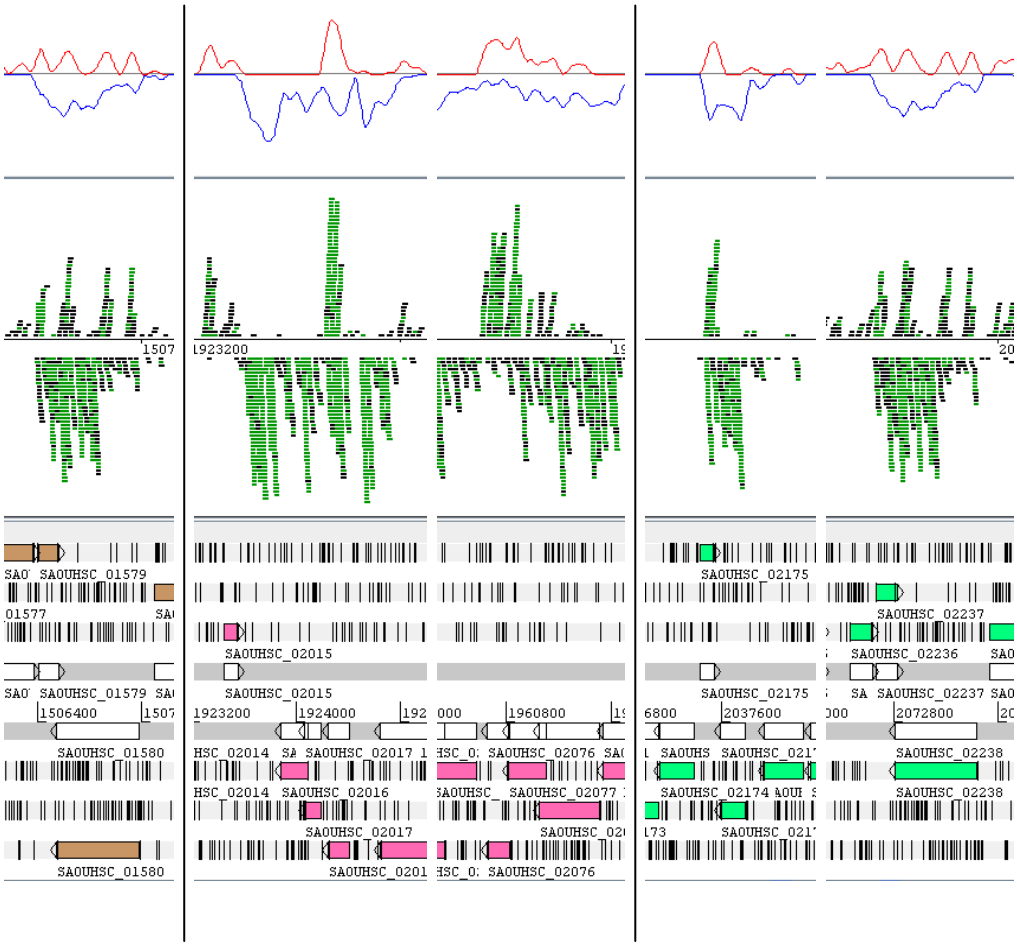
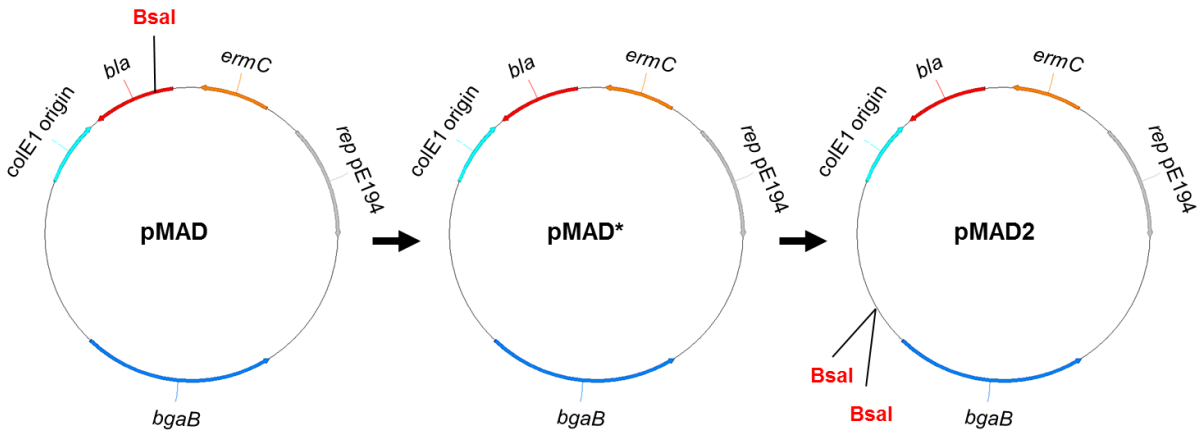


Figure S1. Prophage loci with antisense transcription in HG003. RNA-seq results are visualized with Artemis sequence editor tool.[1] Tracks were added to the classical view as described.[2] (Top) Ln of read coverage track; forward and reverse sequences are in red and in bleu, respectively. (Middle) BamView of mapped reads; forward and reverse sequences are above and under the line, respectively. (Botton) HG003 Artemis representation using NCTC8325 nomenclature. (white boxes) CDSs; (brown boxes) annotated proteins from Φ12; (pink boxes) annotated proteins from Φ11; (green boxes) annotated proteins from Φ13; (small vertical black lines) stop codon for the six DNA reading frames.

