



Characterization and expression of novel small RNAs in Staphylococcus Aureus

Juan Song

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**CHARACTERIZATION AND EXPRESSION OF NOVEL SMALL RNAS IN
STAPHYLOCOCCUS AUREUS**

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RÉSUMÉ

Contexte:

L'expression « petits ARN » est utilisée comme un terme général pour désigner les ARN des bactéries qui ne codent pas des protéines, mais qui sont des éléments clés impliqués dans la régulation de gènes. Cette régulation permet aux bactéries de s'adapter à des environnements complexes en constante évolution. Ils jouent également un rôle dans la régulation de l'expression des facteurs de virulence ^[1]. Les petits ARN sont divisés en différents groupes selon leurs mécanismes d'action ^[2-3] : 1) les ARN antisens *cis/trans*-codés qui possèdent une séquence complémentaire à leurs ARNm cibles; 2) les riboswitches ou thermosenseurs qui modifient leurs propres conformations lors de la détection de divers signaux physiologiques ou des variations de température; 3) les ARNs qui interagissent directement avec les protéines cibles; 4) les CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) qui interfèrent directement avec l'ADN.

Ces dernières années, avec l'utilisation de nouveaux logiciels de bio-informatique, un grand nombre de petits ARN a été trouvé chez diverses bactéries, y compris *Staphylococcus aureus* (*S. aureus*) – un agent pathogène humain important. Un tiers de la population humaine est porteur asymptomatique de *S. aureus* ^[4], mais cette bactérie est également responsable de nombreuses infections hospitalières et communautaires. Il s'agit d'infections liées à la sécrétion de toxine (par exemple: intoxication alimentaire, le syndrome du choc toxique et syndrome de la « peau ébouillantée ») ou de maladies invasives (par exemple: furoncles, pneumonie et endocardite). La pathogénie de *S. aureus* est liée à sa capacité à produire de nombreux facteurs de virulence, telles que les protéines de capsule et de surface, les cytotoxines détruisant les membranes, des toxines superantigéniques et des enzymes. L'expression de ces facteurs de virulence est contrôlée par des systèmes de régulation.

À ce jour, les principaux systèmes de régulation chez *S. aureus* sont des systèmes à deux composants (TCS), des facteurs de transcription ^[5] et des ARN régulateurs. Les TCS sont largement présents chez les bactéries, certains sont sous la dépendance de molécules de signalisation dont la concentration est proportionnelle à la densité de la population bactérienne, c'est le système de Quorum Sensing (QS). Agr est un TCS dont l'activation

dépend du quorum sensing ^[6]. Son effecteur est un ARN régulateur appelé ARNIII. Il est formé de 514 nucléotides et possède une structure secondaire complexe et conservée. En plus de sa capacité de coder l'hémolysine δ , l'ARNIII fonctionne de manière antisens pour réguler l'expression de nombreuses protéines de la paroi cellulaire en phase de croissance post-exponentielle ^[7-10].

En raison de l'importance de l'ARNIII dans la virulence, notre équipe a recherché d'autres ARN régulateurs impliqués dans la régulation de la virulence. Onze nouveaux petits ARN (ARN Rsa pour RNA from *S. aureus*) ont été mis en évidence par une approche bio-informatique ^[11]. Mais leurs fonctions restent encore à définir. Dans ce projet, nous avons entre autre étudié l'expression *in vivo* (à partir de prélèvements d'infection et de colonisation) de ces petits ARNs afin d'évaluer la pertinence des études *in vitro* qui montrent leur implication potentielle dans la régulation de la virulence.

Objectifs :

Durant ma thèse :

- j'ai participé à la construction des outils génétiques nécessaires à l'étude des fonctions de 4 des ARN Rsa (RsaA, E, G, H) (construction de mutants)
- j'ai étudié la relation entre l'expression *in vitro* de ces petits ARN et la pression antibiotique
- j'ai analysé pour la première fois l'expression des petits ARN dans différents types de prélèvements issus d'infections aiguës ou chroniques à *S. aureus* et dans des prélèvements de nez de patients colonisés à *S. aureus*.
- enfin, j'ai étudié l'influence de l'interférence avec *Pseudomonas aeruginosa* sur l'expression des ARN Rsa et explorer le mécanisme possible.

Méthodes :

1. Construction d'outils génétiques : Chaque ARN a été inactivé par la technique de remplacement allélique en utilisant le vecteur pMAD. Chaque mutant a ensuite été complété en électroporant un vecteur exprimant l'ARN inactivé. Ces constructions ont été vérifiées par Northern-blot.

2. Expression des ARN Rsa et la pression antibiotique : La concentration minimale inhibitrice (CMI) pour l'oxacilline et la lévofloxacine ont été déterminées pour les souches RN6390 et HG001. Ensuite, une série de concentrations (de 1/16 CMI à 4 CMI) d'antibiotiques a été ajoutée dans les cultures bactériennes en milieu liquide. L'expression de chaque Rsa a été déterminée par RT-PCR en présence et en absence d'antibiotiques.

3. Expression des ARN Rsa en situation clinique : Des prélèvements d'abcès, des crachats de patients atteints de mucoviscidose et des prélèvements de nez de patients colonisés à *S. aureus* (20 sujets par condition) ont été collectés. Les souches cliniques correspondantes à chaque prélèvement ont été isolées et génotypées en utilisant des puces à ADN. L'ARN total a été extrait directement à partir des prélèvements cliniques et de la culture *in vitro*. Le niveau d'expression des ARN Rsa a été mesuré par RT-PCR.

4. Influence de *Pseudomonas aeruginosa* (*P. aeruginosa*) sur l'expression des ARN Rsa : *S. aureus* a été co-cultivée avec *P. aeruginosa* (rapport de volume = 9:1) ou des molécules chimiques (AHL ou LS) qui sont sécrétées par *P. aeruginosa*. L'ARN total a été extrait et le niveau d'expression des petits ARN a ensuite été analysé par RT-PCR.

5. Recherche du régulateur impliqué dans la dysrégulation de l'expression des ARN Rsa en présence de *P. aeruginosa* : Le rôle de plusieurs régulateurs majeurs de *S. aureus* a été criblé à travers la co-culture de mutants de *S. aureus* (Δagr , Δarl , Δrot , $\Delta sarA$, $\Delta sarHI$) avec *P. aeruginosa* souche de type sauvage (PAO1), suivie par l'analyse de l'expression des petits ARN. Un extrait protéique total a été extrait de la co-culture de RN6390/PAO1 et la mono-culture de RN6390. Une chromatographie d'affinité ADN a été réalisée pour rechercher d'éventuelles protéines qui peuvent se lier aux petits ARN.

Résultats:

1. Construction d'outils génétiques : les mutants pour 4 petits ARN Rsa ont été construits avec succès dans différents contextes génétiques (RN6390, HG001, et NEWMAN). La complémentation a été réalisée en clonant chaque ARN sous le contrôle du promoteur P3.

2. Expression des ARN Rsa et de l'ARNIII en présence d'antibiotiques : les CMI de l'oxacilline et de la lévofloxacine pour RN6390 et HG001 sont identiques, i.e. 0.25 µg/ml, quelque soit le milieu de culture (MH et BH). La croissance bactérienne est inhibée par les deux antibiotiques de manière dose-dépendante de 1/4 CMI à 4 CMI. Les niveaux d'expression des petits ARN diminuent lorsque les concentrations de lévofloxacine/

oxacilline sont $\geq 1/4$ CMI. Dans la souche RN6390, $1/16$ de la CMI et $1/8$ de la CMI de lévofloxacine augmente l'expression de RsaH.

3. Expression des ARN Rsa et de l'ARNIII dans des prélèvements cliniques : Les 5 petits ARN étudiés (RsaA, RsaE, RsaH, RsaG et ARNIII) sont codés et exprimés dans tous les isolats cliniques de *S. aureus*. L'expression globale de ces petits ARN est extrêmement variée dans les prélèvements d'abcès, plus homogènes dans les crachats de patients atteints de mucoviscidose, et très uniforme dans les prélèvements de nez chez les porteurs sains colonisés à *S. aureus*. Le niveau d'expression relatif par rapport au gène de ménage *gyrB* est similaire à celui obtenu en milieu de phase exponentielle de croissance pour l'ARNIII, RsaA et RsaE. Par contre, le niveau d'expression de RsaH est similaire à celui obtenu en fin de phase exponentielle de croissance. Enfin, RsaG présente un niveau d'expression toujours plus élevé *in vivo* que *in vitro*.

4. Influence de *P. aeruginosa* sur l'expression de des ARN Rsa : Le niveau de RsaG dans la souche HG001 est augmenté plus de 8 fois en présence de *P. aeruginosa* vivants. Le niveau d'expression de RsaH dans RN6390 et HG001 est augmenté de 8 à 15 fois en présence de *P. aeruginosa* vivants ou de surnageant de *P. aeruginosa*. Les molécules sécrétées par *P. aeruginosa* AHL et LS n'ont aucun effet sur l'expression de RsaG et RsaH.

5. Recherche des régulateurs de *S. aureus* impliqué dans la dysrégulation de l'expression des ARN Rsa en présence de *P. aeruginosa* : Le niveau d'expression de RsaH est augmenté dans tous les mutants de *S. aureus* (Δagr , Δarl , Δrot , $\Delta sarA$, $\Delta sarHI$) en présence de la culture vivante de PAO1. Par contre, aucune augmentation de l'expression de RsaG a été observée dans les mutants de Δagr , Δarl , Δrot et $\Delta sarHI$.

Conclusions:

1. Les mutants et les souches correspondantes complémentaires pour les petits ARN ont été construits avec succès pour les analyses futures transcriptomiques et protéomiques.

2. Des concentrations supérieures à $1/4$ de la CMI de lévofloxacine et d'oxacilline ont un effet inhibiteur sur la croissance bactérienne, mais ont aussi tendance à induire une réduction de l'expression des petits ARN. Le facteur σ^B n'est pas impliqué dans la modification de l'expression de RsaH par certaines concentrations subinhibitrices ($<1/8$ MIC) de lévofloxacine.

3. L'expression de petits ARN “*in vivo*” est très différente de leur expression “*in vitro*”. Surtout dans le cas de la colonisation nasale, il montre un profil d'expression uniforme des petits ARN qui reflète probablement l'état de commensalisme du *S. aureus*.

4. L'échange d'informations entre *S. aureus* et *P. aeruginosa* peut influencer l'expression de RsaG et RsaH. Le mécanisme de cette dysrégulation n'implique pas les molécules AHL et LS. Nous n'avons pas mis en évidence que les systèmes Agr, Rot, SarA, SarH1 participent directement à la régulation de RsaH, mais Agr, Arl, Rot, SarH1 semblent liés à l'augmentation de l'expression de RsaG.

En résumé, dans cette étude nous avons développé plusieurs approches pour étudier le rôle de nouveaux petits ARN régulateurs dans la virulence de *S. aureus*. L'originalité de nos travaux est l'étude de l'expression de ces petits ARN en condition “*in vitro*” et durant le processus d'infection ou de colonisation “*in vivo*”. Cette étude participe à l'enrichissement des connaissances actuelles sur la régulation des petits ARN bactériens.

Mots clés : *Staphylococcus aureus*; petit ARN; Rsa ARN; ARNIII ; Expression; *in vivo*; *in vitro*

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Abbreviations

Agr: Accessory gene regulator
Arl: Autolysis related locus
BLAST: Basic Local Alignment Search Tool
B. subtilis: *Bacillus subtilis*
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
DEPC: Diethylpyrocarbonate
DNA: Deoxyribonucleic acid
E. coli: *Escherichia coli*
IGR: Intergenic region
MIC: Minimal Inhibition Concentration
mRNA: messenger ribonucleic acid
MRSA: Methicillin-resistant *Staphylococcus aureus*
MSSA: Methicillin-sensitive *Staphylococcus aureus*
ncRNA: non-protein coding RNA
PCR: Polymerase Chain Reaction
PVL: Panton-Valentin Leucocidin
QS: Quorum Sensing
RBS: Ribosome Binding Site
RNA: Ribonucleic acid
Rot: Repressor of toxins
rRNA: ribosomal ribonucleic acid
Rsa: RNA from *Staphylococcus aureus*
RT-PCR: Reverse Transcription Polymerase Chain Reaction
Sae: *Staphylococcus aureus* exoprotein expression
Sar: Staphylococcal accessory regulator
S. aureus: *Staphylococcus aureus*
SCCmec: Staphylococcal Chromosomal Cassette mec
Spr: *Staphylococcus aureus* pathogenicity island RNA
sRNA: small regulatory RNA
Srr: Staphylococcal respiratory response
tmRNA: transfer-messenger ribonucleic acid, also called SsrA RNA and 10Sa RNA
tRNA: transfer ribonucleic acid
UTR: Untranslated region

General Introduction

Bacterial pathogenicity depends on the expression of numerous virulence factors and stress response ways. Besides proteins, more and more studies have established sRNA as an important role in gene regulation ^[12]. They could majorly exert regulatory functions through base pairing with mRNA, binding to proteins and metabolites.

Staphylococcus aureus belongs to the commensal flora and is carried by 30% of healthy human beings in their nasal caves. It could also cause a large diversity of clinical infections as an opportunistic pathogen. Great concern has been aroused about this bacterium due to the appearance of serious drug-resistance ^[13]. Gene regulation in *S. aureus* involves a lot of coordinated regulators. One paradigm of them is RNAIII, responsible for the repression of cell-wall associated proteins and activation of transcription of several exoproteins ^[8,10,14].

Recently, more than 90 sRNAs have been discovered in *S. aureus* through a combination of bio-informatics and experimental approaches, including newly confirmed Spr RNA and Rsa RNA ^[11,15-20]. They reside either in the core *S. aureus* genome or on mobile elements, as single or multiple copies. Among them, RsaA to RsaK were identified by a collaborative work of our team and teams in Strasbourg and Toulouse. Our team focuses on the molecular cloning, to supply mutants and complementation strains for other teams to further study the potential targets and functions of these sRNAs. Our team also performs phenotypic and functional studies in cellular and animal models to characterize the biological functions of these ncRNAs. Until now, RsaE has been proved to down regulate numerous metabolic enzymes by blocking the formation of ribosomal initiation complex ^[11,15]. Other functional characterization mainly surrounds their responses to simulated stresses, like pH variation, heat/cold shock, nutrient deficiency and oxidative stress. RsaA and RsaE are expressed under various stresses. RsaA is induced by alternative sigma-B factor (σ^B), while RsaE is agrA-dependant ^[11].

The clinical importance of *S. aureus* prompted our team to investigate whether these sRNAs are expressed in such complicated host environment and how they react to various pressures from host, other co-infecting (or colonizing) bacteria and antibiotics in real clinical situations. Last but not least, could we extend those laws obtained *in vitro* directly to *in vivo*?

The objective of my thesis is to elucidate the characteristics of 4 Rsa RNAs, namely RsaA, E, G, H, especially their expression levels under various conditions that are encountered in the human host. Firstly, besides the work on genetic construction, effects of several antibiotics on expression of these RNAs have been studied *in vitro*. Secondly, direct transcript analysis

for these sRNAs has been done for different types of *S. aureus* infection or colonization. Thirdly, we postulated that other bacteria present in the same niche in the host (i.e. *P. aeruginosa* in bronchial specimens from cystic fibrosis patients) could interfere with *S. aureus*, and thereby we analyzed the sRNA expression of *S. aureus* in the presence of *P. aeruginosa*. Finally, we tried searching for potential targets of these sRNAs by several experimental techniques.

Chapter 1 Literature Review

1.1 Small regulatory RNAs in bacteria

After Miescher discovered nucleic acids in 1868, it has not been considered as the major genetic material in cells until the famous *Streptococcus pneumoniae* conversion test conducted by Avery after 40 years ^[21]. When the genetic central dogma was established by Crick in 1958 ^[22], the only recognized role of RNA was to transmit the information from DNA to the functional proteins, which is to realize the transcription and translation process of genetic information. And this RNA was known as messenger RNA (mRNA) later. However, with the graduate discovery of transfer RNA (tRNA), ribosome RNA (rRNA), a hot wave of RNA research was started. In particular, the discovery of ribozymes liberating people's thought, RNA's biological function is not limited to the transmission of genetic information any more ^[23].

Cellular RNAs could be divided into two major groups according to their functions, protein-coding RNAs and non-protein-coding RNAs (ncRNA). The former group refers to the mRNAs, taking the classic responsibility of encoding protein synthesis, whereas the members of latter group are unexpectedly numerous, and still increase at high speed in both prokaryotic and eukaryotic cells ^[24-25]. Usually, the term small RNA – 'sRNA' is predominated for bacterial ncRNAs, although other terms, such as non-messenger RNA (nmRNA) or functional RNA (fRNA), are also occasionally used. Why so large number of sRNAs exist if they are useless? People started to question that and the phrase 'RNA world' was put out ^[26-27].

1.1.1 Definition

Among the enormous sRNAs, some bear with house keeping functions, like 4.5S essential for forming signal recognition particle, tmRNA consisted of both tRNA and mRNA nature, tagging targets the unfinished protein for proteolysis and releases stalled ribosome, RNase P acting as a catalyst on tRNA and other RNAs. Besides house-keeping sRNAs, recent studies revealed that lots of sRNAs are capable to regulate many bacterial physiological activities, either for adapting to the changes of micro-environment or for virulence gene expression ^[1,28-29].

Small RNAs acting as regulators are major interest, so-called 'small regulatory RNAs', which always are noted by these following features: 1) sizes of them are small, usually from 50 to

500 nt; 2) they are usually not coding for proteins; 3) they could be Hfq-dependant for facilitating the formation of duplex, such as the sRNAs in *Escherichia coli* [30-31]; 4) last but most important, they are recognized as regulators involved in many biological processes, controlling different steps of target gene expression [28].

1.1.2 Classification of small regulatory RNAs

Different regulatory sRNAs have distinctive biological functions, and they could realize regulatory functions through diverse mechanisms, such as base pairing with target mRNAs (*cis*- or *trans*-acting RNA), binding to proteins, changing in their own conformation (riboswitches), interfering with DNA (CRISPR) [2,12].

1.1.2.1 *Cis*-encoded antisense sRNAs

Most of the discovered sRNAs regulate gene expression through base pairing with target mRNAs, and they could be further classified into two categories. The *cis*-encoded antisense sRNAs are usually transcribed in an inverse direction with their target mRNAs, capable to base pair with them in extensive complementarity; meanwhile, *trans*-encoded antisense sRNAs are located in the regions distinct from the genes encoding target mRNAs, and their complementarity with targets is more limited [12,32-33].

– Plasmid encoded antisense RNAs

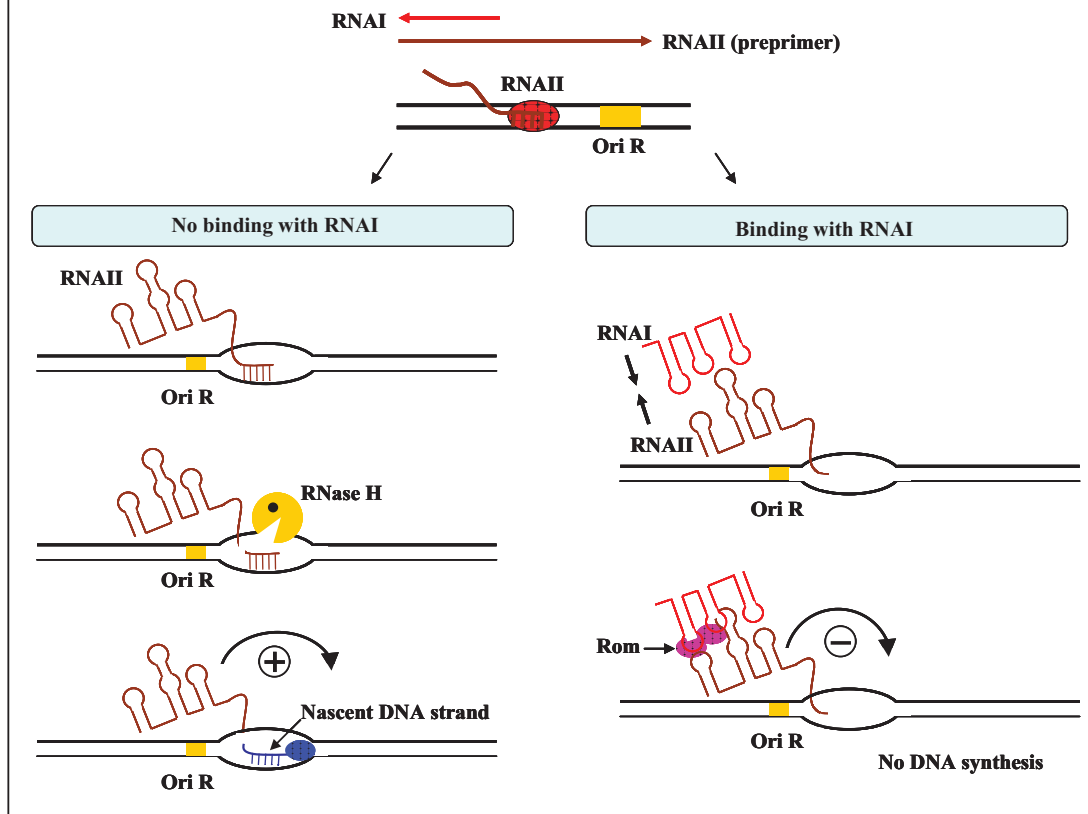
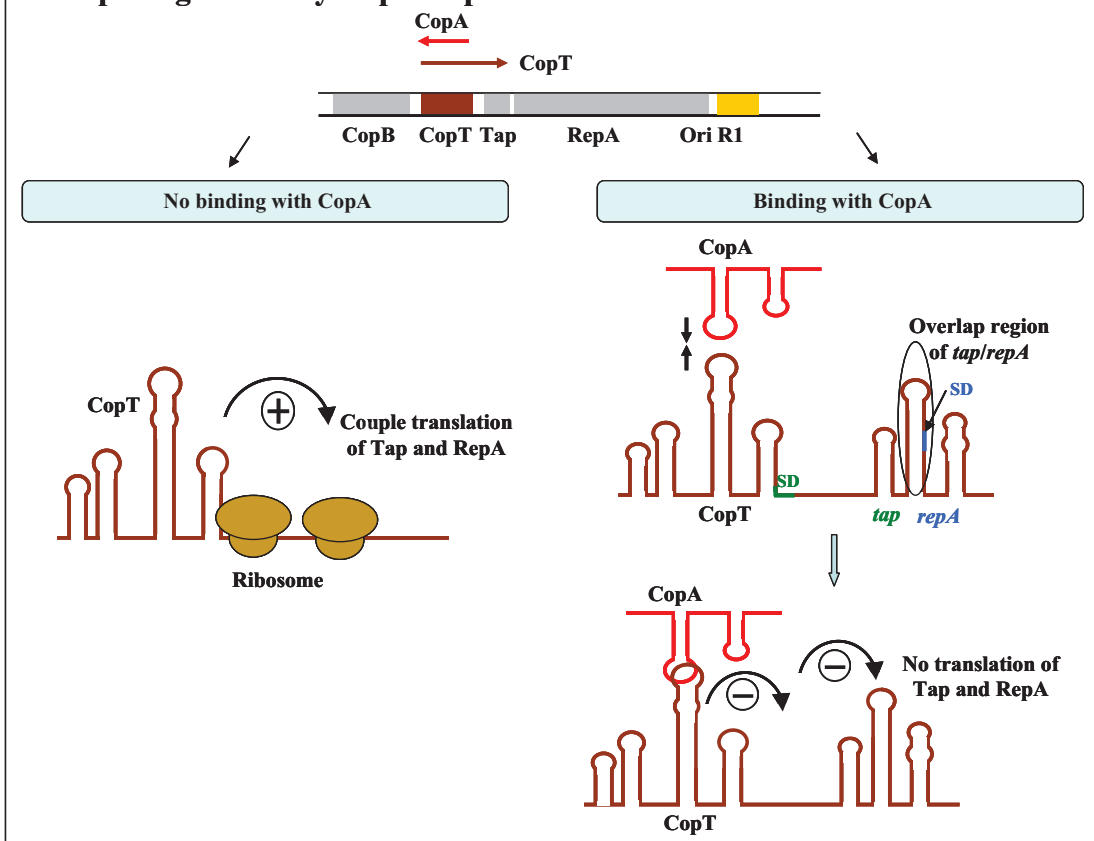
The majority of *cis*-encoded antisense sRNAs are mainly found in bacteriophages, plasmids and transposons, dedicating to control the copy number of these mobile elements [12,32-33]. They not only help prevent excessive reduction, which leads to loss of the plasmids, but also help prevent excessive replication, which may be toxic and kill the bacteria. Regulation on the plasmid replication by these sRNAs is negative. Increased copy number of these mobile elements will induce an augmentation of the related sRNA transcription, which in turn represses the replication; conversely, decreased copy number of these mobile elements will impair sRNA transcription and then promote the replication of its own [34]. The mechanisms for this copy number regulation include inhibition of replication primer formation and transposase translation, most well-studied examples of which are plasmids ColE1 RNA I, R1 CopA and Tn10 pOUT RNA (Figure 1-1).

ColE1, as the prototype of high copy plasmids, uses a primer RNA – RNAII to control its replication [35]. RNAII, synthesized by RNA polymerase, is subject to specific

conformational changes that will enable it form a persistent hybrid with DNA template strand. This complex hybrid is then cleaved by RNaseH to generate a mature primer that will be used for DNA replication. On the other hand, a 108nt ColE1 antisense RNA, called RNAI, is expressed from the same region and strictly complementary to the 5' region of RNAII. The binding of RNAI and RNAII prevents the conformational change of RNAII, which then can not hybrid with DNA, thus inhibiting the replication of the plasmid ^[36-37]. During this process, a plasmid-encoded protein – Rom stabilizes the intermediate product, assisting the binding of RNAI and RNAII ^[35,38] (Figure 1-1A).

R1 is representatively well characterized of low-copy number entero-bacterial plasmid. Its replication requires synthesis of a *cis*-acting protein – RepA, whose expression controls the frequency of replication. The regulation of RepA majorly achieves at two levels: transcriptional inhibition by CopB protein and translational inhibition by CopA antisense RNA. CopA has a strict complementarity with one region of RepA mRNA (CopT). The binding between CopA and CopT blocks translation initiation of protein Tap, and then the complex formed is degraded by RNaseIII ^[39]. Since the 3' end of *tap* overlaps the RBS of *repA*, the translation of these two transcripts is coupled. Thus CopA – CopT interaction inhibits the translation of *repA* indirectly by blocking *tap* translation ^[40] (Figure 1-1B).

Insertion sequence IS10 is the active element in tetracycline-resistance transposon Tn10, encoding a transposase protein – Tnp. IS10 also encodes three promoters: pIN, the promoter for transposase gene *tnp*; pOUT, the strong promoter directing transcription outward toward the outside terminus; pIII, the weak promoter symmetrically related to pOUT ^[41]. The transcript of pOUT is complementary to the 5' end of *tnp* mRNA transcript from pIN. The complementarity region of 36 nt includes the *tnp* RBS, then pIN-pOUT pairing directly blocks ribosome binding of this transposase ^[42]. This negative control prevents the accumulation of these elements in bacteria, thus beneficial for bacterial survival (Figure 1-1C).

A RNAII regulation by RNAI in plasmid ColE1**B CopT regulation by CopA in plasmid R1**

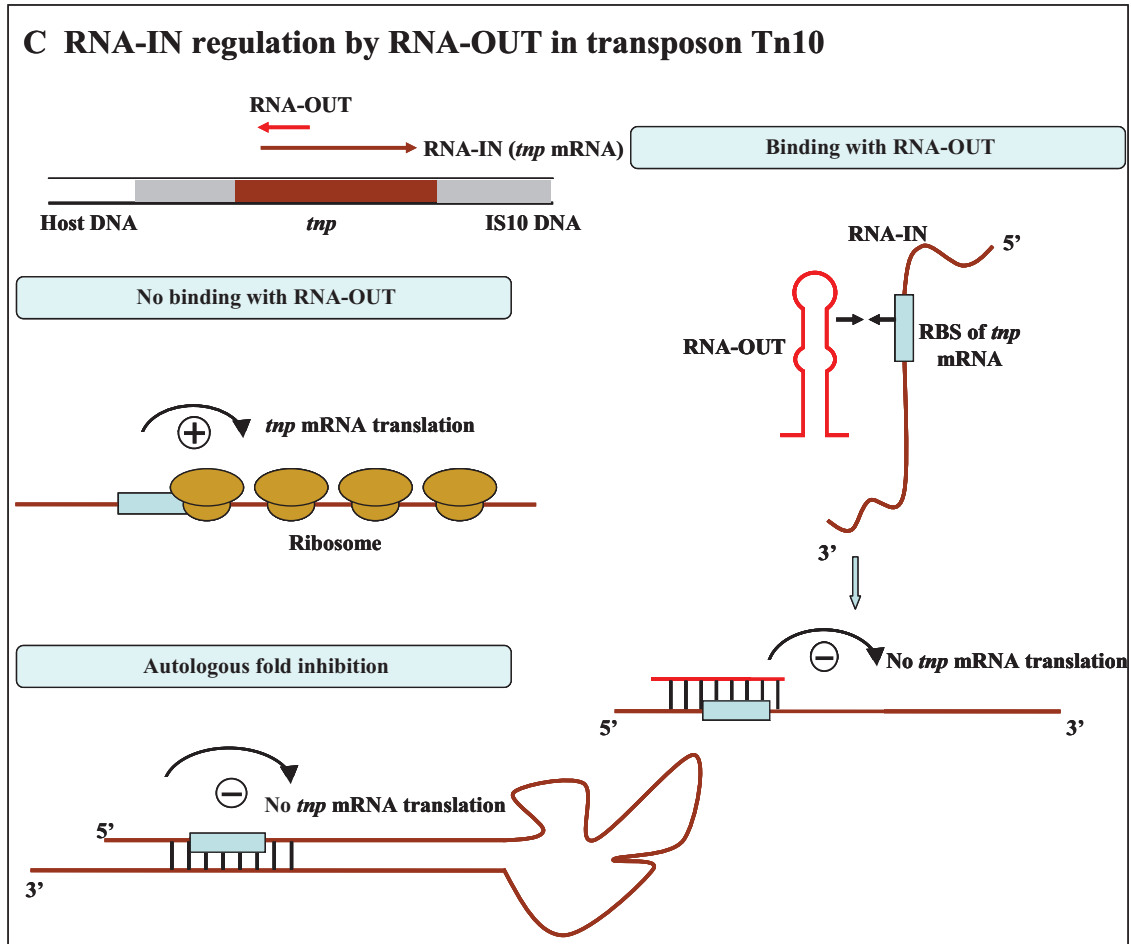


Figure 1-1 Regulation mechanism of plasmid-encoded antisense sRNAs (adapted from Wagner *et al.*, 1994). Regulatory antisense sRNAs are indicated in red and target RNAs in brown. Detailed mechanisms are explained in the text.

– Chromosome encoded antisense RNAs

Fewer antisense RNAs from bacterial genomes are better known compared to those from mobile genetic elements. These RNAs are only partially complementary to their targets. One portion of these sRNAs function as antitoxins repressing translation of mRNAs encoding toxic proteins or inducing their degradation ^[43-44]. There are many antitoxin sRNAs in bacterial chromosome showing homology to plasmid antitoxin sRNAs. For instance, *E. coli* strain K-12 has four long directed repeats (LDR) expressing one mRNA (*ldr*) encoding a toxic protein (LdrD) and an antisense RNA ^[45]. In bacterial cells, low level of toxins caused by the *cis*-encoded antitoxin antisense RNA system may only inhibit cell growth or induce stasis. One speculates that this may be beneficial for cells by letting them have sufficient time to self repair or adapt to stresses ^[46-47].

Besides the toxin-antitoxin modules where antisense sRNAs help maintain low expression of toxin-encoding mRNAs, there is also one sRNA (GadY) which is able to increase the expression of target mRNA (*gadX*)^[28,48-49]. During stationary phase, GadY base pairs with the 3' untranslated region of *gadX* mRNA and confers increased stability, which allows the GadY dependant accumulation of *gadX* mRNA^[48].

Recently, high-throughput techniques like deep-sequencing and tiling arrays revealed that *cis*-antisense RNAs are much more common. It was found that at least 25% of coding regions are transcripts also in antisense. This leads to a processing of the mRNA-antisense RNA complex by RNaseIII^[50]. However, the consequences from this mechanism are not yet well understood.

1.1.2.2 Other *cis*-acting sRNAs

1.1.2.2.1 Riboswitches

Riboswitches are RNA elements situated in 5' untranslated regions (UTRs) of target mRNAs, which can adopt different conformations in response to various physiological signals. These sRNAs are usually both *cis*-encoded and *cis*-acting, however the riboswitches acting in *trans* do exist^[51]. Riboswitches could monitor uncharged tRNAs and a variety of metabolites, such as S-adenosylmethinine (SAM), thiamine pyrophosphate (TPP), flavin mononucleotide (FMN), methionine, lysine, adenine and guanine^[52-54]. Usually they consist of two domains, an aptamer which can bind to small metabolites and a regulatory region or so called 'expression platform' that undergoes a conformational shift after the binding of one ligand. Sequence and structure of aptamer domains are highly conserved for each class of riboswitches that sense every particular metabolite. On the contrary, expression platforms can vary in sequence and structure among riboswitches of the same class.

To date, riboswitches have been found to regulate a wide range of genes involved in metabolism, either at the level of transcription attenuation or translation initiation. Leader region of the genes regulated at transcription level contains one intrinsic transcriptional terminator (Rho-independent terminator) composed of G+C rich helix followed by a series of U residues. In the absence of metabolite, an anti-terminator allows the synthesis of mRNA. Once the metabolite binds to the aptamer, a complete stem-loop structure is formed, and the intrinsic transcription terminator blocks the transcription elongation (Figure 1-2A). On the other hand, in the genes regulated at translation level, the binding of the metabolite causes a

conformational change that makes the SD sequence inaccessible to the ribosome. Thus the translation initiation is blocked (Figure 1-2B). Although most riboswitches are found to exert negative regulation on gene expression, there are also mechanisms of positive control. For example, riboswitch of adenine prevents formation of terminator stem, thus activates transcription. In general, the control systems at the transcription termination predominate in Gram-positive bacteria with low GC%, while systems that regulate translation initiation are more common in Gram-negative and Gram-positive bacteria with high GC%^[55-56].

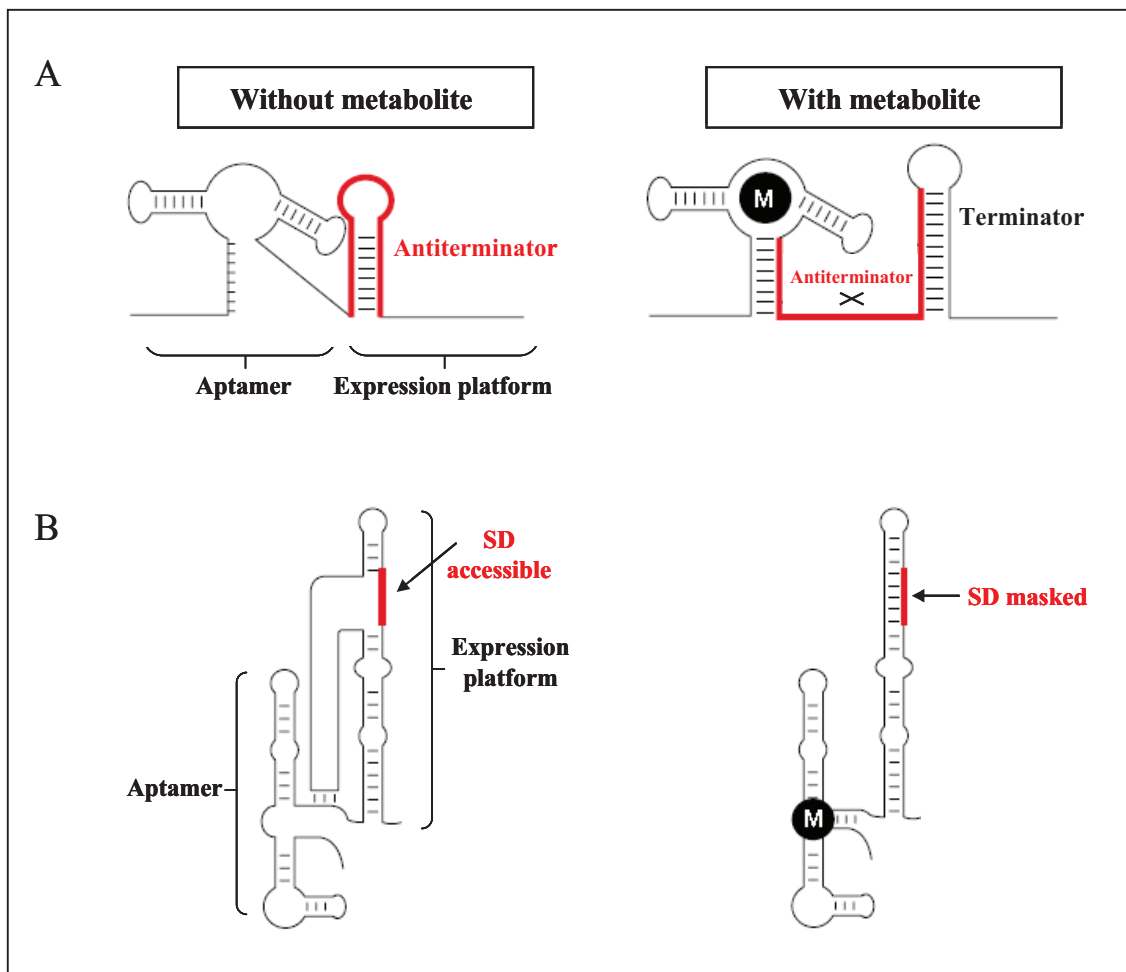


Figure 1-2 Mechanism of riboswitch function^[56] **M** stands for metabolite. (A) Transcription termination induced by metabolite binding, i.e. guanine riboswitch; (B) Translation initiation blocked by metabolite binding, i.e. TPP riboswitch.

