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# Staphylococcus aureus protein S1, an RNA chaperone involved in translation initiation and sRNA regulation

Alessandra Marena

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UNIVERSITÉ DE STRASBOURG



**ÉCOLE DOCTORALE 414 Science de la Vie et de la Santé**

**Architecture et Réactivité de l'ARN – IBMC Strasbourg**

**THÈSE** présentée par :

**Alessandra MARENNA**

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pour obtenir le grade de : **Docteur de l'université de Strasbourg**  
Discipline/ Spécialité : Aspects Moléculaires et Cellulaires de la Biologie

***Staphylococcus aureus* protein S1, an RNA  
chaperone involved in translation initiation and  
sRNA regulation**

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To my Daddy.

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## List of abbreviations

### Proteins and complexes

**30S-PIC** 30S pre-initiation complex

**30S-IC** 30S initiation complex

**70S-IC** 70S initiation complex

**BSA** « bovine serum albumin »

**IF** Initiation factor

**OB** Oligonucleotide/oligosaccharide binding

**ORF** Open reading frame

**PNPase** Polynucleotide phosphorylase

**RNase** Ribonuclease

### RNA

**mRNA** messenger RNA

**sRNA** regulatory RNA

**tRNA** Transfert RNA

**itRNA** Initiator tRNA

**RBS** Ribosomal binding site

**SD** Shine and Dalgarno sequence

**UTR** Untranslated region

### Metabolites/Ions

**Ca** Calcium

**Cl** Chlorine

**Fe** Iron

**K** Potassium

**Mg** Magnesium

**Na** Sodium

**Ni** Nickel

**NH<sub>4</sub>** Ammonium

**ROS** Reactive oxygen species

### Bacteria

***B. subtilis*** *Bacillus subtilis*

***E. coli*** *Escherichia coli*

***S. aureus*** *Staphylococcus aureus*

Media and antibiotics

**LB** Lysogeny broth

**BHI** Brain heart infusion

**NZM** Minimal media

**Amp** Ampicillin

**Cam** Chloramphenicol

**Kan** Kanamycin

Chemical reagents

**EtBr** Ethidium bromide

**DTT** Dithiothreitol

**EDTA** Ethylenediaminetetraacetic acid

**IPTG** Isopropyl  $\beta$ -D-1-thiogalactopyranoside

**P32** Phosphorus-32

**SDS** Sodium dodecyl sulfate

General

**Å** Angström

**°C** Celsius degrees

**cpm** Count per minute

**OD** Optical density

**g** grams

**K<sub>D</sub>** Dissociation constant

**L** Litre

**M** Molar

**mol** Mole

**nt** nucleotide

**pb** base pair

**pI** isoelectric point

**UV** ultraviolet

**V** volt

**vol** volume

**W** watt

**WT** wild-type



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**Synopsis de la thèse en Français**



# **La protéine S1 chez *Staphylococcus aureus*, une protéine chaperonne de l'ARN impliquée dans l'initiation de la traduction et la régulation médiée par des ARN non codants**

## **I. Introduction**

*Staphylococcus aureus* est une bactérie pathogène opportuniste à Gram-positif, responsable d'un grand nombre d'infections communautaires et nosocomiales qui peuvent être bénignes comme les furoncles, ou beaucoup plus graves telles que des pneumonies, des ostéomyélites, et des endocardites. Cette bactérie est aussi capable de s'adapter rapidement à divers environnements. Cette adaptation rapide requiert un changement de l'expression de gènes qui s'effectue aussi bien au niveau transcriptionnel, traductionnel que post-traductionnel. Contrôler la traduction a l'avantage d'apporter une réponse rapide nécessaire pour les processus adaptatifs. Les régulations de la traduction s'effectuent majoritairement à l'étape de l'initiation qui est l'étape limitante de la synthèse des protéines, et au cours de laquelle l'ARN messager (ARNm) se lie à l'ARN de transfert (ARNt) initiateur sur la petite sous-unité du ribosome (30S). L'ARNm exerce un rôle clé dans ces mécanismes de régulation, en présentant des structures particulières dans leurs régions 5' non codantes qui peuvent soit directement influencer sur la reconnaissance du ribosome, soit être reconnues par des protéines régulatrices ou des ARN non codants (ARNnc). Ainsi, les ARNm régulés sont souvent fortement structurés. Néanmoins pour être activement traduit, le site de liaison du ribosome sur l'ARNm doit être accessible. Chez *Escherichia coli*, l'initiation de la traduction des ARNm structurés est facilitée par l'action de la protéine ribosomique S1 qui est une protéine chaperonne de l'ARN capable de déstabiliser les structures des ARNm et de favoriser l'adaptation du codon d'initiation sur le canal de décodage du ribosome (Duval *et al.*, 2013). La protéine S1 d'*E. coli* (*EcoS1*) est constituée de six domaines OB-fold et est ancrée à la sous-unité 30S grâce aux deux premiers domaines, alors que la protéine S1 de *S. aureus* (*SauS1*) est plus courte (quatre domaines OB-fold) et ne contient pas le premier domaine qui sert à l'ancrage sur le ribosome. La fonction de cette protéine chez ce pathogène majeur de l'homme n'était pas encore connue au début de ma thèse.

Les objectifs de ma thèse ont été (1) d'élucider les mécanismes moléculaires qui permettent au ribosome de *S. aureus* de reconnaître ces ARNm structurés pour initier leur traduction et (2) de caractériser les fonctions de *SauS1* dans le métabolisme des ARN.

Nous avons récemment démontré que les sous-unités 30S purifiées de *S. aureus* ne contiennent pas S1, et ne sont pas capables de former efficacement des complexes d'initiation avec des ARNm structurés de *S. aureus* (Introduction, §V.4. Article 1 : Khusainov, Marena *et al.*, 2016). Pendant ma thèse, j'ai démontré que la protéine *SauS1* n'interagit pas directement avec la sous-unité 30S mais est capable de stimuler *in vitro* et *in vivo* l'initiation de la traduction de certains ARNm structurés par une interaction directe. Par l'utilisation de diverses approches incluant des études d'interactome, de mutagenèse, de FRET, de cartographie en solution, de toe-printing (pour analyser la formation des complexes ribosomiques) et de retard sur gel, j'ai également montré que *SauS1* agit comme une protéine chaperonne de l'ARN et qu'elle peut former différents complexes cellulaires impliqués dans des régulations dépendantes des ARNnc et dans les processus de maturation/dégradation de l'ARN.

## II. Résultats

### II.1. Fonction de *SauS1* dans la traduction

Le premier objectif de mon projet de thèse a porté sur la caractérisation de l'impact fonctionnel de la protéine *SauS1* sur l'initiation de la traduction d'ARNm structurés spécifiques. Du fait que j'ai montré que *SauS1* n'est pas strictement une protéine ribosomique, mon objectif a été de caractériser les cibles *in vivo* pour mieux comprendre l'étendue de ses fonctions biologiques. Dans un premier temps, en collaboration avec I. Caldelari, j'ai utilisé l'approche RIP-Seq qui est basé sur l'expression d'une protéine *SauS1* portant à son extrémité C-terminale une étiquette Flag. Après immunoprécipitation, les ARN co-purifiés à *SauS1* ont été identifiés par séquençage haut débit.

Nous avons ainsi identifié plusieurs classes d'ARN enrichis avec *SauS1* dont plusieurs ARNm, ARN régulateurs (ARNnc, « riboswitch ») et ARNt. Les interactions directes entre certains des ARN cibles et *SauS1* ont été validées par des expériences de gel retard et par la détermination de la structure des complexes par cartographie en solution. De manière intéressante, parmi les ARNm, nous avons identifié l'opéron

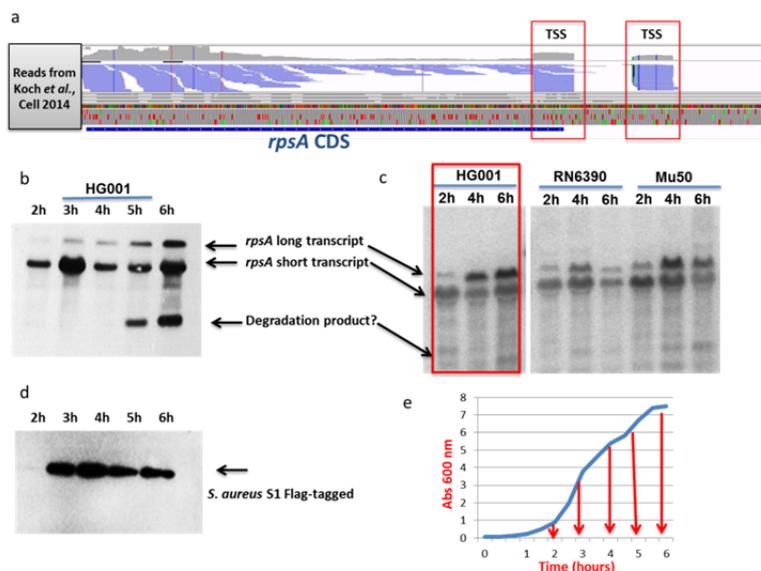
$\alpha$ psm1-4 qui code pour quatre peptides appelés « Phenol-soluble modulins » ou PSM, qui sont des exo-toxines dont la synthèse est activée par le système de densité cellulaire *agr*. Les PSM (« Phenyl Soluble Modulins ») appartiennent à une famille de peptides amphipathiques composés d'hélices alpha. Cet ARNm est fortement structuré et les quatre sites de reconnaissance du ribosome sont tous engagés dans des structures en tige-boucle. L'analyse du transcriptome comparatif provenant des souches HG001 sauvage et  $\Delta rpsA$  montre une régulation négative de l'opéron  $\alpha$ psm1-4. Nous avons émis l'hypothèse que la délétion de *SauS1* a entraîné une chute de la traduction de l'ARNm et par conséquent une déstabilisation de l'ARNm. De manière intéressante, d'autres ARNm cibles identifiés codent aussi pour des exo-toxines impliquées dans la virulence et la structure secondaire de leurs ARNm sont prédites pour adopter des structures dans leurs régions 5' non traduites qui pourraient moduler l'accessibilité de la sous-unité 30S.

Dans ce manuscrit, nous avons analysé en détail le mécanisme d'action de *SauS1* sur l'opéron  $\alpha$ psm1-4. Nous avons ainsi démontré que *SauS1* active la formation du complexe d'initiation impliquant la sous-unité 30S, l'ARNm  $\alpha$ psm1-4, et l'ARNt initiateur. Des expériences *in vivo* ont confirmé le rôle important de *SauS1* pour recruter l'ARNm dans les polysomes. Dans ce travail, j'ai bénéficié de l'expertise de plusieurs membres de l'équipe. Isabelle Caldelari a construit plusieurs souches mutées au niveau du gène *rpsA*, et a introduit l'étiquette Flag au niveau de l'extrémité C-terminale de la protéine pour effectuer les expériences de co-immunoprécipitation. Celle-ci a également mesuré le temps de demie vie de l'ARNm  $\alpha$ psm1-4 dans les souches sauvage HG001 et la souche mutant  $\Delta rpsA$ . Lucas Herrgott a suivi la traduction *in vivo* des ARNm  $\alpha$ psm1-4 et *hu* en analysant les profils des polysomes, suivi de l'analyse des ARNm par des expériences de Northern blot. Iskander Khusainov a préparé les sous-unités 30S de *S. aureus*, qui ont été utilisées pour des études structurales (Khusainov *et al.*, 2017). Dans ce qui va suivre, j'ai résumé les résultats que j'ai obtenu et qui ont été décrits dans le manuscrit présenté dans la partie résultat (§ I.1).