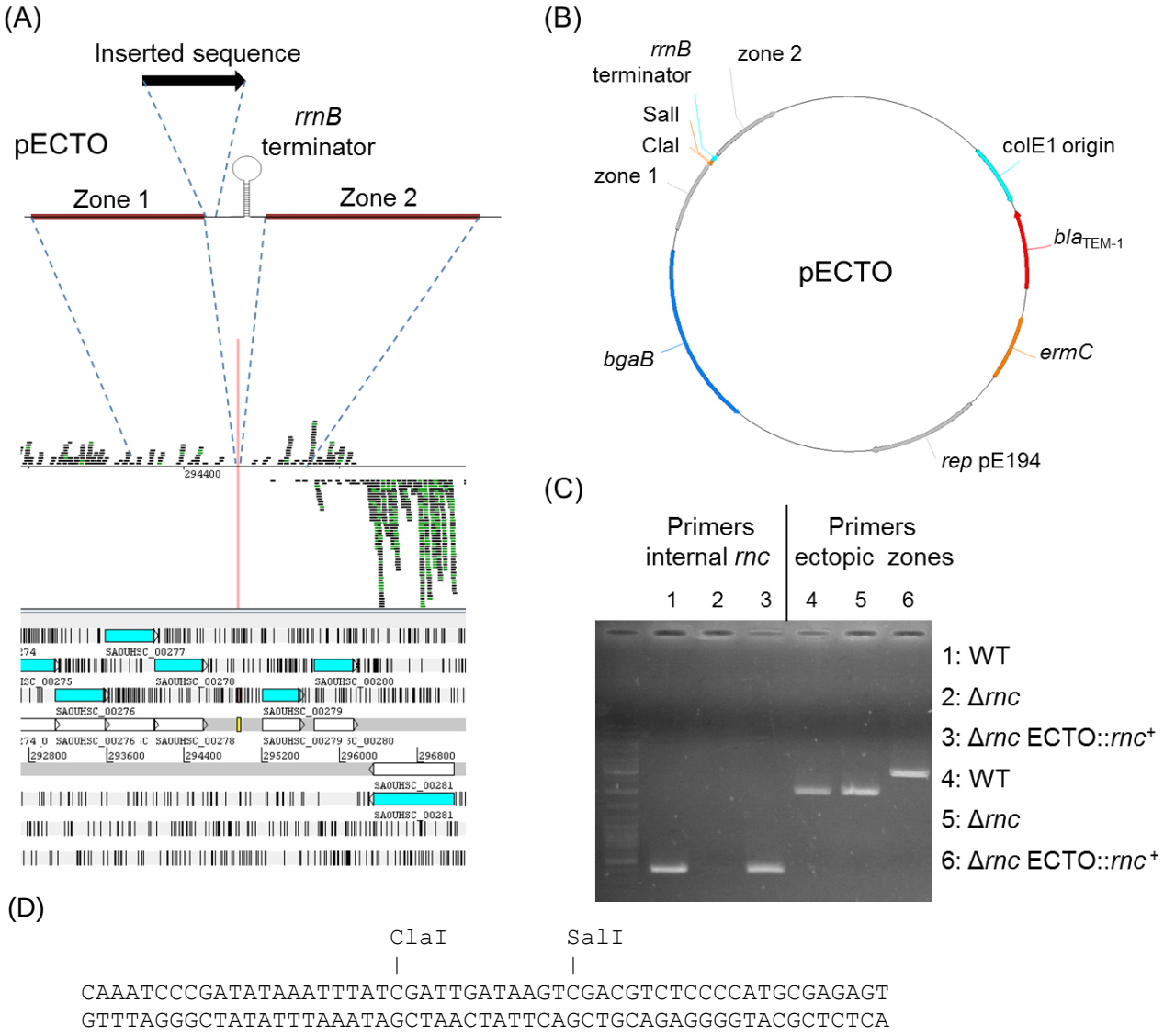
(A)



(B)

```
            SmaI                                  XhoI
             |          |                          |
 NcoI     XmaI        EcoRI                NotI    ApaI          BamHI
  |         |          |                    |     | |            |
CCATGGTACCCGGGAGCTCGAATTCGAGACCGCTAGCGCGGCCGCGGGCCCCTCGAGGGTCTCGGATCCG
ATATCGCC
GGTACCATGGGCCCTCGAGCTTAAGCTCTGGCGATCGCGCCGGCGCCCGGGGAGCTCCCAGAGCCTAGGC
TATAGCGG
                      BsaI                                BsaI
```