Other novel mechanisms of riboswitches are recently recognized (Figure 1-3). For instance, in *Vibrio cholerae*, 5' UTR of *VC1422* contains two nearly identical conserved domains. After glycine binds to one domain, it will improve the binding efficiency of glycine to the

other domain by 100-1000 folds ^[57]. One riboswitch residing in the leader region of the glucosamine-6-phosphate synthase gene (*glmS*) has been demonstrated to control mRNA processing as ribozyme. When GlcN6P bound, a self-cleavage event happens at the specific site at its 5' end, leading to repression of *glmS* gene ^[58-59].

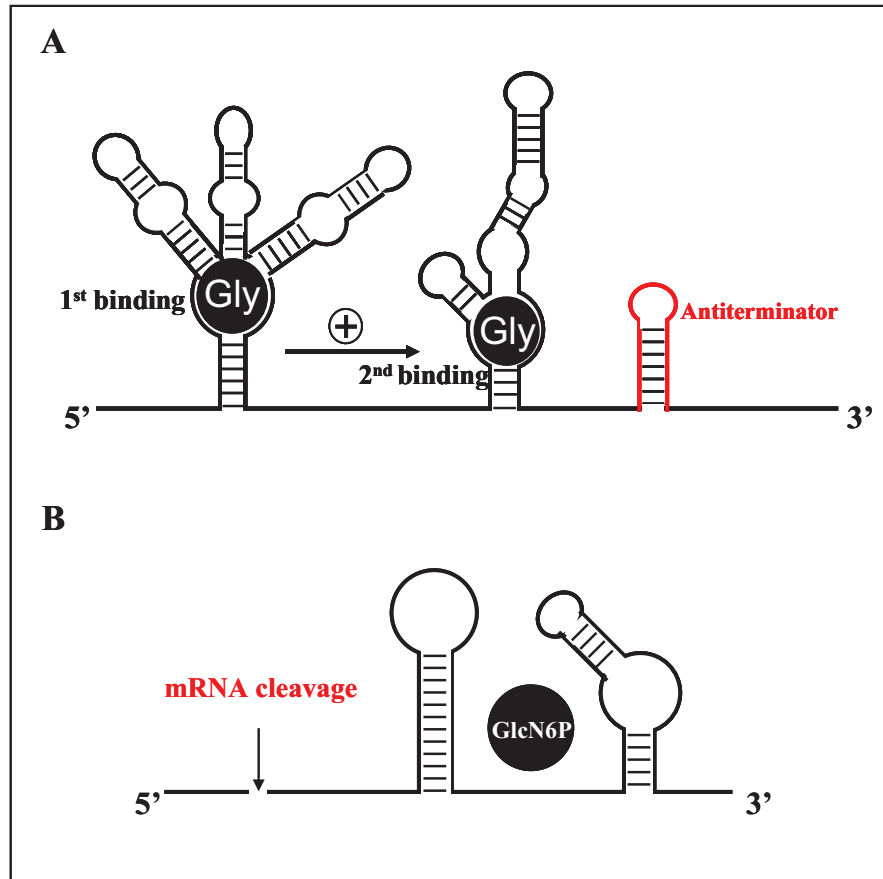


Figure 1-3 (A) The glycine cooperative riboswitch (adapted from Lioliou *et al.*, 2010): The first binding of glycine promotes the second binding, and then stabilizes the antiterminator structure. (B) The GlcN6P ribozyme switch: Self cleavage is induced by GlyN6P binding at the 5' UTR of mRNA.

1.1.2.2.2 Thermosensors

To survive in host, bacteria need to response to changing environmental factors and accordingly adjust their gene expression. One major variation they face is the temperature change. RNA thermosensors are those mRNAs responding to temperature changes by altering conformations to allow or prevent binding of the ribosome to translation initiation site, thus modulate the heat-shock or cold-shock protein expression. In *Listeria monocytogenes*, 5' UTR of the *prfA* mRNA forms a secondary structure masking ribosome

binding region at lower temperatures (e.g. 30°C). At human body temperature (37°C), another structure is formed where SD region is exposed, allowing the translation start ^[60]. In contrast, *cspA* mRNA in *E. coli* undergoes a structure rearrangement beneficial for translation activation at lower temperature (e.g. 15°C) rather than high temperature (37°C). The structure folded at lower temperature is more stabilized as well, which in turn promotes translation ^[61].

1.1.2.3 *Trans*-acting sRNAs

Trans-acting sRNAs are antisense RNAs regulating distant target mRNAs by base pairing to them in limited complementarity. Usually identified in intergenic regions, they may down regulate target activity through inhibiting translation initiation, or induce complex degradation, or both (Figure 1-4A). They often bind to the 5' UTR of mRNAs and occlude the ribosome-binding site. Then the duplex formed are tended to be degraded by RNaseE. For many *trans*-acting sRNAs, induced degradation is frequently the major contributor of their negative control on target synthesis, except for few sRNAs, such as RyhB silencing *sodB* mRNA and SgrS silencing *ptsG* mRNA in *E.coli* ^[62]. However, positive regulation by *trans*-acting sRNAs also exists. An anti-antisense mechanism is adopted during the activation of target mRNA translation (Figure 1-4B). In this case, base-pairing between sRNA and mRNA could disrupt an inhibitory structure and thereby releasing the ribosome-binding site ^[63-65]. Sometimes one sRNA can both inhibit and activate target synthesis ^[65].

Given the partial complementarity between *trans*-acting sRNAs and targets, the Hfq protein is usually required to facilitate RNA-RNA interactions ^[30-31]. Hfq, originally discovered in *E. coli*, has homology with Sm or Sm-like proteins in eukaryotes and archaea. Hfq mutants show various stress response related phenotypes, such as slow growth, hypersensitivity to osmotic shock and ultraviolet radiation ^[66]. It is essential for the expression of sigma factor σ^S , global regulator of many genes under stress conditions ^[67]. Present as a hexamer, it can bind to AU rich single-stranded RNA on the proximal side, poly (A) tail on the distal side. This protein is revealed as a chaperone to accelerate strand exchange and annealing between sRNA and target mRNA. It may also modulate RNAs to melt inhibitory secondary structures. On one hand, it can protect sRNAs from degradation in the absence of base pairing with mRNAs. On the other hand, after the base pairing between sRNAs and mRNAs, it may help

RNaseE target and degrade mRNA by forming the RNaseE-Hfq-sRNA complex ^[68]. In addition, Hfq may also protect mRNA against degradation. For instance, it protects *rpoS* mRNA from hydrolysis of exoribonucleases and RNaseE by binding to its poly (A) ^[69].

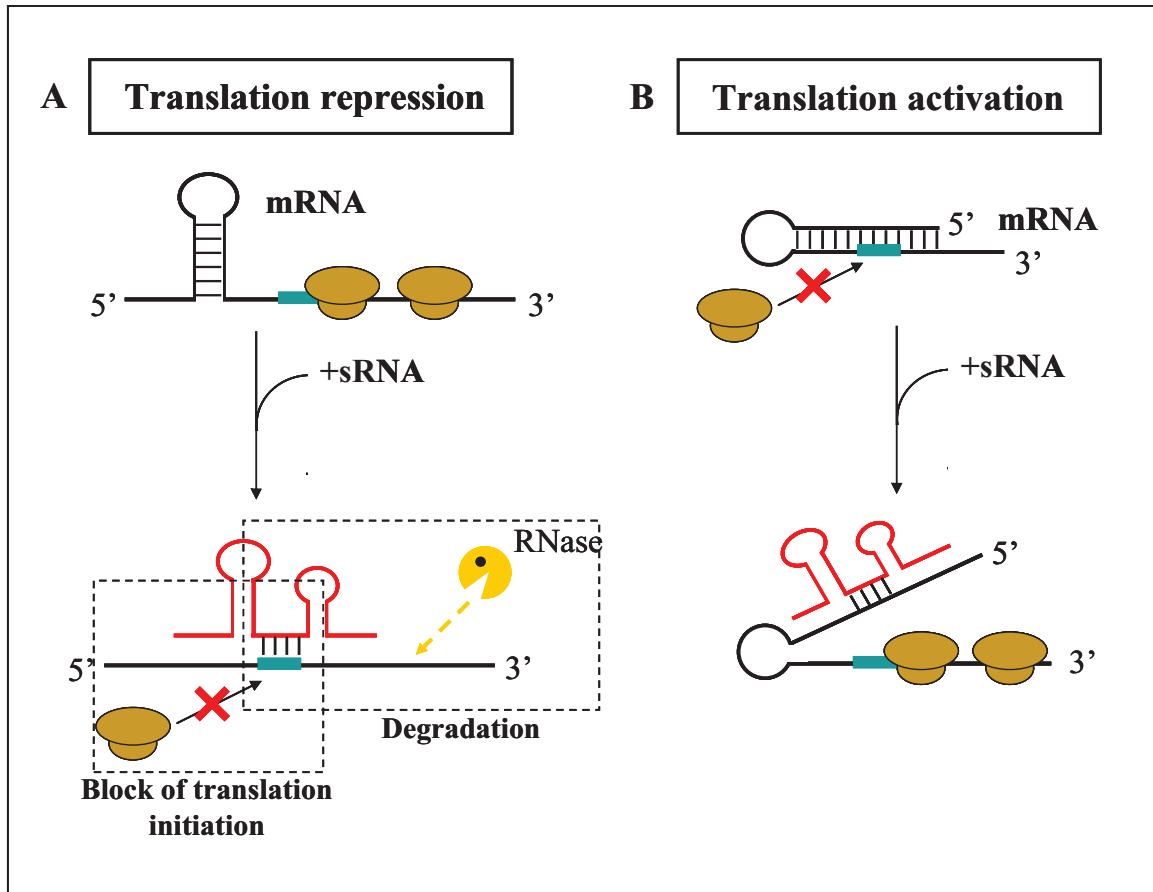


Figure 1-4 Mechanism of regulation by *trans*-acting sRNAs. (A) Most regulation is negative: sRNA (stem-loops in red) base pairs with SD region (represented in blue box), then makes the translation initiation aborted; or the complex induces irreversible degradation by RNase. (B) Positive regulation: Binding of sRNA and mRNA makes the SD region on mRNA accessible by ribosome.

It has been found that until now all the characterized *trans*-acting sRNAs in *E. coli* need the presence of Hfq to exert their regulation roles on targets, however, this requirement for a chaperone protein is not universal among bacteria. For example, in *S. aureus*, although Hfq binds to RNAIII tightly *in vitro*, it has no detectable effect on RNAIII-target mRNA complex formation. Another example is VrrA RNA in *Vibrio cholerae*, it can repress the OmpA protein expression in Hfq mutant strains ^[70].

1.1.2.4 Protein-binding sRNAs

There is one class of regulatory RNAs having the ability to bind to proteins and regulate their activities. In *E. coli*, three protein-binding sRNAs are demonstrated to antagonize activities of their target proteins by mimicking the structures of other nucleic acids. They are CsrB sRNA modulating carbon usage related protein CsrA, GlmY sRNA targeting the YhbJ protein, and 6S RNA targeting the σ^{70} -containing RNA polymerase [12,71-73]. For example, the RNA-binding protein CsrA normally base pairs with the GGA motifs in 5' UTR of target mRNAs, thus influences its stability or translation. CsrB family of sRNAs, CsrB and CsrC contain several GGA repeats which mimic the binding sites on CsrA target mRNA, and thereby sequester the CsrA from its target mRNA. Homologs of CsrB sRNA have been identified in many other bacteria where they antagonize corresponding CsrA homologs, such as the RsmZ and RsmY antagonize RsmA protein which is involved in carbon metabolism, biofilm formation, motility, virulence in *P. aeruginosa* [74-76].

Worth to mention, the housekeeping sRNAs, as part of ribonucleoprotein complexes (RNPs), have also the protein-binding functions and are crucial for bacterial metabolism and adaptation to stresses. In bacteria, 4.5S RNA and Ffh protein constitute the signal recognition particle (SRP), which target polypeptides to bacterial cell membrane by a co-translational way; tmRNA binds to SmpB protein, then interacts with the translational ribosomal complexes stalled at the 3' end of truncated mRNA, followed by a proteolysis-induced tag added to the truncated protein facilitating rapid degradation for abnormal mRNA; RNase P, consisting of M1 RNA and C5 protein, has the primary role to cleave 5' end of precursor-tRNAs to produce mature 5' end tRNAs.

1.1.3 Advantages and limits

In the last decades, RNAs have been recognized as a key effector of gene regulation among all living bacteria. They have several advantages over proteins. First of all, compared to proteins, RNA regulators are more economic and rapid to produce, since they are much shorter and do not need the step of translation. Secondly, the regulation by RNAs is quite fast, especially for *cis*-acting sRNAs, such as riboswitches affecting the same mRNA where they reside when sensing appropriate metabolites. Furthermore, one sRNA can simultaneously regulate multiple genes (i.e. RNAIII regulates *spa* mRNA, *hla* mRNA and *rot* mRNA), as the partial complementarity allows sRNA interact with more than one mRNA

target in many cases. Moreover, several sRNAs can regulate one single target (i.e. DsrA, RprA and OxyS regulate RpoS translation directly or indirectly), which permit bacteria integrate various environmental signals.

Although many sRNAs are unveiled recently, there are still lots of sRNAs missed, because that they are only expressed under specific conditions, or some *cis*-encoded antisense sRNAs whose sequences are hard to distinguish from 5' UTR or 3' UTR of bacterial genomes.

1.2 Virulence gene regulation of *Staphylococcus aureus*

Staphylococcus aureus, a Gram-positive coccal bacterium, could be considered as a 'commensal flora' since about 20-30% of human population are long term carriers of this bacterium on skin or nasal passages ^[4]. However, it is also a great harm to public health, responsible for many community-acquired and hospital-acquired infections. It can cause a wide variety of diseases, from minor skin infections to severe life-threatening infections. The diversity and severity of *S. aureus* infection depends on the coordination of different virulence factors expression (Table 1-1), which in turn relies on the fine cooperation of complicated virulence regulators.

1.2.1 Pathogenicity of *S. aureus*

1.2.1.1 Invasive infection

Once the human skin or mucosal barrier is breached, the bacterial cells from colonization sites enter into the adjacent tissues or bloodstream. Then the combat between *S. aureus* and host defense system will decide whether this infection localizes or spreads. Several high-risk conditions have been associated with invasive infection. For instance, host immunity is compromised or deficient by underlying diseases, such as newborn, old age, diabetes, HIV infection; or foreign material/equipment is used inside human body, such as intravenous catheter, feeding tube and artificial pacemaker.

Table 1-1 Major known virulence factors of *S. aureus* and their regulation by Agr (adapted from Novick, 2003)

Gene	Location	Virulence factor	Function/Disease	Time _a	Agr ^b	Reference
Capsule and Surface proteins						
<i>cap5, 8</i>	chrom	Polysacch serotypes 5, 8	Anti-phagocytosis	pxp	+	[77]
<i>spa</i>	chrom	Protein A	Adhesion, immunomodulation	exp	—	[78-79]
<i>cna</i>	PT island	Collagen binding Protein	Collagen binding	pxp	0	[80]
<i>fnbA, B</i>	chrom	Fibronectin binding PA, B	Fibronectin binding	exp	-	[81]
<i>clfA, B</i>	chrom	Clumping factor A, B	Fibrinogen binding	exp	0	[82]
<i>coa</i>	chrom	Coagulase	Adhesion (plasminogen to plasmin conversion)	exp	-	[82-83]
Cytotoxins (membrane-damaging)						
<i>hla</i>	chrom	α - haemolysin	Cytolysin	pxp	+	[84]
<i>hlb</i>	chrom	β - haemolysin	Cytolysin, sphingomyelinase	pxp	+	[78,85]
<i>hld</i>	chrom	δ - haemolysin	Cytolysin	xp	+	[86]
<i>hlg</i>	chrom	γ - haemolysin	Cytolysin	pxp	+	[87]
<i>lukD/E</i>	phage	Leucocidin	Cytolysin			[88]
<i>lukS/F</i>	phage	P-V leucocidin	Cytolysin	pxp	+	[84]
Superantigenic toxins						
<i>sea</i>	phage	Enterotoxin A	Food poisoning, TSS	xp	0	[89]
<i>seb</i>	SaPI3	Enterotoxin B	Food poisoning, TSS	pxp	+	[84]
<i>sec</i>	SaPI4	Enterotoxin C	Food poisoning, TSS	pxp	+	[90]
<i>sed</i>	plasmid	Enterotoxin D	Food poisoning, TSS	pxp	+	[91]
<i>eta</i>	phage	Exfoliatin A	Scalded skin syndrome	pxp	+	[92]
<i>etb</i>	plasmid	Exfoliatin B	Scalded skin syndrome	pxp	+	[93]
<i>tst</i>	SaPI1,2	Toxic shock toxin-1	Toxic shock syndrome	pxp	+	[84]
Enzymes						
<i>splA-F</i>	chrom	Serine protease	Protease		+	[85]
<i>sspA</i>	chrom	V8 protease	Diffusion	pxp	+	[94]
<i>sspB</i>	chrom	Cysteine protease	Enzyme		+	[94]
<i>scpA</i>		Staphopain protease	Diffusion, nutrition	pxp	+	[94]
<i>geh</i>	chrom	Glycerol ester hydrolase	Diffusion, nutrition	pxp	+	[85]
<i>lip</i>	chrom	Lipase	Diffusion, nutrition	pxp	+	[95]
<i>aur</i>	chrom	Metallo protease (aureolysin)	Enzyme	pxp	+	[96]
<i>nuc</i>	chrom	Nuclease	Nutrition	pxp	+	[97]
<i>hys</i>	chrom	Hyaluronidase	Diffusion	xp		[98]
<i>sak</i>	phage	Staphylokinase	Plasminogen activator	pxp	+	[78]
<i>fme</i>	chrom	FAME	Fatty acid esterification	pxp	+	[95]

a. exp: early exponential phase, pxp: post exponential phase, xp: exponential phase;

b. +: upregulation, -: downregulation, 0: no regulation

The invasive *S. aureus* infection has a broad range of clinical presentations from bacteremia with or without primary focus to many complications, such as endocarditis, metastatic infection or sepsis syndrome. Breakthrough towards endothelial cells plays a key role during these pathogenic processes. After adherence to endothelial cells, *S. aureus* are phagocytized by them ^[99]. Then the intracellular milieu protects bacteria from both the host defense mechanism and the bactericidal effect of antimicrobial agents. Besides, if the endovascular tissue is invaded, it will become more convenient for bacteria to spread to other tissues.

1.2.1.2 Toxin-mediated disease

– Staphylococcal foodborne disease

Foodborne disease (FBD) is the illness caused by consumption of contaminated food (i.e. pathogens, chemical toxins). Each year, the FBD resulted from bacteria contamination takes up more than two thirds of recorded outbreaks ^[100]. Some kinds of bacteria are particularly arousing attention because of their frequency or seriousness, and *S. aureus* is one of them.

At first, *S. aureus* could grow in a wide range of temperatures, pH and sodium chloride concentrations. Together with its high carrier rate among normal population, this bacterium can contaminate various food products either during the step of food preparation or processing. Most importantly, the staphylococcal enterotoxins (SEs) secreted by *S. aureus* contribute to the foodborne disease. Typical symptoms appear rapidly, including nausea, vomiting and abdominal cramps, sometimes followed by diarrhea.

– Toxic shock syndrome

S. aureus is a major cause of toxic shock syndrome (TSS), the dangerous and potentially fatal illness due to bacterial toxin. Patients with staphylococcal TSS often have high fever, low blood pressure, soon progress to coma and multi-organ dysfunction.

The superantigen toxins (i.e. TSST-1) produced by *S. aureus* could improperly stimulate host immunology mechanisms and are responsible for TSS. These proteins are resistant to heat denaturation and proteases. They bound firstly with MHC class II molecules then are recognized by an antigen specific T-cell receptor. Formation of this trimolecular complex activates the expansion of T-cells, thus induces a massive cytokine release causing various symptoms of TSS.

– Scalded skin syndrome

Scalded skin syndrome (SSS), also called Ritter disease or Staphylococcal scalded skin syndrome, is a life-threatening infection characterized by skin damage like burn or scald. It usually affects infants and children younger than 5 years or patients with repressed immune system or renal failure. The disease starts with fever and redness of skin, followed by formation of fluid-filled blisters which rupture easily making the top layer of skin peel off in sheets.

Formation of blisters is caused by the production of two exotoxins – exfoliative toxins A and B. They can digest one of the intracellular adhesion molecules, desmoglein1 (Dsg1), whose function is to hold the granulosum and spinasum layers together, thus induce intraepidermal cell-cell dissociation.

1.2.1.3 Positive and adverse effects of host response

Outcomes of infections usually depend on the complex and dynamic interactions between pathogens and host defense mechanisms. During the process of abscess formation, one of the typical *S. aureus* pathological representations, bacterial cells elicit a series of inflammatory responses ^[101]. After phagocytosis, endothelial cells express adhesion molecules (vascular-cell adhesion molecules and intercellular adhesion molecules), and release interleukin-1, 6, and 8. Then leukocytes migrate to the site of infection and adhere to these endothelial cells ^[102]. Macrophage activation occurs after the release of interferon-g by T cells. Cytokines released into bloodstream, from monocytes or macrophages, as well as endothelial cells, contribute to the manifestations of the sepsis syndrome and vasculitis associated with systemic staphylococcal disease ^[103].

Nevertheless, it has been recognized that the host immune system is like a double edged sword. When working within a proper range, it is essential to protect the host by activation of antimicrobial defenses. If overacting, a bursting swarm of cytokines are released, thus induce severe inflammatory responses which are deleterious to host. However, this uncontrolled inflammatory host response does not take the full responsibility for sepsis mortality. Depressed immune system also plays a crucial role in the prognosis of sepsis ^[104]. Many studies have reported that leucocytes from sepsis patients may have impaired capacity to release proinflammatory cytokines ^[105-106]. And in many sepsis death cases, a large number of immune cells, such as B cells, CD4 T cells, dendritic cells, and gastrointestinal

cells, are found lost because of an apoptosis-induced mechanism ^[107-109].

1.2.2 Various virulence factors

A large number of virulence factors are encoded on the *S. aureus* core genome and mobile genetic elements, such as surface proteins, exotoxins (Figure 1-5) and extracellular enzymes. They participate in the different steps of staphylococcal infection (adhesion, invasion and escape from host defense) and are responsible for the diversity of diseases.

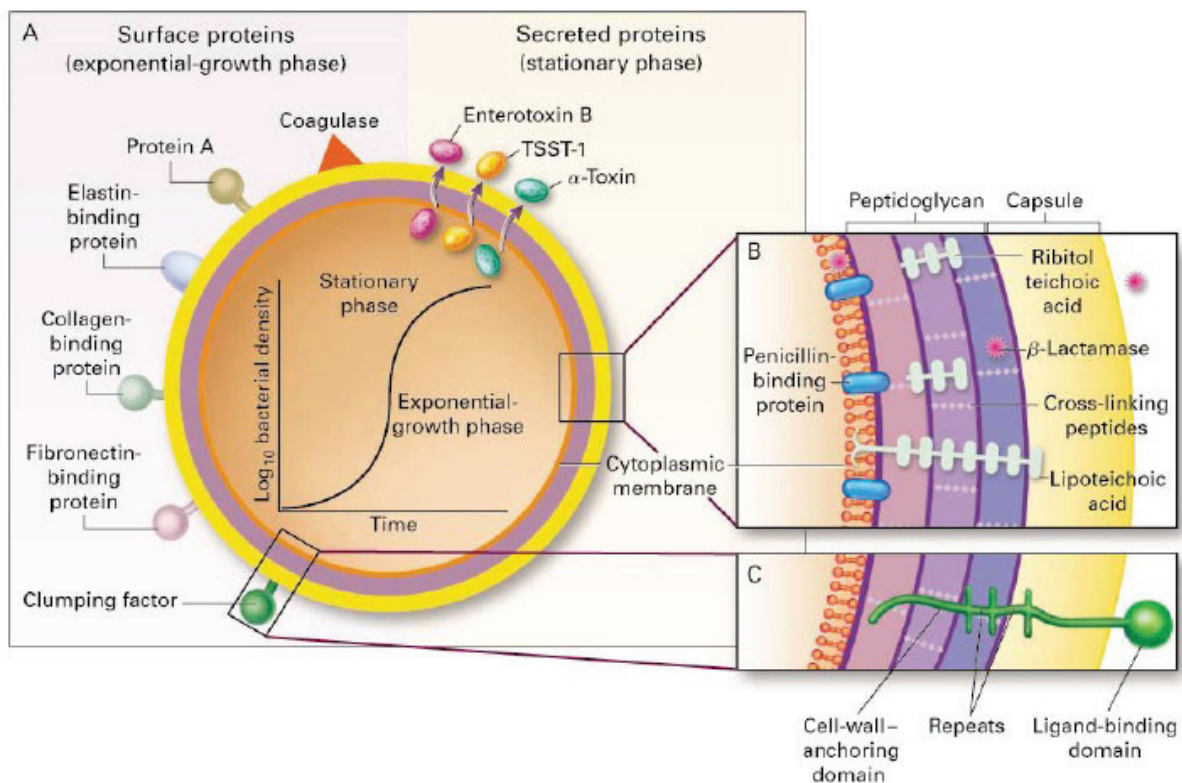


Figure 1-5 Structure of *S. aureus* ^[101]. A. Surface proteins and secreted proteins: surface proteins (also called cell wall-associated proteins) are expressed in early exponential phase whereas the synthesis of secreted proteins (also called exoproteins) is activated in the post-exponential and stationary phase; B. the cross section of cell envelope; C. the typical constitution of clumping factor, like other surface proteins.

1.2.2.1 Capsule and cell wall

Capsule and cell wall are two important structures in pathogenesis of *S. aureus*. Up to now, 11 capsular polysaccharide types have been identified in this pathogen, most common ones causing human infection are type 5 and type 8 ^[101,110]. Capsule can not only counterwork phagocytosis, but also promote biofilm constitution and abscess formation ^[101,111]. *S. aureus*

has a typical Gram-positive bacterial cell wall composed of many layers of peptidoglycan and teichoic acids, which dedicate to maintain bacterial integrity and mediate evasion of host immune system. For instance, peptidoglycan may covalently link adhesive proteins, whereas lipoteichoic acids play an important role in adhesion and colonization through their hydrophobic part ^[99,112]. In a rabbit model of endocarditis, the cell wall teichoic acids deficient mutants have been demonstrated to have reduced interactions with endothelial cells ^[113].

1.2.2.2 Surface proteins

– MSCRAMMs

Many staphylococcal surface proteins are those cell wall-anchored proteins with certain structure features. They majorly consist of a long signal sequence at N terminal, and a hydrophobic membrane-spanning domain and a cell-wall anchoring region at C terminal ^[101]. Some of these surface proteins could function as adhesions to initiate colonization by adhering to components of extracellular matrix (ECM) of host, so named as 'Microbial Surface Components Recognizing Adhesive Matrix Molecules' – MSCRAMMs ^[114].

MSCRAMMs, secreted via the Sec system, are associated with the peptidoglycan by covalent bonds. Its signal peptide (S) N-terminal position allows the addressing of the protein neo-synthesized at the plasma membrane. C-terminal hydrophobic regions are W and M, separated by the consensus sequence LPXTG (Leu-Pro-X-Thr-Gly). The M-rich region positively charged amino acids corresponds to a region in the cytoplasmic membrane anchor (Figure 1-6). The LPXTG motif is the target of the enzyme sortase, whose role is to anchor the protein adhesin to the cell wall peptidoglycan by a transpeptidation mechanism. The sortase can cleave between threonine and glycine residues of the LPXTG motif and catalyzes the formation of an amide bond between the carboxyl group of threonine and the amino group of the molecules of the wall peptidoglycan ^[115-117].

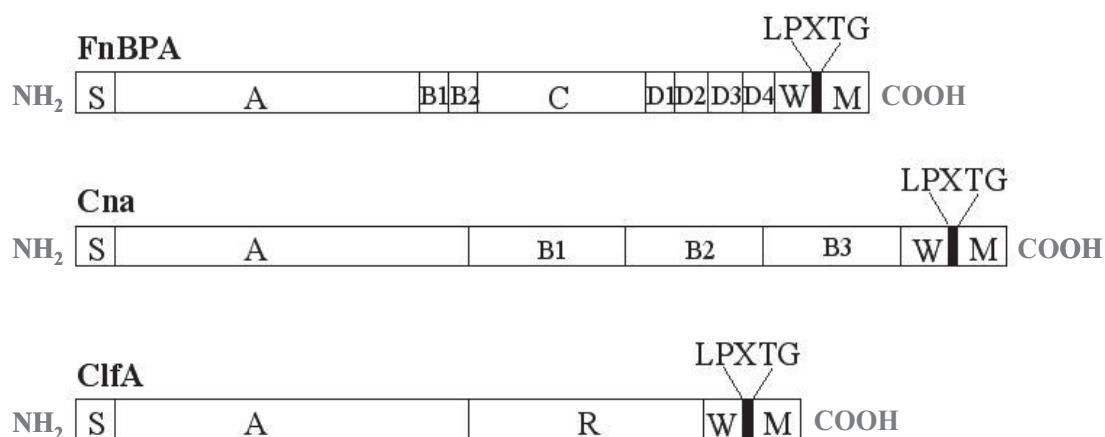


Figure 1-6 Schematic model of MSCRAMMs – domain organization of fibronectin binding protein A (FnBPA), collagen binding protein (Cna) and clumping factor A (ClfA) (from Foster 1998 and thesis of Sandrine 2006). "S" represents the signal sequence, "A" corresponds to the fibrinogen-binding domain, "R" and "D" are involved in binding to fibronectin, the functions of the regions "B" and "C" are unknown, "W" represents the wall-spanning region, "M" represents the membrane-spanning region and positively charged residues. The position of the LPXTG motif is indicated.

More than 20 members of MSCRAMMs have been identified until now, and the most well-studied models are still the fibronectin binding proteins (FnBPA and FnBPB), collagen binding proteins (Cna), fibrinogen binding proteins (clumping factors, ClfA and ClfB) [118-122]. However, studies about other surface proteins have also advanced. For example, Serine-aspartate repeat proteins (Sdr) are similar structured with ClfA and ClfB, just different in including an additional region of B repeats between the A-domain and R-domain, and thereby have the same fibrinogen-binding ability [123]. Iron-regulated surface determinant (Isd) can be expressed by *S. aureus* in iron limited conditions. IsdA is able to adhere to a range of host proteins, such as loricrin and cytokeratin K10, whereas IsdB interacts with platelet through directly binding to integrin GPIIb / IIIa [124-126].

– Protein A

Protein A (Spa) is a major surface protein of *S. aureus*, which is primarily known for its capacity to bind to Fc region of immunoglobulin G. Lately it is referred to as pleiotropic virulence factor, due to its multiple roles in the interaction with the host during infection.

Structure organization of this 42-kDa protein has a specific feature, which is a tandem repeat of five homologous domains (E, D, A, B and C) following the N-terminal signal sequence (Figure 1-7A). Polymorphism in the region W, upstream of the LPXTG motif, consists of a

repeating pattern of eight residues. The sequence and number of repetition of this pattern are now used as an epidemiological marker ^[127]. The extracellular domains (E, D, A, B or C) are responsible for binding to the Fc region of immunoglobulin G or binding to the Fab variable regions of immunoglobulin M ^[128-129]. The crystal structure of complex Spa-IgM has shown that the helices II and III of Spa domain D interacts directly with VH3 region of IgM. And the residues responsible for Fab binding are separate from those mediating Fc binding (Figure 1-7B) ^[128].

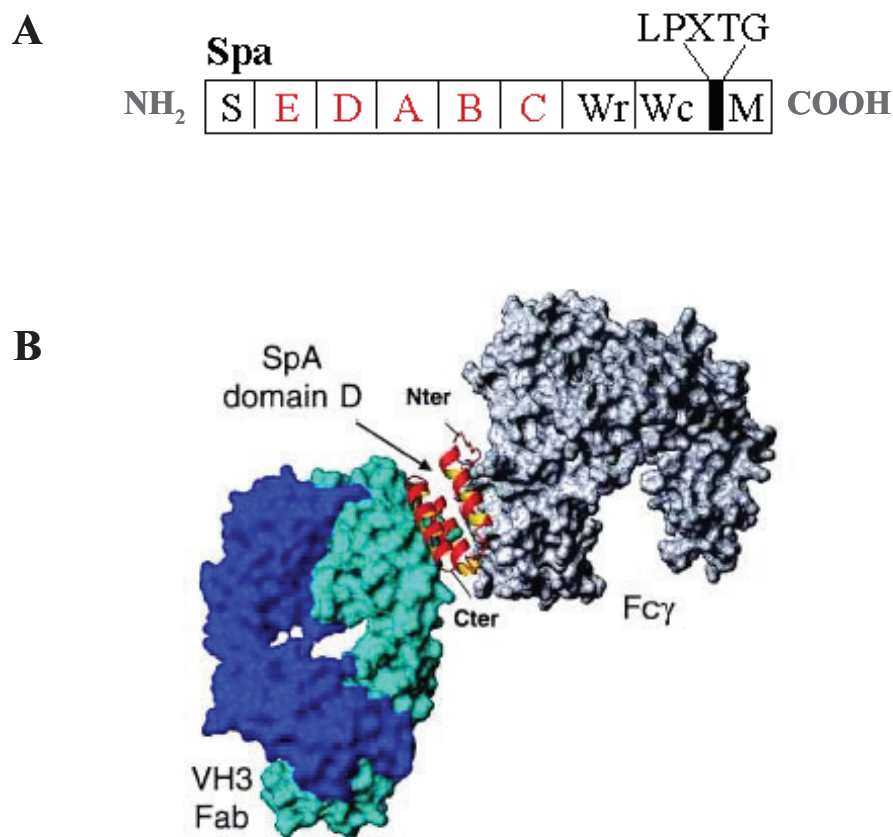


Figure 1-7 (A) Schematic structure of protein A; (B) Crystal structure of complex Spa, IgG and IgM (from Graille *et al.*, 2000). The domain D of protein A (red) binds to VH3 fragment of IgM (blue) and the Fc fragment of IgG (gray).

In addition to its capacity of binding to immunoglobulins, Spa can activate complement ^[130]. It can also act as an activator of the immune response by its ability to bind to TNFR1, the receptor of TNF α . This interaction is particularly important in respiratory infections such as pneumonia, where the TNF α is essential in the signaling of the infection in respiratory

epithelium. Moreover, Spa plays a role in staph-associated endocarditis by binding to von Willebrand factor, the plasma glycoprotein involved in platelet adhesion to injured vascular endothelium ^[131]. And these are the same amino acids involved in binding to von Willebrand factor and Fc fragments of IgG ^[132]. Spa may also interact directly with osteoblasts, preventing proliferation and inducing apoptosis, to accelerate bone weakening in osteomyelitis ^[133].

– Coagulase and SERAMs

Coagulase is a protein of 60 kDa useful for detection of different staphylococcus isolates, which is always positive for *S. aureus*. It possesses a conserved region with multiple domain repeats and an N-terminal binding domain of prothrombin. The complex formation between coagulase and prothrombin is called staphylothrombine, causing the polymerization of fibrinogen to fibrin, and then leading to thrombus formation ^[134]. Its C-terminal domain is demonstrated to be involved in the bacterial adherence to immobilized platelets by using phage display assays ^[135].

Coagulase belongs to the family of 'Secretable expanded Repertoire Adhesive Molecules' – SERAMs. This family also includes Eap – "extracellular adherence protein", Emp – "extracellular matrix binding protein" and Efb – "extracellular fibrinogen binding protein". No significant homology in structure has been shared among these bacterial proteins, but they do have common functions, which may be illustrated as i) binding to host proteins to mediate bacterial adhesion, and ii) binding to a broad array of host ligands to interfere with host defense mechanisms ^[136].