### ***II.1.1. SauS1 ne suit pas le profil d'expression des protéines ribosomiques mais est synthétisée dès le milieu de la phase exponentielle de la croissance.***

Au cours de la phase exponentielle de la croissance bactérienne, la synthèse des protéines ribosomiques est liée à la transcription des ARN ribosomiques et est régulée selon les changements environnementaux et aux différentes phases de croissance

(Nomura, 1999; Kaczanowska and Rydén-Aulin, 2007). *SauS1*, codée par le gène *rpsA*, a un profil d'expression caractéristique puisque sa synthèse n'est pas stimulée dans les conditions où les autres protéines ribosomiques sont activement produites, par exemple en réponse aux stress antibiotiques, comme il a été récemment démontré par une analyse de spectrométrie de masse (Bonn *et al.*, 2016). Afin de comprendre le mécanisme de régulation, j'ai analysé l'expression de l'ARNm *rpsA* en fonction de la phase de croissance (Figure 1) par des expériences de Northern et suivi la synthèse de la protéine par Western. L'ARNm *rpsA* présente deux isoformes à cause de deux sites d'initiation de la transcription (TSS) (Figure 1a). Le transcrit court est constitutivement exprimé alors que le transcrit long commence à être exprimé à 3 h et s'accumule en fin de croissance (Figure 1b). Un profil de transcription similaire est observé dans d'autres souches de *S. aureus* (Figure 1c) indiquant un mécanisme de régulation conservé. Le profil d'expression de la protéine *SauS1*, analysée par Western, montre que sa synthèse est en phase avec l'expression du transcrit long (Figure 1d).



**Figure 1 : Profils d'expression de *rpsA*.** a) L'ARNm *rpsA* a deux TSS (jeux de données SRR949025 ENA-EBI obtenu par Koch *et al.*, 2014 réaligné sur le génome HG001 et visualisé avec IGV). b) et c) Les expériences de Northern montrent l'expression de l'ARNm *rpsA* dans différentes souches. Les ARN totaux ont été préparés à partir de cultures bactériennes en milieu BHI arrêté à différents moments de la croissance (2h, 3h, 4h, 6h). d) Analyse en Western de *SauS1* à partir d'un extrait protéique de cultures en BHI à 2h, 3h, 4h, 5h et 6h. e) Courbe de croissance de *S. aureus* qui montre les phases où les échantillons ont été prélevés.

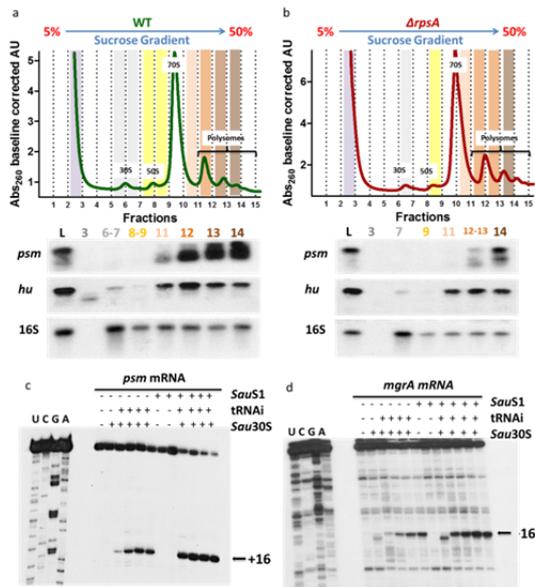
### II.1.2. *SauS1* n'est pas une protéine essentielle mais est liée au métabolisme des ARN

Afin de mieux comprendre la fonction de *SauS1*, j'ai délété le gène *rpsA* par remplacement allélique. Chez *E. coli*, le gène *rpsA* est essentiel et la protéine ribosomique *EcoS1* est requise pour la traduction de la majorité des ARNm (Duval *et al.*, 2013). L'absence de phénotype de croissance observée dans la souche de *S. aureus* mutante dans des conditions de culture optimale de laboratoire indique que *SauS1*, contrairement à *E. coli*, n'est pas essentielle. Toutefois, nous avons effectué une étude comparative des protéomes et des transcriptomes de la souche sauvage (HG001) et de la

souche mutante (HG001  $\Delta rpsA$ ). Dans la souche mutante, le niveau d'expression de nombreux ARNm et ARNnc est altéré alors qu'un défaut de la maturation de certains ARNt a été détecté. Des expériences de rifampicine ont été réalisées pour suivre la stabilité de l'ARNm  $\alpha psm1-4$ . Le temps de demie-vie est clairement diminué dans la souche mutante. Ce résultat pourrait suggérer un rôle de *SauS1* dans la traduction (voir manuscrit dans la partie résultat, Figure 13).

### ***II.1.3. SauS1 n'interagit pas avec le ribosome mais active la synthèse des PSM***

Pour caractériser l'impact de *SauS1* sur la traduction de l'ARNm *psm*  $\alpha psm1-4$ , nous avons analysé les profils des polysomes couplés à des expériences de Northern. De cette façon, nous avons évalué le taux de traduction de l'ARNm dans les souches sauvage (WT) et mutante  $\Delta rpsA$ . Une différence importante est observée dans la quantité de l'ARNm *psm*  $\alpha psm1-4$  trouvée dans les polysomes entre les deux souches: en absence de *SauS1*, beaucoup moins d'ARNm (2,5 fois moins) est engagé sur les ribosomes pour être traduit. Ce comportement est spécifique de l'ARNm *psm*  $\alpha psm1-4$ , puisque l'ARNm *hu* est légèrement mieux traduit (1,78 fois plus) (Figure 2a et b). Pour confirmer l'implication directe de *SauS1* dans la traduction des PSM, j'ai purifié *SauS1* et ai testé son activité sur la formation du complexe d'initiation de la traduction par des expériences de Toe-printing. Les résultats ont montré que l'addition de *SauS1* augmente fortement la formation d'un complexe d'initiation actif sur l'ARNm *psm*  $\alpha psm1-4$  (Figure 2c). Cet effet est spécifique puisqu'aucun effet n'a été observé pour un ARNm non structuré tel que l'ARNm *spa* codant pour la protéine A (manuscrit dans partie résultats, Figure S3).



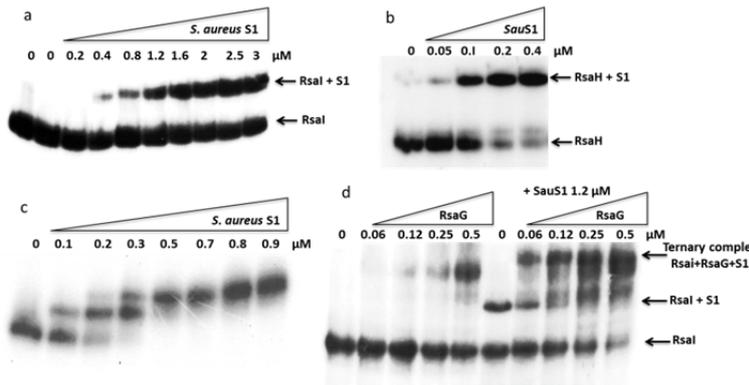
**Figure 2** Effet de *SauS1* sur la traduction de l'ARNm *psm*, *hu* et *mgrA*. a) et b), les profils des polysomes sont couplés à une analyse par Northern des ARNm *psm* et *hu* dans les souches WT et  $\Delta rpsA$  (a et b, respectivement). L= lysat cellulaire. l'ARN 16S en ligne L est utilisé pour la normalisation. c) et d), effet de la protéine *SauS1* sur la formation des complexes d'initiation impliquant les ARNm de *S. aureus* *psm* c) et *mgrA* d). Les expériences montrent que l'addition de la protéine S1 induit la formation d'un complexe d'initiation actif surtout à des concentrations très faibles de 30S. L'arrêt de la reverse transcriptase en position +16 indique la présence du ribosome fixé à l'ARNm.

## II.2. *SauS1* est une protéine chaperone de l'ARN

### II.2.1. *SauS1* interagit avec d'autres ARN

Afin d'identifier les partenaires protéiques et les ARN de *SauS1* *in vivo*, nous avons effectué des expériences d'immunoprécipitation suivies par des analyses protéomiques et de séquençage haut débit des ARN. Cela a permis de caractériser les protéines et les ARN qui ont été enrichis avec la protéine étiquetée. Parmi les ARN, nous avons trouvé plusieurs ARNnc dont *RsaI*, *RsaH*, *RsaG*, *RsaE* et *RsaD*, des ARNm hautement structurés comme les *psm*, des éléments régulateurs agissant *in cis* appelés « riboswitch » incluant celui de la flavine mononucléotide (FMN), et de nombreux ARNt.

Pour valider *in vitro* l'interaction directe entre S1 et les ARN, j'ai visualisé la formation des complexes par des expériences de gel retard. Celles-ci montrent que *SauS1* forme un complexe stable avec *RsaI* et *RsaH* (Figure 3a et b), et une interaction faible avec *RsaE* (données non montrées), alors qu'aucune interaction n'a été observée avec les autres ARNnc (*RsaA* et *RsaG* ; partie résultats, Figure 20). J'ai également vérifié l'interaction directe entre S1 et l'ARNm *psm* (Figure 3c).



**Figure 3. Analyse de la formation des complexes S1-ARN par gel retard.**

Formation du complexe entre les ARNc RsaI (a) et RsaH (b), et l'ARNm *psm* (c). les ARN sont marqués radioactivement en 5' et les concentrations croissantes de S1 ont été ajoutées.

(d) A gauche, la formation du complexe entre RsaI et RsaG en absence de S1. A droite, la même expérience en présence de *SauS1*. Un complexe ternaire entre RsaI, RsaG et S1 est observé.

Parmi les protéines potentiellement partenaires de *SauS1*, nous avons détecté deux machineries cellulaires majeurs, le dégradosome avec tous ses composants hautement enrichis (dont les ribonucléases J1, J2, Y, la PNPase et l'hélicase CshA) et le ribosome.

### II.2.2. Quel est le mécanisme d'action de *SauS1*?

Des expériences effectuées par Delphine Bronesky dans notre laboratoire ont démontré une interaction directe entre RsaI et RsaG, et comme nous n'avons pas mis en évidence de formation de complexe entre *SauS1* et RsaG, nous avons vérifié la possible formation d'un complexe ternaire qui pourrait expliquer les résultats de la co-immunoprécipitation. De manière intéressante, des expériences de formation des complexes par gel retard montrent clairement une bande retardée spécifique qui correspondrait au complexe formé entre RsaI, *SauS1* et RsaG (Figure 3d). De plus, la présence de S1 augmente de manière significative l'affinité entre RsaI et RsaG.