Figure S2. pMAD2, a pMAD [3] derivative that allows one-step cloning with BsaI.[4]. pMAD2 contains two BsaI sites and additional unique restriction sites. When pMAD2 is digested by BsaI, the two BsaI sites do not remain associated with the vector and two cohesive non-complementary overhang sequences compatible with BamHI and EcoRI cloning sites are created. pMAD2 allows to clone multiple fragments using solely BsaI with properly designed PCR products.[4] (A) pMAD2 was constructed as follow: i) an unwanted BsaI site in the pMAD *bla* gene was removed; the resulting plasmid was named pMAD*. ii) a cloning site generating two cohesive non-complementary overhang sequences upon BsaI digestion was created; the resulting plasmid was named pMAD2. To remove the BsaI site from pMAD, we used the property that BsaI does not cut within its recognition site, which allowed to exclude the BsaI site from a pMAD reconstructed plasmid. We amplified a part of the *bla* gene using the mutagenic primer (BsaImut) introducing a codon change GGG to GGA (gly to gly). This primer contained a BsaI site that generates a cohesive end compatible with the BsaI pMAD generated end. The second primer (upScaI) primed downstream a ScaI site. The PCR amplification using the two primers BsaImut/upScaI and pMAD generated a fragment of 500 bp. This PCR product and pMAD were ScaI digested and then ligated together leading a linear fragment, which was subsequently incubated with BsaI and the T4 DNA ligase together. The ligation mix was used to transform DH5αZ1. The presence of the expected mutation in the resulting pMAD* was confirmed by DNA sequencing. To construct pMAD2, two complementary oligonuclotides (except at their 5' ends) (BNNAXB / BNNAXB-as) were assembled together to generate a double stranded DNA as described.[5] The none-complementary 3' ends generated cohesive ends were compatible with BamHI and EcoRI sites, respectively. The assemble fragment (containing the two BsaI sites) was inserted between the pMAD* BamHI and EcoRI restriction sites giving rise to pMAD2. pMAD2 was fully sequence (accession number pending). (B) pMAD2 multicloning site sequence. Above the sequence, unique pMAD2 restriction sites are indicated. The two BsaI recognition sites are highlighted in yellow; the remove sequence upon BsaI digestion is underlined, and plasmid cohesive ends compatible with EcoRI and BamHI are highlighted in green and blue, respectively.



(D)

```
                          ClaI          SalI
                          |             |
CAAATCCCGATATAAATTTATCGATTGATAAGTCGACGTCTCCCCATGCGAGAGT
GTTTAGGGCTATATTTAAATAGCTAACTATTCAGCTGCAGAGGGGTACGCTCTCA
```

Figure S3. pECTO, a plamid for ectopic complementation in *S. aureus*. pECTO is a pMAD2 derivative containing two contiguous fragments of *S. aureus* (named here zone 1 and 2) separated by terminator T1 of the *rrnB* operon. pECTO is use to integrate any DNA sequence between SAOUHSC_00278 and SAOUHSC_00279 (NCT8325 nomenclature). This region is conserved and this plasmid can be used with different *S. aureus* strains. If properly cloned, transcriptions originating from inserted sequences are stopped by the *rrnB* terminator. DNA sequences to integrate in the chromosome are inserted using the unique ClaI and SalI restriction sites of pECTO or more practically by Gibson assembly method.[6] (A) pECTO integration site. (Top) Scheme of pECTO loci integrating by homologous recombination in *S. aureus* chromosome. (Middle) BamView of HG003 RNA-seq mapped reads; the vertical red line indicates a region with no transcription in 16 tested growth conditions. (Bottom) Artemis representation of pECTO integration region. (B) pECTO plasmid map; major relevant featured are indicated. (C) PCR results showing the successful ectopic insertion of *rnc*$^{+}$ between SAOUHSC_00278 and SAOUHSC_00279 in RN4220 Δ*rnc*. (D) pECTO cloning site sequence. Two unique sites are available. This sequence can be used for insertion by a Gibson assembly.[6]

Table S1: Primers.

Name*	Sequence
Site1_F	CTGCAGGTCGACTCTAGAGGGAAAGAAAAGGAAATTGTTTATTC
Site1_R	CCATTCAGGCTGCGCAACTGACACTTACGCGCTTCCATTT
Site2_F	CTGCAGGTCGACTCTAGAGGTCTCTTCGGCAACTTTGC
Site2_R	CCATTCAGGCTGCGCAACTGTTCTGCCCCACCTAATCAG
Site3_F	CTGCAGGTCGACTCTAGAGGACCCAATAGCTTTTGCGATG
Site3_R	CCATTCAGGCTGCGCAACTGAATGCTCAGCACACTCAACG
Site4_F	CTGCAGGTCGACTCTAGAGGTTTCAGCAGTGTTTGAAAGG
Site4_R	CCATTCAGGCTGCGCAACTGGACCGAATAGCACCGTTTG
pCN38-lin_F	CAGTTGCGCAGCCTGAATGG
pCN38-lin_R	CCTCTAGAGTCGACCTGCAG
SapT-mutF	TTTTCACCTCCTTACATCAAATTTGTAAGTCATCAACTAACCTAC
SapT-mutR	GAGGTGAAAAGCCTCTAACTAGACATAATAAAAACACTTCTAG
Rnc-upF	GAATTCGAGACCGCTAGCGCATTCCGTTAGCACGTTTTGG
Rnc-upR	GCGTATGGACCTAGGTATATCTCCTTTATGTGTTGCTCTTGAATC
Rnc-dwF	ACCCCACAACCTAGGTATATACAACGTGCTGCTGAAAGTG
Rnc-dwR	GATATCGGATCCGAGACCCTTCTACGCGACCTTCCAAATC
Rny-upF	GAATTCGAGACCGCTAGCGCGGGAGACACTCACGTTGGTT
Rny-upR	GCGTATGGACCTAGGTATATCACCTCCTTTTCTAGGGTTTGC
Rny-dwF	ACCCCACAACCTAGGTATATCACAAATTAGTGAGGGAGCTTTTT
Rny-dwR	GATATCGGATCCGAGACCCTCGGAAAATTCGCTGGTCTTA
TagF	GGTCTCATGTGTTGTGGGGTACAGCAATGAC
TagR	GGTCTCTGAGATCCATACGCAGCTATGCAAT
pMAD-lin F	GCGCTAGCGGTCTCGAAT
pMAD-lin R	AGGGTCTCGGATCCGATATC
pECTO_A_F	GAACAAAAGCTGGGTACCAGTTTGATCAATTAGGCCGTGAAAAC
pECTO_A_R	ACTTATCAATCGATAAATTTATATCGTGATTTGTTAATTAGTTG