SERAMs are fixed by non-covalent bonds to proteins of the extracellular matrix such as fibrinogen, fibronectin, prothrombin, collagen, laminin, sialoprotein, elastin, or vitronectin ^[136]. These bonds could be either hydrophobic, such as Emp (composed of 25% hydrophobic residues) with lipoteichoic acid, or electrostatic, such as Emp and Eap (cationic) interact with the negatively-charged compounds of the wall. Finally, these adhesins may be recognized by receptors on the surface of the bacterium ^[137].

Except from their functions as adhesins, interaction of these molecules and host components permit them possess immunomodulatory properties. They are involved in the pathogenesis of endo- and extra-vascular staphylococcal diseases ^[136]. Eap favors the internalization of the bacteria into eukaryotic cells ^[138], and adherence to the degradation products of ECM. *S.*

aureus binds preferentially to the damaged tissues, and masks the receptors of leukocytes limiting their infiltration. On one hand, Eap may activate the secretion of proinflammatory cytokines, such as IL-6 and TNF- α , from CD14 leukocytes ^[139]. On the other hand, it can reduce leukocyte recruitment and T cell proliferation and response *in vitro* ^[140-141]. Efb binds to the complement fragment C3, and prevents its activation and opsonization ^[142]. Efb also interacts with platelets, either indirectly by binding to fibrinogen or directly via the receptors GPIIb / IIIa. This interaction inhibits platelet aggregation ^[143]. Interaction between Efb and host fibrinogen blocks the fibrinogen-driven leukocyte adherence ^[144].

1.2.2.3 Toxins

1.2.2.3.1 Membrane-damaging toxins

These toxins destroy target cells either through the formation of membrane channels allowing free access of ions, or destabilize the membrane structures through enzymatic action.

– Alpha hemolysin (α -toxin)

Alpha toxin, encoded by the *hla* gene, represents the archetype of membrane-damaging toxins. This toxin is secreted as monomers, which are grouped into a lytic heptamer in the plasma membrane of target cells such as erythrocytes, lymphocytes, platelets, keratinocytes and human fibroblasts ^[145-146]. Molecular components of target cells then leak through the pore formed under osmotic pressure. Erythrocytes from some species (e.g. rabbit) are more susceptible than others (e.g. human) ^[147]. In one species certain cell types are preferentially attacked by α -toxins, such as human platelets and monocytes ^[148].

Expression of α -toxin is repressed in the early exponential growth of bacteria by transcription factors SarT and SarS ^[149-150]. Two-component systems Agr, Sae, and transcription factor SarA can activate the transcription of *hla* in the late exponential phase ^[96,151-152], simultaneously RNAIII (effector of Agr system) can activate the translation of *hla* mRNA ^[153].

– Beta hemolysin (β -toxin)

Beta toxin is produced by most isolates of *S. aureus*, causing a partial hemolysis on sheep blood agar at 37°C. However, this halo of hemolysis increases significantly at 4°C, hence the name of hemolysis "hot-cold". It acts as a phospholipase C, which has specific

sphingomyelinase activity in the outer phospholipid membrane layer of erythrocytes. Efficiency of their hemolysis functions on erythrocytes of different species depends on the content of their sphingomyelin. Sheep erythrocytes are more sensitive than those of rabbits. This toxin is usually produced in post-exponential growth *in vitro* and totally repressed in early-exponential growth ^[154]. Viability of human polymorphonuclear leukocytes and lymphocytes could be reduced by β -toxin ^[155].

– Delta hemolysin (δ -toxin)

Delta toxin is a small peptide of 26 amino acids, presented as an alpha helix with hydrophobic domains ^[156]. With this structural feature, it can readily insert itself into hydrophobic membrane structures and form an ion channel. Produced by 97% of *S. aureus* isolates, δ -toxin is able to target different cell types. This toxin, encoded by RNAIII, is expressed maximum at the end of exponential growth phase and self controlled by the Agr system ^[154].

– Bi-component synergohymenotropic cytolyisin

Gamma hemolysin (γ -toxin) and Panton Valentine leukocidin (PVL) are pore-forming toxins consisting of two separate components, S for "slow" and F for "fast", so named according to their elution rate in ion exchange column. They act synergistically on cell membranes. Component S binds primarily to the surface of target cells and then a secondary interaction of component F leads to membrane permeabilization ^[157]. γ -toxin is produced by nearly all strains of *S. aureus*, while PVL is produced only by 2% - 3% of the strains. Studies revealed that γ -toxin and PVL may share a conserved three dimensional structure with α -toxin, constituting the beta-barrel membrane protein folding, although they are different in sequence ^[158-159]. Another two-component toxin called LukDE has also been characterized, which has a dermonecrotic activity but no hemolytic and poorly leucotoxic compared to other leucotoxins of *S. aureus* ^[88].

1) Gamma hemolysin (γ -toxin)

Gamma toxin was first isolated from *S. aureus* 5R Smith strain, which has high γ -toxin productivity and free from α -, β - and δ - toxins. Gamma hemolysis is not observable on blood agar plates as result of the inhibitory influence of agar on γ -toxin ^[160]. This toxin is composed of two distinct water-soluble protein elements S and F ^[154]. These elements are derived from 3 *hlg* genes, two of which encode S (*hlgA* and *hlgC*) and one (*hlgB*) encodes F.

Active cytotoxic function of γ -toxin is based on the synergistic combination of components S and F. It can target erythrocytes from human and many other mammalian species, and leukocytes as well. Expression of HlgB and HlgC is activated by SarA and Agr, whereas HlgA is the least expressed compared to the other two proteins and is not regulated by Agr^[87]. The Sae system can positively regulate the expression of Hlg proteins in *S. aureus* 5R Smith strain, independent of Agr and SarA^[161].

2) Panton Valentine Leucocidin (PVL)

PVL, encoded by two contiguous and cotranscribed genes – *lukF-PV* and *lukS-PV*, is involved in chronic furunculosis and necrotizing pneumonia^[162-163]. It can be associated with virtually all the community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains that cause soft-tissue infections^[164]. The *pvl* genes are found on the genome of several different phages, i.e. Φ PVL, Φ 108PVL, Φ SLT, Φ Sa2958, Φ Sa2MW and Φ Sa2USA^[165-168]. There is 20% to 27% sequence homology between the two PVL protein compounds LukS-PV and LukF-PV. The two components are secreted separately and then assemble into octamer on the surface of target cells, leading to the formation of a β -barrel molecular complex and creating a pore. *In vitro*, PVL induces the lysis of several cell types involved in host defense such as neutrophils, monocytes, and macrophages. However, PVL is not hemolytic^[167]. High concentration (200 nM) of PVL induces polymorphonuclear cell (PMN) necrosis by activation of calcium channels and osmotic leakage from the cytosol. Conversely low concentration (10 nM) of PVL induces the formation of pores in the mitochondrial membrane, and thereby cell apoptosis^[169]. Expression of LukS-PV and LukF-PV could be positively controlled by SarA, Agr and SaeRS systems, negatively regulated by Rot^[87,170-171]. SarA and Rot are essential regulators for the inducing effect of beta-lactams on PVL production^[171].

1.2.2.3.2 Superantigenic toxin

Superantigens are one class of highly immuno-stimulatory molecules, which bind first to MHC class II then coordinate with T-cell receptors, thus induce profound T cell activation and massive cytokines release. Staphylococcal superantigens include the toxic shock syndrome toxin-1 (TSST-1) and enterotoxins (SEs), genes of which are all located on mobile genetic elements. To date, more than twenty different enterotoxins have been described and named following the alphabet letter, such as SEA to SEE and SEG to SEV^[172-173]. Five of

these enterotoxins (SEG, SEI, SEM, SEN, SEO) are encoded in the same operon, thereby this gene locus is called enterotoxin gene cluster (*egc*)^[174]. SEs, heat stable, frequently target the mucosal cells of gastrointestinal tract and are responsible for food poisoning. As superantigenic toxins, TSST-1 and some enterotoxins can stimulate non-specific T-cell proliferation and are involved in the onset of toxic shock syndrome. Very low concentrations of these superantigenic toxins (0.1 pg/ml) can activate T-cell proliferation in an uncontrolled manner resulting the occurrence of toxic shock^[175]. SEs have also a potential role in allergic respiratory diseases^[176-177]. *In vitro*, the Agr system can upregulate the synthesis of these superantigenic toxins, such as SEB, SEC, SED and TSST-1. Only the expression of SEA is constitutive, independent of Agr^[151,178].

1.2.2.3.3 Exfoliative toxins

The highly contagious, blistering *S. aureus* skin infections – bullous impetigo and staphylococcal scalded-skin syndrome (SSSS) are caused by the staphylococcal exfoliative toxins (ETs). Currently, there are four exfoliative toxins (ETA-ETD) have been described, among which ETA and ETB are the two major isoforms responsible for human disease. Crystal structure of ETs has indicated that they are serine proteases that become active once binding to specific receptor – desmoglein (Dsg1)^[179]. These toxins act by membrane-tropic intra-epidermal cleavage of Dsg1 between the stratum spinosum (spinous layer) and stratum granulosum (granular layer) of keratinocytes, causing disruption of intercellular adhesion and formation of blisters^[93,180]. Like most other exotoxins in *S. aureus*, synthesis of ETs is induced by the Agr system^[14].

1.2.2.4 Enzymes

S. aureus is capable of producing various enzymes that aid the extension of infection from the initial site to adjoining tissues, such as proteases, lipases, and hyaluronidase, which will degrade the connective tissue^[101,181].

1.2.2.4.1 Proteases

S. aureus produces three major catalytic classes of extracellular proteases – metalloprotease named aureolysin (Aur), serine protease (SspA) and popain-like cysteine protease^[182]. In the third class, there are two cysteine proteases that have been characterized and referred to as staphopain A (ScpA) and staphopain B (SspB). These two cysteine proteases, both of 20 kDa,

have almost identical three-dimensional structures, despite their limited similarity in sequence. Serine protease (SspA) and cysteine protease staphopain B (SspB) are translated from the same mRNA ^[183]. All these proteases are secreted in an inactive form and undergo cascade-like activation before exercise of their roles in bacterial adhesion. Aur is responsible for a proteolytic cleavage of clumping factor B (ClfB) ^[184]. SspA, requiring Aur to be activated, is found to decrease the release of cell-bound fibronectin binding proteins (FnBPs) and protein A ^[185]. Both two staphopains can degrade the fibrinogen and prolong the thrombin time, however SspB is more efficient than SspA ^[186]. The *in vitro* studies have demonstrated that SspA and SspB can not only disturb clotting and kinin systems and destruct connective tissue, but also interact with host immune cells directly ^[187].

Transcription of these proteases is activated by Agr, yet repressed by SarA ^[188], so that the proteases are mainly produced during the post-exponential growth phase. Sigma B factor plays a negative control on their transcription ^[189]. In addition, MgrA, SaeRS, SarR, SarV and ArlRS systems are also involved in the synthesis of these proteases ^[190-194]. Rot can inhibit the transcription of these proteases by binding at their promoters. The positive control on Aur and SspA by Agr system is mainly due to its counteraction towards Rot ^[195].

1.2.2.4.2 Staphylokinase

Certain *S. aureus* strains express a 135 aa plasminogen activator called staphylokinase. It is associated with lysogenic bacteriophages. Staphylokinase can convert plasminogen into its active form – plasmin, which digests fibrin clots thus facilitates the bacterial penetration into adjoining tissue. Moreover, staphylokinase is involved in bacterial resistance to host defense mechanisms. Human neutrophils secrete bactericidal peptides, α -defensins, during infection. By binding with staphylokinase, the bactericidal properties of α -defensins are abolished ^[196]. Staphylokinase also has the property of cleaving IgG and complement C3b fragment that can bind to the bacterial cell wall to prevent phagocytosis ^[181].

1.2.2.4.3 Other extracellular enzymes

Apart from the enzymes described above, certain *S. aureus* can also express lipase and fatty acid modifying enzyme (FAME). The FAME is an esterifying enzyme, which inactivates bactericidal lipids ^[197]. Together with lipase, FAME can overcome the inhibition by glycerides, and negate the bactericidal activity of lipids in lesions ^[198]. Therefore, they both

have a role in prolonging bacterial survival in lesions and abscess formation.

1.2.3 Regulation systems

As described above, the pathogenicity of *S. aureus* is determined by the coordinated expression of various virulence factors, which is controlled through the network of different regulatory factors. These factors can either act directly on target genes (usually the promoters), or act via other factors in a cooperative way. They are sensitive to environmental signals and their regulation always depends on certain growth phase or nutritional conditions. To date, there are many regulatory systems that have been proven important in *S. aureus*, such as the two-component signal transduction systems (i.e. Agr, SaeRS, SrrAB, ArlSR, LytRS and WalKR), and transcriptional factors (i.e. Sar, Rot, MgrA and sigmaB) ^[14,199]. The diagram describing the complex relationship among these regulators, which have been found so far, is shown below (Figure 1-8).

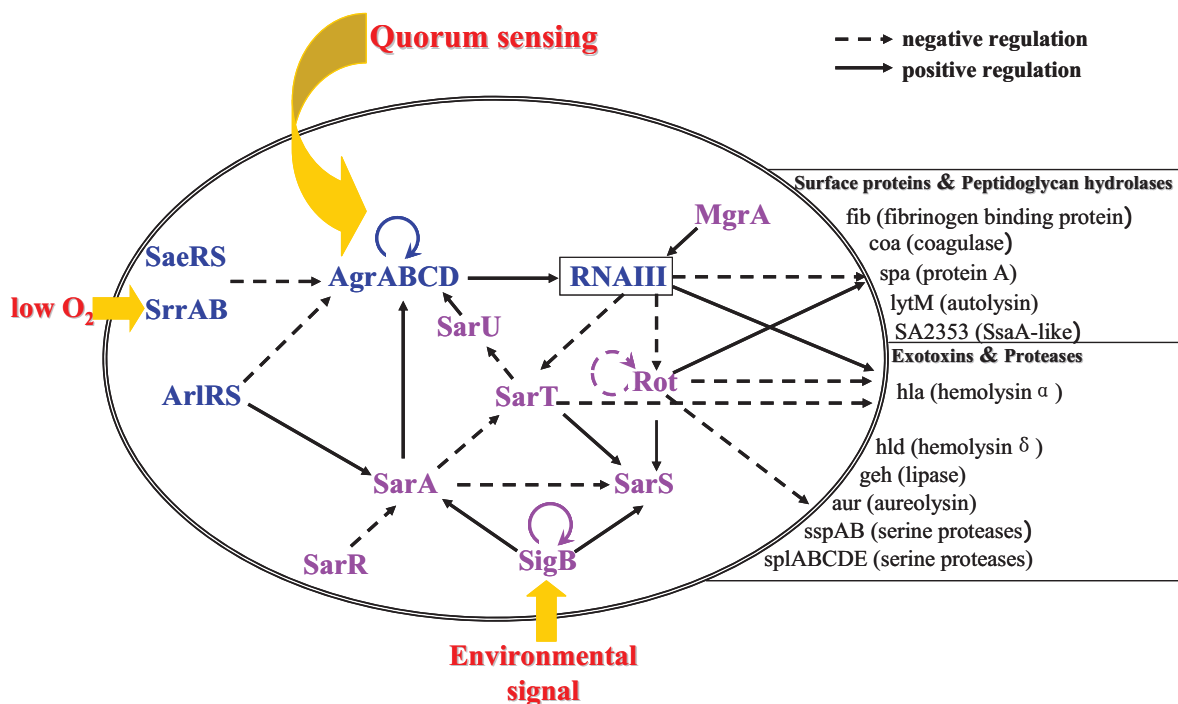


Figure 1-8 Global regulation of staphylococcal virulence factors. Two component systems are indicated in blue, while transcriptional factors in violet. RNAIII, the major effector of Agr system is shown in box. Arrows in solid line stand for positive control, and those in dashed line for negative control.

1.2.3.1 Two-component systems

When bacteria move to a new niche, it is necessary for them to sense and respond rapidly to a large number of environmental signals. Two-component systems (TCS) are often used in this process of adaptation by regulating the expression of various virulence genes. These systems consist of two proteins: a histidine kinase protein called sensor and a response regulator. The sensor is a transmembrane protein that interacts directly with a signal ligand or a receptor that binds to the signal ligand. This binding event leads to the autophosphorylation of the histidine residue in the intracellular C-terminal. A phosphotransferase reaction then happens by transferring the phosphate group to an aspartate residue on the N-terminal region of the cognate response regulator. This cascade of phosphorylation results in the modification of the allosteric regulatory DNA-binding protein, which is also part of response regulator. TCSs are ubiquitous in bacteria, also in archaea, fungi, yeast and some plants, however none of them found in mammals. Among different bacterial species, these TCSs have large sequence or structural homology^[200-201].

In *S. aureus*, there have been more than sixteen potential TCSs identified^[202-203], and six well-studied systems among which are Agr, SaeRS, SrrAB, ArlRS, LytRS and WalKR.

1.2.3.1.1 Agr – accessory gene regulator

Agr, with its property of autoinducer, is an important quorum sensing system (QS) in *S. aureus*. This system, whose effector is an RNA molecule – RNAIII, is demonstrated to regulate the synthesis of many extracellular and cytoplasmic proteins during the post-exponential growth.

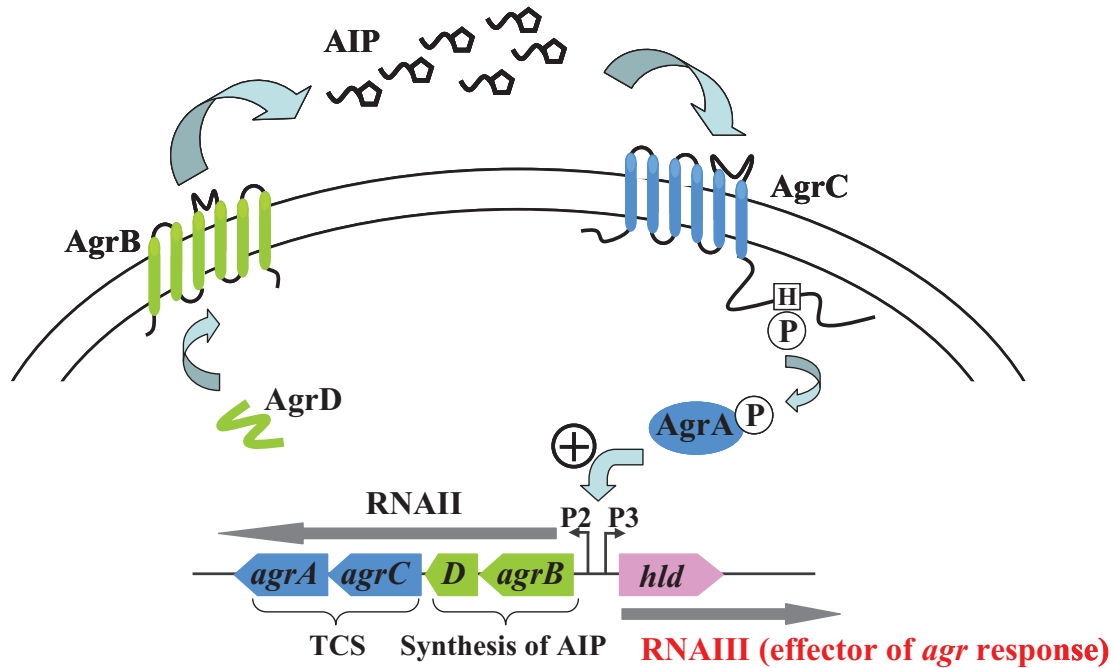


Figure 1-9 Schematic model of *agr* response loop (adapted from Novick and Geisinger, 2008). AgrB and AgrD (in green) cooperate to generate AIP: the immature peptide encoded by *agrD* is processed by AgrB located in cytoplasmic membrane and secreted. During exponential growth phase, the AIPs accumulate in the extracellular medium and reach to a certain concentration, thus activate two-component system AgrA / AgrC. AIPs bind to the transmembrane receptor AgrC, and induce autophosphorylation of the histidine residue C-terminal of the latter. The phosphate residue is then transferred to an aspartate residue of the cytoplasmic protein AgrA that enables the expression of RNAII and RNAIII.

– Organization of *agr* system (basis of an autoactivation circuit)

The *agr* system contains two divergent transcription units, driven by promoters P2 and P3 respectively ^[204-205]. The P2 operon consists of four genes – *agrB*, *agrD*, *agrC* and *agrA*. AgrA and C form a classical two component module, whereas AgrB and D together complement the QS system and allow for the generation of a self-inducing ligand ^[6]. This ligand (also called autoinducing peptide, AIP) contains a thiolactone macrocycle formed between the cysteine sulfhydryl group and the α -carboxylate. It binds to the N-terminal transmembrane domain of AgrC that in turn binds to the response regulator – AgrA, whose activation then upregulates its own promoter P2, as well as the promoter P3 ^[205-206]. In brief, this is a positive feedback regulation (Figure 1-9). Another fruit of this signal transduction system is that its P3 operon transcribes a 514 nt RNA molecule – RNAIII, which is responsible for post-transcriptionally regulating expression of various virulence factors ^[151].

– Genetic variability of the *agr* locus

In *S. aureus*, the variable regions of genes *agrB*, *agrD* and *agrC* result in at least four *agr* specificity groups – AgrI, II, III and IV ^[207-208]. Within a group, each strain produces a peptide capable of activating the Agr response of other strains of the same group, while between the different groups, the peptides self-inducers are mutually inhibitory ^[209]. Even single substitution of amino acid can interfere with the AIP ability to identify its specific receptor ^[210]. This *agr* differentiation is correlated with the phylogeny of *S. aureus*, and the four groups reflect the genetic drift of Agr system during the evolution of the species. AgrI, II, III were assumed to arise from one common ancestor, whereas AgrIV from AgrI ^[6]. There is some linkage between *agr* allele and certain staphylococcal infections. For instance, all the glycopeptides intermediate-level resistant *S. aureus* (GISA) from various geographic origins belong to the Agr group II ^[211]. Most strains causing toxic shock syndrome are those of Agr group III ^[209], while strains of Agr group IV are usually associated with exfoliatin production ^[212]. Correspondingly, SaPI2 encoding TSST-1 and the phage encoding PVL are mostly found in Agr group III strains, whereas the prophage and plasmid encoding exfoliatin A and B, mostly in Agr group IV strains ^[6,207].

– *agr* effector: RNAIII

A 514 nt RNA – RNAIII, transcribed by P3 promoter, is the only known effector of the *agr* system. It can regulate the expression of multiple virulence genes. Indeed, the introduction of RNAIII gene under the control of an independent promoter in a strain deleted of the entire *agr* locus, is sufficient to restore the *agr* + phenotype in strain complemented ^[151]. RNAIII encodes also for a 26 amino-acid peptide, the δ -hemolysin.

RNAIII Structure

Highly abundant and stable in *S. aureus*, RNAIII has a complex and conserved secondary structure. In 1993, the structure model was proposed by Novick *et al.* The authors suggest that the regulatory mechanisms involve different regions of the molecule and are probably related to conformational changes of RNAIII ^[151,213]. Benito *et al.* mapped the secondary structure of this molecule *in vitro* and *in vivo* by using chemical and enzymatic probes. It is characterized by 14 stem-loop motifs and 3 long distance interactions that give its general conformation of the molecule (Figure 1-10) ^[214]. This folded RNA is divided into three distinct domains: 1) the 5' domain containing the forward 31 nucleotides which form stem-loop 1; 2) the central domain consisting of stem-loops 2-11, not only responsible for

encoding δ -hemolysin and initiating the translation of α -hemolysin, but also involved in the negative regulation of *rot* gene; 3) the 3' domain containing stem-loops 12-14, whose task is primarily negative regulation of target genes ^[215]. Indeed, loops 7, 13 and 14 are rich in cytosine, an unusual feature for an AT-rich organism. These sequences are potentially complementary to the SD regions of target mRNAs, and initiate an antisense pairing with them.

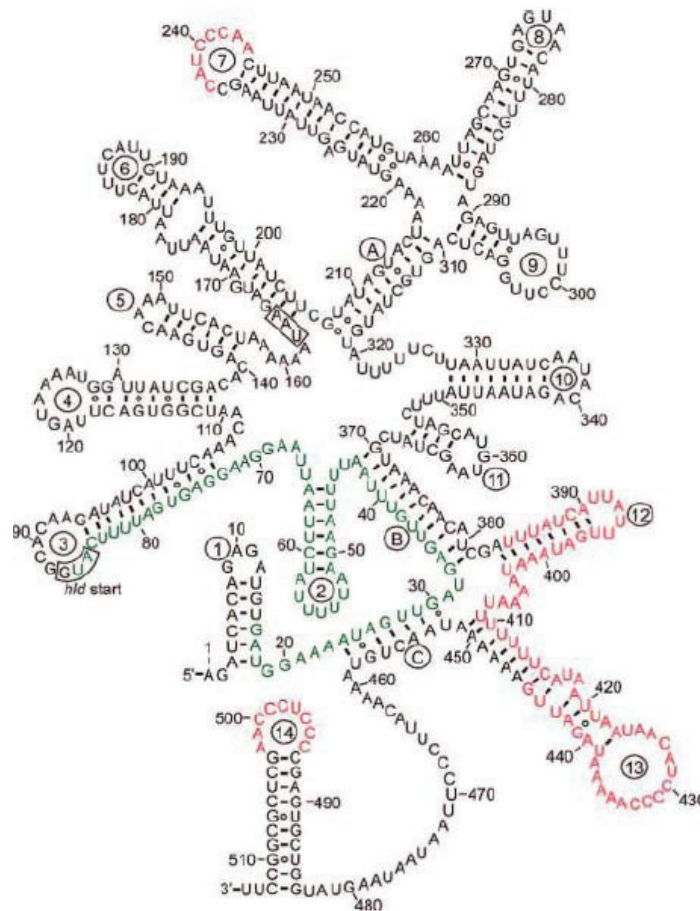


Figure 1-10 Secondary structure of RNAIII ^[214-215]. The stem-loop motifs are numbered from 1 to 14. Three long distance interactions are signified with A, B and C. Nucleotides that base pair with and activate α -hemolysin are indicated in green, while those dedicating to inhibit gene translation are in red. Start and stop codons of *hld* gene are highlighted with box.

Counterparts of RNAIII of *S. aureus* were also found in other staphylococcal species, such as *S. epidermidis*, *S. simulans*, *S. warneri* ^[216], *S. lugdunensis* ^[217-218], *S. saprophyticus* ^[219] and *S. intermedius* ^[220]. Although sharing a conserved secondary structure, their primary sequence is only partial conserved at the first 50 and last 150 nucleotides ^[214,216]. Except for

S. lugdunensis and *S. saprophyticus*, these RNAs encode one or two toxins. These RNAs are able to restore at least partially the expression of virulence factors in Agr-mutant strains of *S. aureus*.

Regulation of target genes

The major mechanism of RNAIII regulation is to base pair with target mRNAs. It can either enhance or inhibit the translation of various mRNAs (Figure 1-11). In the former case, the binding of RNAIII and target mRNA can release the SD sequence that is masked before by a stem-loop structure, thus allow the initiation of translation ^[153]. In the latter condition, RNAIII (especially stem-loops 7, 13 and 14) base pairs with the SD region of target mRNA, thereby hides the ribosome-binding site by forming double-stranded RNA and blocks the translation ^[7-10]. A highly conserved C-rich motif is found in each of these three stem loops, which allows them bind to the G-rich SD region ^[7]. Many other novel sRNAs in *S. aureus* are demonstrated to carry the C-rich motif as well, suggesting that they may exert regulatory roles through the similar mechanism ^[11].

In addition, RNAIII can mediate the degradation of the RNAIII-target complex through RNaseIII, an endoribonuclease specific for double stranded RNAs. The destabilization of target mRNA depends on both the RNAIII paring and RNase III.

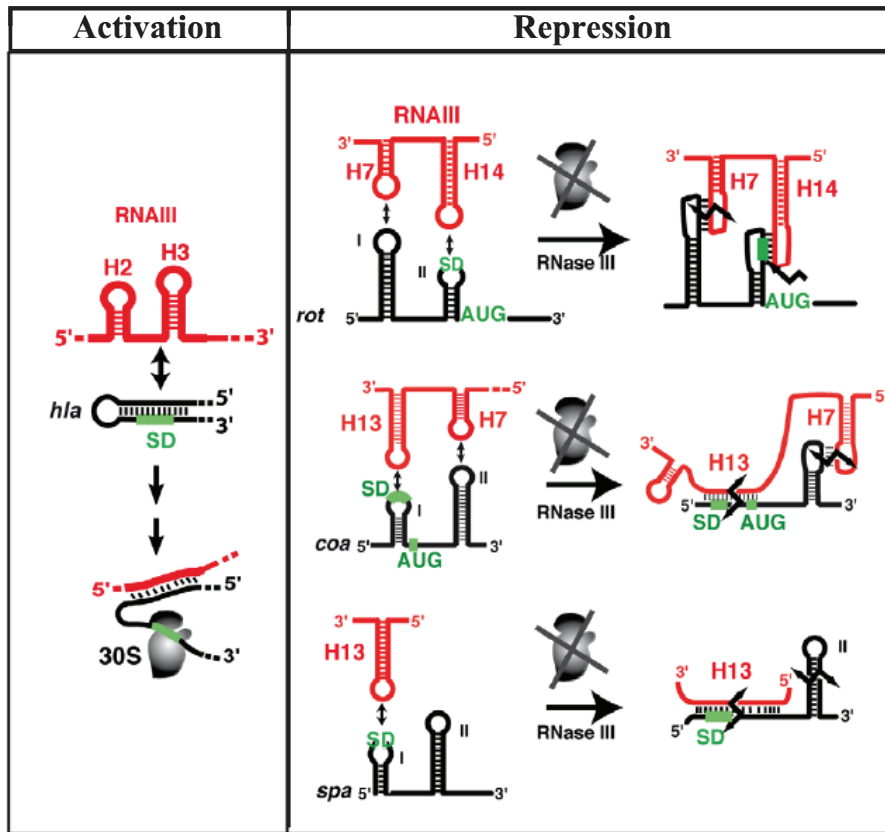


Figure 1-11 The major targets of RNAIII and mechanisms ^[16]. Left part shows the translation initiation activation of *hla* mRNA by RNAIII, and hairpin loops H2 and H3 are involved. Right part displays the translation initiation repression of *rot*, *coa* and *spa* mRNAs, concerning the hairpin loops H7, H13 and H14. Start codon and SD sequence are in green, and hairpin loops in red. Cleavage induced by RNaseIII is shown by broken black arrows.

1.2.3.1.2 SaeRS – *S. aureus* exoprotein expression

Firstly described by Giraudo *et al.* ^[221], the system Sae regulates gene expression at the transcriptional level. It is a staphylococcal two-component regulatory system. The *sae* locus consists of four open reading frames (*saeP*, *Q*, *R* and *S*), two of them encoding a sensor histidine kinase (by *saeS*) and a response regulator (by *saeR*), respectively ^[222]. The *saeP* and *saeQ* genes, located upstream of *saeRS*, are predicted to encode a lipoprotein and a membrane protein respectively, however no direct evidence has shown their involvement in signaling transduction ^[223].

The *sae* operon has two promoters – P1 (also called P_C) and P3 (also called P_A). P3 resides in the *saeQ*, responsible for the synthesis of a transcript encoding SaeR and SaeS, while P1 locates in the upstream of *saeP*, able to produce all the four genes. P1 is the stronger

promoter, whose activity is 2-30 times higher than P3 promoter. Moreover, by having two SaeR binding sites, P1 is positively autoregulated ^[223-225]. P1 is also sensitive to environmental stresses, for example, low pH and high NaCl concentrations can depress its activity whereas subinhibitory concentrations of beta-lactam, H₂O₂ and α -defensins activate it ^[224,226].

The SaeRS TCS is required for the expression of many virulence factors in *S. aureus* ^[194,227]. Target genes of SaeRS could be divided into two groups – class I (e.g. *fnbA*, *coa* and *eap*) and class II (e.g. *hla*, *hly* and *cap*), according to whether they need a high level of phosphorylated SaeR for induction ^[228]. SaeRS has no measurable effect on the transcription of *agr*, *sarA*, *arlRS* and *sigB* ^[229]. Recently, one study has shown that either deletion of the *saePQ* region including promoter P1 or chromosomal mutation in P1 did not significantly influence the expression patterns of coagulase and α -hemolysin, implying that P1 is not involved in the activation of target genes, and the transcription of *saeRS* from P3 is sufficient in target gene regulation ^[230].

1.2.3.1.3 SrrAB – Staphylococcal respiratory response

The SrrAB system was described simultaneously by two groups as being involved in the expression of virulence factors influenced by environmental oxygen levels ^[199,231-232]. Its locus consists of two open reading frames that overlap by 20 bp. The two genes – *srrA* (762 bp) and *srrB* (1752 bp) are co-transcribed, but *srrA* can be transcribed independently of *srrB*. The *srrA* encodes the response regulator, while *srrB* encodes a sensor histidine kinase ^[232]. The *srrAB* has similar sequences to *resDE* that forms a two-component system identified in *Bacillus subtilis* as regulator of aerobic and anaerobic respiration ^[233].

S. aureus has a facultative anaerobic nature, and SrrAB is required for fermentative growth of the bacteria. It can increase the expression of enzymes involved in fermentation (e.g. alcohol dehydrogenase, L-lactate dehydrogenase). This system is also discovered overexpressed in anaerobic conditions ^[231]. Indeed, *srrAB* can influence the expression of many virulence genes in response to oxygen availability. For example, it is able to bind to the promoters of *agr*, *srr*, *spa*, and *tst* *in vitro*, and repress their transcription under anaerobic conditions. The overexpression of *srrAB* decreases the bacterial virulence in a rabbit endocarditis model ^[234]. It can also bind to the promoter of *icaADBC* (intercellular adhesion cluster) and activate its transcription, thus leading to increased PIA (polysaccharide

intercellular adhesion) production in low-oxygen environments ^[235].

1.2.3.1.4 ArlSR – Autolysis related locus

The ArlSR system was originally described as a regulator of autolysis by Fournier *et al.* The *arl* locus is composed of two overlapping open reading frames – *arlS* and *arlR*. These two genes are cotranscribed in an mRNA of 2700 bp. ArlS (52.4 kDa) is the sensor protein, while ArlR (25.5 kDa) is the response regulator and belongs to the PhoB-OmpR family. ArlR contains a conserved C-terminal domain that can bind to specific sequences upstream of target promoters ^[236].

Disruption of the *arlS* gene leads to increased activity of the peptidoglycan hydrolases, causing autolysis. During the growth cycle, the autolysins allow the bacteria to break the cell wall at the time of cell division. Thus ArlS and ArlR are involved in cell growth and division. Expression of a multidrug resistance efflux pump, NorA, is increased in an *arlS* transposon insertion mutant ^[236]. ArlSR is involved in the downregulation of several virulence factors, such as protein A, α -haemolysin, β - haemolysin, lipase, coagulase and serine protease ^[190]. A transcriptomic analysis on *arl* mutants and wild-type strains conducted by Liang *et al.* has shown that this system positively or negatively modulates the transcription of more than one hundred genes involved in autolysis (*lytSR*, *lrgAB*), cell growth and pathogenesis (*lukD*, *LukE*, *hld*, *ssaA*) ^[237]. In addition, it has been demonstrated that ArlR can upregulate *agr* and *rot*, indicating that the down regulation of virulence factors above might be directly mediated by Arl or indirectly through Agr or Rot ^[237].

1.2.3.1.5 LytRS

Like Arl, the LytRS system is also a two-component system involved in autolysis. The *lytR* and *lytS* genes are co-transcribed on a mRNA of 2500 bp, and separated by 5 nt. Their sequences are similar to those of other two-component systems. However, unlike other sensor histidine kinase, LytS contains six transmembrane domains at N-terminal instead of two commonly described. This structure is composed of alternating hydrophilic and hydrophobic domains similar to those of transport proteins, suggesting that signal transduction by LytS involves the transportation of proteins ^[238].

Two target genes identified are *lrgA* and *lrgB*, two additional open reading frames located immediately downstream of *lytS* and *lytR*. Their transcription is positively regulated by

LytRS. Recent study has suggested that LytRS can sense a collapse in membrane potential and then promote the transcription of *lrgAB* operon ^[239]. LrgA, and possibly LrgB are homologous to bacteriophage-encoded anti-holins, and involved in the regulation of murein hydrolase activity and cell death ^[240].

1.2.3.1.6 WalKR

Originally identified in *Bacillus subtilis* ^[241], the WalKR system is essential for cell viability to closely related and low G+C Gram-positive bacteria, such as *S. aureus* and *S. pneumoniae*. It used to be referred to various designations (YycGF, VicKR, MicAB), now renamed as WalKR, because this system has a crucial role in cell wall metabolism.