J'ai ensuite effectué *in vitro* des expériences d'empreinte en utilisant la cartographie en solution SHAPE qui modifie les riboses, DMS pour modifier les adénines en position N1 et les cytosines en position N3, et diverses ribonucléases qui coupent les régions en simple brin (RNase T1 pour les guanines) et en double brin (RNase V1). De cette façon, j'ai obtenu la structure de RsaI et identifié le site d'interaction avec RsaG. Les résultats sont en faveur de l'existence d'une structure en pseudoknot dans RsaI qui implique deux régions hautement conservées chez tous les staphylocoques: un motif riche en guanines exposé dans une boucle et une séquence interhélicoidale riche en uridines. Le motif riche en guanines se lie à un motif conservé riche en cytosines de RsaG (Geissmann *et al*, 2009). Par des expériences de pontage, j'ai montré une interaction possible entre *SauS1* et la séquence riche en uridines de RsaI. Il est possible que S1

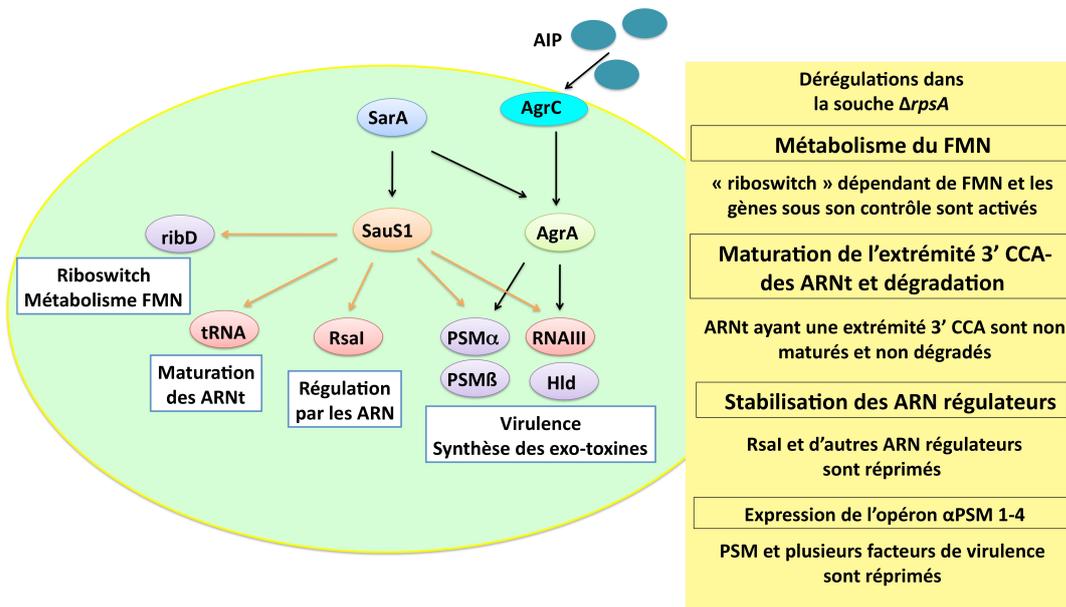
pourrait perturber la structure en pseudoknot de RsaI afin de faciliter l'interaction avec RsaG (Partie résultats, Figure 24).

### II.2.3. *SauS1* est une protéine chaperone de l'ARN

*SauS1* interagit directement avec ses ARNm et ARNnc cibles et stimule l'interaction ARN-ARN probablement en modifiant leur structure secondaire. Pour vérifier si *SauS1* stimule la cinétique de l'interaction ARN-ARN et fonctionne comme une protéine chaperone de l'ARN, j'ai effectué des expériences préliminaires de « time resolved FRET » avec des oligonucléotides d'ARN modèle couplés à des fluorophores. La présence de *SauS1* accélère la formation des appariements ARN-ARN de 5 fois (Partie résultats, Figure 27).

## III. Discussion et perspectives

Malgré le fait que la protéine *SauS1* n'est pas une protéine essentielle, elle est impliquée dans plusieurs processus cellulaires fondamentaux résumés dans la figure 4.



**Figure 4. Les différentes fonctions de la protéine *SauS1*:** La protéine kinase membranaire AgrC active le régulateur de réponse AgrA lorsque le peptide signal sécrété AIP a atteint une concentration seuil. Les trois locus *PSMs* sont contrôlés à la fois au niveau transcriptionnel par AgrA et au niveau traductionnel par *SauS1*. SarA est un des facteurs activant la synthèse de *rpsA* et des transcrits *psm*. Des expériences de co-immunoprécipitation utilisant une version modifiée de *SauS1* portant une étiquette Flag suivies d'un séquençage des ARN a permis l'identification *in vivo* de différentes cibles ARN incluant divers ARNm, ARN régulateurs, riboswitch et des ARNt dont le CCA est aussi présent dans le gène. Toutes ces ARNs ont un taux affecté dans la souche mutante *ΔrpsA*. Des exemples de dérégulations pour chaque classe d'ARN sont donnés dans l'encadré de couleur jaune.

Même si elle n'est pas associée directement au ribosome, *SauS1* favorise l'initiation de la traduction d'ARNm structurés. Le mécanisme de cette stimulation n'est pas encore connu mais il est possible que *SauS1*, comme *EcoS1*, puisse fragiliser les structures d'ARNm au niveau des sites d'initiation de la traduction, facilitant l'accès au ribosome. Nous avons démontré que l'ARNm *psm*  $\alpha$ psm1-4 nécessite *SauS1* pour faciliter l'initiation de sa traduction. A forte concentration ( $> 1 \mu\text{M}$ ), les PSM $\alpha$  possèdent une activité lytique *in vitro* sur les neutrophiles (Löffler *et al.*, 2010) et sur les érythrocytes (Cheung et Otto, 2012). Ce pouvoir lytique est lié à leur propriété amphipathique. En fait, les PSM $\alpha$  s'agrègent à la surface des membranes pour former des pores transmembranaires. En outre, il a été montré que PSM $\alpha$ 1 et PSM $\alpha$ 2 présentaient une activité bactéricide contre les bactéries d'un genre différent de *Staphylococcus*. Ainsi ces toxines confèreraient à *S. aureus* la capacité d'entrer en compétition avec d'autres espèces bactériennes pour la colonisation de l'hôte (Joo et Otto, 2011).

*SauS1* peut-elle moduler la virulence de *S. aureus* en agissant sur la production des PSM? Pour aborder cette question, en collaboration avec l'équipe de François Vandenesch (Lyon), nous caractériserons l'impact fonctionnel de la protéine S1 sur la pathogénicité de *S. aureus*, en utilisant différentes approches *in vivo* et *in vitro*: dosage par spectrométrie de masse des PSM dans les surnageants de culture, infection des cellules immunitaire (macrophages, neutrophiles), analyse de la formation de biofilms entre les souches sauvage et mutante  $\Delta rpsA$  et la souche mutante complémentée avec un plasmide qui exprime le gène *rpsA* sous son propre promoteur, et analyse de l'effet de *SauS1* dans divers modèles d'infection chez la souris.

Des résultats plus récents montrent que *SauS1* est aussi requise pour faciliter la traduction de l'ARNIII qui code pour l'hémolysine delta, un autre peptide de type PSM induit par le système *agr*. Il a été suggéré que la traduction de *hld* est retardée de 1h par rapport à la transcription de l'ARNIII. Ce décalage entre transcription et traduction a aussi été observé pour les autres PSM $\alpha$ . Du fait que la protéine *SauS1* est exprimée principalement à haute densité cellulaire, nous proposons que la protéine serait responsable de cet effet de décalage pour activer de manière coordonnée la traduction des PSM. Nous envisageons d'utiliser un promoteur constitutif pour exprimer *SauS1* et analyser si, dans ce contexte, le délai entre la transcription et la traduction des PSM est aboli.

S1 est-elle impliquée dans des processus de régulation des ARNnc? Dans diverses bactéries à Gram-négatif, les protéines chaperones, dont Hfq et ProQ, participent à la régulation des ARNnc en les stabilisant et en facilitant leurs appariements avec leurs ARNm cibles. Chez *S. aureus*, aucune protéine n'a encore été impliquée dans ces mécanismes. ProQ n'est pas présente, et Hfq a une composition en acides aminés différente au niveau du site de liaison de l'ARN, ce qui ne permet pas de former des complexes ARN-ARN. Nous avons trouvé que *SauS1* est directement associée à plusieurs ARNnc et avons montré que cela pourrait, au moins dans un cas, stimuler l'hybridation entre l'ARNnc et sa cible (RsaI-RsaG). Bien que l'activité chaperone à l'ARN de *SauS1* a été démontrée, les mécanismes détaillés par lesquels elle favorise l'interaction ARN-ARN doivent encore être élucidés. Des résultats préliminaires suggèrent que celle-ci favorise la cinétique d'appariement entre deux fragments d'ARN dans un système modèle. Pour montrer que la cinétique de liaison de l'ARNnc aux cibles est stimulée par S1, il sera possible d'utiliser l'appareil SwitchSense disponible dans notre unité. Nous pourrions tester différents systèmes ARNnc-cibles en présence ou en absence de *SauS1*.

Est-ce que S1 est impliquée dans la stabilité et la maturation des ARN? *SauS1* a été retrouvée en association avec le dégradosome, la machinerie responsable pour la dégradation/maturation des ARN. Les nombreux ARNt identifiés dans les expériences de co-immunoprécipitation suggèrent un défaut de maturation qui devra être confirmé par des expériences de Northern.

Ainsi, pour la première fois, l'ensemble de mes résultats ont permis de démontrer le rôle clé d'une protéine fixant l'ARN pour la traduction d'ARNm structuré et pour la régulation de l'expression des gènes. Il sera intéressant de vérifier si ces fonctions sont conservées dans les bactéries de type Gram-positif et qui sont éloignés dans l'évolution telles que *Bacillus subtilis*. L'utilisation d'autres approches telles que le Grad-seq devrait aider à mettre en évidence d'autres protéines impliquées dans la régulation post-transcriptionnelle de l'expression des gènes de *S. aureus* (Smirnov *et al.*, 2016).

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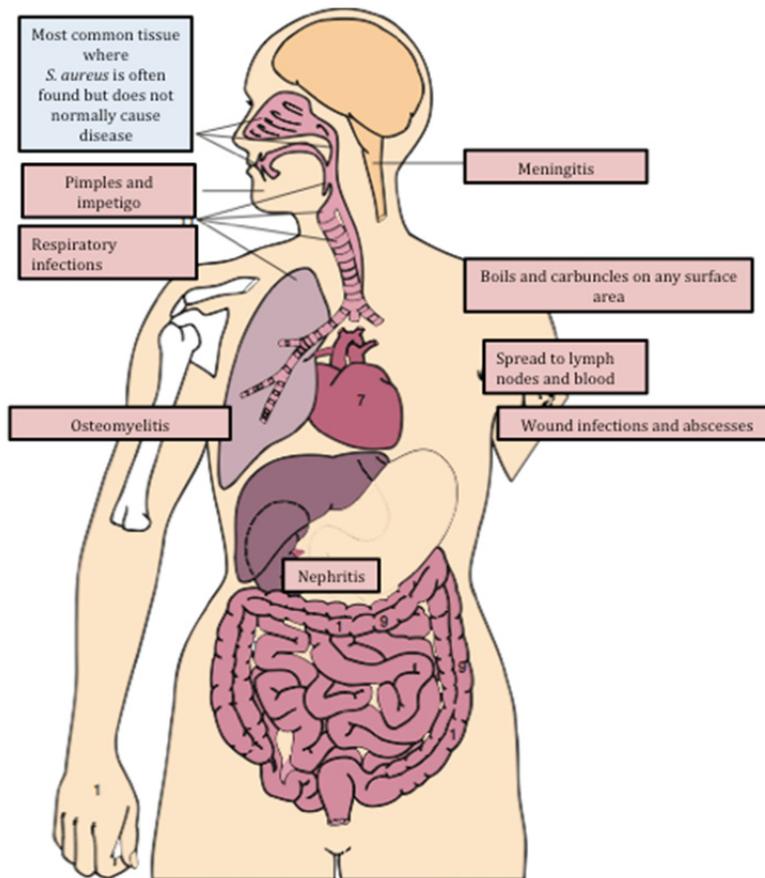
# Introduction



## I. *Staphylococcus aureus* is a versatile opportunistic human pathogen

*Staphylococcus aureus* was discovered in 1880 by Sir Alexander Ogston (Ogston, 1881). He observed grape-like clusters of bacteria when examining a purulent discharge from patients with post-operative wounds and he named them “staphyle”, the greek expression for a bunch of grapes. Few years later, in 1884, Friedrich Julius Rosenbach succeeded in isolating yellow bacterial colonies from abscesses and named them *Staphylococcus aureus*, “aureus” from the latin word referred to golden color caused by the presence of carotenoids (Rosenbach, 1884). 121 years later, this golden pigment staphyloxanthin, has been demonstrated to be one of the many virulence factors produced by *S. aureus*. It shields the microbe from oxidation-based clearance promoted by the neutrophil oxidative burst, thus allowing its survival in blood (Liu et al., 2005).