pECTO_C_F	CTGAGTAGGATTGTCATTAATTGAAAAGGTTATTG
pECTO_C_R	TATAGGGCGAATTGGAGCTCTCATCTTCATAATCTGCTTTATG
pECTO_B_F	ATTGATAAGTCGACGTCTCCCCATGCGAGAGTA
pECTO_B_R	TTAATGACAATCCTACTCAGGAGAGCGTTC
rnc-comp-F	GATATAAATTTATCGATTGAAAATCTTACATCTGGGTCGTCA
rnc-comp-R	GGGAGACGTCGACTTATCCTTAAATCCTAAATCGAACAC
Rnc-int F	TAACAAAAATGCGTGCCACT
Rnc-int R	TCAGCTATTGCTTCCCCTTG
Checkup DErnc	AAATCTTACATCTGGGTCGTCA
checkdw_DErnc	TCCTTAAATCCTAAATCGAACA
pECTO-lin-F	TAAGTCGACGTCTCCCCATGC
pECTO-lin-R	TCAATCGATAAATTTATATCGGGATTTG
BsaImut	ATATGGTCTCTACCGCGAGATCCACGCTCACCGGCTCCA
upScaI	CTGCTATGTGGCGCGGTATTAT
BNNAXB	5′P-AATTCGAGACCGCTAGCGCGGCCGCGGGCCCCTCGAGGGTCTCG
BNNAXB-as	5′P-GATCCGAGACCCTCGAGGGGCCCGCGGCCGCGCTAGCGGTCTCG

* F and R indicate forward or reverse orientation with respect to the chromosome annotation, respectively.

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DISCUSSION & PERSPECTIVES

The function of bacterial genes is often found via the identification of phenotypes associated with gene inactivations. However, in most cases, no phenotypes are found with sRNA genes deletions. An important part of the manuscript concerns the development and application of a method to detect phenotypes associated with sRNA gene deletions in *S. aureus*. It is based on a competitive assay between several strains.

We constructed 39 mutants having sRNA-gene deletions marked with tag-specific sequences. The proportion of each mutant was determined in a mix population by DNA sequencing of their specific DNA barcode. Mutant phenotypes were considered relevant when the barcode fold change (ratio of barcode between tested to mock conditions) was less than 0.2 (underrepresented) or more than 5 (overrepresented).

With this method, we are able to detect mutants leading to advantageous or disadvantageous growth phenotypes. In addition, some mutants having moderate phenotypes can be characterized. Phenotypes associated with sRNA-deletion found via fitness experiment can sometime be confirmed by compairing mutant and parental strains growing individually in the tested conditions. However, some phenotypes cannot be observed by simple growth curves. The fitness test allows the identification of subtle differences cumulating over the experience. In addition, some phenotypes may depend on the presence of other strains.

Thirteen growth conditions (12 stresses and mouse model) were tested and analyzed. We obtained 11 strains having significant altered fitness in at least one of the tested conditions. Among them, mutants *sau60*, *teg147*, *ssrS* have an effect in only one condition (Table 1, Figure 18).

Table 1: Fitness experiments in different stress conditions

No	Conditions / sRNA mutants	20°C	42°C	pH 5.4	pH 8.68	NaCl 1.5M	H_2O_2 0.1mM	RPMI	RPMI no folate	BHI no O_2	RPMI no O_2	BHI + DIP	BHI + 10% human serum	Blood	Spleen	Kidney	Liver
1	Sau60	↓↓															
2	Teg147					↓↓											
3	SsrS						↓↓										
4	Sau6528	↓↓				↓↓											
5	RsaD	↓↓				↓↓	↓/↓↓										
6	RsaOV	↓↓					↓↓									↓↓	
7	Sau30		↓	x						↓↓							
8	RsaH		↓					↓↓									
9	Sau6836		↓		↓/↓↓			x	↓↓								
10	Teg49	↓↓									↑↑						
11	Sau6428		↓↓	↓↓													

↓	Under-represented in exponential phase
↓↓	Under-represented in exponential-stationary-exponential phase
↑	Over-represented in exponential phase
↑↑	Over-represented in exponential-stationary-exponential phase
x	Disappearance