In *S. aureus*, there is a five-cistronic operon (*walR*, *walK*, *yycH*, *yycI* and *yycJ*) consisting the whole *walKR* locus. The first two genes encode the WalR response regulator and the WalK histidine kinase, respectively. WalK has a typical structure of the sensor histidine kinase, with two transmembrane domains and an extracellular loop, while WalR is a member of OmpR/PhoB family. YycH and YycI, whose presence is associated with the WalK essentiality, do not share similarities in sequence, yet have a conserved tertiary structure. They both are extracellular proteins anchored by an amino-terminal transmembrane domain ^[242-243]. For the last gene, *yycJ*, its corresponding deletion strain does not display any distinguishing phenotypes and cell morphology ^[244].

Bioinformatics and transcriptional studies have shown that WalKR system is involved in the regulation of nine cell-wall metabolism genes, including two genes encoding two major *S. aureus* autolysins (*atlA* and *lytM*), two genes encoding lytic transglycosylases (*isaA* and *sceD*), as well as five genes encoding for proteins with a CHAP amidase domain (*ssaA*, SA0620, SA2097, SA2353, SA0710) ^[245]. Among them, it has been identified that the expression of only two WalKR regulated genes, *lytM* and *ssaA*, can restore cell viability in the absence of WalKR. Thereby, the peptidoglycan crosslinking relaxation through crossbridge hydrolysis plays a crucial role for WalKR-dependent cell viability ^[246].

1.2.3.2 Transcription factors

1.2.3.2.1 Sar – Staphylococcal accessory regulator

– SarA

The SarA system was firstly described by Cheung *et al.* ^[247]. Insertion of the transposon

Tn917 LTV1 in a region distinct from the Agr system, leads to a pleiotropic effect on the expression of many extracellular and cell wall-associated proteins. SarA operon consists of three overlapping reading frames with a common 3'-end. The transcripts *sarP1* (0.58 kb), *sarP3* (0.84 kb) and *sarP2* (1.15 kb) are driven by three distinct promoters P1, P3 and P2, respectively. The three transcripts together encode a 14.5-kDa protein, SarA ^[248].

Transcriptional regulation involving the three promoters is complicated. SarA activates its own expression via P1. P1 and P2 are controlled by the transcription factor σ^A while P3 is a σ^B dependent promoter. Because of this different σ factor dependence, SarA is expressed during all growth phases. Promoters P1 and P2 permit maximum transcription in the exponential growth phase, and the P3 promoter induces the expression of SarA during the stationary phase ^[249-250].

The protein SarA binds as a dimer to conserved AT-rich regions at the promoters of target genes. A most well-known target of SarA is *agr* operon. SarA binds to the *agr* P2 and P3 promoter, and increases the transcription of both RNAII and RNAPIII, thus alters the synthesis of virulence factors indirectly ^[251]. Moreover, SarA can regulate the expression of several cell wall associated proteins and exoproteins by directly binding conserved regions within their promoters ^[79,252-253]. Two virulence factor transcripts, *cna* and *spa*, are found to be stabilized in a SarA-dependant manner, suggesting that SarA may regulate target gene expression via post-transcriptionally modulating mRNA lifetime ^[254].

Recently there were at least 10 SarA homologs identified in the *S. aureus* N315 genome, and named "SarA protein family". Except SarA, four of them (SarR, SarS, SarT and SarU) have been more characterized than others.

– SarR

The SarR protein (13.6 kDa) shows 51% similarity with SarA. SarR binds to the upstream of the promoter P1 and represses the transcription of SarA at the end of exponential growth phase and stationary phase ^[203,255]. Like SarA, SarR regulates also the *agr* expression by direct binding to the same *agr* promoter region ^[256].

– SarS (SarH1)

Isolated by Tegmark *et al.*, the SarH1 protein (29 kDa) has high affinity to the promoter regions of four genes (*hla*, *hld*, *spa*, and *ssp*), among them *hla* transcription is repressed whereas *spa* transcription activated ^[86]. This same protein was identified in parallel by

Cheung *et al.* and named "SarS" ^[149]. The gene *sarS* is located just upstream of the gene *spa*. It is transcribed from two distinct promoters, one promoter controlled by transcription factor σ^A and the other by σ^B . Protein SarS is composed of two homologous but non identical parts, SarS1 and SarS2, with 34.5% and 28.3% homology with SarA, respectively ^[203].

Given that the expression of *sarS*, an activator of *spa*, is repressed by *agr*, SarS may in part play as an intermediary factor during the down regulation of *spa* by *agr* locus ^[149]. Transcriptional analysis also showed that *sarS* is repressed by *mgrA*, and activated by ClpXP protease ^[257].

– SarT

The SarT protein (16.1 kDa) shows 35% similarity with SarA and 20% similarity with SarR. There are complex interactions between the systems Agr, SarA and SarT. The SarT expression is repressed by Agr and SarA, and SarT represses the transcription of RNAIII. These interactions can modulate the expression of α -hemolysin: on the one hand, SarA activates transcription of *hla* by directly interacting with its promoter or indirectly by repressing the SarT expression; On the other hand, SarT represses the expression of α -hemolysin by repressing the transcription of RNAIII ^[150].

– SarU

The SarU protein (29.3 kDa), encoded by a mRNA adjacent but transcribed divergently to *sarT*, has two homologous domains to SarA, thus its length is twice of SarA. SarU is an activator of Agr system, and the transcription of SarU is increased in a SarT mutant. Thereby, SarU participates in a regulatory network involving Agr and SarT. The tandem SarT-SarU is a second mechanism of Agr autoactivation, in addition to the mediation by "quorum sensing" autoinducer peptide. When the Agr system represses SarT, the expression of SarU increases, and this augmentation in turn activates the Agr system ^[258]. This regulation circle is consistent with the finding that *agr* promoter can be activated *in vivo* in an *agr* deletion mutant ^[259].

1.2.3.2.2 Rot – Repressor of toxins

The Rot protein (15.6 kDa), which has limited homology to global regulators AgrA and SarA, was initially identified by McNamara *et al.*, as a repressor of toxin transcription ^[260]. It can interact with the promoter regions of target genes during the exponential phase ^[260]. An

analysis by DNA microarrays has shown that Rot negatively regulates sixty genes, but also positively regulates more than 80 genes. Rot and Agr appear to have opposite regulatory effects on gene expression. Indeed, the protein A gene (*spa*) and serine protease genes (*sspB*, *sspC*) are activated by Rot and repressed by Agr, while the α -hemolysin gene (*hla*) and γ -hemolysin gene (*hlgB* and *hlgC*) are repressed by Rot and activated Agr^[85]. Translation of *rot* mRNA is repressed by RNAIII, through a limited base pairing involving two loop-loop interactions^[7-8]. Manna *et al.* have revealed that *rot* transcription is repressed by only SarA at the post exponential phase, through using gel shift and transcriptional analysis^[261]. However, Hsieh *et al.* have demonstrated that both SarA and SarS are able to bind to the *rot* promoter region. This divergence could be explained by that the putative *sarR* binding site is not included in the promoter construct of Manna team^[262].

1.2.3.2.3 MgrA

The *mgr* locus was originally identified from an unknown mutation, which affects the expression of multiple genes^[192]. This locus consists of only one gene, *mgrA*, encoding protein MgrA. This protein is involved in the regulation of cell lysis and virulence. It activates the expression of capsular polysaccharide type 8 (CP8), nuclease, and represses that of α -hemolysin, coagulase, protease and protein A^[192]. The inactivation of gene *mgrA* leads to the repression of RNAIII and *hla*, but the activation of *sarS* and *spa*^[191]. MgrA affects the bacterial resistance to antibiotics by controlling the expression of multiple efflux pumps, e.g. NorA, NorB, NorC and Tet38, which involve the resistance to multiple drugs including quinolone, tetracycline and chemical compounds^[263-264]. It is also a direct activator of *abcA*, which encodes an ATP-dependant transporter conferring resistance to β -lactams^[265]. Moreover, MgrA has been shown to be a repressor of biofilm formation, and the biofilm formed by *mgrA* mutants majorly relies on surface proteins and extracellular DNA instead of polysaccharide intercellular adhesin (PIA)^[266].

1.2.3.2.4 Sigma B factor

The transcription factor σ^B was well known for its involvement in gene regulation upon changes in environment and conditions of stress, such as starvation, heat shock and osmotic shock, *etc.* Wu *et al.* firstly identified a chromosomal gene cluster (*rsbU*, *rsbV*, *rsbW*, *sigB*) in methicillin resistant strain of *S. aureus*, encoding products highly homologous with those of *sigB* operon in *B. subtilis*. The similarities suggest that the staphylococcal *sigB* operon

may perform similar physiological roles^[267]. The gene products of *rsbU*, *rsbV* can positively modulate the activity of σ^B in *S. aureus*^[268].

In *S. aureus*, the σ^B factor influences the expression of many genes, including virulence factors^[269-271], and regulatory systems^[249,272]. In addition, σ^B affects the bacterial resistance to methicillin and glycopeptides^[273], biofilm formation^[274], internalization into endothelial cells^[275], and pathogenesis in some infection models^[276]. A study by DNA microarray indicated that 122 genes of *S. aureus* are positively regulated by σ^B , only 12% of which have homology with σ^B regulon in *B. subtilis*. It suggests that the function of σ^B in *S. aureus* is different with that in *B. subtilis*^[277]. Another recent study showed that, upon subinhibitory concentration of aminoglycoside exposure, the σ^B factor induces the appearance of *S. aureus* small-colony variants (SCVs) and biofilm formation, both of which are linked to chronic infections^[278].

1.3 sRNA discovery in *S. aureus*

S. aureus generates a large quantity of virulence factors, the timing and expression of which are finely controlled by regulatory proteins and RNAs^[12]. Except from its regulatory role, RNA helps bacteria adapt rapidly to the environmental changes and various stresses. In the last few years, the number of sRNAs identified in bacteria has considerably increased, thanks to the combinational use of disparate approaches^[1,279].

1.3.1 Computational and experimental approaches

Computational approaches are frequently used as one important initial step to search for the sRNAs in bacterial genome. For a long time, scientists keep trying to find the universal characteristics of sRNAs that could be used as criteria. For instance, a program was described for the analysis of RNA secondary structure by calculating free energy^[280]; Schattner *et al.* presented the feasibility to use local base-composition statistics (G+C%) to distinguish the sRNA-rich regions and sRNA-poor regions^[281]; QRNA, a sRNA genefinder program, employs the comparative sequence analysis algorithm to detect novel structural RNA^[282]. However, applications of these approaches are still limited by the great diversity of sRNAs (their size, sequence, structure and position in genome, *etc.*). They usually focus on the intergenic regions, and thereby miss the sRNAs located in protein-coding regions. They

may also rely on the sequence conservation and thus neglect those species specific sRNAs [28].

Experimental approaches are also powerful tools in the prediction of novel sRNAs. For sRNA that exists abundantly in genome, it could be directly identified by size fractionation of total RNA labeled *in vivo*. Moreover, direct cloning of small sized RNAs after size fractionation and shotgun cloning of small RNAs is adopted by several groups [25,283-284]. There are some sRNAs which have been identified through the co-purification with proteins, based on their association with RNA-binding proteins (such as the Sm-like protein, Hfq) [285]. At last, the microarray expression studies, as well as the analysis of mRNA libraries and expressed sequence tags, allow the detection of antisense sRNAs, even there are sometimes false-positive hybridizations [286-287].

Finally, all the predicted sRNA genes should be confirmed *in vivo* and *in vitro* by direct detection of these transcripts, notably by Northern-blot analysis.

1.3.2 Novel sRNAs of *S. aureus*

In recent years, approximately 100 *trans*-encoded sRNAs, 100 *cis*-encoded antisense RNAs, and more than 30 *cis*-acting regulatory regions of mRNAs have been discovered in *S. aureus* (Table 1-2) [16]. Most of them are encoded by the core genome while several sRNAs are expressed from mobile elements, pathogenic islands [18-19] and plasmids [288]. The expression of most of these sRNAs was confirmed by several methods, such as northern blot, qRT-PCR, and the high-throughput sequencing study [19,289]. Worth to mention, in the HTS study performed by Beaume *et al.*, nearly all the sRNAs discovered in *S. aureus* strains (in most cases, N315) were confirmed, except from the sRNAs identified in clinical isolates by Abu-Qatouseh *et al.* [16]. It might suggest that the sRNA profiles are distinct among the different isolates, and we can not totally apply the data obtained from reference strains to the clinical isolates.

Table 1-2 Small RNAs discovered in *S. aureus*

Year	Name of sRNA	Approach	Reference
1993	RNAIII	Transposon mutagenesis	[151]
2005	7 SprRNA (SprA-G)	Bioinformatics	[18]
2006	13 non-protein-coding transcripts	Transcriptome	[254]
2006	5 non-protein-coding transcripts	Transcriptome	[291]
2009	11 Rsa RNA (RsaA-K)	Bioinformatics	[11]
2009	7 RsaO RNA(RsaOA-OG)	Bioinformatics	[292]
2010	18 non-protein-coding transcripts	Bioinformatics	[20]
2010	195 non-protein-coding transcripts	Illumina- High throughput sequencing	[19]
2010	16 RsaO RNA (RsaOH-OW)	RNomics based on pyrosequencing of cDNA libraries	[15]
2011	3 Sbr RNA (SbrA-C)	Bioinformatics	[293]
2012	SSR42	Mutagenesis	[294]

Among the numerous sRNAs in *S. aureus*, only the functions of a few of them (e.g. RNAIII, SprD, RsaOX, and RsaE) are more understood than others. SprD, a small pathogenicity island RNA firstly identified by Pichon and Felden, interacts directly with the 5' end of *sbi* mRNA including the SD region, and thereby negatively regulates the expression of the Sbi immune-evasion molecule [18,290]. RsaOX, a *cis*-acting sRNA, is complementary to the coding sequence of *tnp* mRNA, and is suspected to induce its rapid degradation [15,19]. RsaE (RsaON), a sRNA also present in *Bacillaceae* and primarily described by Geissmann *et al.*, binds to cognate target mRNAs and prevents the formation of ribosome initiation complex. It can regulate several enzymes involved in various metabolic pathways, such as amino acid and peptide transport, cofactor synthesis, lipid metabolism, carbohydrate metabolism and the TCA cycle [11,15].

Recently, a novel long-chain regulatory RNA – SSR42, which modulates approximately 80 mRNAs, was described in *S. aureus* strains. This RNA molecule, consisting of 891

nucleotides, is required for the pathogenesis of USA300, such as hemolytic ability, resistance to human polymorphonuclear leukocyte killing, ability to cause skin and soft tissue infection in a murine model ^[294]. Moreover, it was revealed that many short RNA fractions are generated through the digestion of overlapping sense/antisense transcripts mediated by RNaseIII, which contributes to adjust mRNA levels in diverse Gram-positive bacteria ^[50].

1.4 Conclusion

The regulation of the virulence genes in *S. aureus* depends on a complex network comprised of various regulatory factors. These factors could be divided into two major groups of regulatory factors, the quorum sensing system Agr and the broad family of SarA-like proteins. The latter group includes the classical SarA proteins and other transcription factors which have homology to SarA (e.g. Rot, MgrA and sigmaB). In this complicated regulatory network, RNaseIII, the major effector of Agr, plays a crucial role by up-regulating exoproteins and down-regulating cell wall-associated proteins, as well as exerting regulatory functions on other regulators.

With a large number of sRNAs identified and our increasing knowledge about them, the importance of sRNAs in gene regulation of bacteria has aroused more and more attention. Thus, an urgently needed further step is to investigate their functions both *in vitro* and *in vivo*. Identification of their cellular interaction partners could be carried out by using a variety of methods, such as screening the regulated targets in sRNA overexpression or deletion strains, co-purification of proteins with sRNAs and subsequent analysis by mass spectrometry. Furthermore, it is important and necessary to study these factors *in vivo*, as the infection environment presents much more signals that can not be simulated in laboratory conditions.

Chapter 2 Construction of genetic tools for studying Rsa

2.1 Introduction

Since the discovery of RsaA to RsaK, their sizes and structures have been studied by using experimental techniques like RACE, primer-extension and enzymatic hydrolysis, followed by using Northern-blot to reveal their expression conditions in several *S. aureus* strains ^[11]. Like the strategy used in the study about RNAIII ^[7-8,214], to investigate further the characteristics and functions of these novel sRNAs, we firstly constructed their respective deletion mutants and complemented strains.

2.2 Methods and Materials

2.2.1 Bacterial strains and plasmids

All the strains and plasmids used in this project are listed in table 2-1.

E. coli DH5 α was grown in Luria-Bertani (LB) broth or on LB-agar (bacteriological tryptone 1%, yeast extract 0.5%, NaCl 1%, bacteriological agar 1.5% for solid phase, pH 7.0) with 100 μ g/ml ampicillin when appropriate.

S. aureus strains were grown in the Brain-Heart Infusion broth (BHI) or on BH agar (peptone mixture 1%, beef-heart infusion 1%, calf-brain infusion 0.75%, Na₂HPO₄ 0.25%, NaCl 0.5%, bacteriological agar 1.5%, pH 7.4), with 5 μ g/ml erythromycin when appropriate. Trypticase Soy (TS) broth or agar (trypticase peptone 1.5%, phytone peptones 0.5%, NaCl 0.5%, bacteriological agar 1.5% for solid phase, pH 7.4) were also used, with 1.5 μ g/ml erythromycin when appropriate.

Table 2-1 Strains and Plasmids used in this study

Strains/Plasmids	General Characteristics	Source/reference
Strains		
<i>E. coli</i>		
DH5 α	Cloning strain	Promega
<i>S. aureus</i>		
8325-4	NCTC8325 cured of three prophages	[295]
RN4220	Restriction mutant of 8325-4, with ability to accept DNA from <i>E. coli</i>	[296]
RN6390	Derivative of 8325-4, <i>agr</i> positive	[297]
Newman	Wild type strain	[298]
HG001	rsbU+ strain derivative of RN1	[299]
LUG1376	RN4220/pLUG749	Team work
LUG1397	RN4220/pLUG766	Team work
LUG1411	RN4220/pLUG754	Team work
LUG1476	RN6390 Δ <i>rsaE</i> /pLUG790	Team work
LUG1630	HG001 Δ <i>rsaA</i>	My work
LUG1640	NEWMAN Δ <i>rsaH</i>	My work
LUG1644	HG001/pLUG754	My work
LUG1656	HG001/pLUG766	My work
LUG1657	NEWMAN/pLUG749	My work
LUG1658	NEWMAN/pLUG766	My work
LUG1664	NEWMAN/pLUG754	My work
LUG1670	NEWMAN Δ <i>rsaE</i>	My work
LUG1691	RN6390 Δ <i>rsaE</i> /pLUG848	Team work
Plasmids		
pBLUEScript	Cloning vector, size of 2.9 kb	Stratagene
pCN37	<i>E. coli-Staphylococcal</i> shuttle vector with an inducible promoter cassette	[300]
pGEM-T	Cloning vector, size of 3.0 kb	Promega
pMAD	<i>E. coli-Staphylococcal</i> shuttle vector with the temperature sensitive origin of replication	[301]
pLUG749	pMAD:: <i>rsaH</i> upstream region (1056 nt)- <i>kana</i> [*] - <i>rsaH</i> downstream region (1069 nt)	Team work
pLUG752	pBS:: <i>rsaA</i> upstream region (1045 nt)- <i>kana</i> [*] - <i>rsaA</i> downstream region (949 nt)	Team work
pLUG754	pMAD:: <i>rsaA</i> upstream region (1045 nt)- <i>kana</i> [*] - <i>rsaA</i> downstream region (949 nt)	Team work
pLUG766	pMAD:: <i>rsaE</i> upstream region (1802 nt)- <i>kana</i> [*] - <i>rsaE</i> downstream region (1052 nt)	Team work
pLUG790	pCN37:: <i>rsaE</i>	Team work
pLUG848	pLUG920:: <i>rsaE</i>	Team work
pLUG920	pCN37 <i>Pst</i> I-BamHI::TT-Pcad	Team work

* *kana*: gene coding for kanamycin resistance

Table 2-2 Oligonucleotides used in this study

Primers Sense(+/-)	5' to 3' sequence ^a	T _m ^b	Restriction Site	Utilization ^c
Kcr3(+)	CGTTTCTGCG GACTGG	56	-	Inactivation control
Kcr4(-)	CTATCGCCTT CTTGACG	54	-	Inactivation control
RsaA-76(+)	AGCCCGGGAT CCAAAACATA GTC	46	<i>SmaI</i>	Construction pMAD
RsaA-1120(-)	TGGATATCTA ATATTATTTT AACCTATTG	47	<i>EcoRV</i>	Construction pMAD
RsaA-1246(+)	ACATCGATAT TGATAATACA TTAGC	44	<i>ClaI</i>	Construction pMAD
RsaA-2194(-)	TTGTCGACAA GGAGCGATAA ACATG	50	<i>SalI</i>	Construction pMAD
RsaA-24(+)	GGTGCATCAA AAATGACG	52	—	Inactivation control
RsaA-1109(+)	TAAGGATCCA TTAGTTAACC ATTACAAAAA TTGTATAG	53	<i>BamHI</i>	Inactivation control
RsaA-1157(+)	CTATTGAGGT TAACGTTTAT ATG	53	-	Inactivation control
RsaA-1257(-)	ATGGAATTCC AATAACAAAG TACACTTTGC TCATAG	57	<i>EcoRI</i>	Inactivation control
RsaA-2270(-)	GCCACGTCAC CAATTTG	54	-	Inactivation control
RsaE-140(+)	TTTCCCGGGAGGT TTACTIONACATA TG	50	<i>SmaI</i>	Construction pMAD
RsaE-2041(-)	TAAGATATCG TTCATAATAT AACATGCTAT C	50	<i>EcoRV</i>	Construction pMAD
RsaE-2163(+)	CGTGTGCGACA TGTTCTTTTT TAATAAGAGA G	48	<i>SalI</i>	Construction pMAD
RsaE-3214(-)	TGACTCGAGA TGGCTGGAGA ATTAC	52	<i>XhoI</i>	Construction pMAD
RsaE-70(+)	G TTCAGATAG AGGTAATGAC	53	-	Inactivation control
RsaE-2063(+)	ATTGGATCCA TGAAATTAAT CACATAACAA ACATACC	54	<i>BamHI</i>	Inactivation control
RsaE-2065(+)	GAAATTAATC ACATAACAAA CATACC	54	-	Inactivation control
RsaE-2162(-)	AAAGAATTTC AAAAAAACGT CGTGTCTGAA TACAC	57	<i>EcoRI</i>	Inactivation control
RsaE-3283(-)	GATTAAGTGC TAATCATATG GTG	54	-	Inactivation control
RsaH-435(+)	TTCCCGGGCT CATTAATGGT ATTG	47	<i>SmaI</i>	Construction pMAD
RsaH-1490(-)	CGAGATATCA CCTTTATTAT AACTTATATC	48	<i>EcoRV</i>	Construction pMAD
RsaH-1633(+)	GTCAAGCTTA TTTATTTCTT ATTCCCATTA TAC	50	<i>HindIII</i>	Construction pMAD
RsaH-2701(-)	TCTGTGACC TGGTTCATTT TTGTTTGAC	51	<i>SalI</i>	Construction pMAD

RsaH-387(+)	<u>CCTTTGTTT</u> G TAAGTCGC	52	-	Inactivation control
RsaH-1511(+)	GTACCTTCGA <u>TAACGAATAA</u> AC	54	-	Inactivation control
RsaH-1629(-)	CGACCCGCAC <u>GATTAAC</u>	57	-	Inactivation control
RsaH-2762(-)	CCAATATTCG <u>AGACAACC</u>	52	-	Inactivation control

^a Restriction sites underlined

^b Optimal melting temperature regardless of restriction sites

^c Oligonucleotides used in the different steps: construction pMAD – to construct pMAD derivatives to inactivate sRNA; inactivation control – to check the deletion of sRNA gene after homologous recombination

2.2.2 Primers and PCR

Polymerase chain reaction (PCR) was carried out in a standard system: 1.25 units of GoTaq® Hot Start Polymerase (Promega, Madison, USA), 1×reaction buffer, MgCl₂ 2.5 mM, dNTP 200 µM (Eurobio, Courtaboeuf, France), primers 0.2 µM, and DNA 1 ng. PCR was performed with a thermo cycler (Biometra, Göttingen, Germany), programmed as follows:

- a) The initial denaturation step at 94°C for 15 min
- b) 30 cycles of amplification (containing 3 steps)
 - Denaturation at 94°C for 30 s
 - Annealing at the temperature according to the optimal melting temperature (T_m) of primers, for 30 s
 - Elongation at 72°C for 30 s - 1 min 30 s, depending on the length of PCR products
- c) The final elongation at 72°C for 10 min

Primers are described in table 2-2.

2.2.3 Vector construction and homologous recombination

For homologous recombination, sequences upstream and downstream of sRNAs to inactivate and a kanamycin resistance cassette were cloned into the *E. coli* cloning vector pBluescript (Annex I). The construction was then transferred into the *E. coli*-*S. aureus* shuttle vector pMAD (Annex II). This pMAD derivative was then used for homologous recombination in *S. aureus*.

2.2.3.1 Vector construction

The constructions of vectors (pLUG752 & pLUG754) to inactivate RsaA were already done by Benito and Boisset of our team in 2007. However, as an important preliminary work for the latter part of this project, the procedure should be described. As an example, the process of constructing RsaA inactive vector will be explained briefly as follows (Figure 2-1). Benito and Boisset applied a similar method for the construction of other knock-outs (i.e. RsaE, RsaH and RsaG).

At first, DNA fragments corresponding to the upstream and downstream regions of RsaA were amplified with the two oligonucleotides pairs, RsaA-76/RsaA-1120 and RsaA-1246/RsaA-2194, from the chromosome DNA of *S. aureus* RN6390. Then the PCR products were digested with restriction enzymes *SmaI/EcoRV* and *ClaI/SaI* respectively (step ①). Next, plasmid pBLUEScript containing a kanamycin resistance gene was cut with the *SmaI/EcoRV* (step ②) and ligated with the fragment RsaA 76-1120 (F1) (step ③). Afterwards, the recombinant plasmid was transformed into *E. coli* strain DH5 α and cloned (step ④). Amplified recombinant plasmid was extracted and digested with restriction enzymes *ClaI/SaI* (step ⑤). The second fragment RsaA1246-2194 (F2) was ligated within the plasmid (step ⑥). This whole plasmid (pLUG752) was then transformed and amplified in DH5 α (step ⑦). pLUG752 was extracted and digested with restriction enzymes *SmaI/SaI*, generating a fragment containing kanamycin resistance gene flanked by F1 and F2 (step ⑧). Subsequently pMAD vector was digested with *SmaI/SaI* (step ⑨). At last, the fragment of *rsaA* inactivation was ligated with pMAD, forming pLUG754 (step ⑩).

This plasmid has three relevant features for the future recombination steps: a) ampicillin and erythromycin resistance genes allowing for antibiotic selection; b) thermo sensitive origin of replication; c) β -galactosidase gene allowing for blue-white discrimination.

Before transfer of this plasmid into various *S. aureus* strains, it was introduced into strain RN4220, which is a *S. aureus* strain able to accept DNA of *E. coli*, to obtain strain LUG1411. The detailed electroporation procedure is presented in the following part.

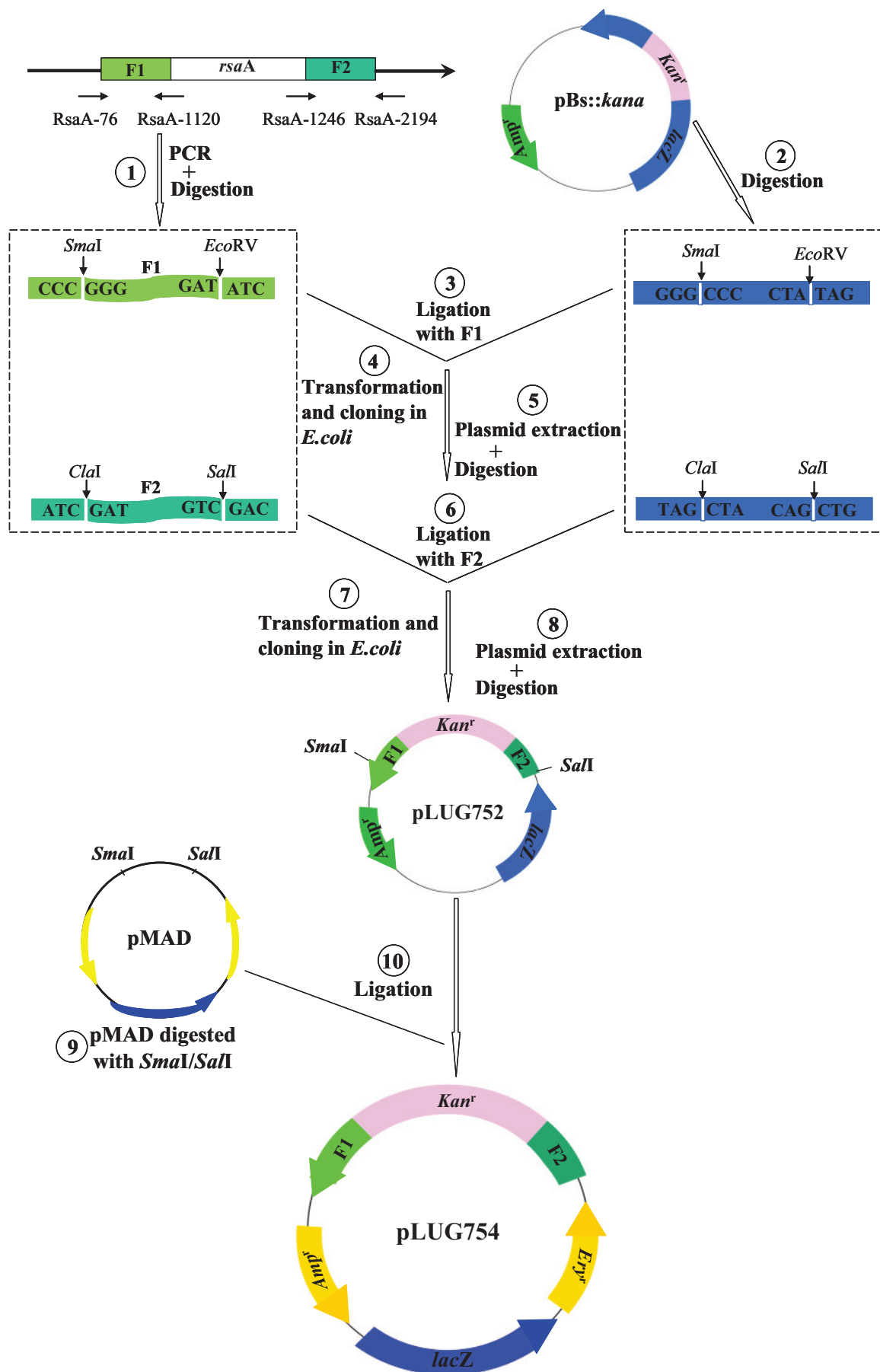


Figure 2-1 Construction of RsaA inactivation vector. F1 and F2 stand for the upstream and downstream sequences of RsaA amplified with primers Rsa-76/RsaA-1120 and RsaA1246/Rsa2194 respectively.

DNA electroporation into *S. aureus* was done as follows: Five μl plasmid DNA ($1.3 \mu\text{g}/\mu\text{l}$) was added into the tube containing 100 μl electrocompetent bacteria cells, then homogenized by gently mixing with pipette several times. Afterwards the bacteria/DNA mixture was transferred to a pre-chilled electroporation cuvette, and incubated for 15-30 min on ice. The mixture was then pulsed within the electroporation system (BIO-RAD): Voltage-2900 V, capacitance-25 μF , resistance-100 Ω , time-2.3 ms. Then the suspension was diluted with 300 μl BHI and incubated at 30°C for 1 hour with moderate shaking. Two hundreds and fifty μl of the above culture was spread on TS agar plate with 5 $\mu\text{g}/\text{ml}$ erythromycin and 40 $\mu\text{g}/\text{ml}$ X-gal. Transformants were selected after 2 days at 30°C ; those electroporated successfully with the pMAD Rsa-inactivation plasmid produced blue colonies.

Preparation for the electroporation:

1) Plasmid extraction was done with commercial kit (QIAGEN® Plasmid Midi Kit).

2) Produce electro-competent cells

Freshly streaked blood agar plate with the appropriate cells (e.g. HG001) was prepared and incubated at 37°C overnight. Next morning, several colonies were picked up into 100 ml TSB medium. The mixture was incubated for about 2.5 h at 37°C with vigorous shaking. When the OD_{600} reached 0.3-0.5, the mixture was centrifuged at 4,000 rpm for 10 min at 4°C . After that, the pellet was washed with 10 ml ice-cold sugar solution (Saccharose 0.5 M, sterilised and filtered with 0.22 μm filter), then centrifuged again. This washing step was repeated 5 times in total. Care should be taken to keep the cells cold during the entire procedure. At last, the pellet was suspended with 600 μl pre-chilled sugar solution. The solution (100 μl per tube) was stored at -80°C , except the cells used for electroporation immediately.

2.2.3.2 Gene recombination

The key factor in this process is the thermo-sensitive origin of replication, which allows the plasmid replicating at 30°C and not at 42°C . In the presence of erythromycin at 42°C , pale blue colonies due to integration of the vector by a single crossover event could be selected. The second crossover happens at 30°C without antibiotic stress: candidate colonies are white

because of the lost of erythromycin resistance gene and β -galactosidase gene.

Seven-day double-recombination (Figure 2-2):

Day 1: One blue colony was added into 5 ml TSB (with the final concentration of erythromycin 1.5 $\mu\text{g/ml}$) and incubated at 42°C overnight.

Day 2: Fifteen μl of the above mixture was spread onto an agar plate (TS + erythromycin 1.5 $\mu\text{g/ml}$ + X-Gal 40 $\mu\text{g/ml}$) and incubated at 42°C overnight.

Day 3: Pale blue colonies (avoiding the dark blue ones) were chosen and streaked on another agar plate (TS + erythromycin 1.5 $\mu\text{g/ml}$ + X-Gal 40 $\mu\text{g/ml}$) and incubated at 42°C overnight.

Day 4: The pale colony was resuspended in 5 ml TSB medium without antibiotics and incubated at 30°C with moderate shaking.

Day 5: Serial dilutions were made until 10^{-7} or 10^{-8} , and then applied onto the TS plates with 40 $\mu\text{g/ml}$ X-Gal. These plates were incubated at 37°C for at least 48 h.

Day 6: Each white colony on the plate was picked and streaked on two agar plates, one TS plate with 40 $\mu\text{g/ml}$ X-Gal and 1.5 $\mu\text{g/ml}$ erythromycin, the other TS plate with 40 $\mu\text{g/ml}$ X-Gal but without any antibiotics. The plates were incubated at 37°C for 24 h.

Day 7: White colonies growing on the agar plate without antibiotics and not surviving on the plates with erythromycin simultaneously were chosen. Their chromosome DNA was extracted using DNeasy Tissue Kit (QIAGEN) and checked with PCR using the oligonucleotides for inactivation control.

2.2.4 Complementation

After the construction of RsaA, E, G and H mutants, our team started to construct their corresponding complemented strains. Northern blot was used to detect the sRNAs in the complemented strains (with RsaE complementation in RN6390 as an example).

2.2.4.1 Construction of complementation strains

Our team has tried 2 different strategies to construct the vector for complementation (Figure 2-3).

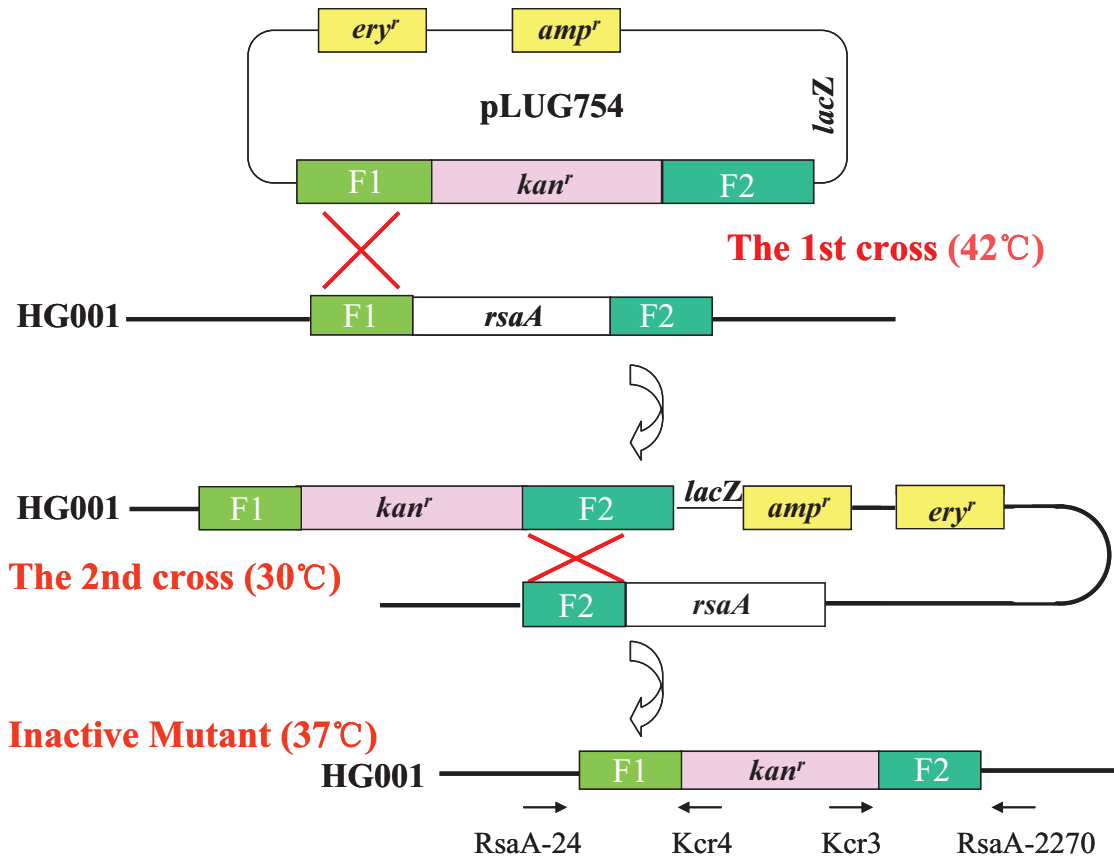


Figure 2-2 The schematic of homologous recombination: example of HG001 Δ *rsaA*. Plasmid pLUG754 is inserted into the chromosome of HG001 after the 1st cross (at 42°C), and the *rsaA* gene is replaced with kanamycin resistance sequence after the 2nd cross (at 30°C). Mutants that grow at 37°C are checked with PCR (primers indicated as small black arrows).