Among staphylococci, *S. aureus* is the most virulent and pathogenic for humans, being responsible of wide range of diseases from a variety of skin, wound and deep tissue infections to more life-threatening conditions such as pneumonia, endocarditis, septic arthritis and septicemia (**Figure 1**). This bacterium is one of the most common species responsible for nosocomial infections and it might also cause food poisoning, scalded-skin syndrome and toxic shock syndrome, through production of several toxins. Intriguingly, despite its invasive opportunism, *S. aureus* replicates and evolves in a large proportion of the human population as a harmless colonizing organism that might never cause diseases. Approximately 30% of the population is asymptotically colonized by *S. aureus* (Wertheim et al., 2005). The anterior nasal mucosa is the most frequent site for the colonization of healthy human carriers (Kluytmans et al., 1997); other anatomical sites are throat (Mertz et al., 2007), perineum, gastrointestinal tract (Yotis, 1963), axillae, groin (Gordon and Lowy, 2008), and vagina (Bourgeois-Nicolaos et al., 2010). Although humans are the primary natural reservoirs, domestic animals, livestock, and fomites may serve as adjunctive reservoirs.



**Figure 1. Sites of colonization and diseases caused by *S. aureus*.** *Staphylococcus aureus* can be a human commensal or a potentially lethal opportunistic pathogen. Indeed, it is able to survive and multiply within the human body by creating microenvironments that protect it from host immune attack (Rooijackers et al., 2005). It can cause life-threatening diseases, as well as minor diseases such as soft tissue infections.

*S. aureus* is a Gram-positive bacterium, has a cell diameter of 0.6  $\mu\text{m}$ , is non-motile, non-spore-forming, and is a facultative anaerobe. It belongs to the genus *Staphylococcus* of the *Micrococcaceae* family. This genus is traditionally divided in two groups based on the bacteria ability to produce coagulase, an enzyme that causes blood clotting. The coagulase-positive staphylococci includes *Staphylococcus aureus*, and the coagulase-negative staphylococci (CoNS) are common commensals of the skin. Both coagulase and staphyloxanthin are contributing to the host immune system evasion strategies, part of *S. aureus* impressive armory which relies on antigens (adhesins and capsule) that facilitate adhesion to host cells, enzymes (coagulases, lipases, hyaluronidases, staphylokinases, nucleases) for tissue degradation and nutrient acquisition, and toxins ( $\alpha$ -,  $\beta$ - and  $\delta$ - haemolysins, P-V leukocidins, enterotoxins, exfoliative toxins, Toxic Shock Syndrome toxin) for the evasion of host defenses. Their production requires the need for *S. aureus* to make physiological adjustments for energy conservation. Thus, the virulence

factors are primarily targets of gene regulation, which reprogram the *S. aureus* lifestyle upon infection.

## II. Virulence determinants in *Staphylococcus aureus* play central role in its pathogenesis

*S. aureus* has a circular chromosome of 2.8 M base pairs (bp) with low G+C composition (32.8%). Its genome has about 2700 coding sequences among which approximately 23% have still unknown functions (data from the most recent annotation of the strain HG001 (Caldelari et al., 2017)). Several virulence factors are chromosomally encoded while others are parts of pathogenicity islands (PIs), which are the repository of many toxins, adherence and invasion factors, superantigens, and secretion systems (Novick et al., 2010; Novick and Ram, 2016; Novick and Subedi, 2007).

The pathogenicity of *S. aureus* is a complex process based on extremely coordinated expression of virulence factors at appropriate time among the different stages of host infection (**Table 1**): colonization, escape host immuno-defense, growth and cell division, and spreading (**Figure 2**). Their expression also responds to a plethora of environmental cues including bacterial cell density, amino acid limitation, metal depletion, decreased pH, and oxidant production. Through integration of these environmental cues, *S. aureus* can simultaneously coordinate expression of the genes coding for surface proteins involved in adhesion and defense against the host immune-system and only later during the post-exponential phase, *S. aureus* starts to secrete toxins able to disrupt host cells and tissues facilitating the spread of the infection (Thammavongsa et al., 2015).

Studying the expression pattern of virulence factors during the infection in the host could be quite difficult (Burian et al., 2010; Cheung et al., 2004; Montgomery et al., 2008; Que et al., 2005) and many factors, including cellular immune factors and nutrient conditions, might add an extra layer of regulation. In a few studies, the use of serum in *S. aureus* cultures to induce the expression of virulence factors (Ishii et al., 2014; Oogai et al., 2011) has produced promising results, but most commonly, bacterial media, such as Trypticase soy broth (TSB), brain heart infusion (BHI) broth, and Luria-Bertani (LB) broth, have been used for *S. aureus* growing. In the following description of several

major virulence factors, their timed expression is referred to different phases of the growth in these media.

VIRULENCE FACTORS	PUTATIVE FUNCTIONS
<b>Cell surface factors</b>	
Staphylococcal protein A (SpA)	Bind to IgG, interfering with opsinization and phagocytosis
Fibronectin-binding proteins (FnbpA and FnbpB)	Attachment to fibronectin and plasma clot
Clumping factor proteins (ClfA and ClfB)	Mediate clumping and adherence to fibrinogen in the presence of fibronectin
Capsular polysaccharides	Reduce phagocytosis by neutrophils; enhance bacterial colonization and persistence on mucosal surfaces
Staphyloxanthin	Resistance to neutrophil reactive oxidant-based phagocytosis
<b>Secreted factors</b>	
<u>Superantigens</u>	
Staphylococcal enterotoxins (SEA, B, C, D, E, G and Q)	Massive activation of T cells and antibody presenting cells
Toxic shock syndrome toxin-1 (TSST-1)	Massive activation of T cells and antibody presenting cells
<u>Cytolytic toxins</u>	
$\alpha$ -hemolysin	Induce lysis on a wide spectrum of cells, mainly platelets and monocytes
$\beta$ -hemolysin	Hydrolysis of sphingomyelin of the plasmatic membrane of monocytes, erythrocytes, neutrophils and lymphocytes; make cells susceptible to other lytic agents
$\gamma$ -hemolysin	Induce lysis on erythrocytes and leukocytes
<u>Leukocidin family</u>	
Leukocidins E/D and M/F-PV	Induce lysis on leukocytes
Panton-Valentine leukocidin (PVL)	Induce lysis on leukocytes
<u>Various exoenzymes</u>	
<u>Lipases</u>	Inactivate fatty acids
<u>Proteases</u>	
Serine (e.g. exfoliative toxins ETA and ETB)	Inactivate neutrophil activity; activate T cells (only ETA and ETB)
Cysteine (e.g. staphopain)	Block neutrophil activation and chemotaxis
Aureolysin	Inactivate antimicrobial peptides
Hyaluronidase	Degrade hyaluronic acid
Staphylokinase (SAK)	Activate plasminogen; inactivate antimicrobial peptides
<u>Coagulase</u>	
CoA	Coagulation promoting virulence factor
<b>Miscellaneous proteins</b>	
Staphylococcal complement inhibitor (SCIN)	Inhibit complement activation
Extracellular fibrinogen binding protein (Efb)	Inhibit complement activation
Chemotaxis inhibitory protein of <i>S. aureus</i> (CHIPS)	Inhibit chemotaxis and activation of neutrophils
Formyl peptide receptor-like 1 inhibitory protein (FLIPr)	Inhibit chemotaxis of neutrophils
Extracellular adherence protein (Eap)	Inhibit neutrophil migration

**Table 1 Major virulence factors involved in the pathogenesis of *Staphylococcus aureus* and respective putative functions.**

## II.1. Tissue colonization

Interaction with endothelial cells is a critical primordial step of infection and several bacterial proteins have been shown to be involved. They include secreted proteins and Cell Wall Associated proteins (CWA). Hereafter, I provide few examples of different mechanisms with which they promote adhesion and prevent clearance by the host immune system.

The extracellular adherence protein (Eap) of *S. aureus* participates in a wide range of protein-protein interactions that facilitate the initiation and dissemination of *Staphylococcal* disease. Eaps are secreted proteins involved in adherence and internalization of bacteria in eukaryotic cells (Hagggar et al., 2003; Palma et al., 1999). They have strong anti-inflammatory properties resulting in a decreased recruitment of neutrophils at the sites of infection. These immunomodulating proteins are also able to inhibit T and B cells proliferation (Hagggar et al., 2005).

The cell-surface proteins are the frontline of infection, involved in adhesion, internalization, colonization (Malachowa et al., 2011; Sibbald et al., 2006), invasion of non-phagocytic host cells (Foster et al., 2014), and immune evasion. *S. aureus* produces up to 24 CWA, which are covalently anchored to the cell wall peptidoglycan. Among them the most abundant are the protein A (*spa*), Sbi and the fibronectin binding proteins (FnBPs) (Foster et al., 2014). *Spa* is expressed during the exponential growth phase (Gao and Stewart, 2004). It is involved both in adhesion to host cells and in evading innate immune responses mediated by immunoglobulins binding, bacterial opsonisation and stimulation of TNF- $\alpha$  pro-inflammatory response (Atkins et al., 2008; DeDent et al., 2007; Gonzalez et al., 2015; Thammavongsa et al., 2015; Zecconi and Scali, 2013). Protein A also impacts the normal function of B-cells and induces their apoptosis, thus preventing the host from developing immunological memory (Goodyear and Silverman, 2004). Staphylococcal binder of immunoglobulin (Sbi) is both a secreted and CWA protein that, as protein A, binds to IgG (Smith et al., 2011). Both forms of the proteins participate in immune evasion whereas only the secreted form triggers the activation of the complement (Smith et al., 2012). Indeed, the secreted form of Sbi interacts with the antigen recognition of B-cells, rendering the pathogen undetectable and as a result, opsonins are not released and the immune response is not triggered (Markiewski and Lambris, 2007; Smith et al., 2012; Toapanta and Ross, 2006). Binding to the IgGs

contained in the human serum stimulates its expression, this is why in laboratory growth conditions, Sbi expression is very low. Fibronectin binding proteins (Fnbps) are adhesins, which as other cell wall-associated proteins, are expressed during the exponential phase of bacterial growth (Fitzgerald et al., 2012). They form fibronectin bridges required to stabilize the adhesion of the pathogen to host tissues (Martin et al., 2012; Piroth et al., 2008). Moreover, FnbpA and FnbpB are able to bind platelets whose activation and aggregation promotes thrombus formation (Kerrigan et al., 2002). The formation of platelet-fibrin thrombi protects bacteria from neutrophil recognition (Hartleib et al., 2000).