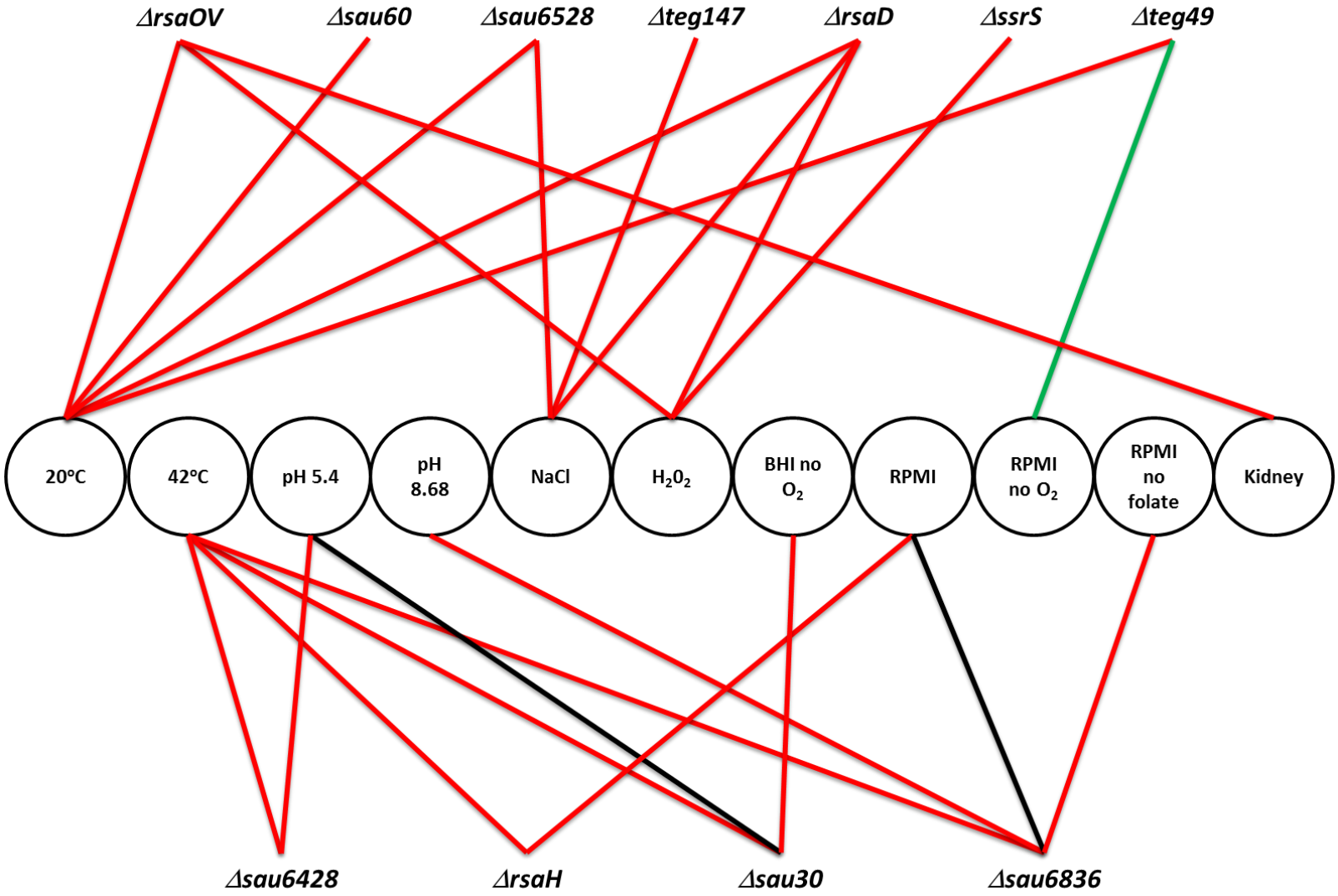

Figure 18: Summary result of fitness experiments in different growth conditions and in a mouse model.

Red line: under-represented. Green line: over-represented. Black line: disappearance.

1. Acid and alkaline adaptation

In acidic condition, mutant *sau6428* grew badly and mutant *sau30* disappeared from the mutant set. In addition, both mutants also had growth defects at 42°C (see chapter A.1).

Sau30 (Abu-Qatouseh et al. 2010) was also reported as a regulatory RNA under SSR154 name (Anderson et al. 2010). The gene is located between SAOUHSC_02483 (*cbiO* - cobalt transporter ATP-binding subunit) and SAOUHSC_02484 (*rplQ* - 50S ribosomal protein L17). In HG003, our transcriptome data indicates that Sau30 is probably a part the *rplQ* 3'UTR rather that a sRNA *per se* and indeed, the *rplQ* gene is downregulated in acid-shocked cells (Anderson et al. 2010). The constructed *sau30* deletion encompasses a large region that could also affect *cbiO* gene. Therefore, the two phenotypes observed at 42°C and in acidic pH could be link to *rplQ* and/or *cbiO*.

Sau6428 (Abu-Qatouseh et al. 2010) also known as Teg109 (Beaume et al. 2010) was initially classified as a sRNA located between SAOUHSC_00775 (hypothetical protein) and SAOUHSC_00776 (*uvrB* - excinuclease ABC subunit B). However, Sau6428 is likely the 5'UTR of *uvrB* mRNA (http://srd.genouest.org). UvrB is involved in nucleotide excision repair, a mechanism to repair DNA damage which is enhance by enviromental stress such as acidic growth media (see chapter II.1.4.1) or heat shock. The phenotypes observed for mutant *sau6428* are possibly related to the altered expression of *uvrB*.