– Construction of pCN37::*rsaE*

rsaE was amplified by PCR from the chromosome DNA of RN6390 with oligonucleotides RsaE-2063/RsaE-2162. This PCR product and plasmid pCN37 were digested with same double restriction enzymes (*Eco*RI/*Bam*HI) separately, and then ligated by using T4 DNA ligase (Fermentas). Next, this complementation plasmid (pLUG790) was electroporated into the *S. aureus* RN4220 (LUG1472), and then into RN6390 Δ *rsaE* (LUG1476).

– Construction of pLUG920::*rsaE*

A transcription terminator (TT) and another copy of inducible promoter (*Pcad*) without *cadC* were introduced downstream *cadC*, forming pLUG920. *rsaE* was cloned into this modified plasmid, forming pLUG848 in *E.coli* DH5 α . Then, it was electroporated in *S.*

aureus RN4220 (LUG1689), followed by electroporation into RN6390 Δ *rsaE* (LUG1691).

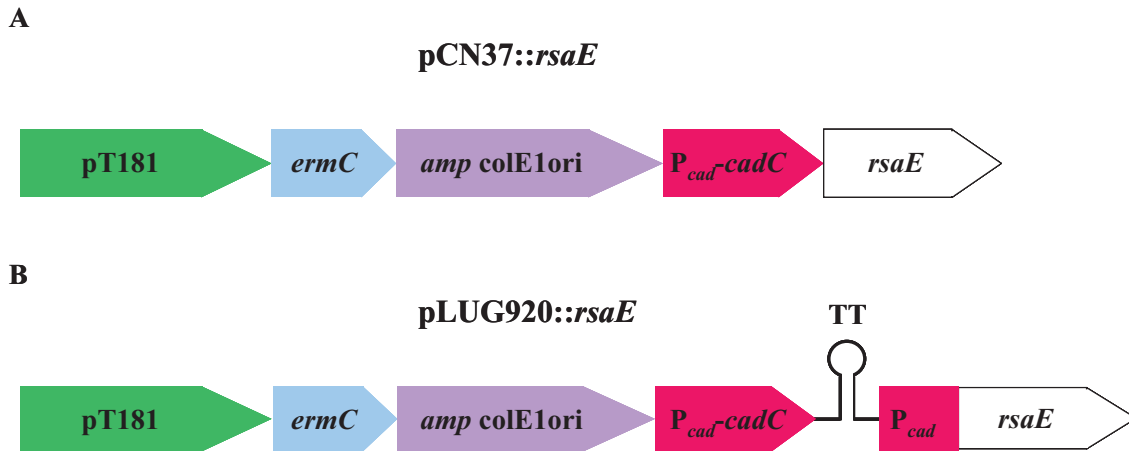


Figure 2-3. Vector constructs for complementation. (A) pCN37, containing an inducible promoter cassette *Pcad-cadC* (in red), is represented in a linear form. Gene *rsaE* (in white) is inserted in the MCS site, downstream of *Pcad-cadC*. (B) pCN37 is modified and renamed as pLUG920, where a transcription terminator (TT) and a 2nd *Pcad* are cloned. *rsaE* is inserted in the downstream region of the additional *Pcad*.

2.2.4.2 Total RNA extraction

S. aureus wild type strain (RN6390), RsaE mutant and complemented strains were cultivated in BH medium and OD₅₅₀ value was checked each hour of the incubation time. When the OD₅₅₀ reached 0.5, 5 μ M cadmium was added into the culture of complemented strain. Five ml of bacterial culture after 0.5 h and 1.5 h, and 1 ml after 3 h were collected. They were standardized with TES (20 mM Tris, 10 mM EDTA, 50 mM NaCl) to an OD₅₅₀ of 1.5. Then, 1 ml was centrifuged at 6,000 rpm for 2 min. Each pellet was resuspended in 100 μ l TES with 10 μ l Lysostaphin (2.5 mg/ml), then incubated on ice for 30 min. Subsequently, 100 μ l SDS (2%) and 5 μ l Proteinase K (10 mg/ml) were added. After the addition of 20 μ l tracking dye, the mixture was vortexed for 3 min at room temperature to make sure that bacteria were well lysed. Finally, total RNA was conserved at -80°C.

2.2.4.3 Probe labeling

rsaE gene was amplified by PCR from the chromosomal DNA of RN6390 with oligonucleotides RsaE-2063/RsaE-2162, then purified with MinElute PCR purification Kit

(QIAGEN).

Next, *rsaE* PCR product was cloned into pGEM[®]-Teasy (Promega, Annex III) through the ligation step: 3 µl PCR product, 1 µl plasmid and 1 µl T4 DNA ligase (Promega) within 1×ligation buffer, 16°C overnight. This ligation product was introduced into CaCl₂ treated *E. coli* DH5α cells through heat-shock method. Transformed clones should be firstly picked out by blue/white selection on the LB agar plates with 2% X-gal and 100 µg/ml ampicillin because insertion of foreign gene lead to the inactivation of β-galactosidase gene. The presence of insert was checked again by PCR.

Recombinant plasmid was linearized by the restriction enzyme *SalI* (Fermentas, Burlington, Canada), 37°C for 1 h. Later, DNA of this linearized plasmid was transcribed into digoxigenin-labeled RNA by *in vitro* transcription system (Roche): 1 µg DNA, 2 µl T7 RNA polymerase, 2 µl NTP labeling mixture, 2 µl 10×buffer and 1 µl RNase inhibitor, at 37°C for 2 h. Afterwards, RNA product was processed with RNase-free DNase (Roche) for 15 min at 37°C, with reaction stopped then by 0.2 M EDTA. It was precipitated by incubating with 3 M sodium acetate, pH 5.2 and cold absolute ethanol for 30 min at -80°C. Pellet was resuspended in 50 µl DEPC H₂O, with 1 µl RNase inhibitor to prevent RNA degradation.

2.2.4.4 Northern-Blot

Twenty µl of total RNA was mixed with 10 µl fresh new-made denaturation solution (deformaldehyde 5 µl, 10×MOPS 3 µl, deionized formamide 2 µl, Ethidium bromide (BET) 10 mg/ml), then incubated at 65°C for 10 min. Twenty five µl of denaturized mixture was loaded on gel (1% agarose, 1×MOPS, Formaldehyde 2.2 M). Electrophoresis was carried out with 5 V/cm of voltage (*i.e.* 20 cm = 100 V) for 1~2 min first to let samples enter into gel, then 5 V/cm for 1.5 h, in the migration buffer (1×MOPS, Formaldehyde 2.2 M).

The gel was laid on nylon membrane (Roche), with 20×SSC (NaCl 3 M, sodium citrate 0.3 M, pH 7). Isolated RNA was transferred with vacuum gene pump during 1 h at 50 mbar of vacuum (Pharmacia LKB). Membrane was then dried at 37°C for 5 min, and fixed by UV (1.5 KJ/cm²), dried again at room temperature for 1 h.

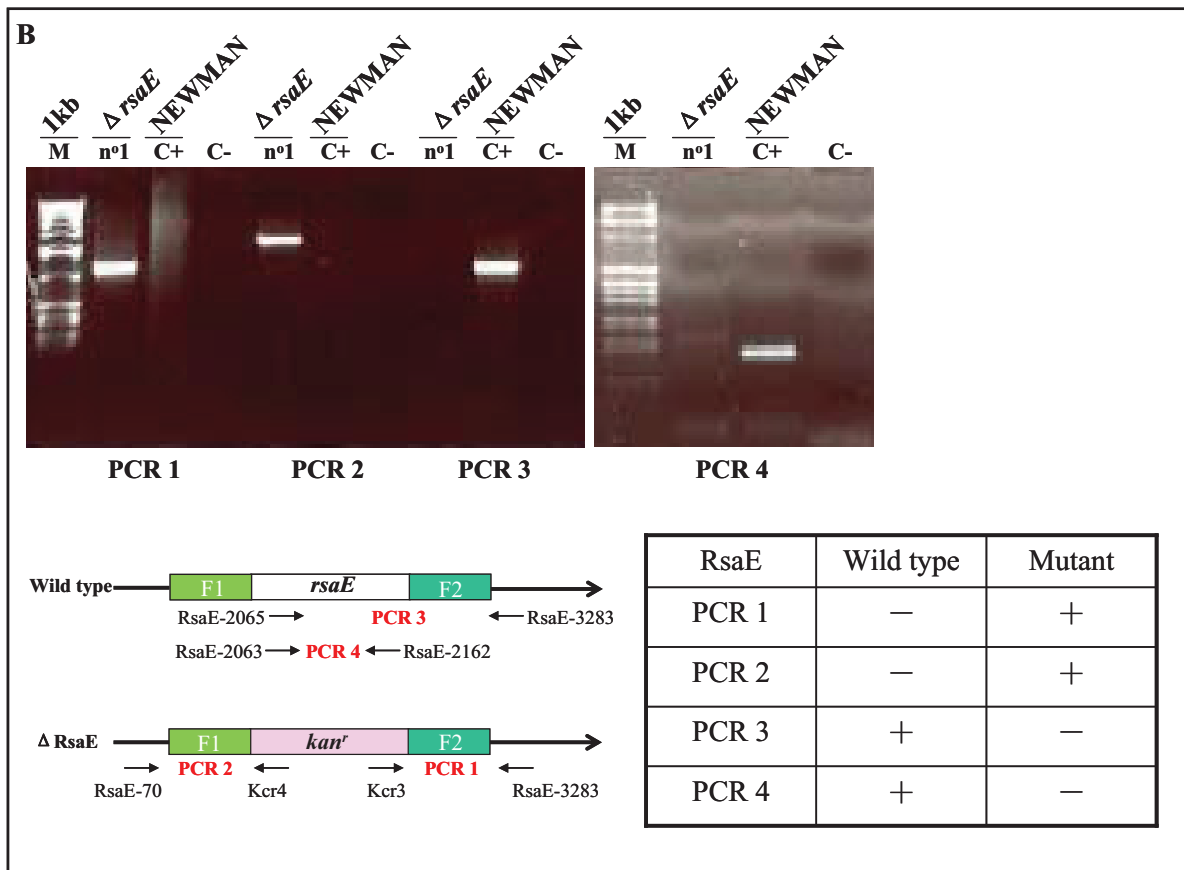
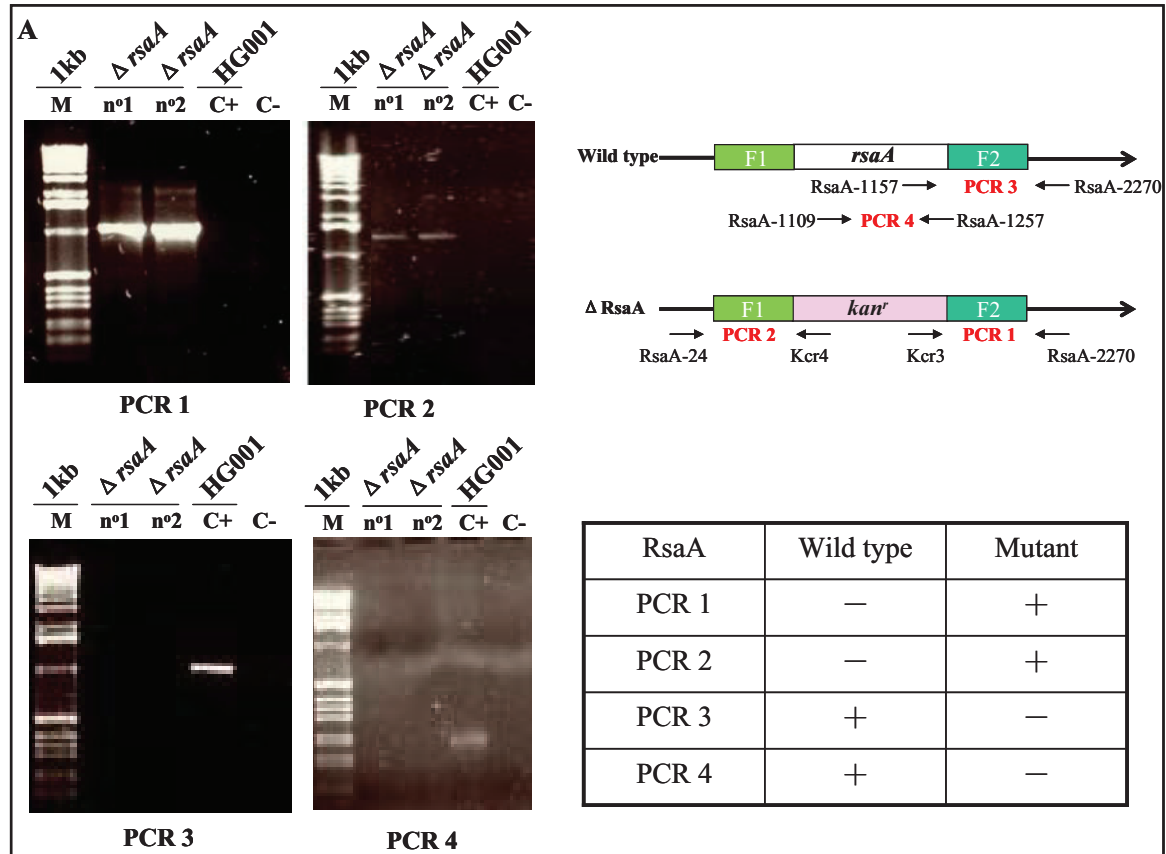
Then, the membrane was soaked in 30 ml hybridization solution (5×SSC, deionized formamide 50%, 1×blocker reagent, N-laurylsarcosine 0.1%, SDS 0.02%), and incubated at 42°C with rotation for 1 h. Afterwards, the membrane was soaked again in 5 ml fresh hybridization solution, where 50 ng of marked probe was added. The hybridization was done

overnight at 42°C with rotation. The membrane was washed several times (with 2×SSC, 0.1% SDS for 5 min, 2 times, at room temperature; with 0.1% SSC, 0.1% SDS for 15 min, 2 times, at 68°C) and rinsed thoroughly with DEPC water. Then, it was washed again with washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 2 min at room temperature. Following, one step of saturation was done with 30 ml fresh blocking buffer (10% blocking reagent, 90% maleic acid buffer) for 30 min, at room temperature. Anti-digoxigenin antibody coupled to alkaline phosphatase (1.5 µl antibody diluted in 15 ml blocking buffer) was added and incubated for 30 min, at room temperature. The excess of antibody was removed by two-time washing with 30 ml washing buffer, each time 15 min. Detection buffer (0.2 M Tris-HCl, 0.1 M NaCl, pH 9.5) was used to rinse membrane for 5 min, at room temperature. The luminescent substrate (Roche) was diluted 1:100 in detection buffer and added, 5 min at room temperature. Excess liquid was removed by putting the membrane on whatman paper (whatman, maidstone, Angleterre). Finally membrane wrapped within SARAN film was incubated at 37°C for 10 min to activate the alkaline phosphatase, then visualization was carried out by capturing the chemi-luminescence signal on a film (lumi-film, Roche)

2.3 Results

2.3.1 Mutants of Rsa

Until now we have constructed mutants of 5 sRNAs (RNAIII, RsaA, E, G, and H) in 3 genetic backgrounds (RN6390, HG001 and NEWMAN), 3 among which was built personally by me: HG001 Δ *rsaA* (LUG1630), NEWMAN Δ *rsaE* (LUG1670), NEWMAN Δ *rsaH* (LUG1640). To obtain these strains, I used plasmids for inactivation constructed by Benito and Boisset. Then the plasmids were electroporated into different genetic backgrounds of *S. aureus* (e.g. pLUG754 into HG001, pLUG766 into NEWMAN, pLUG749 into NEWMAN). Finally mutants were obtained after double recombination and selected by using blue/white screen. PCR was carried out to check if the correct mutation happened (Figure 2-4).



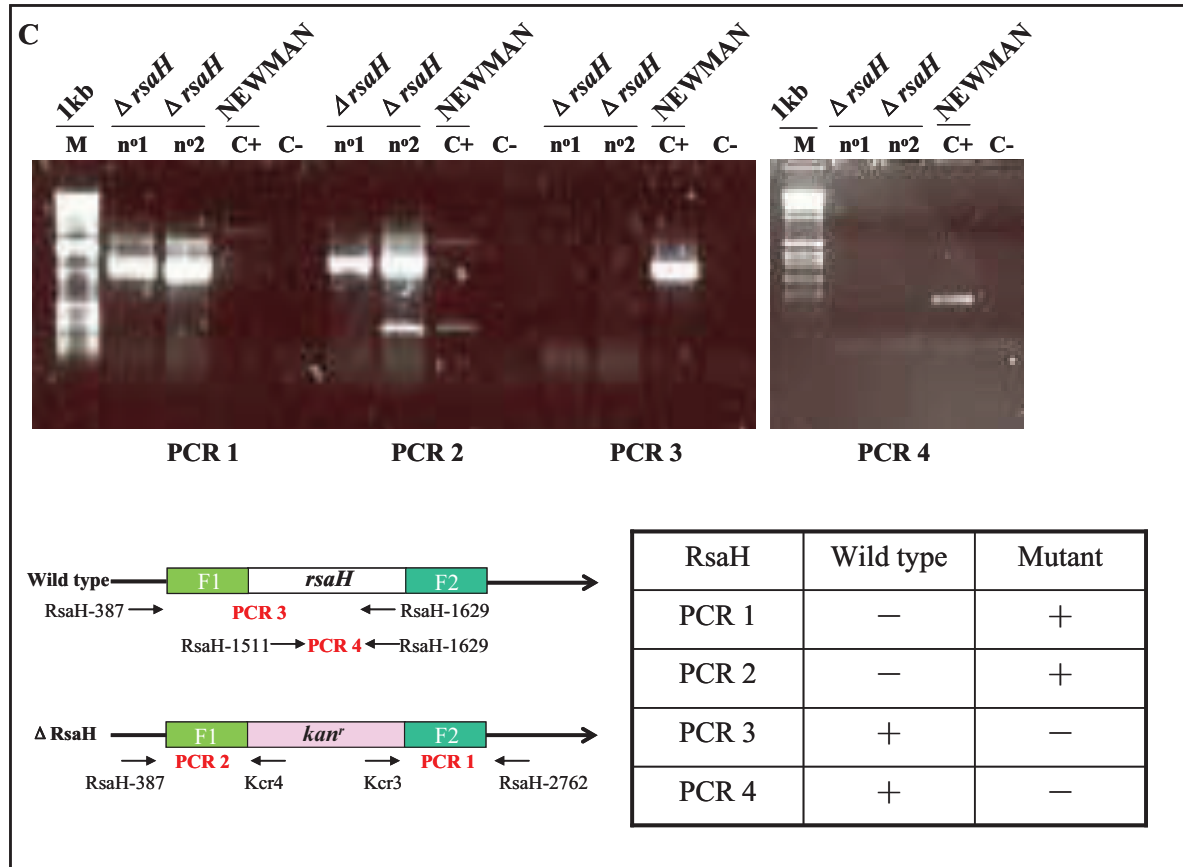


Figure 2-4 Migration bands of PCR results on 1% and 2% agarose gel, to control the inactivation of Rsa gene. Schemas of wild type and mutant show clearly positions and orientations of the oligonucleotides used for PCR, together with one table for each sRNA giving the proper results we should have. A. Two colonies of HG001 Δ *rsaA* were confirmed by PCR using 4 pairs of primers: PCR1 (Kcr3/RsaA-2270); PCR 2 (Kcr4/RsaA-24); PCR 3 (RsaA-1157/RsaA-2270); PCR 4 (RsaA-1109/RsaA-1257). B. One colony of NEWMAN Δ *rsaE* was confirmed by PCR: PCR1: Kcr3/RsaE-3283; PCR2: Kcr4/RsaE-70; PCR3: RsaE-2065/RsaE-3283; PCR4: RsaE-2063/RsaE-2162. C. Two colonies of NEWMAN Δ *rsaH* were confirmed by PCR: PCR1: Kcr3/RsaH-2762; PCR2: Kcr4/RsaH-387; PCR3: RsaH-387/RsaH-1629; PCR4: RsaH-1511/RsaH-1629.

2.3.2 Complementation of RsaE mutant strain

RsaE is chosen as an example to explain the strategy used for complementation. Theoretically, when we use the plasmid pCN37, the inducible promoter *Pcad-cadC* is activated by micromolar concentrations of cadmium, which also allows transcription of the gene cloned downstream *cadC*. To test the first strategy used (Figure2-3A), RN6390 and LUG1476 (RN6390 Δ *rsaE* strain harboring the pCN37::*rsaE*) were cultivated in BH medium with shaking at 37°C, in the presence or in the absence of 5 μ M cadmium. Bacterial cultures

were collected at 0.5 h, 1.5 h and 3 h after cadmium induction respectively, and then total RNA was extracted. We tested the expression of RsaE by northern-blot, and no band of correct size for RsaE was shown for LUG1476 (Figure 2-5A, done by Geissmann). We supposed that transcripts of *cadC* and *rsaE* formed a hybrid, which damaged the normal secondary structure of RsaE, which also explain the longer RNA size shown by Northern-blot.

For the second strategy (Figure 2-3B), the plasmid was modified to retain the *Pcad-cadC* module which is required for proper induction of the promoter in the presence of cadmium, and a second promoter devoid of any ORF was inserted downstream the *cadC*, separated by a transcription terminator. Thus, RsaE DNA cloned downstream *PcadC* should lead to an inducible transcription of RsaE without additional sequences. RNA from RN6390, RN6390 Δ *rsaE* mutant and LUG1691 (RN6390 Δ *rsaE* strain harboring the pLUG920::*rsaE*) were analyzed according to the same procedure described above. Northern blot showed weak bands of some products with larger sizes for LUG1691 both with cadmium induction and without cadmium induction (Figure 2-5B). This suggests that the constructs with inducible promoter cassette did not work, maybe the promoter alone does not function.

Eventually, it was decided by my supervisor and colleagues to use a derivative of plasmid PE194 in which a strong promoter (*agr* P3) was cloned. RsaE and other sRNA DNA were cloned in this plasmid for complementation experiments. This work was accomplished by Dr. S. Boisset.

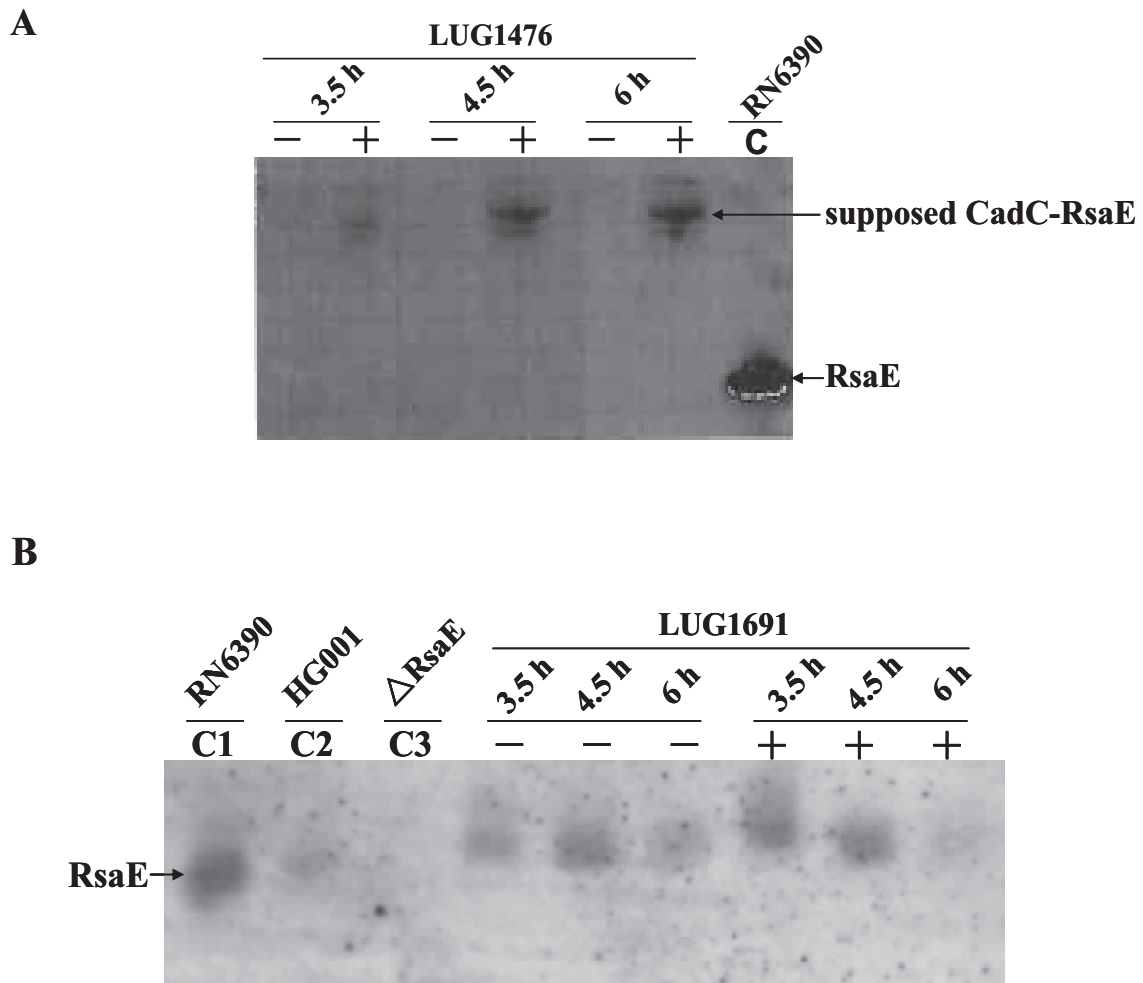


Figure 2-5. Northern blot for RsaE, used to verify complementation in RsaE mutant. Cultures of LUG1476 (shown in A) and LUG1691 (shown in B) were collected at 3.5 h, 4.5 h and 6 h of growth in the presence (+) or absence (–) of cadmium. Addition time of cadmium was when OD₅₅₀ arrived at 0.5 (approximately 3 hours of growth). RN6390 (*agr*⁺) was adopted as positive control, while HG001 (*agr*[–]) and Δ RsaE as negative controls.

2.4 Discussion

In the recent decades, a large number of sRNAs have been identified in bacteria, followed by much effort in unveiling their specific functions and mechanisms. Although regulation functions for some sRNAs have already been found ^[2], there are still lots of sRNAs whose cellular roles are unknown. To elucidate the functions of the arising amount of sRNAs, it is quite critical to adopt appropriate approaches. Previous studies give us two major conventional types of strategies: sRNA knockout followed by complementation ^[302] or sRNA overexpression ^[48]. In this project, we have chosen the former strategy to construct

mutants and complemented strains for RsaA, RsaE, RsaG and RsaH, which is feasible since there is only a single copy of each sRNA in the core genome of *S. aureus*. Comparison of phenotypes, gene expression profiles and proteomic analysis of mutant and wild type strains will be helpful for establishing the functions of the sRNAs. Then by function analysis of the associated mRNA and proteins, it is possible to find the potential biological effects of this sRNA, such as *rsaE* ^[11].

2.5 Conclusion

The chapter introduced the genetic tools and methods to build knock-out strains and corresponding complementation strains. Four Rsa (RsaA, RsaE, RsaG and RsaH) mutants in 3 genetic backgrounds (RN6390, HG001 and NEWMAN) have been successfully constructed by our team. Construction of their complementation strains with inducible plasmid failed, but we have succeeded with classical staphylococcal plasmid PE194.

Subsequent transcriptomic and proteomic analyses could be carried out on the mutants, complementation strains and wild type strains. They will help us better understand the functions of these sRNAs and find out their potential targets. Our collaboration team (P. Romby, Strasbourg) is operating these *in vitro* studies. In the meantime, a series of studies have been already started by our team, focusing on characteristics and roles of Rsa in biological environments, such as in the biofilm formation process and internalization of *S. aureus* by human epithelial cells. Mutants and complementation strains shall be extensively used in these projects.

Chapter 3 The influence of antibiotics on Rsa expression

3.1 Introduction

The production of many virulence factors of *S. aureus*, like staphylococcal enterotoxin A and B (SEA, SEB), autolysin, protein A, and alpha-hemolysin, delta-hemolysin, are reduced by some antibiotics, such as clindamycin, linezolid, and fusidic acid, *etc* ^[303-306]. However, subinhibitory concentration of clindamycin stimulates coagulase and fibronectin binding protein B expression, and Panton-Valentine Leukocidin (PVL) is enhanced by subinhibitory concentration of oxacillin (DUMITRESCU *et al.* 2008; DUMITRESCU *et al.* 2007).

As RNA molecules have recently emerged as major players during the bacterial gene regulation and stress response ^[2,307-309], the objective of this chapter is to investigate whether the expression levels of 5 sRNAs in *S. aureus* react to antibiotics.

3.2 Methods and Materials

3.2.1 Strains and antibiotics

Laboratory strains RN6390 (agr positive) and HG001 (σ^B factor positive) were used, and their characterization could be seen in table 2-1 (seen in chapter 2).

Antibiotics used:

- Oxacillin, a penicillinase-resistant β -lactam similar to methicillin; of note, strain resistant to this class of antibiotic are called methicillin resistant *S. aureus* (MRSA).
- Levofloxacin, one of fluoroquinolone class, interfering with bacterial cell division by inhibiting DNA gyraseA.

3.2.2 Minimum inhibitory concentration (MIC) test

The MIC for oxacillin and levofloxacin were determined on strain RN6390 and HG001 according to Clinical Laboratory Standards Institute (CLSI) recommendations. The two strains were streaked on blood-agar plates and incubated at 37°C, overnight. Oxacillin and levofloxacin were diluted to the initial concentration for MIC test, 16 $\mu\text{g/ml}$ and 64 $\mu\text{g/ml}$ respectively. On the 96-well plate, 100 μl Mueller-Hinton (MH) medium or Brain-Heart (BH) medium was added into each well. For each line of 12 wells, 100 μl antibiotic initial solution was applied in the 2nd well and mixed well, and then 100 μl above mixture was added into

the 3rd well, serial dilution (each time 1: 2) should be continually until the 12th well. So the 1st well would be the negative control without antibiotics. Next, 100 μ l fresh inoculum (2×10^6 UFC/ml) was added in each well. Plates were incubated overnight at 37°C, and checked visually to determine MIC.

Accuracy of inoculums for each strain was controlled by spiral inoculator in duplicate. To prevent growth of the inoculum before the inoculation, no more than 15-20 min should be taken from suspensions prepared to the final incubation step.

3.2.3 Bacterial culture

RN6390 and HG001 were cultivated in BH at 37°C, and antibiotics were added when OD₆₀₀ reached 0.5. A series of concentrations of 0 MIC, 1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2 MIC, 1 MIC, 4 MIC were adopted in this experiment. After the antibiotic addition, bacterial culture was incubated at 37°C with vigorous shaking for 30 min, and 5 ml bacterial culture was collected.

3.2.4 RNA extraction

When the bacteria grew to the desired growth phase, the pellet was standardized until its OD₆₀₀ \approx 1.0 (approximately 10^9 cells). Cell lysis and RNA isolation was performed with the QIAGEN RNeasy Plus kit, following the manufacturer operating instructions.

3.2.5 Reverse transcription PCR

To choose the most suitable internal control, 16S rRNA and *gyrB* were tested and expression of *gyrB* of *S. aureus* was validated to be very stable as our proper endogenous reference gene (not shown).

Specific reverse-transcription was carried out with the Enhanced Avian Reverse Transcriptase (eAMV, Sigma). Firstly, 1 μ l dNTP (10 mM, Promega), 2 μ l mixed reverse primers (10 μ M) and constant amount of RNA (1 μ g) were mixed and adjusted to a volume of 10 μ l, and then were incubated at 70 °C for 10 min. Next, 1 U eAMV, 1 U Ribonuclease Inhibitor from human placenta (Sigma) and 2 μ l 10 \times buffer were added into the former mixture of 10 μ l and adjusted to a total volume of 20 μ l with DEPC water. Afterwards, the RT reaction was performed in 20 μ l reaction volume at 55 °C for 50 min.

After RT reaction, the quantitative real-time PCR was carried out with Light-Cycler instrument and LightCycler® FastStart DNA Master SYBR Green I kit (Roche). Two μl cDNA (1:5 diluted) was mixed with 8 μl master mix that consisted of 3.8 μl H_2O , 1.2 μl MgCl_2 , 1.0 μl Mixtaq, 1.0 μl Primer forward (5 $\mu\text{M/L}$), 1.0 μl Primer reverse (5 $\mu\text{M/L}$). The Primer list can be seen in table 3-1. The following system was used for amplification: initial denaturation at 95°C for 8 min (temperature transition, 20 °C/s); 40 cycles at 95 °C for 15 s, 52 °C for 5 s and 72 °C for 10 s (temperature transition, 20 °C/s); Melting curve analysis was done between 65°C and 95°C (temperature transition, 0.1 °C/s); Samples cooled at 40°C for 30 s (temperature transition, 20 °C/s). Peaks of melting-curves were analyzed to make sure the specificity of reactions. Moreover, the amplification efficiencies of sRNAs and internal control *gyrB* were checked to be almost equal.

Table 3-1 Primers used in RT-PCR.

Primers	Sense (+/-)	5' to 3' sequence	T _m (°C)
<i>gyrB</i> -F	+	GGTGGCGACT TTGATCTAGC	59
<i>gyrB</i> -R	-	TTATACAACG GTGGCTGTGC	57
<i>Agr-sa1502</i>	+	GGAAGGAGTG ATTTCAATGG	55
<i>Agr-sa1329</i>	-	GGGATGGCTT AATAACTCAT AC GTTAACCATT ACAAAAATTG	55
<i>RsaA</i> -F	+	TATAGAG	54
<i>RsaA</i> -R	-	CCGAGTAGTC TTCCTTGG	56
<i>RsaE</i> -F	+	AATCACATAA CAAACATACC C	52
<i>RsaE</i> -R	-	TCTGAATACA CGACGCTAAA	53
<i>RsaG</i> -F	+	ATGTGCGTGA AGAGATGAAA GA	56
<i>RsaG</i> -R	-	CTAAGTCGGG CAAATAAGGA TAC	58
<i>RsaH</i> -F	+	TAAAACGCTG CATGATACAA AC	54
<i>RsaH</i> -R	-	CCACTACTAA AGGGAGTCAA A	55

3.2.6 Data analysis

The relative gene expression ratios for quantitative Real-time PCR were calculated based on

cycle threshold (Ct) and the equation $2^{-\Delta Ct}$, where ΔCt stands for the difference between Ct of the gene studied and the Ct of *gyrB* in the same condition. When comparing the expression level of sRNA under antibiotics pressure with that growing without antibiotics, the method $2^{-\Delta\Delta Ct}$ was used, where $\Delta\Delta Ct = (Ct \text{ target} - Ct \text{ } gyrB) \text{ with antibiotics} - (Ct \text{ target} - Ct \text{ } gyrB) \text{ without antibiotics}$ (LIVAK and SCHMITTGEN 2001; SCHMITTGEN *et al.* 2000; WINER *et al.* 1999).

3.3 Results

3.3.1 MIC of the antibiotics

MIC test was carried out for selected antibiotics on RN6390 and HG001 with both MH and BH media. MH medium was chosen as the standard microbiologically growth medium for antibiotic resistance test, because its components are more stable than other media and recommended by CSLI, while rich medium like BH medium favors the bacterial growth and virulence factors production. As shown in table 3-2, for oxacillin and levofloxacin, MICs obtained with the two media were always identical.