## II.2. Immune evasion (non CWA or Eap)

Normally, the blood coagulation system is a process resulting in the formation of a blood clot, which closes the injured part of the vessel. However, this process can also be activated by the immune system in response to infection. In this case, the bacteria are trapped in a blood coat preventing their dissemination in other sites of the body. Many organisms, including *S. aureus* have developed strategies able to convert the blood coagulation as an advantage to ensure their survival. Indeed, when a clot or eventually an abscess is formed, the bacteria are protected from the host immune attack. Coagulase (Coa) is the main coagulation-promoting factor produced by *S. aureus*. In combination with other proteins, it activates the host prothrombin inducing fibrin formation that protects the pathogen against phagocytosis by immune cells (Friedrich et al., 2003; Kroh et al., 2009) and promotes abscess formation (Cheng et al., 2010; McAdow et al., 2012)).

*S. aureus* has also other specific proteins that affect the innate and adaptive immune system. The innate immune modulators such as chemotaxis inhibitory protein (CHIPS) and the staphylococcal complement inhibitor (SCIN), are employed in the inactivation of the human complement (van Wamel et al., 2006).

## II.3. Tissue invasion and dissemination

*S. aureus* secreted proteins are involved in host tissue damage, inflammation, invasion and disruption of the host immune system in order to facilitate bacteria dissemination (Foster, 2005; Lin and Peterson, 2010; Malachowa et al., 2011). These secreted factors

can be divided into four groups: superantigens, exoenzymes, miscellaneous proteins and cytolytic (poreforming) toxins.

### **II.3.1 Superantigens**

Superantigens (Sags) are secreted immune-stimulatory low-molecular weight (19,000 to 30,000 Da) enterotoxins involved in many human diseases, including allergy, autoimmune diseases, food poisoning and toxic shock syndrome (TSS) (Chesney et al., 1997; Parrillo, 1993; Reingold et al., 1982). These proteins are exceptionally resistant to heat, to proteolysis and acidic conditions and they are highly resistant to desiccation (Dinges et al., 2000; McCormick et al., 2001). The production of superantigens interferes with antibody production and phagocytic cell chemotaxis. Indeed, irrespective of their antigen specificity and function, Sags induce massive activation of T cells resulting in a cytokine overproduction, aggravation of allergic inflammation and shock (Xu and McCormick, 2012).

### **II.3.2 Exoenzymes**

*S. aureus* secretes several extracellular enzymes whose principal goal is to disrupt host tissues, to inactivate host antimicrobial mechanisms (e.g. antibodies and complement mediators), and to produce nutrients for bacterial growth and facilitate dissemination. In the large group of exoenzymes are included lipases, proteases and staphylokinase (SAK).

Lipases are deoxyribonuclease (DNase) and fatty acid modifying enzymes that catalyze the hydrolysis of the ester bonds between glycerol and fatty acids to form triglycerides and this is believed to aid the bacteria to breakdown host tissue resulting in nutrients availability (Lu et al., 2012). Among the proteolytic enzymes produced by *S. aureus* there are the metalloproteinase (aureolysin, Aur), the serine glutamyl endopeptidase referred to as the V8 protease (serine protease, SspA) and two related cysteine proteinases, the staphopain (ScpA) and the cysteine protease (SspB) (Arvidson et al., 2000). Their deletion resulted in increased abundance of secreted and surface-associated virulence factors. Indeed, these proteases work to degrade indiscriminately both "self" and "host" proteins.

The metalloprotease, Aureolysin (Aur) is responsible for the proteolytic cleavage and activation of SspA (Drapeau, 1978) as well as for the cleavage of the surface-associated clumping factor ClfB, which protects the bacteria from phagocytosis (McAleese et al., 2001). The SspA protease, produced from a polycistronic operon with *sspB* (Reed et al., 2001), was shown to induce the cleavage of fibrinogen-binding protein (McGavin et al., 1997), of surface protein A (Spa) (Karlsson et al., 2001), and host proteins such as the heavy chain of all human immunoglobulin classes (Prokesova et al., 1992).

The secreted cysteines Staphopain A (ScpA) and Staphopain B (SspB) are known to induce cleavage of different host substrates including fibrinogen, collagen and elastin (Potempa et al., 1988). ScpA degrades fibers composed of elastin in the connective tissues, and both ScpA and SspB contribute to the turnover of collagen, the main component of the connective tissue (Potempa et al., 1988). In the bloodstream, fibrinogen cleavage by ScpA and SspB interferes with plasma clotability, resulting in a tendency to induce bleeding (Ohbayashi et al., 2011). ScpA can also inhibit neutrophil activation and chemotaxis (Laarman et al., 2012). The proteolytic susceptibility of cell-wall associated proteins such as FnBP and Protein A, both involved in adherence to host cells, could suggest that these extracellular proteases are important for the release of *S. aureus* from colonization sites to other sites of the human body. It has also been proposed that the degradation of toxins, such as  $\alpha$ -haemolysin, induced by the proteases may downregulate *in vivo*, the virulence of *S. aureus* (Shaw et al., 2004).

Staphylokinases (SAKs) are potent extracellular prothrombin activators (Osamu Matsuo, Masashi Sakai, 2017 - (Dinges et al., 2000) able to convert human plasminogen (plg) to active plasmin (Kwiecinski et al., 2010), a serine protease with a broad-spectrum of substrates such as fibrin, collagen and elastin. Plasmin-mediated proteolysis of different extracellular substrates potentially contributes to bacterial virulence by facilitating staphylococci entry into the deeper host tissues (Bergmann and Hammerschmidt, 2007; Bhattacharya et al., 2012; Sanderson-Smith et al., 2012). The role of this factor in virulence is however controversial. Indeed, Jin *et al.* showed that staphylokinase-deficient *S. aureus* strain is more virulent compared to wild-type strain, whereas Piechowicz *et al.* did not find such a phenotype (Jin et al., 2003; Piechowicz et al., 2010).

### ***II.3.3. Cytolytic (poreforming) toxins***

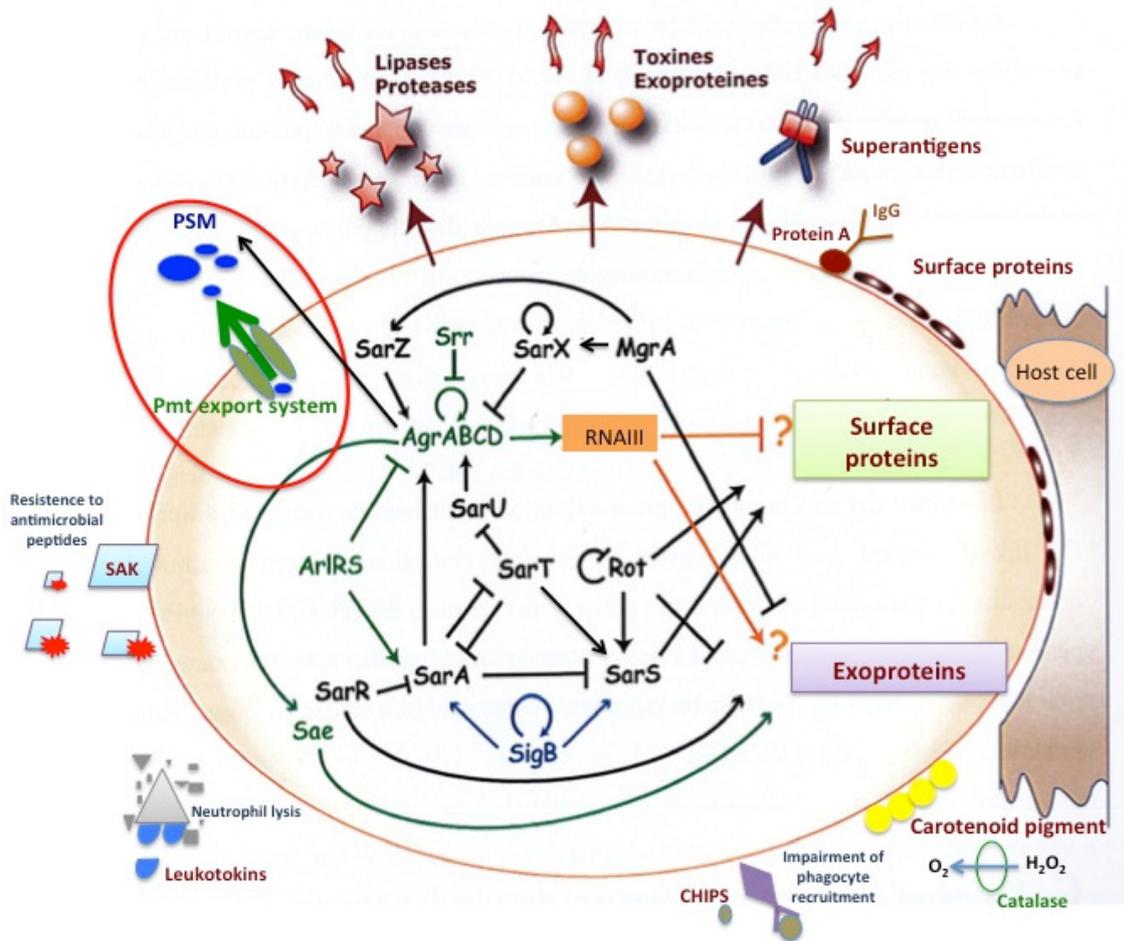
Immune evasion is, to a large extent, due to cytolytic toxins which are secreted to kill immune cells, among which the most important are the bi-component leukotoxin family,  $\alpha$ -toxin and the phenolsoluble modulins (PSM) peptides (Otto, 2014).

#### ***II 3.3.1 Leukotoxins***

*S. aureus* strains associated with human infections produce four types of leukotoxins: the Pantone-Valentine Leukocidin (PVL), gamma ( $\gamma$ )-haemolysin (HlgACB), Leukotoxin ED (LukED), and Leukotoxin AB/GH (LukAB/GH). They are bi-component proteins composed of two subunits secreted separately and then assembled in hexameric or heptameric oligomers having high affinity for leukocytes. They mainly act by forming pores in the membranes of leukocytes leading to their lysis, all four leukotoxins have also been demonstrated to kill human neutrophils (Löffler et al., 2010). Moreover, the  $\gamma$ -haemolysin can cause the lysis of red blood cells thus promoting the survival of *S. aureus* in bloodstream (Kaneko et al., 1997; Malachowa et al., 2011).