Only mutant *sau6836* was underrepresented in alkaline condition. The *sau6836* gene is located between SAOUHSC_00646 (penicillin binding protein 4, *pbpD*) and SAOUHSC_00647. Sau6836 initially consider as a sRNA is now categorized as a putative 5'UTR of SAOUHSC_00647 (http://srd.genouest.org). The *abcA* gene encoding an ABC transporter component is the SAOUHSC_00647 homolog in strain KB400. Its disruption was shown to increase resistance to cefoxitin and methicillin (Domanski & Bayles 1995). This phenotype was later proved to be due to *phpD* upregulation, resulting in increased peptidoglycan crosslink in *S. aureus* cell wall (Domanski et al. 1997). *abcA* and *pbpD* share an overlapping 80 nt promoter region and are controlled by common regulatory mechanism. However, *abcA* and *pbpD* expressions are different in strain NCTC8325 (Schrader-Fischer & Berger-Bächi 2001). *abcA* is regulated by the Agr TCS,

MgrA is a direct activator and NorG is a direct repressor (Truong-Bolduc & Hooper 2007). Moreover, Rot, SarA, and SarZ are also direct regulators of *abcA* (Villet et al. 2014) (Figure 19). No change of *abcA* and *pbpD* expressions under acidic pH, oxidative stress (H_2O_2), iron limitation was observed while the transcripts of *abcA* increased only under nutrient limitation condition (Villet et al. 2014). Therefore, Sau6836 is likely the 5'UTR of SAOUHSC_00647 an may affect *pbpD* and *abcA* expression. However, this system is complex and the role of Sau6836 toward growth in alkaline condition remains to be explained.

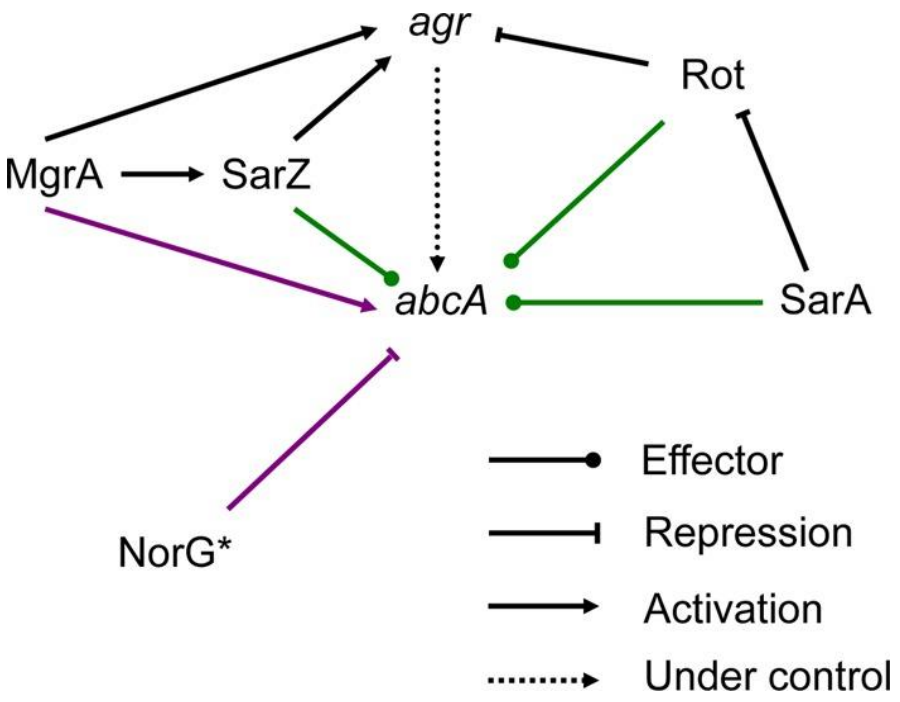

Figure 19: General overview of the predicted directs regulatory elements of *abcA* and their relationship under normal conditions of growth. Black lines are for interactions identified in RN6390 (Schrader-Fischer & Berger-Bächi 2001; Manna et al. 2009). Purple lines are for interactions identified in ISP794 (Truong-Bolduc & Hooper 2007). Green lines are for interactions identified in MW2 background. *, NorG is absent from MW2. Effector indicates a direct regulator that can be a repressor or an activator depending on the strain and the environment [From (Villet et al. 2014)].

2. Adaptation to high osmolarity and oxidative conditions

Mutant *teg147*, *sau6528* and *rsaD* were underrepresented in high osmolarity condition. Mutant *rsaD* is described in Chapter A1. Teg147 is a bona-fide sRNA

expressed from a pathogenicity island (Beaume et al. 2010). Its gene is located between SAOUHSC_00380 (hypothetical protein) and SAOUHSC_00381 (hypothetical protein). So far, the growth defect in high osmolarity condition is the first reported phenotype for mutant *teg147.*

The third deletion mutant, *sau6528*, grew badly at 20^{o}C (see Chapter A.1) and in high osmolarity conditions. *Sau6528* likely affects the 3'UTR of SAOUHSC_01416 (*odhB*) gene encoding a dihydrolipoamide succinyltransferase converting the 2-oxoglutarate to succinyl-CoA in the TCA cycle. *odhB* is upregulated under biofilm condition (Resch et al. 2005). Biofilms protects bacteria better from harsh environments including to high osmolarity. Consequently, high concentration of NaCl induce or increase biofilm formation (Lim et al. 2004; Rachid et al. 2000).We suggest that Sau6528, as a 3'UTR, contributes to *odhB* gene expression.

Resistance to oxidative stress is crucial for bacteria to survive within infected hosts which produce toxic reactive oxygen species (ROS) such as hydrogen peroxide, superoxide and hydroxyl radicals. These ROSs damage DNA, proteins, and other cellular macromolecules. Therefore, all bacteria developed several mechanisms to response to oxidative stress. For example, *S. aureus* produces catalases and superoxide dismutases to degrade hydrogen peroxide and to prevent ROS-associated damages. In oxidative growth condition, *ssrS*, *rsaD* and *rsaOV* mutants grew badly suggesting that the deleted sRNAs may contribute to the expression of ROS protecting enzymes.