Table 3-2 MICs of selected antibiotics on RN6390 and HG001

Antibiotic	MICs ($\mu\text{g/ml}$) in MH/BH medium	
	RN6390	HG001
Oxacillin	0.25/0.25	0.25/0.25
Levofloxacin	0.25/0.25	0.25/0.25

3.3.2 Antibiotic effects on bacterial growth

To observe the influences of antibiotics on bacterial growth, RN6390 was exposed to grading subinhibitory concentrations of oxacillin at different stages of growth. When oxacillin was added at the start of growth ($OD_{600} = 0.05$), RN6390 barely grew with 1/2 MIC and 1/4 MIC, yet inhibited by 1/8 MIC and unaffected by 1/16 MIC (Figure 3-1A). When RN6390 was treated at the exponential phase ($OD_{600} = 0.5$) with oxacillin, bacterial growth was attenuated by 1/2 MIC and 1/4 MIC in a dose-dependant manner, and not affected by

1/8 MIC and 1/16 MIC (Figure 3-1B). Therefore, the second protocol (treatment in exponential phase) guaranteed that enough alive bacteria can be collected for RNA analysis, even with 1/4 MIC and 1/2 MIC.

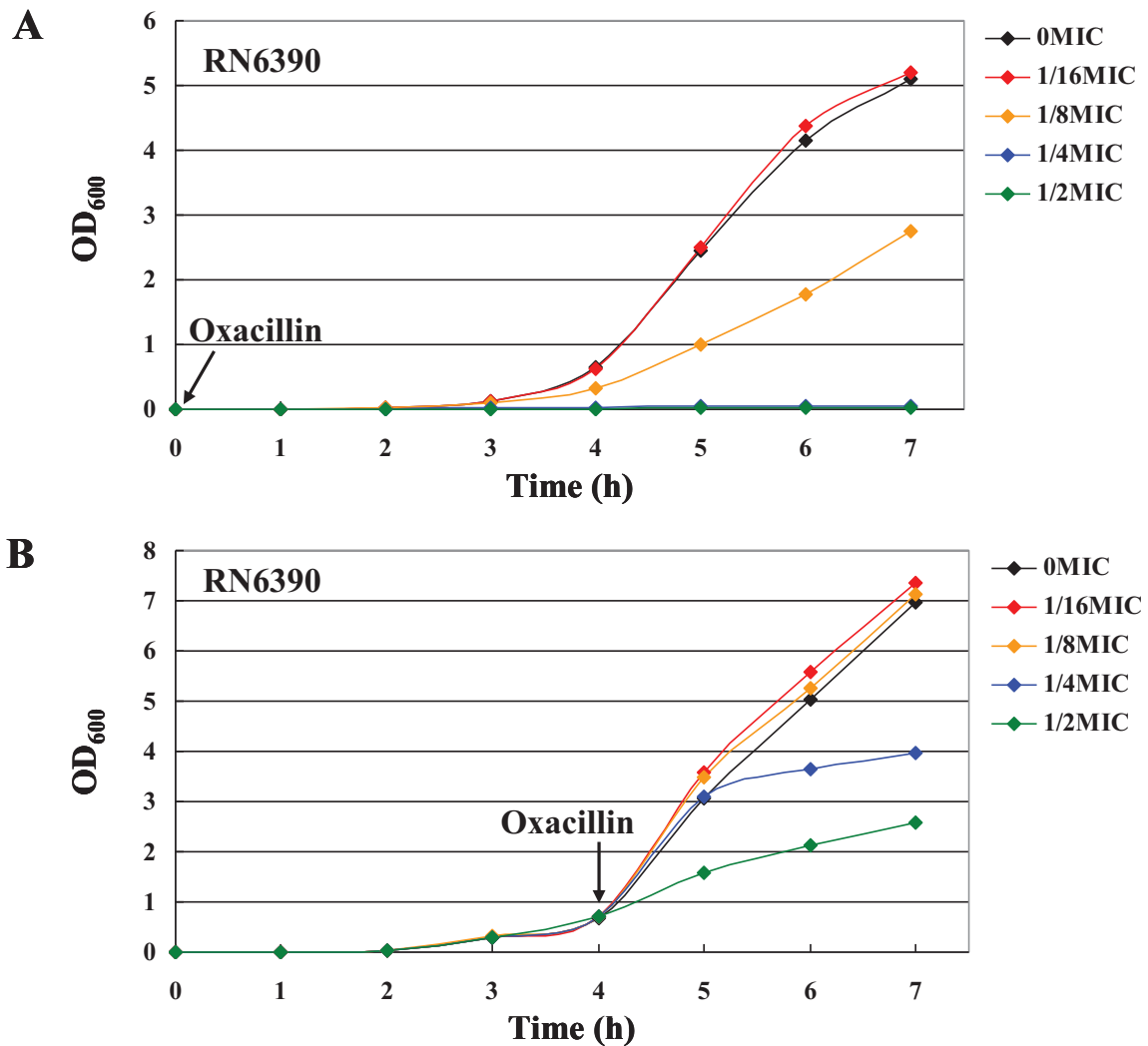
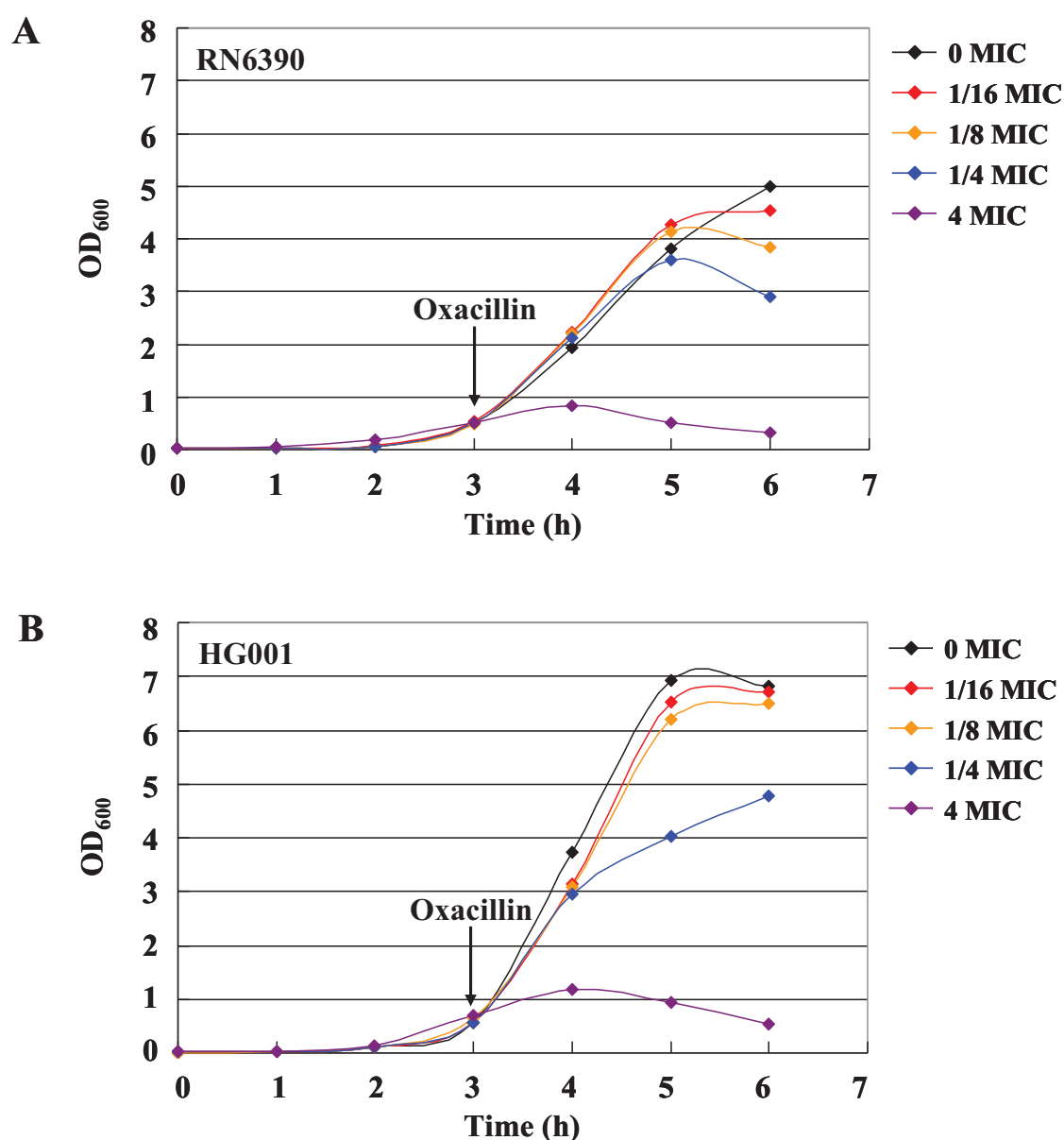


Figure 3-1. Growth curves of RN6390 in the presence of oxacillin (0 MIC-black, 1/16 MIC-red, 1/8 MIC-yellow, 1/4 MIC-blue, and 1/2 MIC-green). Black arrows indicate the time point where oxacillin was added. A. Serial concentration of oxacillin was added at the beginning of bacterial growth. B. As the bacteria grew to exponential phase ($OD_{600} = 0.5$), different quantity of oxacillin was added to the medium.

Then, the different concentrations from 1/16 MIC to 4 MIC of oxacillin and levofloxacin were added at mid-exponential growth phase ($OD_{600} = 0.5$) to interfere bacterial growth of two *S. aureus* strains (RN6390 and HG001) separately. Similar growth patterns were obtained for these two antibiotics. As shown in figure 3-2, in comparison with the situation

of 0 MIC, it was noted that bacterial growth was suppressed in a dose-dependant manner from 1/4 MIC to 4 MIC, and nearly unchanged with 1/16 MIC and 1/8 MIC. In this growth curve figure, the growth repression by 1/4 MIC - 4 MIC antibiotics mostly started to be reflected at two hours after antibiotics addition. However, when 4 MIC of oxacillin was added, its inhibition to bacteria growth happened immediately, which was not the case for levofloxacin.



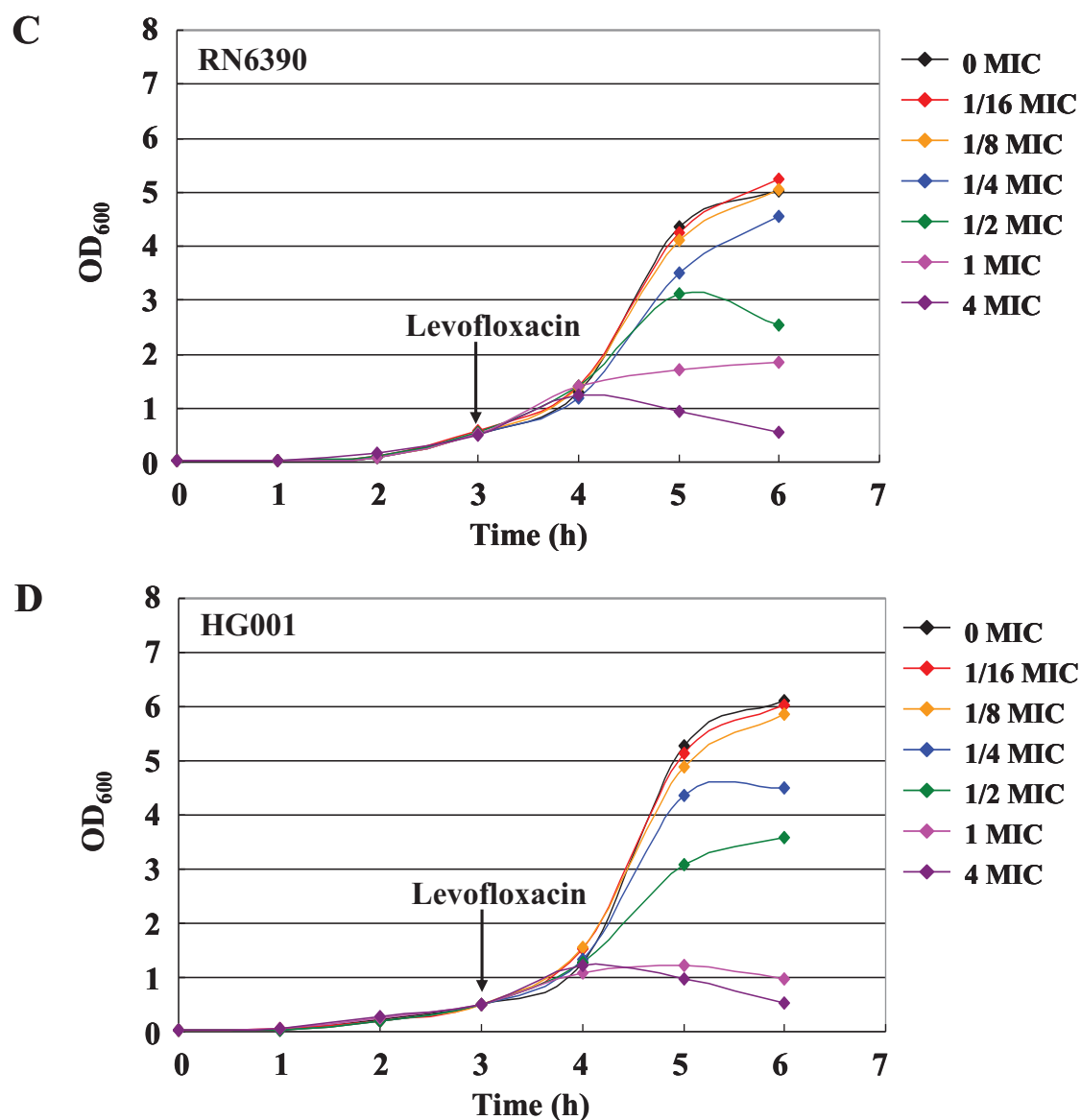


Figure 3-2. Growth curves of RN6390 and HG001 in the presence of oxacillin (A, B) or levofloxacin (C, D). Concentrations of antibiotics are indicated with colors: 0 MIC-black, 1/16 MIC-red, 1/8 MIC-yellow, 1/4 MIC-blue, 1/2 MIC-green, 1 MIC-pink and 4 MIC-purple. Black arrows indicate the time point ($OD_{600}=0.5$) of adding antibiotics. The experiment shown in A is a repetition of experiment shown in figure 3-1B.

3.3.3 Influence of oxacillin on the expression of sRNA

Expression levels of sRNAs were analyzed in the presence and absence of oxacillin. RsaG expression was not tested, because the primers previously designed were not specific enough.

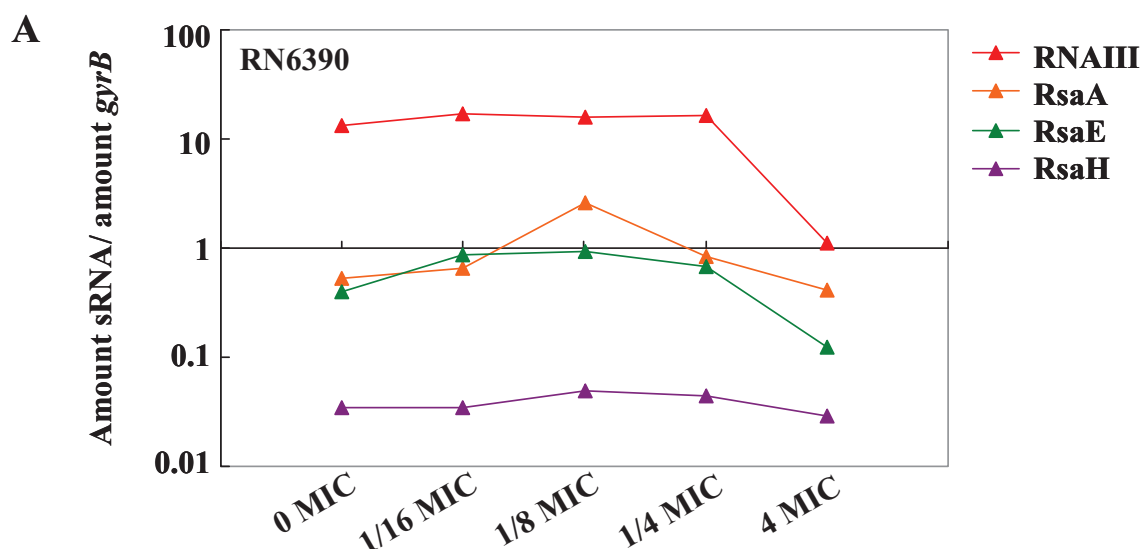
- Expression level difference among the sRNAs

In both strains, RN6390 and HG001, RNAIII was expressed at the strongest level as compared to the other 3 sRNAs, and its level was as high as over 10 folds that of *gyrB*. The expression of RsaA and RsaE were basically comparable to that of *gyrB*, whereas RsaA level was slightly higher than RsaE. The most weakly expressed sRNA was RsaH with a ratio of 0.1 to 0.01 in comparison to *gyrB* mRNA (Figure 3-3).

- Relation between expression level of sRNA and oxacillin concentration

We did not observe a significant reduction of sRNA/*gyrB* ration when concentrations of oxacillin increased gradually from 1/16 MIC to 1/4 MIC. However, in the presence of 4 MIC of oxacillin, there was a visible declination in RN6390 for three sRNAs (RNAIII, RsaA and RsaE), but not for RsaH (Figure 3-3A). In HG001, it was demonstrated that the expression of all four sRNAs were decreased in the presence of 4 MIC of oxacillin (Figure 3-3B).

A comparison for the relative amount of each sRNA (in the presence of oxacillin vs. absence of oxacillin) between RN6390 and HG001 is shown in Figure 3-4. Except for RsaH, we could not detect a difference of expression level of sRNAs between RN6390 and HG001. The relative amount of RsaH, in the presence of a series of concentrations of oxacillin, was higher in RN6390 than HG001 (we use two-fold difference as a threshold in this study).



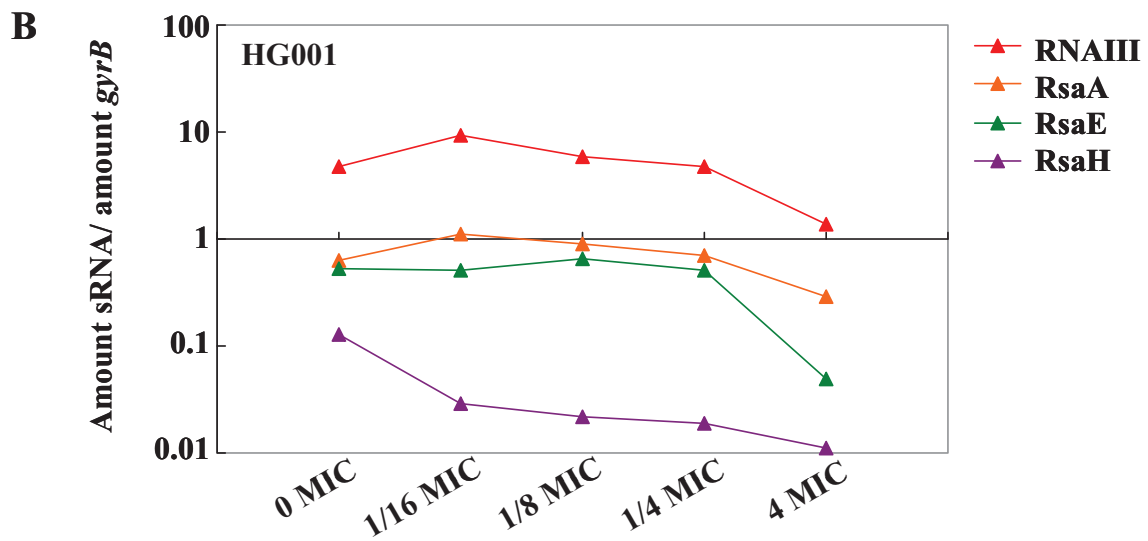
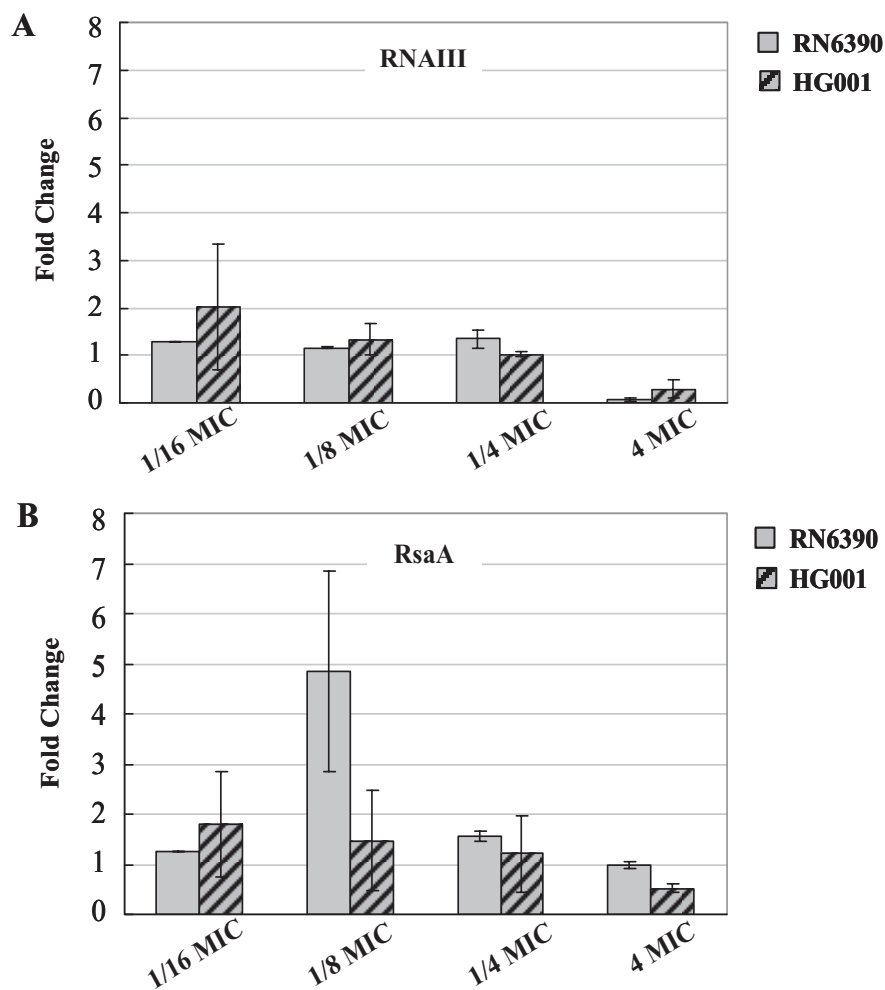


Figure 3-3 Relative sRNA expression ratio to *gyrB* in RN6390 (shown in A) and HG001 (shown in B) under the stress of serial concentrations of oxacillin. Data was the average of two separate experiments.



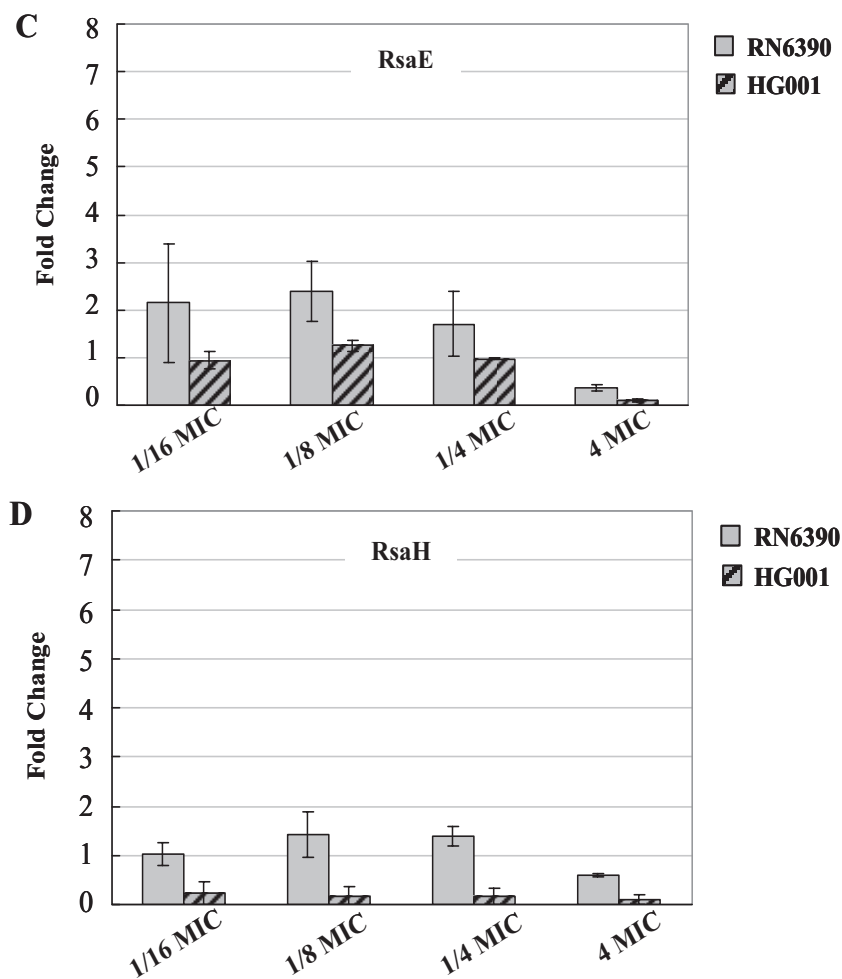


Figure 3-4 Comparison of the sRNA expression after exposure to oxacillin of RN6390 and HG001. Fold changes of sRNA expression levels with/without exposure to oxacillin, shown for RNAIII (panel A), RsaA (panel B), RsaE (panel C) and RsaH (panel D).

3.3.4 Influence of levofloxacin on the expression of sRNA

- Expression level difference among sRNAs

The ratio of sRNA versus *gyrB* was disparate for individual sRNA, even in the same strain and same conditions. Among the five sRNAs analyzed, either in RN6390 or HG001, RNAIII was much more expressed than the other four sRNAs, whose expression was at least ten folds of *gyrB* while growing without any antibiotics, whereas RsaG was the lowest expressed, sometimes close to 0.001 fold of *gyrB*. In RN6390 (σ^B), the ratio of RsaA versus *gyrB* was close to that of RsaE, and slightly less than that of RsaH. In HG001 (σ^{B+}), the ratio of RsaA, E and H versus *gyrB* were very close (Figure 3-5).

- Relation between expression level of sRNA and levofloxacin concentration

One thing noticed was that the amount of sRNA level per amount of *gyrB* did not start to decrease until the concentration of levofloxacin got more than 1/4 MIC (Figure 3-5). With 2 folds as a threshold, no significant increase change of relative sRNA level (in presence of levofloxacin vs. absence of levofloxacin) was observed, except RsaH in the presence of 1/16 MIC and 1/8 MIC of levofloxacin. And this over expression of RsaH happened in strain RN6390 (σ^{B-}) other than HG001 (σ^{B+}) (Figure 3-6).

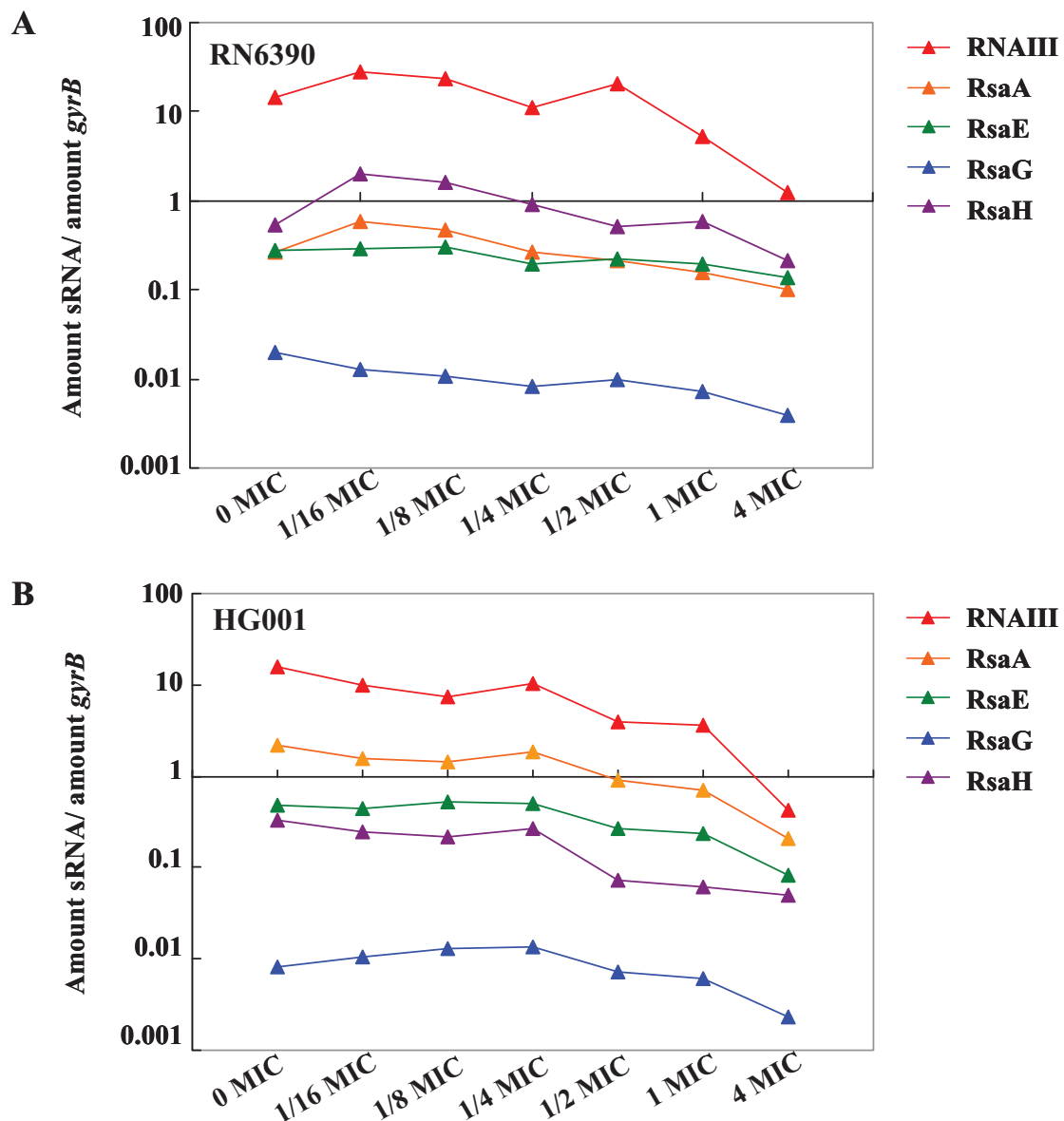
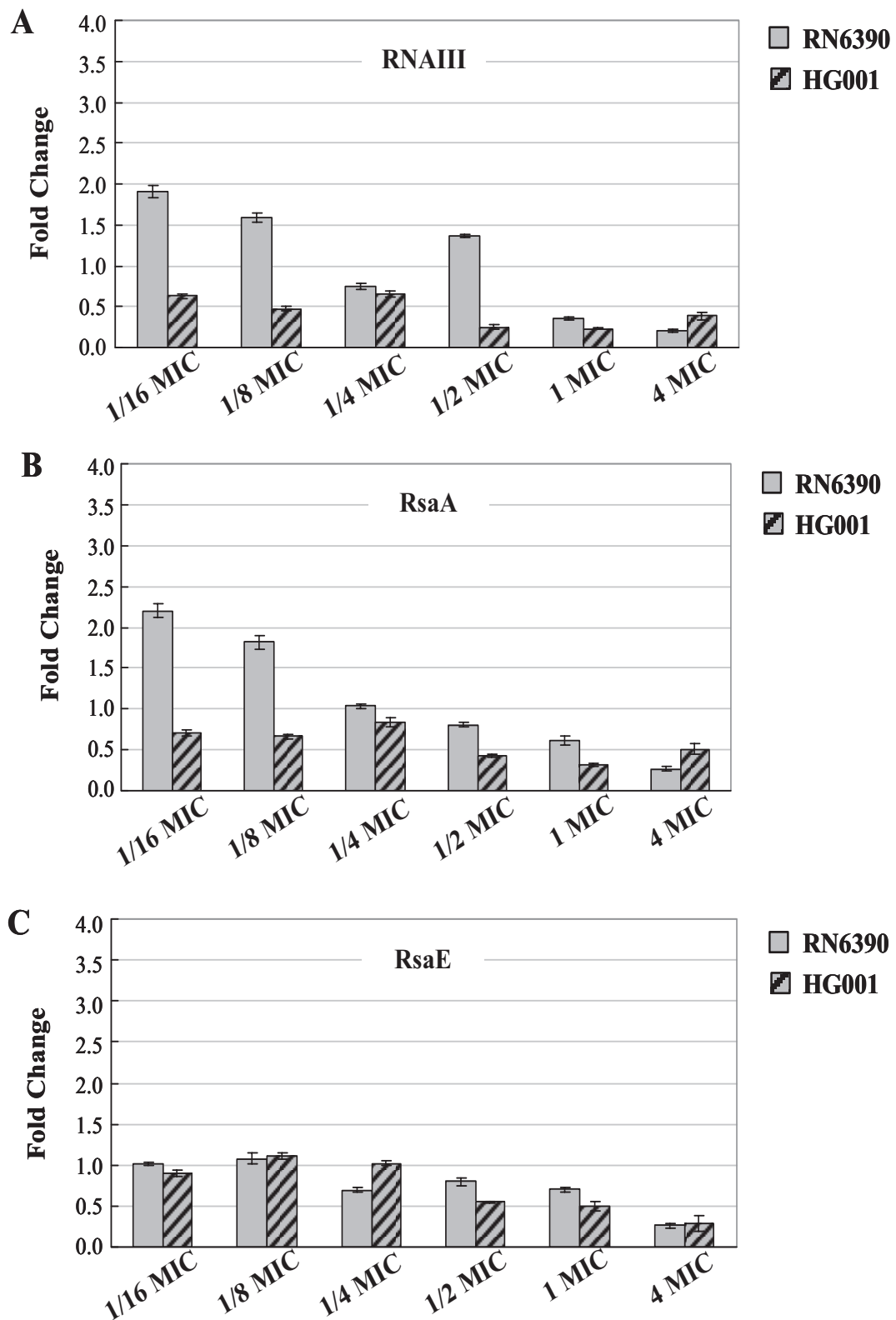


Figure 3-5 Relative sRNA expression ratio to *gyrB* in RN6390 (shown in A) and HG001 (shown in B) under the stress of serial concentrations of levofloxacin. Data was the average of two separate experiments.



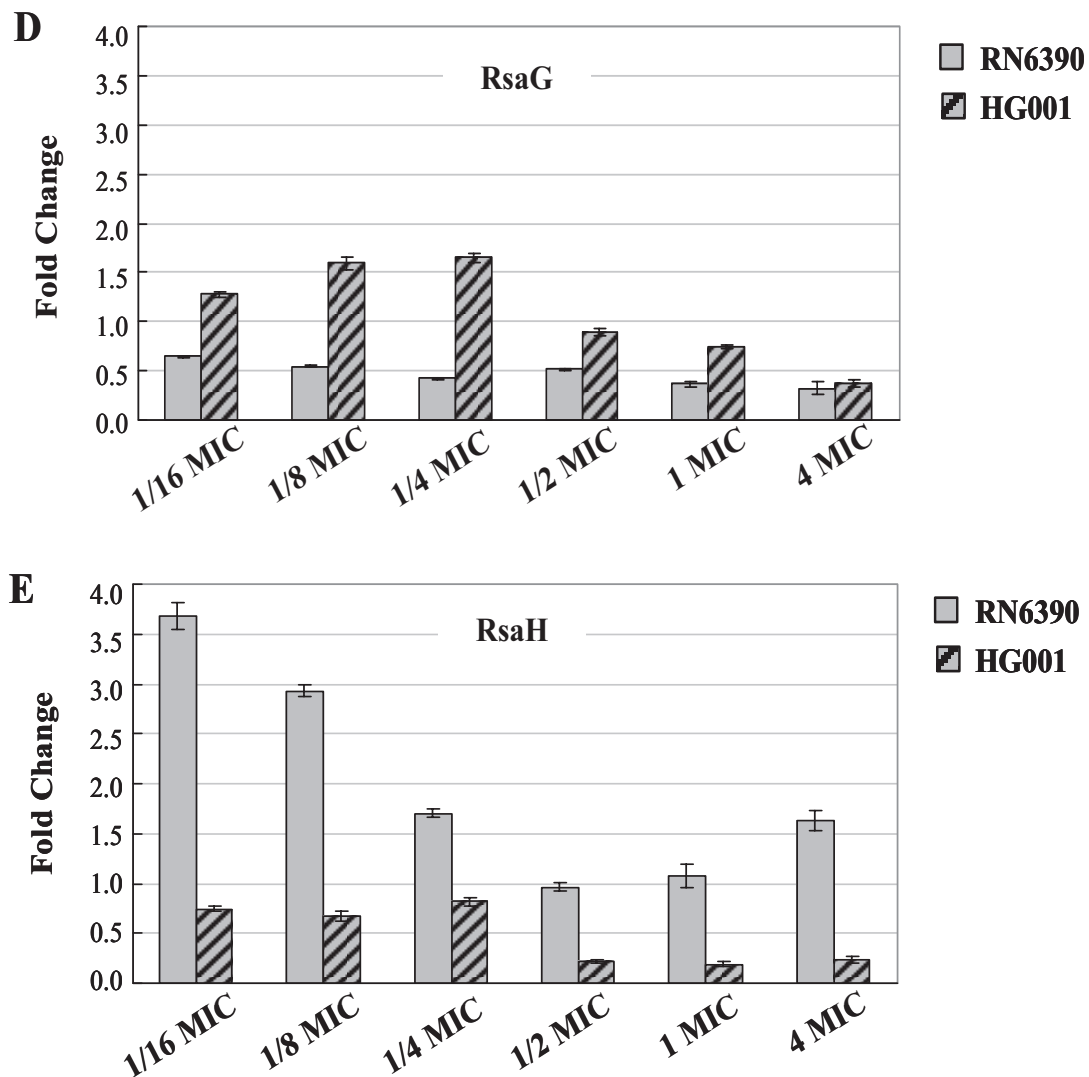


Figure 3-6 Comparison of sRNA expression between RN6390 and HG001 exposed to levofloxacin. Fold changes of sRNA expression levels with levofloxacin, relative to them without levofloxacin were represented-RNIII (shown in A), RsaA (shown in B), RsaE (shown in C), RsaG (shown in D) and RsaH (shown in E).