#### ***II.3.3.2 $\alpha$ -haemolysin***

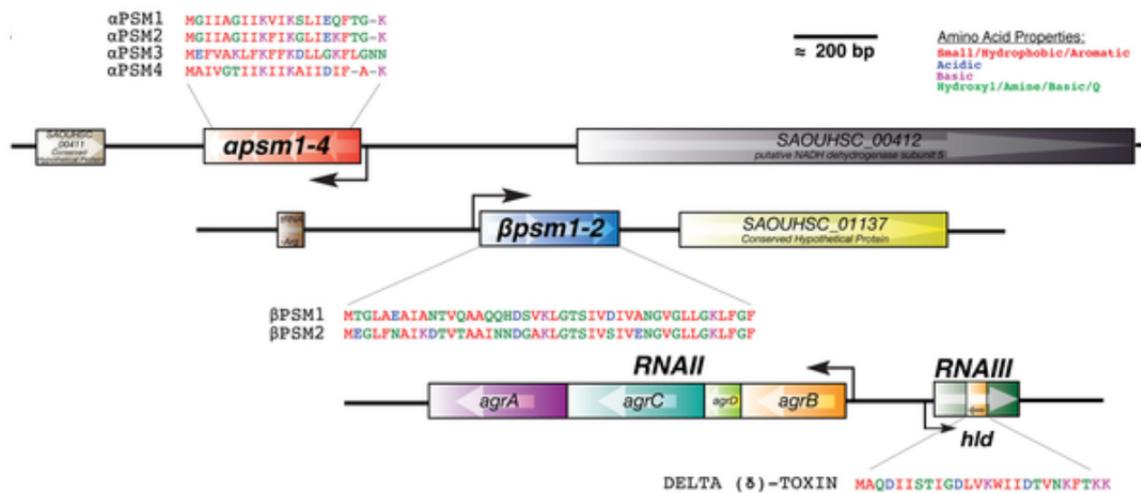
*S. aureus*  $\alpha$ -toxin ( $\alpha$ -haemolysin, Hla) is the prototype for the class of small  $\beta$ -barrel pore-forming cytotoxins (PFTs) (Parker and Feil, 2005). For many years, *Staphylococcus aureus*  $\alpha$ -toxin has been considered as the major virulence factor. Indeed, this peptide is secreted as a water soluble monomer and then oligomerized into a heptameric structure on the host cell membrane to introduce pores and to cause host cell lysis (Berube and Bubeck-Wardenburg, 2013). Following the recent identification of multiple toxin receptors, it is now understood that PFTs exert subtle changes in cell activity and host physiology even at sub-lytic concentrations. Exposure to  $\alpha$ -toxin can cause cellular death by necrosis, apoptosis, or pyroptosis, through activation of different cellular pathways (Craven et al., 2009; Essmann et al., 2003).



**Figure 2 Mechanisms by which *S. aureus* escape host innate immune defense and virulence factors regulation.** The synthesis of virulence factors is under the control of sophisticated mechanisms of regulation. Two component system (green), global transcriptional regulator (black) and the Sigma B factor (Blue). Golden carotenoid pigment provides an antioxidant shield whereas catalase detoxifies hydrogen peroxide. Resistance to cationic antimicrobial peptides is afforded by positive charge modifications of the cell wall, aureolysin-mediated proteolysis, and binding/inactivation by staphylokinase. Protein A binds Fc domains of Igs in a nonopsonic manner. The pore-forming toxins g-haemolysin and Panton-Valentine leukocidin preferentially target leukocyte membranes causing their lysis. The plasminogen (PG) binding protein staphylokinase (SAK) activates the zymogen to the active protease plasmin, which can degrade complement opsonin C3b and the immunoglobulin Fc domain. (Adapted from (Nizet, 2007).

### II.2.3.3 PSMs

PSMs are virtually produced by all staphylococcal species and given their crucial role in *S. aureus* pathogenicity, they have recently received large interest. In *S. aureus*, PSMs constitute a group of seven different peptides that are encoded by three different loci in the bacterial genome (Cheung et al., 2014; Wang et al., 2007) (**Figure 3**).



**Figure 3 Phenol soluble modulins are small peptides expressed from three loci of the *S. aureus* genome.** Phenol soluble modulins (PSMs) are encoded in two operons, the alpha ( $\alpha$ PSM1 to 4) and beta ( $\beta$ PSM1 to 2) operons, and  $\delta$ -toxin is embedded within the regulatory RNAIII (*hld*). (Figure adapted from (Schwartz et al., 2012).

Their coding sequences are small enough to have eluded detection by conventional gene annotation programs, and they are still poorly annotated in public databases (Wang et al., 2007). We have recently sequenced and annotated the genome of the strain HG001, a derivative of RN1 (NCT8325) strain with restored *rbsU* (a positive activator of SigB). Prokka software (Seemann, 2014), used to predict coding sequences (CDS), has failed to include the *psmA* locus which has been manually added (Caldelari et al., 2017). The  $\alpha$ -type peptides are ~20 to 25 amino acids in size and, in *S. aureus*, comprise PSM $\alpha$  1-4 and the  $\delta$ -toxin, which are encoded by the *psmA* and *hld* loci, respectively. *S. aureus* PSM $\beta$ 1 and PSM $\beta$ 2 are members of the larger (~45-amino-acid)  $\beta$ -type PSMs and are encoded by the *psm $\beta$*  locus. The *hld* gene is embedded within RNAIII, the intracellular effector of the accessory gene regulator (*agr*) system ((Novick et al., 1993), see § III). In *S. aureus*, PSM $\alpha$  peptides and  $\delta$ -toxin are highly abundant, with  $\delta$ -toxin usually more strongly expressed than PSM $\alpha$  probably to compensate its only moderate cytolytic capacities. Only small amounts of PSM $\beta$  peptides are produced under common laboratory conditions (Cheung et al., 2010).

PSMs are responsible for the development of *S. aureus* infections, particularly in highly virulent strains. The secretion of these peptides does not occur via canonical system, such as Sec-dependent transport, but requires dedicated secretion mechanisms. The Phenol-soluble modulins transporter (Pmt), present in all staphylococcal species, was recently identified as specific PSM exporter. Pmt consists of four genes (*pmtA*, *pmtB*,

*pmtC*, *pmtD*), encoding an ABC transporter with two separate membrane parts (PmtB, PmtD) and two separate ATPases (PmtA, PmtC). It assists PSM transport in a specific and energy-dependent fashion (Chatterjee et al., 2013). Interestingly, the ABC is essential for bacterial survival. Indeed, its deletion leads to an abnormal accumulation of PSM toxins in the cytoplasm, resulting in growth deficiency, cellular defect and autolysis (Chatterjee and Otto, 2013). Pmt is ubiquitously present among all *Staphylococcus* species and is responsible of exporting all types of PSM peptides. Upstream of the *pmtA-D* genes is a gene predicted to encode a transcriptional regulator named PmtR. Binding of PmtR to the operator of the *pmt* promoter causes repression of the *pmt* cluster. PSMs bind to PmtR and disrupt the PmtR-*pmt* promoter complex, which enables *pmt* transcription. Thus, PSMs positively influence the expression of *pmt* to facilitate their own export (Joo et al., 2016; Joo and Otto, 2016). Moreover, Pmt act to protect *S. aureus* from the antimicrobial activity of the PSM secreted by other co-colonizing staphylococcal bacteria (Cogen et al., 2010).

Despite the fact that these toxins have different structures and different target specificity, their mechanism of action is quite similar. At low doses (nanomolar concentration), they form  $\beta$ -barrel pores in the cytoplasmic membranes of target cells and cause leakage of the cell's content while, when present at high doses (micromolar concentration), they have cytolytic activity. In contrast to  $\alpha$ -toxins and bi-component leukotoxins that induce membrane pore formation by binding to specific receptors, the PSMs are believed to induce the disruption of cytoplasmic membrane in a less specific manner without receptor recognition. Most probably, the phospholipid composition and membrane charge are important for cell susceptibility to PSMs (Otto, 2015).

PSM $\alpha$  of *S. aureus* and *S. epidermidis*, are able to lyse human white and red blood cells (Otto, 2012). Additionally, as soluble molecules they also contribute to biofilm detachment/dissemination acting as surfactant-like peptides (Kong et al., 2006; Periasamy et al., 2012; Tsompanidou et al., 2011). This process allows the spread of the infection in other parts of the body (Periasamy et al., 2012). In contrast to this activity, it has been also demonstrated that, when polymerized into amyloid fibers, some PSMs promote biofilm stability (Schwartz et al., 2012). These PSM fibers share morphological and biophysical characteristics with functional bacterial amyloids such as curli in *Escherichia coli*, TsaA of *Bacillus subtilis*, and the Fap fimbriae in *Pseudomonas*

*aeruginosa* (Chapman et al., 2002; Dueholm et al., 2013; Romero et al., 2010). In this regard, the recent solved crystal structure of full-length PSM $\alpha$ 3 peptide, revealed a distinctive “cross- $\alpha$ ” amyloid-like architecture of the molecules, in which amphipathic  $\alpha$  helices stacked perpendicular to the fibril axis into tight self-associating sheets. It was shown that the “cross- $\alpha$ ” fibrillation of PSM $\alpha$ 3 is responsible for cytotoxicity, confirming that this mode of assembly has specific functions in *S. aureus* (Tayeb-Fligelman and Landau, 2017).

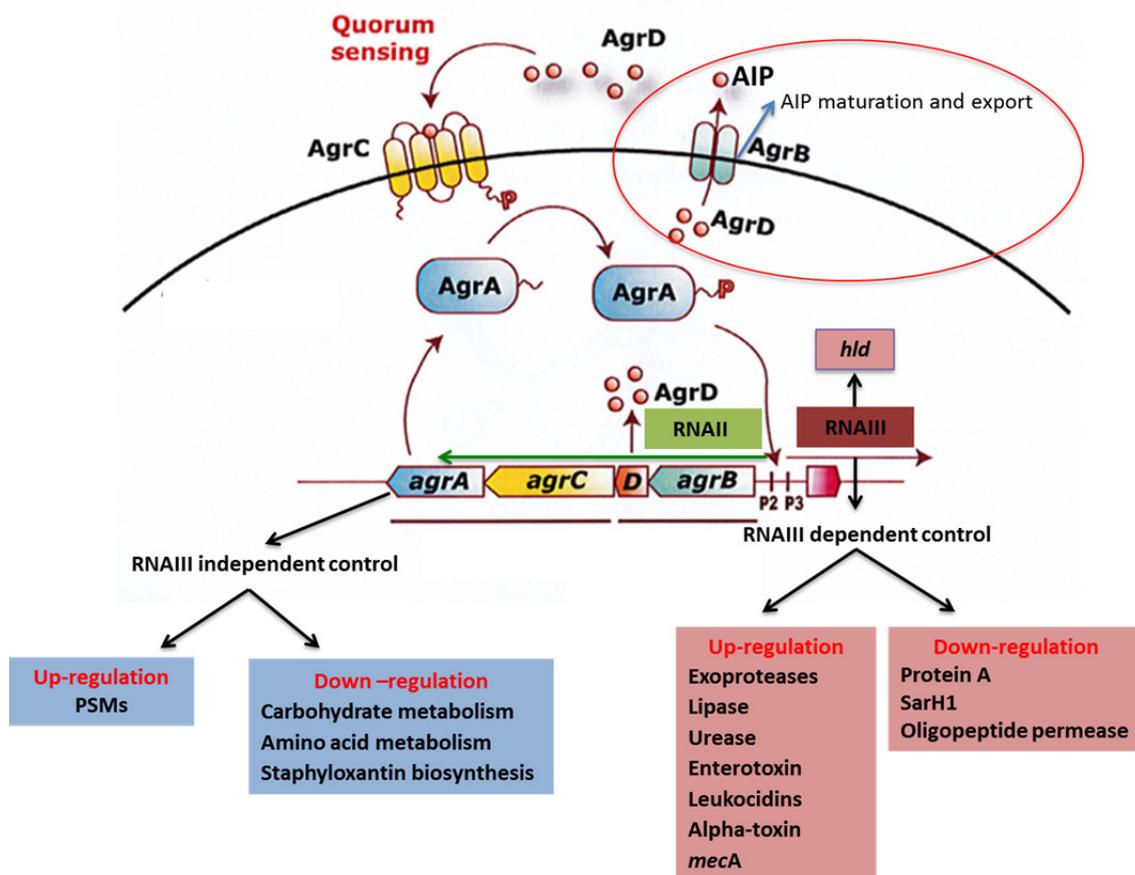
In addition to chromosomally encoded PSM peptides, *S. aureus* secretes PSM-mec toxins belonging to the PSM  $\alpha$ -type. They are encoded on a mobile antibiotic resistance cassette (SCC Staphylococcal cassette chromosome) (Chatterjee et al., 2011; Kaito et al., 2011) that influences cytolytic ability, methicillin resistance, biofilm formation, cell spreading, and the expression of other virulence factors such as other PSMs, resulting in a significant impact on *S. aureus* pathogenicity (Qin et al., 2016). The amount of secreted Psm-mec is highly variable among different MRSA strains and is strongly correlated to the level of synthesis of PSM $\alpha$  peptides (Chatterjee et al., 2011). The *psm-mec* RNA has been shown to alter the stability of *agrA* mRNA most probably through basepairings (Kaito et al., 2013) although these data have not been reproduced in another strain background (Cheung et al., 2014). These data suggested that *psm-mec* RNA has a dual function, acting as an antisense RNA and coding a PSM peptide. Such a dual activity has been well recognized for RNAIII, the intracellular effector of quorum sensing system, which also encodes a PSM (see § III).