The *ssrS* gene is conserved in bacteria. It expresses the 6S RNA that associates with σ^{70} RNA polymerase. Interestingly, many genes directly related to oxidative stress adaptation such as degradation of superoxide and hydroperoxide are upregulated in the *B. subtilis ssrS* mutant (Hoch et al. 2015). In *Burkholderia cenocepacia*, 6S RNA was shown to increase during oxidative stress (Peeters et al. 2010). These results indicate that 6S RNA is related to oxidative stress and support our observation that *S. aureus* 6S RNA is required for a better growth in media containing H_2O_2.

Mutant *rsaD* had a growth defect at 20^{o}C (see Chapter A.1), in high osmolarity and in oxidative conditions. Noticeably, RsaD is induced in cold shock and oxidative stress by H_2O_2 (Beaume et al. 2010; Geissmann et al. 2009). This induction may reflect that RsaD contributes to adaptation to cold and oxidative stress and explains the

defective growth of the *rsaD* mutant in low temperature and in media containing H_2O_2. It contributes to validate our method for identifying sRNA functions by performing fitness experiments.

3. Adaptation to RPMI and RPMI no-folate media

Mutant *rsaH* grew badly in RPMI medium while mutant *sau6836* was underrepresented in RPMI no-folate and completely disappeared in RPMI.

Mutant *rsaH* also had growth defect at 42°C (see chapter A.1). Putative RsaH targets were found to be involved in lactate metabolism, virulence and oxidative stress (see chapter B). However, we have not found yet links between *rsaH* mutant phenotypes and RsaH putative targets.

Mutant *sau6836* grew badly at 42°C (see Chapter A.1), alkaline pH and RPMI (with and without folate). As discussed before, Sau6836 is likely the 5'UTR of SAOUHSC_00647, the observed phenotypes are probably due to an altered expression of *abcA* encoded TCS component (see Figure 20). In addition, the *abcA* mRNA is strongly induced under nutrient limitation condition (Villet et al. 2014) as it may be required for this condition. It could explain the growth defect of mutant *sau6836* in poor medium

In RPMI no-folate medium, we expected to see a phenotype of mutant *rsaE* since RsaE was shown to be involve in TCA and folate metabolism (Bohn et al. 2010), however, no growth phenotype was observed in RPMI and RPMI no-folate.

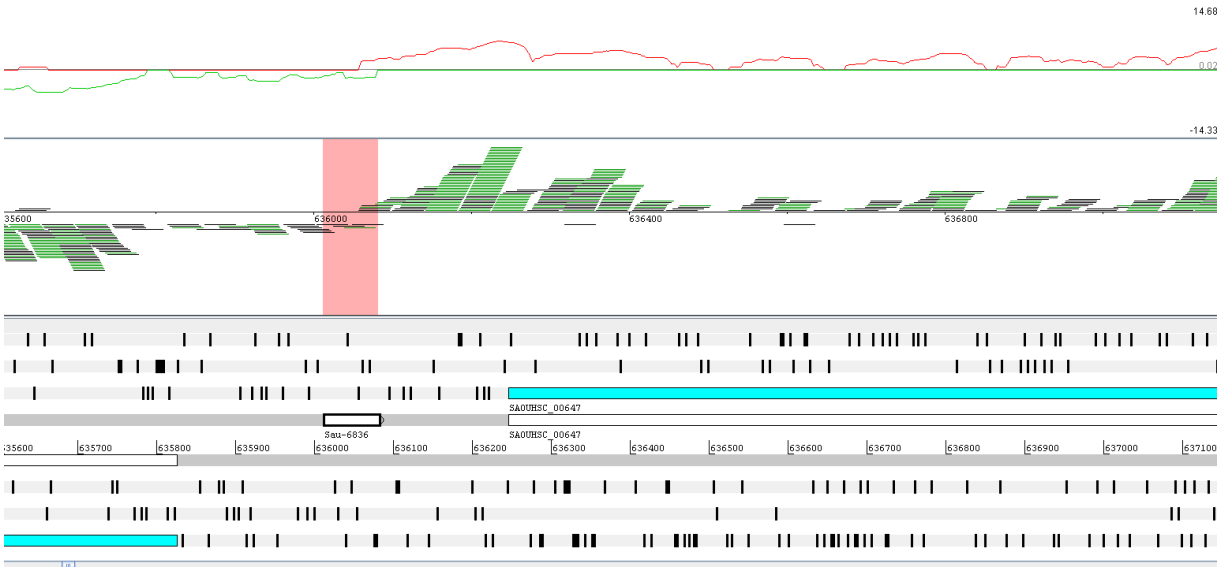

Figure 20: Artemis view of Sau6836 in our transcriptome HG003.

4. Growth in anaerobic BHI and RPMI media

We found mutant *sau30* was under-represented while mutant *teg49* accumulated in the mutant set growing anaerobicaly. As discussed before, the phenotype of mutant *sau30* can be due to an altered expression of its flanking gene.

The *teg49* gene is located between SAOUHSC_00620 (*sarA*, encoding the accessory regulator A) and SAOUHSC_006201. Recently, it was demonstrated that Teg49 correspond to the 5'UTR of *sarA* transcribed from the *sarA* P3 promoter, hence Teg49 also modulate *sarA* expression (Kim et al. 2014). Consequently, the deleted region of our mutant *teg49* also affects *sarA* expression; the accumulation of mutant *teg49* in anaerobic RPMI medium could due to the loss of *sarA* function. This is the only mutant strains that accumulated significantly in our fitness experiments.