3.4 Conclusion

To sum up, there is a reducing trend of sRNA expression by the antibiotic levofloxacin once its concentration is above 1/4 MIC. For oxacillin, similar reduction happens with 4 MIC (the only concentration higher than 1/4 MIC tested with this antibiotic). This phenomenon is in parallel with that of bacterial growth (Figure 3-2), however, we can not conclude that this is just due to the negative impacts of antibiotics on bacterial growth, since enough density of *S. aureus* cells was obtained and standardized in the experimental procedure. It might have a correlation with the DNA damage involved in the function of levofloxacin.

Moreover, we found that subinhibitory concentrations (1/16 and 1/8 MIC) of levofloxacin could enhance the RsaH expression in RN6390, not in HG001. Thus it can be hypothesized that this up-regulation on RsaH is σ^B - independent. Alternatively this up-regulation may be repressed by σ^B since it occurs only in σ^B - strain. To confirm these finding, further experiments on RsaH variation in other strains (e.g. clinical strains) or with other antibiotics of the same family (e.g. moxifloxacin) are necessary.

Chapter 4 The Rsa expression in clinical samples

4.1 Introduction

It is now established that sRNAs are capable to regulate many bacterial physiological activities, which help bacteria to adapt to various environmental changes or participate in complex networks of gene regulation ^[1,3,28-29].

In *S. aureus*, RNAIII is a well studied regulatory RNA responsible for controlling the switch between the expression of adhesin during exponential growth phase and the expression of toxins during stationary growth phase ^[151]. Since the discovery of RNAIII, many other small RNAs have been identified in *S. aureus*, most of them encoded on the core genome while a few are located on mobile genetic elements ^[11,15,18-20,288].

This chapter is dedicated to reveal the features of sRNA expression during *S. aureus* colonization and chronic/acute infection. *In vitro* studies demonstrated that most of the sRNAs are expressed in several *S. aureus* strains and often accumulate in the late-exponential growth phase ^[11]. Nevertheless, their expression in animal models or human infections has not yet been investigated, except for RNAIII. The expression of RNAIII was shown to be reduced both in the sputum of cystic fibrosis (CF) patients and human cutaneous abscesses compared to *in vitro* culturing conditions ^[310-311]. In view of the complicated physical micro environment and the immune defense system of the host, we have hypothesized that the expression of sRNAs would be different between *in vivo* and *in vitro*, which may give us a relevant and visual information about the roles of sRNAs in the staphylococcal pathogenesis in human.

4.2 Results in brief

For the clinical isolates growing in rich medium, a specific expression profile of these sRNAs was observed: RNAIII and RsaA were highly expressed, whereas RsaE and RsaG were poorly expressed. And there was a parallel correlation of the sRNA level between mid- and late-exponential growth phases, e.g. the isolate exhibiting a low expression level at the mid-exponential growth phase, always exhibited a low expression level at the late-exponential growth phase. Furthermore, there was a link between the levels of different sRNAs, e.g. the isolate with low RNAIII expression always showed low levels of RsaA, E, G and H.

For the human samples, first of all, the *in vivo* relevance of all the five sRNAs was

confirmed by their direct detection in samples. However, the expression profile observed *in vivo* differed with that seen *in vitro*. No link was observed between the levels of different sRNAs. Interestingly, when comparing with the highly variable expression levels of the 5 sRNAs in abscess samples, they were more homogeneous in cystic fibrosis samples, and showed a uniform pattern in the nasal samples.

In short, our study revealed that the 5 sRNAs are produced by all the clinical isolates when culturing in BH medium to mid or late-exponential growth phases. And the expression of these sRNAs *in vivo* has its own characteristics distinct to that *in vitro*. The uniform sRNA expression pattern in nasal colonization samples may reflect the state of commensalism of *S. aureus* in the anterior nares.

4.3 Article

The article was published in journal « PloS ONE » (May 2012; volume 7, issue 5; e37294). However, it has been removed from this version, in order to comply with copyright rules.

Chapter 5 Interference between *S. aureus* and *P. aeruginosa*

5.1 Introduction

In the patients with immune deficiency, such as cystic fibrosis (CF), most common cause of death is associated with long-term respiratory infection. The existence of large quantity of mucus in the CF lung is beneficial for polymicrobial infections, mainly represented by *P. aeruginosa* and *S. aureus*, etc^[312-315]. So how do the two pathogens interact with each other while co-existing? It was reported that several quorum-sensing exoproducts secreted from Gram-negative bacteria such as *P. aeruginosa* could exert its influences on Gram-positive bacterial such as *S. aureus*. For instance, long-chain 3-oxo-substituted N-acylhomoserine lactones (AHL) inhibit the *agr*-mediated quorum-sensing system and the growth of *S. aureus* in a concentration dependent manner; 4-hydroxy-2-heptylquinoline-N-oxide (HQNO) induces the biofilm and small colony variant (SCV) formation of *S. aureus* by activating sigma B factor^[316-318]. Moreover, LasA protease (also termed staphylolysin) secreted by *P. aeruginosa*, whose cell wall degrading activity resembles that of lysostaphin (LS), can cause lysis of a wide range of *S. aureus* strains; it is thus considered as a potential therapeutic agent against *S. aureus* infection^[319-320].

Our objective is to study the *S. aureus* sRNAs expression changes when co-cultured with *P. aeruginosa*, and to identify the molecules responsible for these effects.

5.2 Methods and Materials

5.2.1 Strains and chemical molecules

All the strains used in this project are listed in table 5-1. For the growth of *E. coli*, Luria-Bertani (LB) broth or agar (bacteriological tryptone 1%, yeast extract 0.5%, NaCl 1%, bacteriological agar 1.5% for solid phase, pH 7.0) was used.

For the growth of *S. aureus*, *P. aeruginosa* and *Enterococcus faecalis*, brain-heart infusion broth (BHI) or tryptic soy agar with 5% sheep blood agar plates (TSS, Biomerieux SA) were used. M9 Minimal media (1×M9 salts, 2 mM MgSO₄, 0.1 mM CaCl₂, and 0.4% glucose) was also used to check whether interference of bacteria is independent or not of medium.

Two major quorum sensing N-Acylhomoserine lactone (AHL) molecules, N-(3-Oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and N-butanoyl-homoserine lactone (C4-HSL) were kindly provided by Florence Wisniewski (UMR CNRS 5557, Lyon).

They were dissolved by using dimethyl sulfoxide (DMSO) to a stock solution of a concentration of 6 mM and kept at -20°C. Lysostaphin (Sigma) was diluted to 1.25 mg/ml as stock solution and stored at -20°C for use.

Table 5-1 Strains used in this study

Strains / Plasmids	General Characteristics	Source / Reference
Strains		
<i>E. coli</i>		
ATCC25922	Wild type strain, clinical isolate	Laboratory stock
<i>E. faecalis</i>		
ATCC29212	Wild type strain, clinical isolate	Laboratory stock
<i>S. aureus</i>		
RN6390	Derivative of 8325-4, <i>agr</i> positive	[297]
HG001	<i>rsbU</i> ⁺ strain derivative of RN6390	[299]
<i>P. aeruginosa</i>		
ATCC15442	Wild type strain, clinical isolate	Laboratory stock
PAO1	Wild type strain, <i>chl</i> -2 ^a , clinical isolate	[321]
PAO6395	$\Delta lasR$ derivative of PAO1	[322]
PAO6281	<i>gacA</i> :: Ω Sm/Sp ^b	[323]
PAO6711	$\Delta cbrB$ derivative of PAO1	[324]
PT712	PT5 $\Delta rhIA$::Gm ^c	[325]
PAO6344	$\Delta hcnB$ derivative of PAO1	[326]
PAO1-pqsA	$\Delta pqsA$ derivative of PAO1	[327]
PAO1-pvdS	$\Delta pvdS$ derivative of PAO1	[328]

^a *chl*-2 refers to spontaneous chloramphenicol resistance

^b Sm/Sp stands for gene of streptomycin and spectinomycin resistance

^c Gm stands for gene of gentamycin resistance

5.2.2 Co-culture *P. aeruginosa* / *S. aureus*

An overnight culture of *P. aeruginosa* was prepared in BHI at 37°C. The next morning, it

was treated as follows:

- a) 5 ml *P. aeruginosa* live culture
- b) 5 ml *P. aeruginosa* culture was heated to boil for at least 5 min
- c) 5 ml *P. aeruginosa* culture was filtrated with 0.22 µm membrane
- d) 5 ml *P. aeruginosa* culture was firstly filtrated with 0.22 µm membrane and then heated by boiling at least 5 min.

Simultaneously, *S. aureus* strains (RN6390 or HG001) were inoculated from fresh blood agar plates into 250 ml BHI, and then incubated at 37°C, with shaking (190 rpm). OD₆₀₀ value was measured every hour. When OD₆₀₀ of *S. aureus* culture reached 0.5, 0.8 or 2.0, it was divided into 5 flasks, 45 ml each. Within 4 of the 5 flasks, 5 ml of differently pre-treated *P. aeruginosa* culture (as described above) was added, except to the last flask that served as control. After incubating at 37°C with shaking for 30 min, 5~10 ml bacterial culture was collected from each of the 5 flasks. After centrifugation at 4,000 rpm for 10 min, at 4°C, the pellet was stored at -80°C.

5.2.3 *S. aureus* co-culturing with other bacteria or chemical molecules

- Co-culturing with other bacteria

Similar experiments were carried out as described above but with *E. coli* or *E. faecalis* live cultures (volume ratio between *E. coli* or *E. faecalis* and *S. aureus* = 1: 9).

- Co-culturing with AHL molecules

When *S. aureus* culture reached an OD₆₀₀ of 0.8, a volume of 125 µl of 3OC12HSL or C4HSL solution (6 mM) was added to 50 ml of *S. aureus* culture to a final concentration of 15 µM. For the negative control of *S. aureus* culture, 125 µl of DMSO was added.

- Co-culturing with lysostaphin

Serial concentrations of lysostaphin (0.5 µg/ml, 0.2 µg/ml, 0.1 µg/ml, 0.01 µg/ml and 0.001 µg/ml) were used to interfere with the *S. aureus* culture at an OD₆₀₀ of 0.8.

5.2.4 RNA extraction and RT-PCR

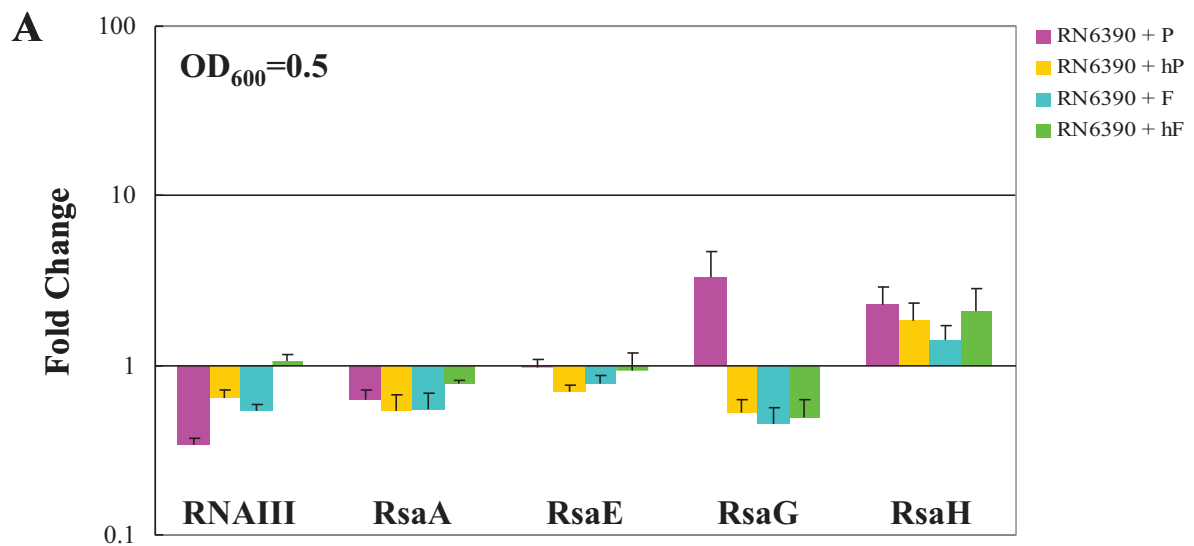
Cell lysis and RNA isolation was performed with the QIAGEN RNeasy Plus kit as recommended by the manufacturer. Reverse-transcription PCR was done as described in

chapters 3 and 4.

5.3 Results

5.3.1 RsaG and RsaH levels are increased by co-culturing with *P. aeruginosa* and its substrates

The expression levels of sRNAs - RNAIII, RsaA, RsaE, RsaG and RsaH in *S. aureus* were analyzed after its exposure to live culture and other pre-treated culture of *P. aeruginosa*. Among the 3 time points ($OD_{600}=0.5$, $OD_{600}=0.8$ and $OD_{600}=2.0$) when *P. aeruginosa* ATCC15442 interference was applied, it was observed that RsaH level was strongly increased (over 12 folds) by the addition of *P. aeruginosa* ATCC15442 live culture at $OD_{600}=0.8$. And the level of RsaG was increased (between 2.8 and 4.3 folds) only when live culture of *P. aeruginosa* had been added. No significant changes were observed for other sRNAs (Figure 5-1).



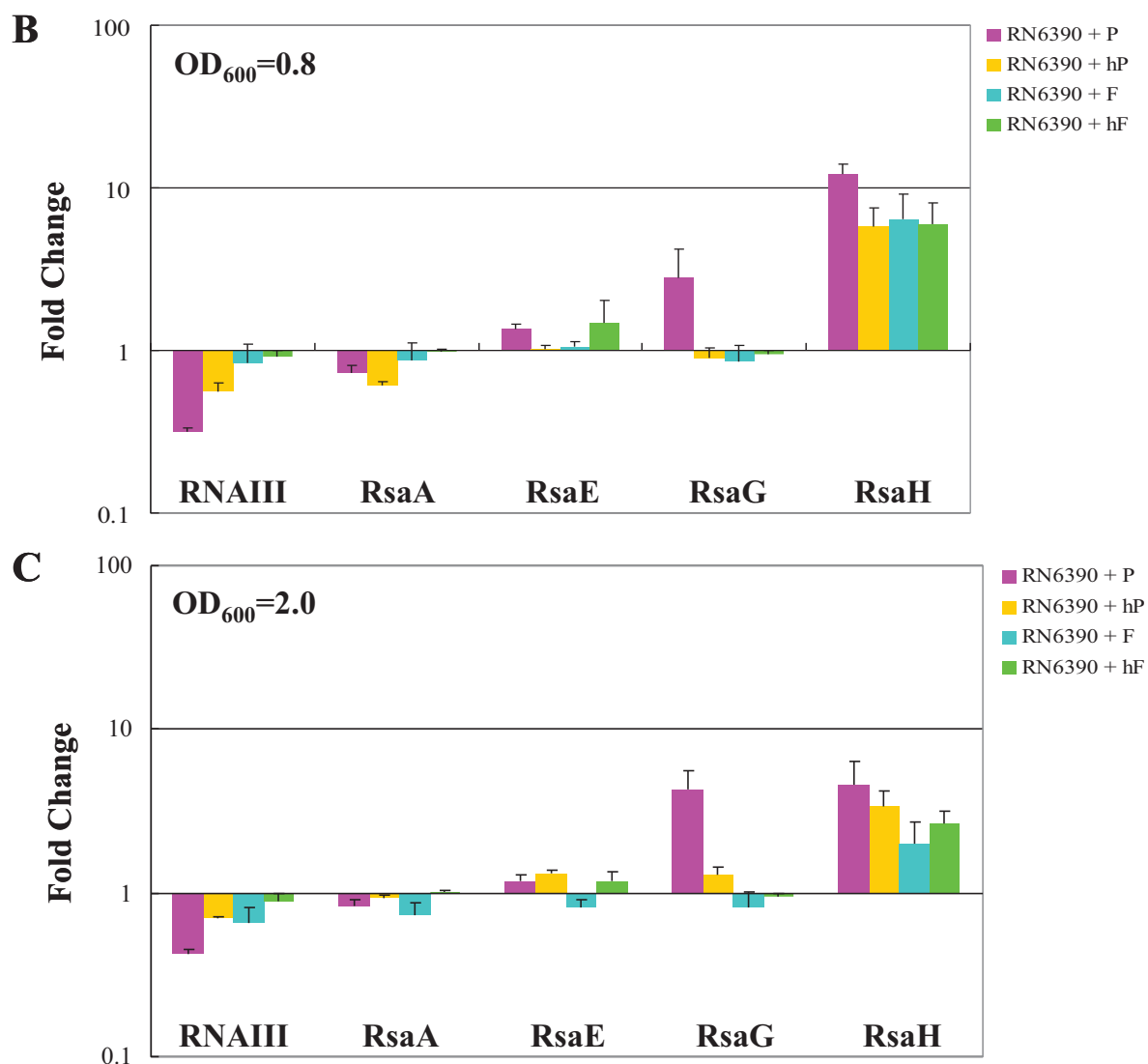


Figure 5-1 Comparison of sRNA expression in RN6390 (sigB-) exposed to *P. aeruginosa* ATCC15442 versus that without ATCC15442. Fold change indicates the ratio of the level of expression in the presence of *P.aeruginosa* vs. control. 6390+P means the interference of live *P. aeruginosa*; 6390+hP means the interference of heated *P. aeruginosa*; 6390+F means the interference of filtrate; 6390+hF means the interference of inactivated *P. aeruginosa*. Three time points were chosen for the ATCC15442 addition, when OD_{600} reached at 0.5 (A), 0.8 (B) and 2.0 (C).

As the signal-sensing system in *S. aureus* usually involves the alternate sigma factor B (sigB), we checked these 5 sRNA expressions in a sigB+ isogenic strain (HG001) in the presence of *P. aeruginosa*. It turned out that the increase of RsaH was similar in both sigB+ and sigB- strains (over 12 folds), and there is also an increase of RsaG in HG001 (over 8 folds) when co-culturing with *P. aeruginosa* live culture (Figure 5-2).

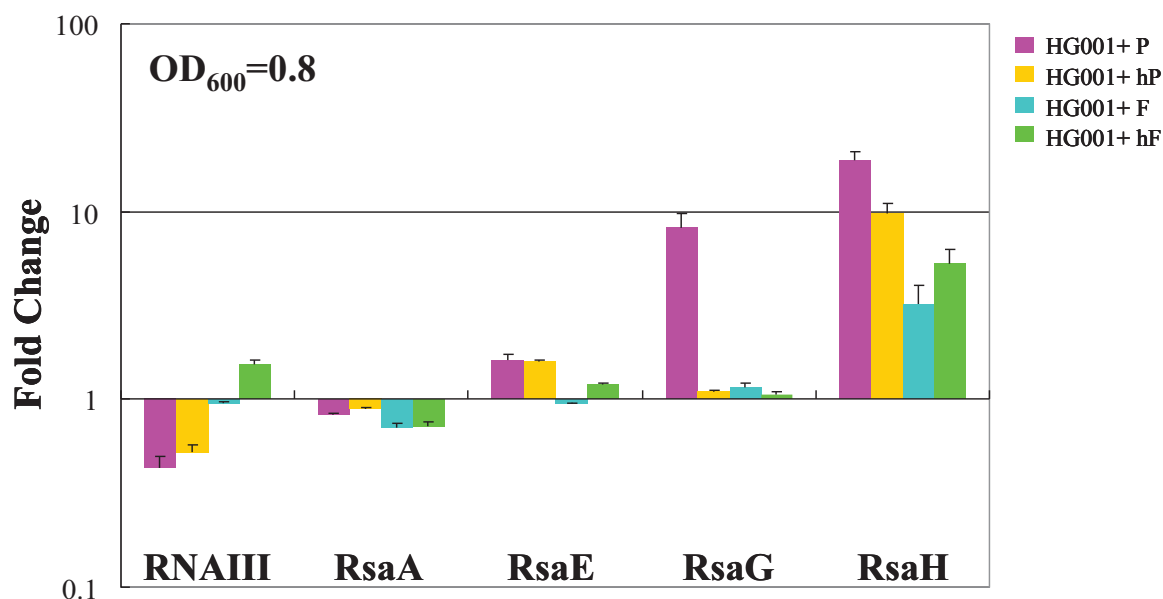


Figure 5-2 Comparison of sRNA expression in HG001 (sigB+) exposed to *P. aeruginosa* ATCC15442 versus that without ATCC15442. Fold change indicates the ratio of the level of expression in the presence of *P.aeruginosa* vs. control. HG001+P means the interference of live *P. aeruginosa*; HG001+hP means the interference of heated *P. aeruginosa*; HG001+F means the interference of filtrate; HG001+hF means the interference of inactivated *P. aeruginosa*.

In addition, another widely used *P. aeruginosa* wild type strain – PAO1, and several major regulator mutants of *P. aeruginosa* were tested as the interference source for RN6390. No RsaG variation was observed. However, PAO1 and PAO6395 ($\Delta lasR$) induced the RsaH expression, while PAO6281, PAO6344, PAO6711, PT712, PAO1 $\Delta pqsA$, PAO1 $\Delta pvdS$ had no effects (Figure 5-3).

All the data obtained above were based on bacterial growth in rich-medium – BHI. We tried to carry out the experiment with poor-medium, like M9 minimal media, unfortunately *P. aeruginosa* grew so badly in this minimal media that we could not proceed with it.

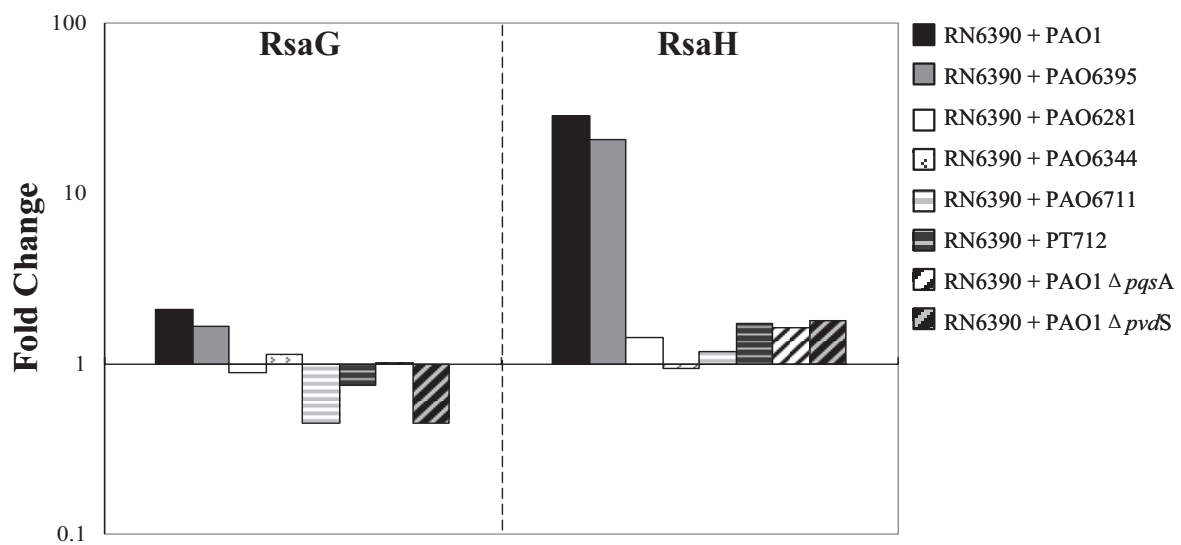


Figure 5-3. Differential expression of RsaG and RsaH after exposure to various *P. aeruginosa* strains. Fold change indicates the ratio of the level of expression in the presence of *P. aeruginosa* vs. control.

5.3.2 RsaH expression is increased by *P. aeruginosa* but not by other bacteria

To determine whether the sRNA response to *P. aeruginosa* was specific, we analyzed these sRNA expressions in RN6390 and HG001 in the presence of either *E. coli* (Gram-) or *E. faecalis* (Gram+). As shown in figure 5-4, there was no apparent effects observed, basically all the sRNA expression stayed the same when comparing their expression with and without *E. coli* / *E. faecalis*. Thus, it seems that the overexpression of RsaH is specific to the presence of *P. aeruginosa*.

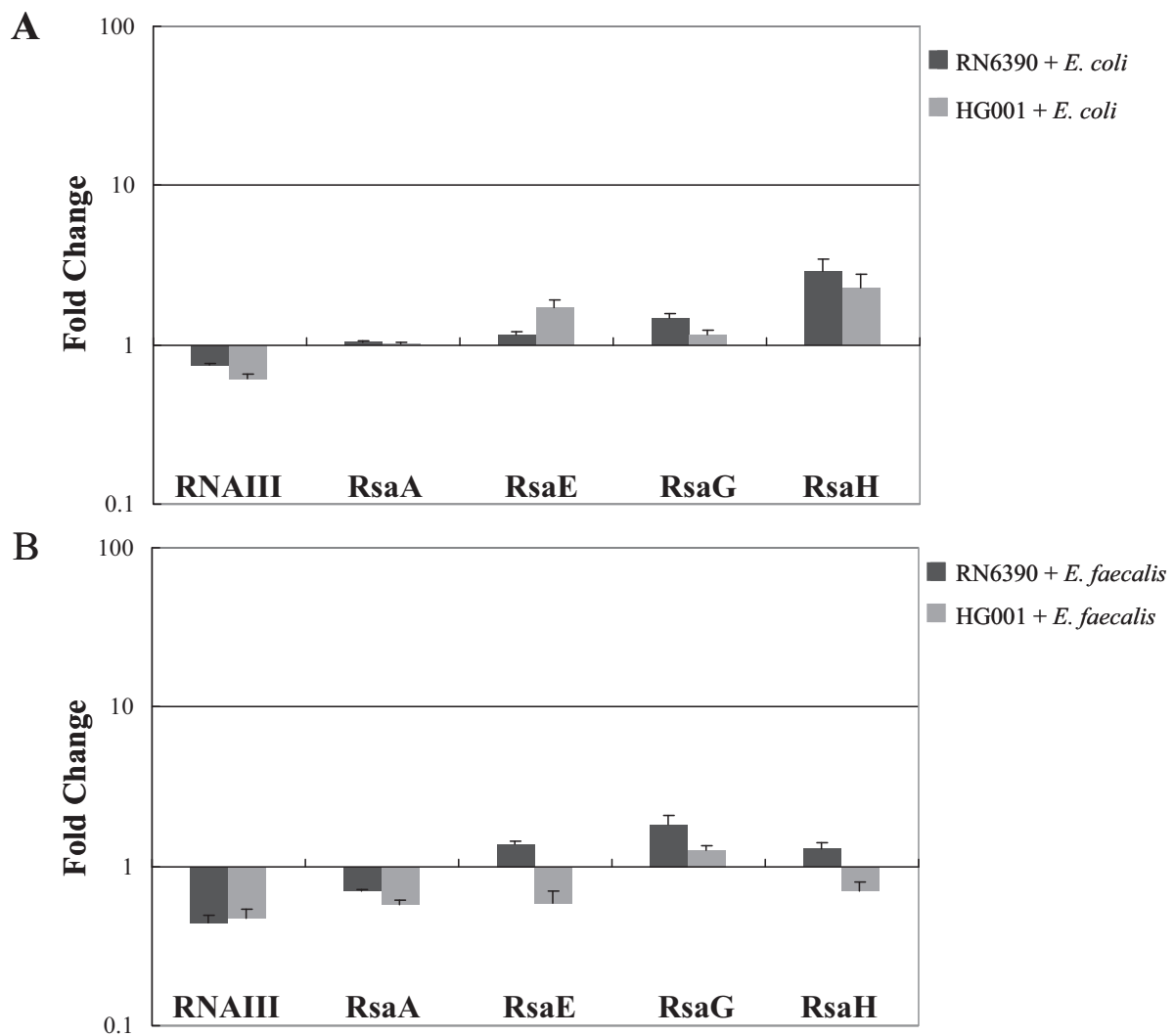


Figure 5-4 sRNA expression changes in RN6390 (sigB-) / HG001 (sigB+) exposed to *E. coli* (A) and *E. faecalis* (B). Fold change indicates the ratio of the level of expression in the presence of bacteria vs. control.

5.3.3 No molecule tested is responsible

The above experiments indicated that the mechanisms of up-regulation of RsaG and RsaH were different: RsaG was increased only by live culture of *P. aeruginosa*, but not by heated and filtrated culture, indicating that the regulator of RsaG was neither heat stable nor secreted substance(s). Conversely, RsaH was increased in RN6390 and HG001 by *P. aeruginosa* live culture and treated culture, suggesting that its regulation probably involves a heat stable secreted substance, and the interaction among live bacteria is more complex than that between bacteria and substance. In addition, the use of various *P. aeruginosa* strains and

their derivative produced inconsistent data in that PAO1 wild type and its $\Delta lasR$ derivative induced RsaH expression but not any of the other five derivatives of this *P. aeruginosa* strain.

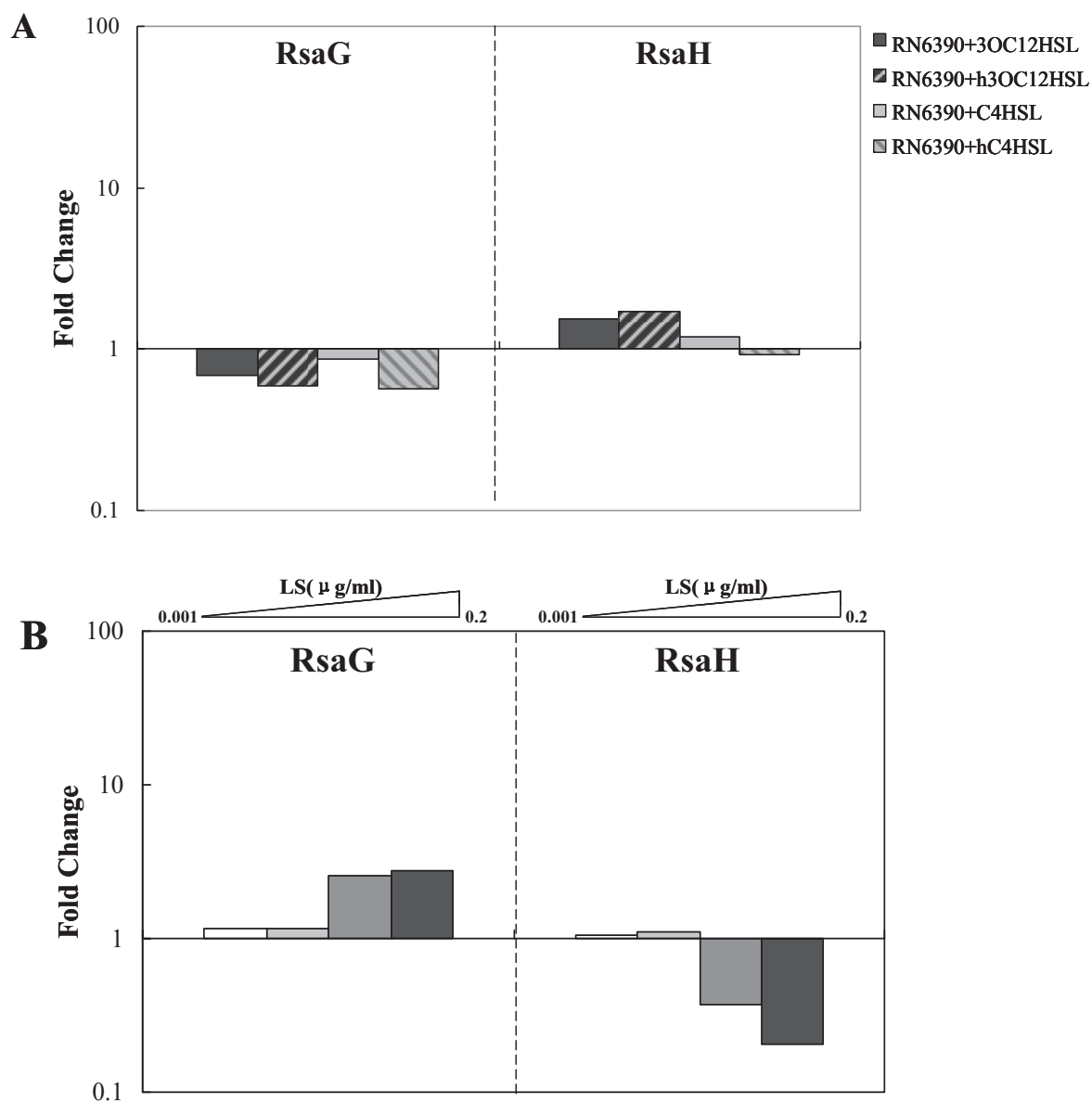


Figure 5-5 RsaG and RsaH expression changes after the interference of AHL molecules (A) and LS (B). A, h3OC12HSL stands for heated 3OC12HSL, hC4HSL stands for heated C4HSL; B, 4 concentrations of LS were used: 0.001 μg/ml, 0.01 μg/ml, 0.1 μg/ml and 0.2 μg/ml.

In an attempt to identify the secreted molecules that could be involved in the above effects (especially those observed with RsaH), we analyzed RsaG and RsaH expression with addition of AHL molecules and LS. None of the AHL molecule enhanced the level of RsaG or RsaH (Figure 5-5A). When LS concentration arrived at 0.1 µg/ml or more, a slight enhancement of RsaG level (2.5-2.8 folds) was observed, whereas the RsaH level was decreased (2.7-4.9 folds). When 0.5 µg/ml LS was added, most of RN6390 bacteria cell died (culture color became very clear).

5.4 Conclusion

In this chapter, we simulated a co-existence circumstance *in vitro* for *S. aureus* and *P. aeruginosa*, and detected the sRNA variation of *S. aureus*. Only the *P. aeruginosa* live cells can promote the RsaG expression in *S. aureus*, and this phenomenon is more obvious in HG001 than RN6390. Both *P. aeruginosa* live bacteria and its secreted molecules could enhance the RsaH expression, although live bacteria had the most striking effect on RsaH expression. However, the mechanism is still undetermined. *lasR* seems not to be involved in the RsaH regulation directly, as shown in our study, neither are AHL nor LS molecules.

Chapter 6 Search for Rsa regulators

6.1 Introduction

Given that RsaH is more expressed when co-culturing with *P. aeruginosa*, it is an intriguing question that which regulators participate in the modulation of RsaH expression. To screen the possible involvement of major regulators from *S. aureus*, we screened different *S. aureus* strains with mutations of some global regulators such as Agr, Rot, SarA, SarH1, Arl. Moreover, in order to search transcriptional regulators involved in RsaH expression, an affinity chromatography approach with the promoter region of RsaH has been undertaken. The knowledge of the regulators of RsaH expression will give us hints about the targets and function of this regulatory RNA.

6.2 Methods and Materials

6.2.1 Strains and media

Both *S. aureus* and *P. aeruginosa* strains were cultured in the Brain-Heart Infusion broth (BHI) or tryptic soy agar with 5% sheep blood agar plates (TSS, bioMérieux SA).

Mutants of major regulators in *S. aureus* are listed in table 6-1.