### **III. Regulation of the expression of virulence factors**

To regulate this coordinated expression of virulence factors, multiple trans-acting modulators, including regulatory proteins, secondary metabolites, small peptides, and RNAs, are brought into play (Novick and Geisinger, 2008; Wyatt et al., 2010). I will thereafter describe only several of these regulators to illustrate the complexity of the regulatory networks, which are aimed to fine tune the expression of the virulence factors in a dynamic manner according to various external and internal stimuli.

### III.1 Regulation of PSM production and other virulence factors by the *agr* locus

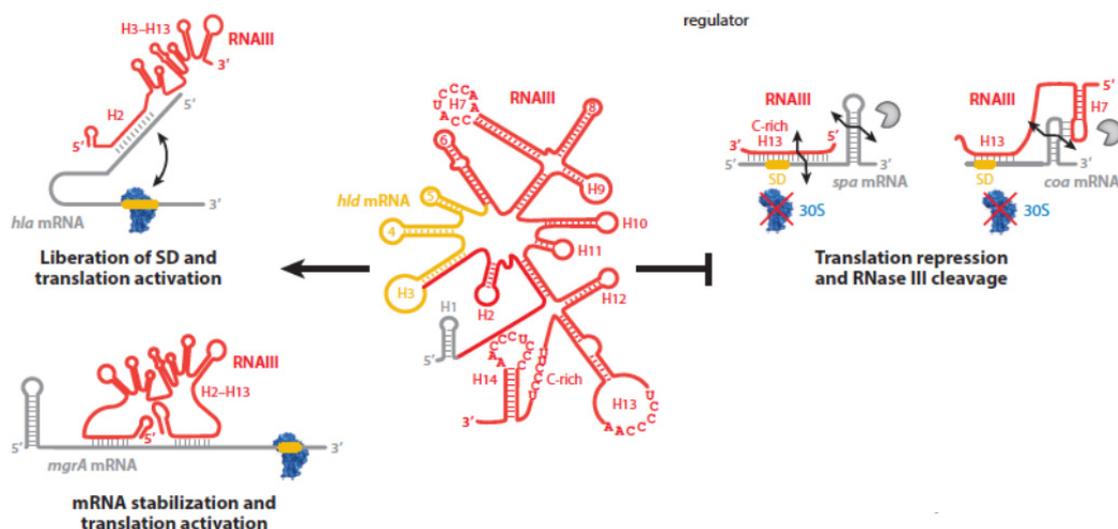
Timing and fine regulation of virulence determinants during infection and development of acute staphylococcal disease might be achieved by putting genes involved in pathogenicity under the control of common regulators such as *agr* quorum sensing system. The *agr* locus is comprised of two adjacent units, RNAII and RNAIII (transcribed in opposite directions) that are under the control of P2 and P3 promoters, respectively. RNAII codes for a cell-density cassette, *agrD* and *agrB*, and for the bacterial two-component signal transduction system (TCS), composed of the sensor histidine kinase AgrC and its response regulator AgrA. The processing of the precursor peptide AgrD by the protease AgrB, produce an autoinducing thiolactone peptide (AIP), which is continuously released in the extracellular environment. AgrA is activated by AgrC through phosphorylation in response to a threshold concentration of the secreted AIP. In turn, the phosphorylated form of AgrA directly enhances the transcription of RNAII and RNAIII by binding to its promoter regions (Queck et al., 2008) (**Figure 4**).



**Figure 4. Schematic representation of *S. aureus* *agr* regulatory system.** The *agr* operon consists of two transcriptional units RNAII and RNAIII, driven by the promoters P2 and P3, respectively. RNAII is an operon of four genes, *agr BDCA*, encoding AgrB responsible for processing and exporting AgrD, the AIP precursor. At threshold levels of AIP, AgrC will be autophosphorylated, leading to the phosphorylation of

AgrA. AgrA activates RNAIII expression. Many RNAIII-dependent agr targets contain a series of key virulence factors such as proteases and toxins while the PSMs are regulated *via* AgrA in an RNAIII-independent way (adapted from Queck et al., 2008).

RNAIII and AgrA are the two main intracellular effectors of the *agr* system. RNAIII is composed of 14 hairpin structures, which are organized in different functional modules (Benito et al., 2000). The RNA is responsible for the regulation of the synthesis of many virulence factors. It positively affects the synthesis of several exoproteins including  $\alpha$ -toxin,  $\beta$ -haemolysin, TSST-1 and leukotoxins (Morfeldt et al., 1995; Novick and Geisinger, 2008; Novick et al., 1993; Oscarsson et al., 2006), while it negatively controls the expression of several cell wall-associated proteins including protein A, coagulase (*coa*) and fibronectin binding proteins (**Figure 5**). The 5' end of RNAIII, activates the translation of  $\alpha$ -haemolysin by disrupting the intramolecular RNA secondary structure sequestering the *hla* ribosomal binding site (Morfeldt et al., 1995; Novick et al., 1993). The RNAIII 3' domain represses the translation of several virulence factors including protein A and of the global regulator of toxins Rot through direct binding to the Shine and Dalgarno (SD) sequence of the mRNAs. The resulting complexes are composed of an imperfect duplex sequestering the SD sequence of mRNAs, and in turn prevent the formation of initiation complexes. The mRNAs bound to RNAIII are then rapidly degraded by the double-strand-specific endoribonuclease III (RNase III) (Chevalier et al., 2010). It was also demonstrated that both the 3' and 5' ends of RNAIII interact with the 5' UTR of *mgrA* mRNA preventing its degradation (Bronesky et al., 2016) (**Figure 5**).



**Figure 5: Mechanism of action of RNAIII on its target mRNAs.** Genomic organization of the quorum-sensing *agr* system is given at top. The schematic secondary structure of RNAIII (red) is from Benito *et al.* The *hld* gene encoding  $\delta$ -haemolysin is yellow. The various C-rich sequence motifs of RNAIII are the seed sequences that bind to the Shine and Dalgarno (SD) sequence of mRNA targets (gray). Various topologies

of RNAIII-mRNA are given. Binding of RNAIII to several mRNAs (*spa*, *coa*) can prevent binding of the 30S small ribosomal subunit, and in several cases this step is followed by rapid degradation initiated by the double strand-specific endoribonuclease III (gray circle). Binding of RNAIII to target mRNAs can also activate synthesis of exotoxins. For *hla* mRNA, interaction with RNAIII prevents the formation of an inhibitory structure sequestering the SD, whereas for *mgrA* mRNA, binding of RNAIII stabilizes the mRNA against an RNase attack. In these latter cases, the seed sequences initiating basepairings between RNAIII and mRNA targets (*hla*, *mgrA*) have not yet been demonstrated. Abbreviation: AIP, autoinducing thiolactone peptide. Adapted from (Bronesky et al., 2016).

Embedded in RNAIII, there is the *hld* gene coding for the PSM  $\delta$ -toxin, the haemolysin delta. In 1995, Novick and Balaban have shown that RNAIII is transcribed at the mid-exponential phase of bacterial growth, while the PSM  $\delta$ -toxin starts to be synthesized during the post-exponential phase. The delay of 1 hour, between the transcription of the RNAIII and the appearance of the PSM  $\delta$ -toxin, was eliminated by the deletion of the 3' non coding part of RNAIII. These data suggested the existence of unknown *trans*-acting factor that would control the expression of  $\delta$ -toxin during the bacterial growth. Moreover, the RNAIII secondary structure showed basepairings between the 3' and the 5' ends of the molecule, suggesting that a conformational rearrangement is required for *hld* translation (**Figure 5**).

AgrA not only activates its own operon and RNAIII, but also the transcription of the *PSM $\alpha$* , *PSM $\beta$* , and *psm-mec* (Chatterjee et al., 2011; Chatterjee et al., 2013; Queck et al., 2008). Based on these data, Queck *et al.* (2008) have suggested that the RNAIII-dependent regulatory circuits and the *agrA* quorum-sensing system have been combined during evolution in order to synchronize the cell density changes to virulence gene expression during infection. Although the regulation of PSM transcription is clearly linked to AgrA, other mechanisms for their regulation also existed.

PSMs are produced at extremely high levels in response to specific external stimuli that could be independent of the cell density, reaching ~ 60% of the total secreted protein mass in *S. aureus* (Chatterjee and Otto, 2013). The quorum-sensing system is activated only in response to sufficient concentration of AIP, however, there is faster regulation of PSM expression via intracellular signaling molecules. When internalized by leukocytes or neutrophils, *S. aureus* produces high level of (p)ppGpp which quickly activate PSM production by a non yet characterized mechanism (Geiger et al., 2012). This data suggested that both *agr*-dependent (Surewaard et al., 2012) and *agr*-independent (Geiger et al., 2012) responses co-exist to ensure immediate and continued production of PSMs, allowing *S. aureus* to escape host immune-response and to induce cell lysis. The

production of the PSM $\alpha$  is also positively modulated by SarA. Indeed, by down-regulating the expression of aureolysin (Aur), SarA decreases the rate of PSM degradation (Antignac et al., 2007; Zielinska et al., 2011).

### III.2. Two component regulatory system SaeRS

The *sae* locus codified for SaeRS TCS regulates the expression of many virulence factors: it up-regulates  $\alpha$ -,  $\beta$ - and  $\gamma$ - haemolysins (Goerke et al., 2005; Liang et al., 2006) and down-regulates Protein A (Giraud et al., 1997). As other typical TCSs, the signaling cascade in the SaeRS TCS starts when the histidine kinase SaeS detects environmental signals coming from human neutrophils or peptides (Geiger et al., 2008). As the consequence, SaeS autophosphorylates and transfer its phosphoryl group to SaeR that binds to SaeR binding sequence (SBS) and, in most cases, activates the transcription of the target genes (Liu et al., 2016).

### III.3. The global regulator SarA

Among the global regulatory systems identified in *S. aureus*, the staphylococcal accessory regulator A (*sarA*) and its several homologues, are the most well characterized. The *sar* locus is composed of three overlapping transcripts *sarA*, *sarC*, and *sarB* originating from the P1, P3, and P2 promoters, respectively. The promoters P1 and P2 are SigA dependent, while the P3 promoter is dependent on SigB. SarA is constitutively expressed and it regulates several genes (Manna et al., 1998). By binding to a consensus motif rich in AT in the promoter regions of target genes (Novick and Jiang, 2003), SarA up-regulates the expression of some virulence factors including Fnbps (for adhesion to the host cells),  $\alpha$ - and  $\beta$ -haemolysins (for tissue spread) and down-regulates other genes encoding Protein A and proteases (Chan and Foster, 1998; Cheung et al., 1994).