5. Adaptation to iron limitation, human serum and in mouse model

In iron limited condition, we did not obtain any significant phenotype but mutant *teg49* was the most overrepresented strain of the mutant set. It is consistent with the accumulation of this mutant in anaerobic RPMI medium which are also iron limited. In addition, we expected to observe a growth phenotype associated with mutant *RNAIII*/*agrB* as we found new putative RNAIII targets that are involved in iron uptake (see Chapter B). However, no RNAIII associated phenotypes were observed in

these media (Figures 19 & 20). Nevertheless, a weak growth defect of mutant *RNAIII* was found in TSM (*Trichoderma-selective medium)* containing the iron chelator EDDHA (ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) in the final concentration 1µM (Tatiana Rochat, unpublished data). The absence of phenotype for the competition experiment could be due to a specific condition associated with the iron chelator that we used.

rsaOV (Sau40) is the 3'UTR of *cwrA*. In addition to a growth defect in media containing H_2O_2, mutant *rsaOV* had a strong growth defect at low temperature (see Chapter A.1). Importantly, this mutant was also underrepresented in kidney in our mouse model. Our results are consistent with the observation that *cwrA* expression in strain SH1000 is required for *S. aureus* virulence in mouse sepsis model (Balibar et al. 2010). These data suggested the role of *rsaOV* acting as 3'UTR in regulating *cwrA* gene expression, leading to pleiotropic effects in adaptation and virulence.

RNAIII and SprD are well-characterized sRNAs in *S. aureus. sprD* is required for bacterial virulence in a mouse infection model (Pichon & Felden 2005; Chabelskaya et al. 2010) (see Chapter IV.3). However, we did not obtain any phenotype of mutant *RNAIII*/*agrB* or mutant *sprD* in any fitness experiment, including the mouse model. This latter negative result could be attributed the high toxicity of the inoculum that we used which consequently reduced the number of mice that we analysed and also shorten the competition time. As indicated before, this experiment should be performed again with a larger sample and less toxicity inoculum.

6. Identification of sRNA targets in *S. aureus*

To understand how sRNAs are related to their phenotypes, it is important to identify sRNAs targets and determine the mechanism of sRNAs action. Several computational methods proposed a list of putative sRNA targets. However, it is often difficult the find the right target within a long list of candidates. Therefore, we developed the strategy inspired from (Douchin et al. 2006), named "Hybrid-trap-seq" to identify reliably sRNA targets. We performed in parallel hybrid-trap-seqs to four *S. aureus* sRNAs RsaA, RsaE, RsaH and RNAIII. Many known sRNA-targets as well as new ones were revealed and confirmed (see chapter B). However, there is still a gap

between hybrid-trap-seq targets and our fitness phenotypes. We did not obtain any phenotype for mutant *rsaA*, *rsaE* and *rnaIII* in the specific condition that these mutants supposed to have effects, such as folate-limited medium for *rsaE* or iron-limited medium for *rnaIII*. Further experiments are needed find deficiencies associated with these deletions.

7. Difficulties

The pMAD plasmid is a tool classically used for disruption gene in *S. aureus* (Arnaud et al. 2004). However, it was shown that the integration step at 42°C of pMAD generates secondary mutations in the *sae* operon during the process [(Sun et al. 2010), our data]. It is likely that the growth at high temperature in presence of erythromycin generates mutations in other genes. When we became aware of this problem, we change the allelic exchanged protocol by performing the integration step at 37°C instead of 42°C. To minimize the effect of secondary mutations associated with the allelic exchange protocol, we propose to associate each deletion with 2 or 3 different barcodes; consequently each mutant will be independent and will not have the same potential secondary mutations. The different tag versions of each deletion mutant will provide an internal control to confirm that the observed phenotypes are directly related to deletions.

We constructed a set of 39 sRNA genes substituted with specific DNA tag sequences in the pathogenic strain HG003. The regions chosen for the sRNA gene disruptions were based on data evaluable at the beginning of the project. As described before, the cartography of the sRNA genes was incomplete and several reported sRNAs correspond in fact to mRNA UTRs. Hence, few constructed sRNA mutants have an effect on the flanking genes rather than on the sRNA gene itself.

A resource for Staphylococcal regulatory RNAs Database (SRD http://srd.genouest.org/) providing a list of curated sRNAs in *S. aureus* was recently created (Sassi et al. 2015). This resource combined with our transcriptome of strain HG003 grown in 13 different conditions allow a more accurate knowledge of HG003

sRNAs than 4 years ago. The data set presented in this work could in the future i) be corrected with adequate deletions and ii) be extended with new sRNA gene deletions.

8. Competitive fitness experiments

Since antibiotic resistance in *S. aureus* is a major problem, one of the most promising experiments will be to perform fitness experiments with a complete sRNA mutant set growing in the presence of sub-lethal concentration of different antibiotics. 409 putative regulatory RNAs were identified in strain multiresistant sequence type 239 (ST239) after it was exposed to 4 antibiotics vancomycin, linezoid, ceftobiprole and tigercycline (Howden et al. 2013). Other antibiotics using for *S. aureus* treatment such as glycopeptides, 'late-generation' cephalosporins, fluoroquinolones, aminoglycosides, oxazolidinones and macrolides can be tested. Importantly, sRNAs contributing to the pathogenicity of *S.* aureus can also be identified by performing competition experiments within macrophages or animal models. These results could help us to understand the functionality of sRNAs related to antibiotic resistance and virulence and to decipher parts of the *S. aureus* regulatory network controlled by sRNAs.

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