Table 6-1 Strains used in this study

Strains	General Characteristics	Source / Reference
<i>S. aureus</i>		
RN6390	Derivative of 8325-4, <i>agr</i> positive	[297]
LUG1054	RN6390 Δ <i>sarHI-kana</i> ^a	Benito and Boisset unpublished
LUG1063	RN6390 <i>sarA::ermC</i> phiSLT	[171]
LUG1064	PMN466 phi SLT ^b Δ <i>agr</i>	Teamwork, unpublished
LUG1066	BF phi SLT ^b Δ <i>arl</i>	Teamwork, unpublished
LUG1160	RN6390 Δ <i>rot-cat</i> ^c	[171]
<i>P. aeruginosa</i>		
PAO1	Wild type strain, <i>chl-2</i> ^d , clinical isolate	[321]

^a *kana*: kanamycin resistance gene

^b phi SLT: Panton-Valentine leukocidin(PVL)-converting temperate bacteriophage

^c *cat*: chloramphenicol resistance gene

^d *chl-2* : spontaneous chloramphenicol resistance

6.2.2 Bacterial interference culture

Culture of *S. aureus* mutants were mixed with overnight culture of *P. aeruginosa* strain PAO1 at mid-exponential growth phase ($OD_{600} = 0.8$). More details are described in chapter 5.

6.2.3 RNA extraction and RT-PCR

Cell lysis and RNA isolation was performed with the QIAGEN RNeasy Plus kit. Reverse-transcription PCR was done as described in chapters 3 and 4.

6.2.4 DNA biotinylation

- Chromosome DNA was extracted from RN6390 by using commercial kit (QIAGEN), following the instructions of the manufacturer.
- Design biotin labeled primers and PCR

Biotin modification was added ^[329] at 5' end of one of the primer pairs for each sRNA. All the primers used are shown in table 6-2.

Conventionnal PCR was done as described in chapter 2, and the annealing temperature was chosen according to their own T_m value. PCR product was checked on 2% agarose gel.

Table 6-2 Primers used in this study

Primers	Sense(+/-)	5' to 3' sequence	T_m (°C)
Prom-RsaA-biotin	+	biotin-CACTATAAAATAACCACATGAGC	59
RsaA-R	-	CCGAGTAGTCTTCCTTGG	56
Prom-RsaE-biotin	+	biotin-CGCTTACATTATGAACAGATTG	58
RsaE-R	-	TCTGAATACACGACGCTAAA	53
Prom-RsaG-biotin	+	biotin-TCGGATTAAAAGTATCGCCAATC	59
RsaG-R	-	CTAAGTCGGGCAAATAAGGATAC	58
Prom-RsaH	+	GGTTATACATTATTTAGACAATAATATGT C	56

RsaH-biotin	-	biotin-CGATTAACGTACGGGTCCAC	55
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– PCR product was further purified by using the High Pure PCR Product Purification Kit (Roche).

6.2.5 Whole protein extraction

– Bacterial culture

RN6390 was cultivated in 100 ml BHI at 37°C with shaking (190 rpm) until its OD₆₀₀ reached 0.8, then 45 ml culture was taken out and mixed with 5 ml supernatant of PAO1 overnight BHI culture, whereas the rest of 50 ml RN6390 culture served as control. After incubation at 37°C with shaking for another 30 min, they were centrifuged at 4,000 rpm, at 4°C, 10 min. Pellet was kept at -80°C.

– Protein extraction

Pellet was re-suspended in 1 ml lysis buffer (1 ml binding buffer + 1 mM Dithiothreitol (DTT) + Protease inhibitor 10 µl). Then 10 µl lysostaphin (Sigma) was added.

The mixture was incubated at 37°C for 30 min, and then bacterial cells were disrupted by sonication for 3 min, on ice. Afterwards, a centrifugation at 13,200 rpm was carried out at 4°C for 10 min.

6.2.6 DNA affinity-chromatography

– Preparation of solution:

Binding buffer (10 mM Tris-HCl, 50 mM HCl, 50 mM KCl, 10 mM MgCl₂, 1mM EDTA, 10% Glycerol).

Elution buffer (same compositions of binding buffer with 2 M NaCl) were prepared and stored at 4°C.

– DNA affinity-chromatography

The supernatant above was mixed thoroughly with 60 µg biotinylated PCR product, and incubated at 4°C with rotation for 20 min. Meanwhile, the BioLogic Duoflow chromatography system (Bio-Rad) was connected, and a HiTrap™ Streptavidin HP column (GE) was used. Binding buffer connected to pump A was referred as Buffer A, and similarly

elution buffer connected to pump B was referred as Buffer B in the program.

The program was set as following:

(1) Pre-run step (30 min, 1 ml/min)

Isocratic flow: buffer A 100%, buffer B 0%, 8 ml

Zero baseline, UV detector

Isocratic flow: buffer A 100%, buffer B 0%, 2 ml

Isocratic flow: buffer A 0%, buffer B 100%, 10 ml

Isocratic flow: buffer A 100%, buffer B 0%, 10 ml

(2) Run step (49 min, 1 ml/min)

Collection Fractions within 1 time window ending at 43 min

Zero baseline, UV detector;

Isocratic flow: buffer A 100%, buffer B 0%, 8 ml

Load sample, auto inject valve, 2 ml

Isocratic flow: buffer A 100%, buffer B 0%, 2 ml

Isocratic flow: buffer A 100%, buffer B 0%, 15 ml

Isocratic flow: buffer A 80%, buffer B 20%, 3 ml

Isocratic flow: buffer A 60%, buffer B 40%, 3 ml

Isocratic flow: buffer A 40%, buffer B 60%, 3 ml

Isocratic flow: buffer A 20%, buffer B 80%, 3 ml

Isocratic flow: buffer A 0%, buffer B 100%, 10 ml

Then 1 ml protein/DNA reaction mixture was injected into the system. At the end of program, protein fractions binding to streptavidin columns were eluted in tubes by elution buffer. They were concentrated and purified by using Vivaspin 6 (0.2 μ m, membrane 10,000 MWCO-PES, Sartorius Stedim Biotech GmbH).

– SDS-PAGE

Purified proteins were separated by SDS-PAGE, and stained by using Coomassie Brilliant Blue R-250 for visualization.

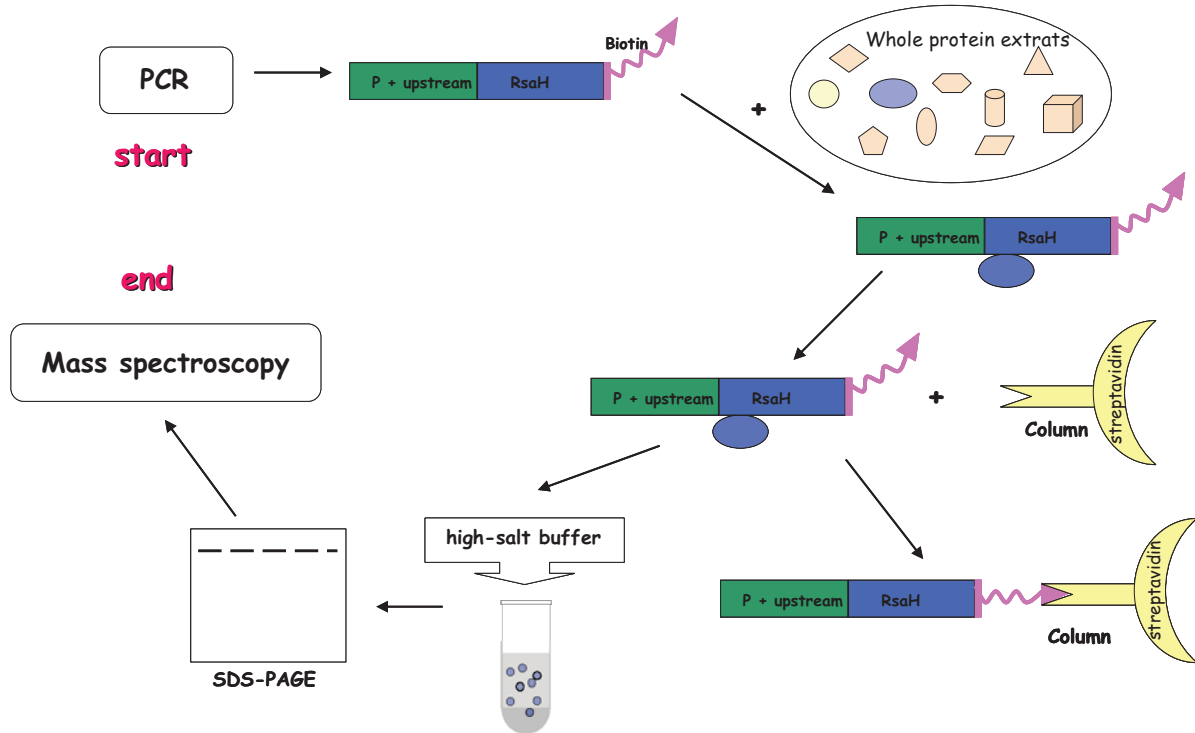


Figure 6-1 Principle of affinity chromatography to search for transcriptional regulators in *S. aureus*, with RsaH as an example.

6.3 Result

6.3.1 RsaH in all the *S. aureus* mutants is increased

Like the *S. aureus* wild type strain RN6390, several *S. aureus* mutants ($\Delta sarH1$, $\Delta sarA$, Δagr , Δarl , Δrot) were treated with the *P. aeruginosa* strain PAO1 live culture. After comparing sRNA expression with vs. without PAO1, RsaH was found more expressed by *P. aeruginosa* interference in RN6390 (12 folds). And the increase of RsaH was also observed in these *S. aureus* mutants, however more in $\Delta sarH1$ (16 folds), $\Delta sarA$ (11 folds), Δarl (12 folds), than in Δagr (7 folds) and Δrot (6 folds).

RsaG was increased by *P. aeruginosa* interference in RN6390 (3 folds), and more increased in $\Delta sarA$ (5 folds). In contrast, no increase of RsaG showed in $\Delta sarH1$, Δagr , Δarl , Δrot (with 2 folds as threshold).

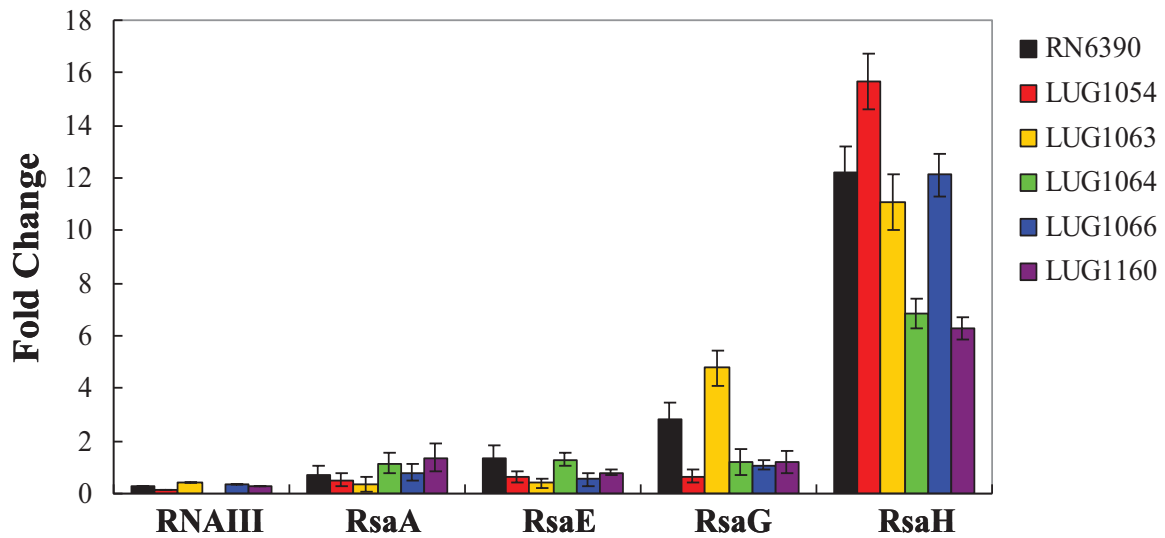


Figure 6-2 Fold changes of sRNA expression of *S. aureus* in presence of *P. aeruginosa* strain PAO1, against to those in absence of PAO1. Data is the average of two separate experiments.

6.3.2 More proteins bind to RsaH in *S. aureus*/*P. aeruginosa* co-culture

We produced the biotinylated sRNA sequence with promoter region by using biotin labeled primers and PCR (*rsaH* as an example, shown in figure 6-3).

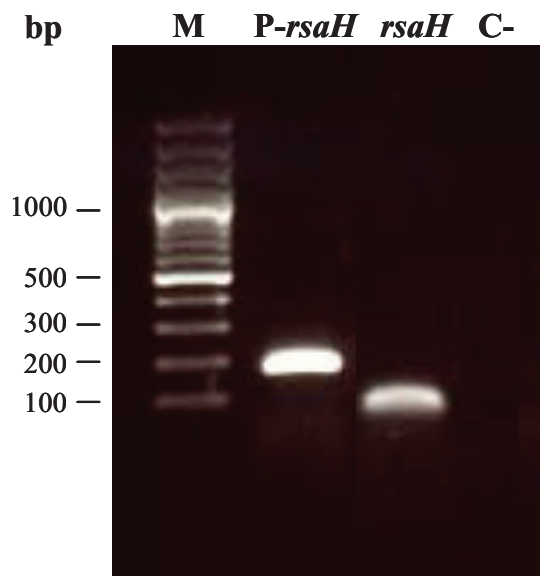


Figure 6-3. Electrophoresis on 2% agarose gel to control the biotinylated *rsaH* sequence with the promotor region. M: 100 bp (Fermentas); P-*rsaH*: biotinylated *rsaH* sequence with promotor region; *rsaH*: normal *rsaH* sequence.

As shown in figure 6-4, *rsaH* affinity chromatography revealed that there was more protein-binding to *rsaH* in *S. aureus*/*P. aeruginosa* co-culture than in *S. aureus* only culture. Unfortunately, the following separation by SDS-PAGE was failed. Thus we could not analyze the purified proteins.

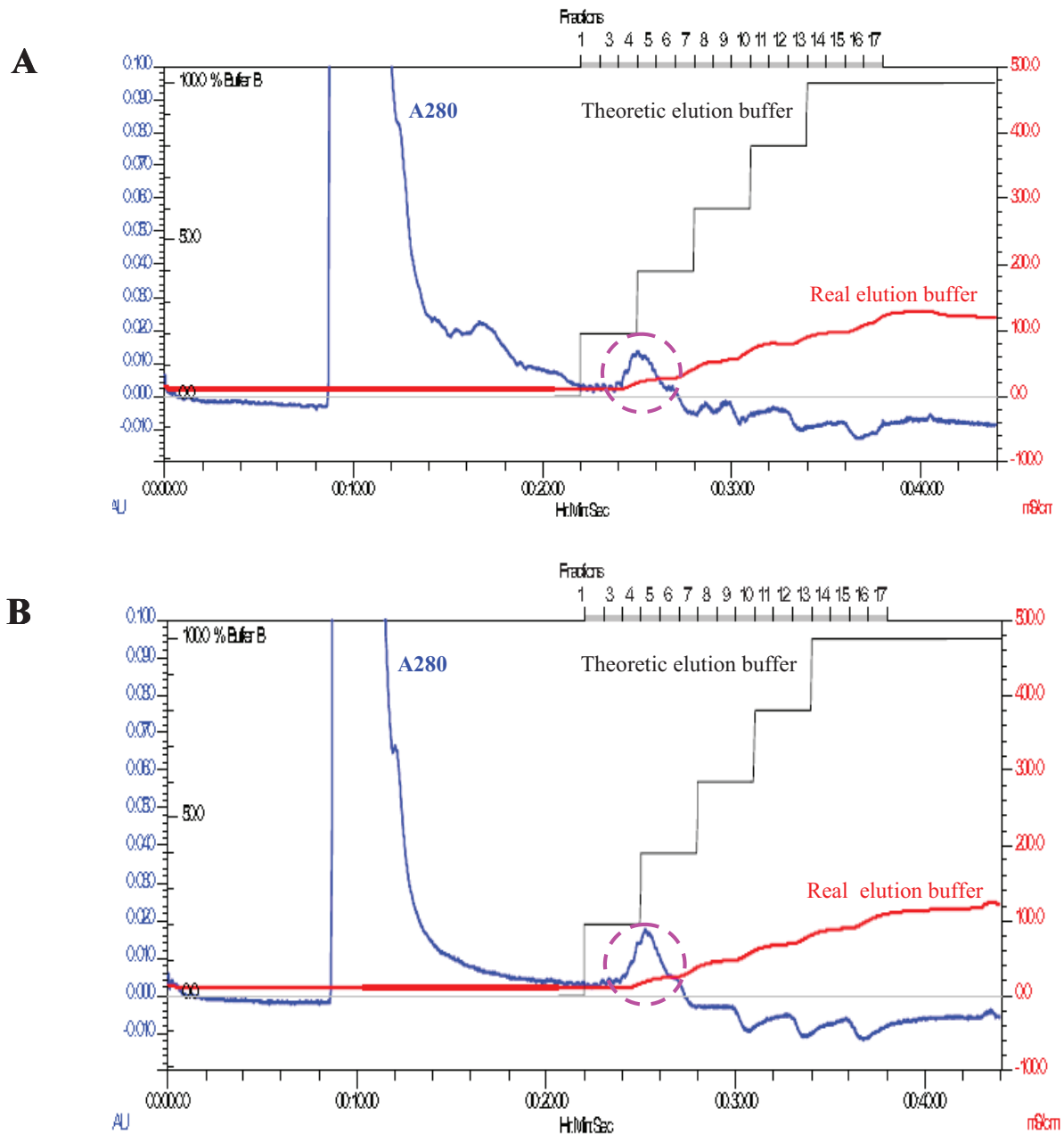


Figure 6-4 The reports of DuoFlow system. A. Proteins of *S. aureus* only culture bind to *rsaH* sequence; B. Proteins of *S. aureus* culture in presence of *P. aeruginosa* bind to *rsaH* sequence. Black stepped curve stands for the theoretical elution buffer, while the red curve for the real elution buffer. Blue curve indicates the A280 curve: the area between it and the baseline (indicated in the pink circle) represents the quantity of proteins.

6.4 Conclusion

Still in a simulated *S. aureus* and *P. aeruginosa* co-existence environment, this chapter is about a research to look for those regulators involved in sRNA regulation. We have focused on RsaH. The screen of different *S. aureus* strains with mutations of some global regulators such as Agr, Rot, SarA, SarH1, Arl, showed no dramatic differences of RsaH expression between these different knock outs vs. the wild type, suggesting that none of these regulators play a determinant role in RsaH induction by *P. aeruginosa*. Although SarA does not seem related with the RsaG upregulation, Agr, Rot, SarH1 and Arl might be involved in the mechanism, since *P. aeruginosa* can not increase RsaG level in their mutants.

In addition, more proteins bind to *rsaH* sequence after the interference of *P. aeruginosa*. Unfortunately we have not got the information about compositions of eluted protein fractions yet. However, further experiments on RsaH and also other sRNAs will be carried out by other members of our team.

General discussion

All my PhD study is aiming at investigating the characteristics and functions of Rsa RNA of

S. aureus, firstly through constructing the genetic tools, then by detection their expression *in vitro*, under various stresses and in clinical samples.

– Genetic strategies to study sRNA functions

With the floods of novel sRNAs unveiled from different bacteria, they are gaining more attention as an important regulatory player controlling diverse biological events ^[12,330]. To identify their targets and mechanisms, there are two major genetic approaches usually adopted: 1) to overexpress the gene of interest; 2) to invalidate the gene of interest followed by its complementation. As sRNAs often fine-tune gene expression, the use of the former strategy can not exclude entirely the influence of endogenous gene expression. In addition, the *rsa* genes exist as a single copy in the core genome of *S. aureus*, which provides the feasibility to choose the second strategy. Thus, we have constructed corresponding mutants and complementation strains for Rsa RNAs. It could provide an indispensable foundation to study the functions of these Rsa RNAs, through a comparison between mutant and wild type strains.

– Structural features and time-dependant characteristics of sRNA

It has been shown that the mechanism of sRNA function is related to structural features enabling them to interact specifically with their target ^[12]. Two main structural features should be noted: one is the existence of a 5' stem-loop structure that could contribute to the stability of sRNA ^[214,331]; the other characteristic is the conserved specific sequence: A / UCCCA / U. The functional analysis performed on the *S. aureus* RNAIII has shown that the crucial role of this sequence located at the apical pole of three loops - the loops 7, 13 and 14 ^[8,10]. These nucleotides involved in the interaction with the targets are typically localized in a single-stranded region and are thus readily available to form complexes sRNA-mRNA. In most cases the kissing-complex leads to the blocking of translation and mRNA degradation by double-stranded ribonuclease, the RNaseIII. Similarly, structural analysis of Rsa RNAs showed the existence of specific structural motifs described above. All the Rsa RNAs, except RsaI and RsaF, contain such a C-rich motif situated in unpaired regions. Especially in RsaG, E and C, there are several copies of this motif ^[11]. And this pattern is not only found in sRNAs of *S. aureus* but also in other bacteria. Indeed, *Salmonella typhimurium*, the sRNA CyaR also has a C-rich sequence, located in a loop structure that binds to the SD sequence of mRNA *ompX* to repress its translation ^[302]. This sequence may therefore represent a common heritage to different sRNAs or may result from convergence. In any case, this

suggests that these sRNAs might interact with the SD sequence of target genes through a shared mechanism.

Another very common feature of sRNA expression is that they often accumulate in the late-exponential growth phase. In several *S. aureus* lab strains, it has been shown that most of Rsa RNAs are predominantly expressed or strongly enhanced in the late-exponential phase, when culturing in rich medium ^[11]. This tightly time-dependant regulation of Rsa RNAs was also observed in *S. aureus* clinical isolates from different dwelling sites (Figure 2 in chapter 4). As for RsaE, the only sRNA conserved in staphylococcal species and *Bacillaceae*, it is preferentially expressed in the exponential phase in *B. subtilis* grown in rich medium ^[11].

– Response of Rsa RNA to stresses *in vitro*

In clinical situations, bacteria could meet various environmental conditions, such as acidic pH, oxidative stress and osmotic stress. The reactions of Rsa RNAs to these stresses were already analyzed in previous study: RsaA, E and H expression are induced under osmotic stress, oxidative stress or acidic pH, while RsaC and D are induced by cold-shock ^[11]. However, *S. aureus* may also encounter the pressures from different antibiotics. The bacterial growth was inhibited once the concentration of oxacillin or levofloxacin reached more than 1/4 MIC, and simultaneously, the expression of these Rsa RNAs started to decrease (Figure 3-2 to 3-6). Given that *S. aureus* cell density was standardized during the experimental procedures, it may be hypothesized that these antibiotics have impacts on bacterial gene transcription. Conversely, an increase of RsaH expression was found in RN6390 (σ^B), not in HG001 (σ^{B+}), under subinhibitory concentrations (1/16 MIC and 1/8 MIC) of levofloxacin, suggesting that this regulation is not σ^B - dependant.

When two or more types of bacteria co-exist, they can cross talk and/or compete with each other. Quorum sensing does not only exist among the bacteria of one species, but also among the members of different species. The signaling molecules generally used during the process of cell-cell communication are N-acyl homoserine lactones (AHLs) in Gram-negative bacteria and oligopeptides in Gram-positive bacteria, respectively ^[332]. In some clinical cases, such as burn wounds, cystic fibrosis and periodontal diseases, *P. aeruginosa* and *S. aureus* are often detected simultaneously. One AHL molecule from *P. aeruginosa* – 3OC₁₂HSL plays a key role during their interspecies interaction, by inducing the up-regulation of staphylococcal surface protein A or down-regulation of *sarA* and *agr* genes in *S. aureus*,

which in turn protects the *S. aureus* from the attack of host immune system or controls the expression of several virulence factors ^[317,333]. In our study, two sRNAs of *S. aureus*, RsaG and H, were found up-regulated in response to the interference with *P. aeruginosa*: RsaH expression was enhanced by both *P. aeruginosa* live bacteria and culture supernatant, while RsaG expression was only promoted by live *P. aeruginosa* live cells (Figure 5-1 and 5-2). Nevertheless, the similar experiments conducted with 3OC₁₂HSL and C₄HSL showed no effects on the expression of RsaG and RsaH (Figure 5-5A), suggesting that the mechanism of up-regulation of RsaG and H by *P. aeruginosa* does not involve one of these two AHL molecules.

– Response of Rsa RNA to human innate environment

The gene expression regulation mediated by sRNAs enables bacteria to adapt rapidly to environmental changes and signals. Except from their involvement in microbial physiology, some of the sRNAs have been implicated in virulence ^[330]. The Rsa RNAs of *S. aureus* were identified from a few reference strains; however, their expression profiles in host remain unknown. Thus, we carried out a direct analysis of sRNA expression in *S. aureus* during differential infection processes in human beings and compared it with *in vitro* isolates. In our study, it is inspiring that all the 5 sRNAs (RsaA, E, G, H and RNAIII) not only proved to be expressed in clinical isolates, but also expressed in the *in vivo* condition, despite of their low abundance.

The observation in our study that RNAIII was expressed in all the clinical isolates after culturing in BH medium to mid or late-exponential phase, was consistent with most previous studies, although it was also reported that *agr* dysfunction can be associated with persistent bacteremia or development of reduced susceptibility to glycopeptides ^[211,334-335]. The other 4 sRNAs (RsaA, E, G and H) were also expressed by all the clinical isolates, suggesting that these sRNAs are encoded in the core genome of *S. aureus*. Concerning their expression kinetics, all these sRNAs accumulate in the late-exponential growth phase, except for RsaE, which reaches the peak in the mid-exponential growth phase (Figure 2 in chapter 4). RsaE is involved in the regulation of protein synthesis of various metabolic pathways ^[11]. The toxicity caused by overexpression of RsaE could be partially alleviated through non-preferred source acetate. It was postulated that the utilization of glyoxylate pathway shunts some RsaE-repressed steps of the tricarboxylic acid (TCA) cycle ^[15].

When measuring directly the expression of 5 sRNAs in clinical samples, it was different

with that observed in clinical isolates grown in BH medium. Firstly, RNAIII level is generally much less expressed *in vivo* than in cultures grown to the late-exponential growth phase. Similar results have been previously reported in various clinical samples ^[310-311,336]. One possible reason is that the cell density in the clinical samples was probably under the threshold for *agr* activation and thereby corresponds to cultures grown to the mid-exponential growth phase other than late-exponential phase. Moreover, some studies have revealed that low *agr* level favors the expression of several proteins involved in biofilm formation, such as fibronectin-binding proteins and protein A ^[337-339].

An interesting fact was that the expression levels of all 5 sRNAs were extremely variable in abscess samples, while the samples obtained from the CF lung infection and nasal colonization showed modest and highly uniform sRNA expression, respectively. This phenomenon might be related with the more quantitatively diverse stresses during acute infection than during colonization. In addition, there was much inherent variability of external conditions in abscesses, such as the time after onset of infection, while such parameters were less critical in samples collected from nasal carriers. Given that the sRNAs of clinical isolates from nasal carriers showed a varied expression pattern similar to other isolates from abscesses or CF infection, we can exclude the possibility that the striking uniformity of the sRNA expression profile in nasal samples is due to the intrinsic properties of isolates. Thereby, it can be proposed that the uniform level of sRNA expression in nasal colonization might reflect a sort of vigilance status of the bacteria during limited stress conditions associated with commensalism.

Overall, my thesis was part of the global project to characterize newly discovered sRNAs in *S. aureus*, for the purpose to understand their mechanism of action. The construction of genetic tools allows us to study further their expression and function in different genetic backgrounds, e.g. to indentify the genes they regulate. Analyze of sRNA expression under stresses (antibiotics, *P. aeruginosa*) has given us the hints about the potential role of sRNAs in an imposed microenvironment. Furthermore, the analysis of these sRNAs expression profiles in clinical samples confirmed that the sRNAs were expressed by all the clinical isolates indicating that they may regulate common targets. The expression levels of these sRNAs *in vivo* showed different patterns compared to *in vitro*, which is also a reflection of the influence from distinct niches and host immune responses during acute, chronic infection or colonization.

For further investigation on potential role of the sRNA, in either ecology or pathogenesis of *S. aureus*, it is essential to assess the sRNA expression through infection/colonization cycle or in animal models. The analyzes of competitive survival ability of wildtype and Rsa deletion mutants, under various complex biological conditions (e.g. blood, cerebrospinal fluid, lung cells), performed by our team, are now in progress. However, on the basis of data obtained in this study, a new angle of view about the communication among bacterial cells or with host during chronic infection or long-term colonization attracts our attention. A comparative genome sequencing of 3 sequential *S. aureus* isolates from the airways of 1 CF patient revealed the genetic polymorphisms correlating with differences in growth, hemolytic activity and antibiotic resistance, which implies an adaptive evolution of *S. aureus* during chronic infection^[340]. Another artificial colonization study in neonatal rat model demonstrated that the established *S. aureus* strains inhibited invasion of new *S. aureus* populations^[341]. The answer to the question ‘How sRNA involves in this co-evolution of human and microorganism’ may broaden the current knowledge about its function and seek novel anti-colonization/infection strategies.

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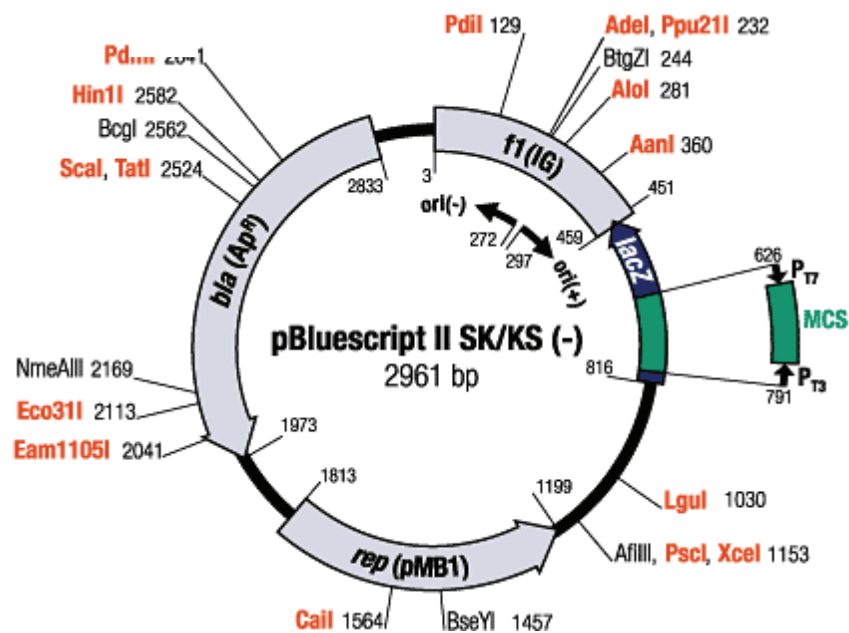
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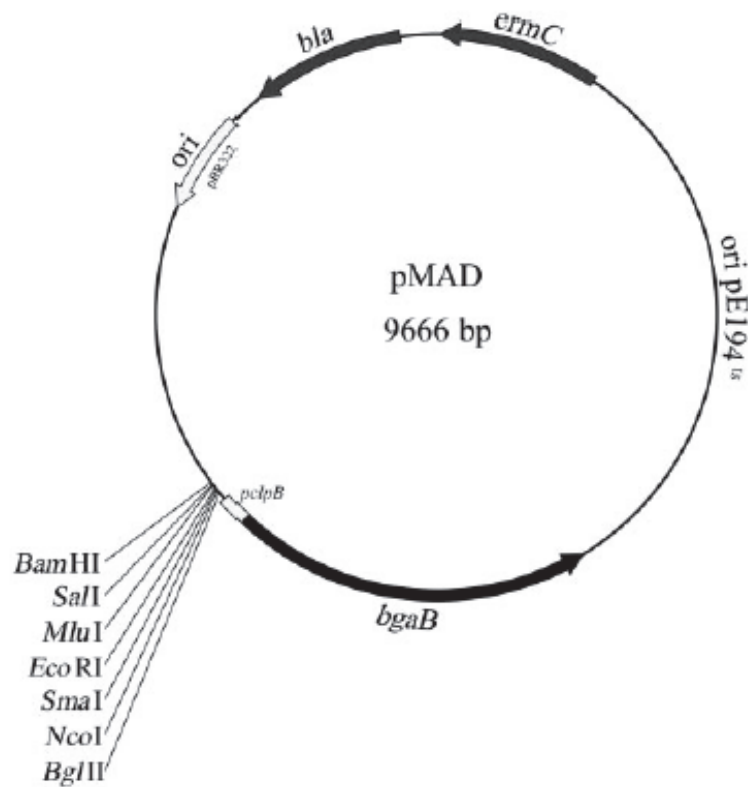
Annex

Annex I



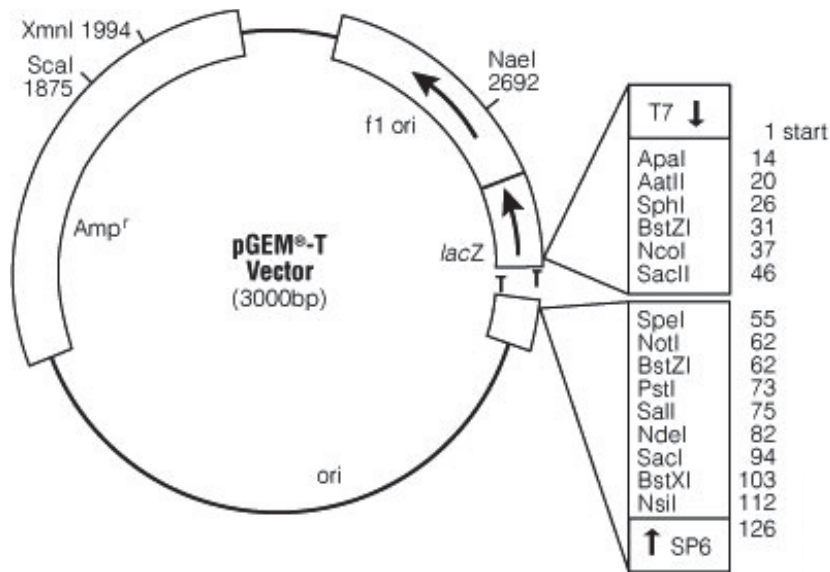
Map of pBluescript plasmid (Fermentas). This cloning vector contains: (1) f1 (IG), the intergenic region of phage f1; (2) *rep* (pMB1), the pMB1 replicon responsible for the replication of plasmid; (3) *bla* (Ap^R), the gene coding for beta-lactamase, that confers resistance to ampicillin; (4) *lacZ*, the gene encoding β -galactosidase allowing blue/white screening of recombinant plasmid.

Annex II



Map of pMAD plasmid (Arnaud *et al.* 2004). This shuttle vector is constructed based on pE194^{ts}::pBR322. It has a thermosensitive origin of replication, erythromycin resistance gene (*ermC*) and ampicillin resistance gene (*bla*), and the gene encoding a thermostable β-galactosidase (*bgaB*) driven by a constitutive promoter (*pclpB*). Multiple cloning sites are situated on the upstream of *bgaB*.

Annex III



Map of pGEM-T (Promega). This cloning vector contains T7 and SP6 RNA polymerase promoters which flank a multiple cloning region (MCS). MCS situates within the coding region for β -galactosidase (*lacZ*). Insertion of foreign gene can inactivate *lacZ*, allowing recombinant clones to be directly identified by white/blue screening. It also has an origin of replication *ori* and the ampicillin resistance gene.

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Dissertation submitted in partial fulfillment of the requirement for Doctor degree of University Lyon 1, 2012

Title: Characterization and effect reaction of novenah amt e Aa in *Staphylococcus aureus*

Resume:

Small RNAs (sRNAs) are involved in the post-transcriptional regulation of metabolic pathways and in responses to stress and virulence. In *Staphylococcus aureus*, RNAIII, the main effector of the Agr system, acts as an antisense RNA to regulate the expression of many cell wall proteins and cytotoxins in late-exponential growth phase. A series of novel sRNAs – Rsa were discovered by our team in 2009. The objective of this study is i) to construct genetic tools to study their functions and ii) to analyze their expression in various conditions *in vitro* and *in vivo*. Thus, we determined the expression level of 4 sRNAs (RsaA, RsaE, RsaG and RsaH) and RNAIII, when the bacterium was exposed to various antibiotics or in contact with other bacteria such as *Pseudomonas*. Subinhibitory concentrations (1/16 and 1/8 MIC) of levofloxacin could enhance the RsaH expression in RN6390, not in HG001. RsaG and RsaH level were increased in the presence of *P. aeruginosa*, however the mechanism of this effect has not been elucidated. Data obtained from human samples showed that the global expression of the five sRNAs was extremely variable in the abscesses, more homogeneous in the sputa of cystic fibrosis patients, and highly uniform in the nasal carrier samples. The unique expression pattern of sRNA associated with nasal colonization might reflect the commensalism of *S. aureus* in this niche.

Key Words:

Staphylococcus aureus; small RNA; RNAIII; Rsa; expression; *in vitro*; *in vivo*

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