Several SarA homologues have been identified, and all of them contribute to modulate the *agr* system (**Figure 2**; (Cheung et al., 2008)). SarR is involved in the inhibition of SarA and in the regulation of several virulence factors during the exponential and stationary phases of growth (Manna and Cheung, 2001; Manna and Cheung, 2006). SarS

is a DNA binding protein involved in the activation of transcription of *spa* during the exponential phase of bacterial growth (Cheung et al., 2004). SarT activates the expression of SarS while it inhibits the  $\alpha$ -haemolysin. The transcription of this factor is also repressed by SarA and AgrA (Cheung et al., 2001). SarU is repressed by SarT, it activates the *agr* operon. In contrast, SarX represses the *agr* operon when activated by MgrA (Manna and Cheung, 2006). MgrA, which activates the *agr* operon, is repressed by SarS. MgrA works as a multiple gene regulator, which inhibits the synthesis of several secreted proteases (Ingavale et al., 2005; Luong et al., 2003). SarZ activates the *agr* operon and the expression of SspA, while it represses SarS. Upon its MgrA-dependent activation, it regulates important genes involved in biofilm formation (Tamber and Cheung, 2009). Finally, the global regulator Rot negatively affects the transcription of numerous factors including lipase, haemolysins, proteases, while positively regulates many other genes including cell surface adhesins. It acts as an antagonist of *agr* which represses its synthesis during the stationary phase of bacterial growth (McNamara et al., 2000; Said-Salim et al., 2003).

#### III.4. *S. aureus* sigma factors

The regulation of virulence factors is also mediated by sigma factors ( $\sigma$ ), which bind to the core RNA polymerase to form the holoenzyme able to recognize specific promoters (Palma and Cheung, 2001). *S. aureus* have four sigma factors ( $\sigma^A$ ,  $\sigma^B$ ,  $\sigma^S$ ,  $\sigma^H$ ).  $\sigma^A$  is responsible for the expression of housekeeping genes essential for growth (Deora et al., 1997).  $\sigma^B$  regulates the expression of genes involved in several cellular functions such as stress responses (Deora and Misra, 1996) and in virulence (Bischoff et al., 2001; Horsburgh et al., 2002). It up-regulates FnbpA and the coagulase (CoA), and downregulates haemolysins and serine protease A (Bischoff et al., 2004; Entenza et al., 2005). However, the effect of  $\sigma^B$  on virulence might result via the regulation of transcriptional factors such as *sarA*, *sarS*, and *rot*. It has also been shown to be involved in antibiotic resistance, biofilm formation and in bacterial internalization into host cells (Nair et al., 2003; Rachid et al., 2000).  $\sigma^H$  has been demonstrated as a key actor for competence development (Morikawa et al., 2012). The factor also modulates prophage integration and excision through the regulation of the integrase expression (Tao et al.,

2010). For the last Sigma factor  $\sigma^S$ , its regulon is still not yet defined but high levels of the factor were observed in cells grown in the presence of serum. Its inactivation decreased the fitness of *S. aureus* at high temperature, and the infectivity of the mutant strain was altered in a murine model of septic arthritis (Miller et al., 2012; Peton et al., 2016).

#### IV. Antibiotic resistances in *Staphylococcus aureus*

*S. aureus* is able to invade and survive within neutrophils and macrophages, leading to its incomplete clearance even in the presence of high levels of antibiotics. The introduction of penicillin in the early 1940s positively influenced the prognosis of patients with staphylococcal infections. However, few years later, penicillin-resistant staphylococci were recognized, first in the hospitals and then in the community. The resistance to penicillin is mediated by *blaZ*, the gene that encodes  $\beta$ -lactamase. This predominantly extracellular enzyme, synthesized when staphylococci are exposed to  $\beta$ -lactam antibiotics, hydrolyzes the  $\beta$ -lactam ring of penicillin deactivating the antibacterial properties of the molecule (Chambers and Deleo, 2009; Lowy, 2003). With the emergence of resistance to the penicillins, a semi-synthetic antibiotic methicillin, which is derived from penicillin but resistant to  $\beta$ -lactamase inactivation, became the molecule of choice for the treatment *S. aureus* infections. However, immediately after its introduction, methicillin-resistant (MRSA) bacteria were isolated (Jevons, 1961). The gene responsible for methicillin resistance is named *mecA*, it is located on a mobile genetic element known as *mec* element (SSCmec) (Chambers, H. F. 1997) that serves as a vehicle for gene exchange among staphylococcal species (Hacker et al., 2004). *MecA* is responsible for synthesis of the penicillin-binding protein 2a (PBP2a), a membrane-bound enzyme located at the membrane-cell wall interface and involved in the peptidoglycan synthesis (Ghuysen, 1994). PBP2a substitutes for other PBSPs and, given its low affinity for all  $\beta$ -lactam antibiotics, allows staphylococci to survive even in the presence of high concentrations of this drug. Under the pressure of intensive emerging antibiotic resistant strains, the last remaining antibiotic to which MRSA strains were susceptible was the vancomycin, able to inhibit *S. aureus* cell wall. The reduced susceptibility to vancomycin was due to the synthesis of an unusually cell wall containing dipeptides (D-Ala-D-Ala) capable of binding the antibiotic, sequestering it

and thereby reducing availability of the drug for intracellular target molecules. Fortunately, these resistant strains did not spread substantially, possibly due to increased fitness cost. The ability of *S. aureus* to develop resistance to antibiotic treatments is dynamic and has changed significantly over the years. It could virtually acquire resistance against all antimicrobial agents available in hospitals and communities (DeLeo et al., 2010). Hence, the reengineering of existing antibiotics and the synthesis of new therapeutic alternatives are urgently needed.

## V. Translation and its control in *S. aureus*

### V.1. Translation initiation, a check point for regulation

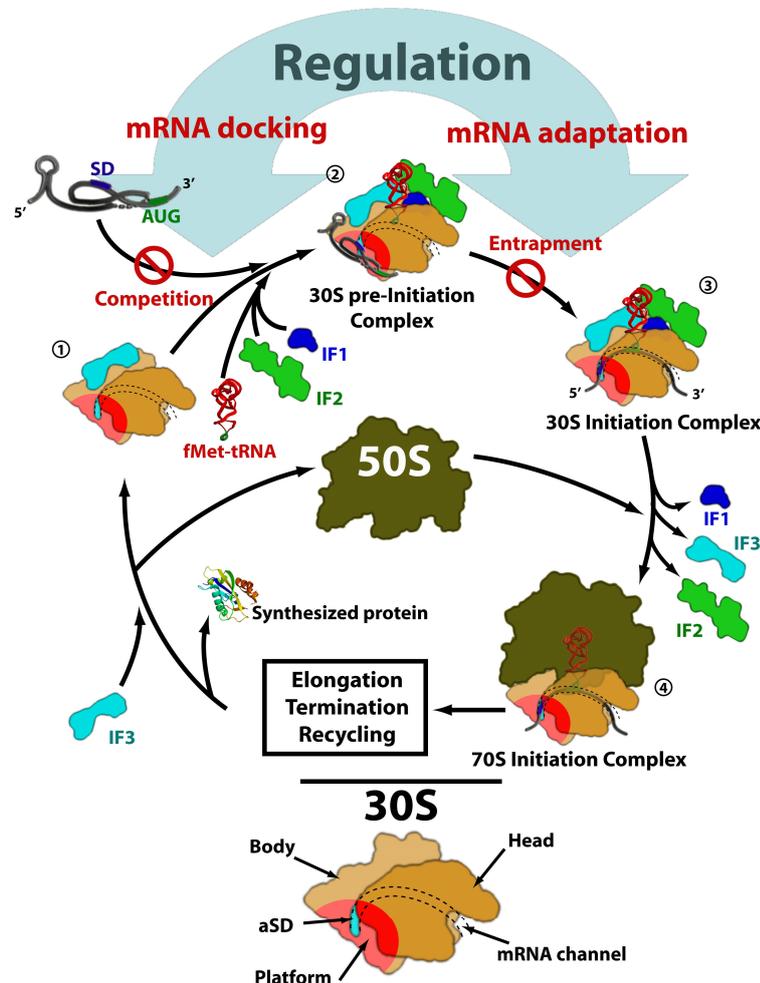
The above described orchestration of *S. aureus* virulence demonstrates the ability that bacteria have to rapidly sense their environment and constantly adapt their physiology in response to its variations. Several extracellular and intracellular signals (e.g. the quorum sensing peptide described for the *agr* system) are detected and associated to multi-step regulations at the transcriptional and post-transcriptional levels, which contribute to reprogramming the bacterial proteome. Among the different regulatory mechanisms, translational control can ensure a rapid and transient. The RNAIII-directed translational silencing or activation mechanisms represent a typical example of how protein synthesis could be regulated. In the vast majority of the cases, changes in translational efficiency are the result of a modulation of its initiation process (Romby, 2007) during which the ribosome assembles on the mRNA in the order of seconds (Gold, 1988; Kennell and Riezman, 1977). This slow, rate-limiting step of protein synthesis provides the time window necessary for regulation between mRNA transcription and active translation or mRNA degradation. Even if in bacteria, transcription, translation and mRNA degradation are interconnected processes and often coupled (Burmann et al., 2010; Campos and Jacobs-Wagner, 2013; Das et al., 1967; Mehdi and Yudkin, 1967; Proshkin et al., 2010; Shin and Moldave, 1966; Stent, 1964), large 5' and 3' Untranslated Regions (5'UTR, 3'UTR) which are often structured, strongly impact translation initiation and are therefore target of several regulatory mechanisms (Geissmann et al., 2009b; Marzi et al., 2008a; Ruiz de los Mozos et al., 2013). Bacterial translation initiation is a highly conserved process, but some differences and specific features among distant bacteria have been observed. This is the case of the Gram-negative bacterium *Escherichia coli* and the Gram-positive low G+C content *S. aureus* which have diverged more than 10 million years ago.

I will thereafter describe the details of the bacterial initiation process mainly derived from studies on Gram-negative bacteria and will illustrate some structural differences between *E. coli* and *S. aureus* ribosomes. The description of the main functional differences between the initiation processes is included in the joined review "A glimpse on *Staphylococcus aureus* translation machinery and its control" (Khusainov et al., 2016) where I am second author.

## V.2. A view on the Translation Initiation in Gram-negative bacteria

In contrast to eukaryotes and archaea (for reviews see (Londei, 2005; Pestova et al., 2007)), the initiation process in bacteria involves a rather low number of *trans*-acting factors. Three initiation factors (IF1, IF2, IF3) kinetically assist the formation of the translation initiation complex, with the 30S small ribosomal subunit, the aminoacylated and formylated initiator tRNA (fMet-tRNA<sup>fMet</sup>) and the mRNA (**Figure 6**; (Simonetti et al., 2008)). The three factors have no direct influence on the recruitment of the mRNA (Milon et al., 2012) but exert specific functions. IF3 acts as “fidelity factor”. It helps in the selection of initiator tRNA (fMet-tRNA<sup>fMet</sup>) by destabilizing the binding of other tRNAs in the P site of the ribosome (Hartz et al., 1990; Milon et al., 2012). It is also known to strongly anchor the 30S subunit preventing the association with the large ribosomal subunit so to maintain a cellular pool of 30S for translation initiation (Grigoriadou et al., 2007). IF2 is a GTPase factor that binds specifically to fMet-tRNA<sup>fMet</sup> and correctly positions it on the 30S to favour the 50S joining (Simonetti et al., 2013; Simonetti et al., 2008). IF1, the smallest of the three initiation factors, binds to the A site of the 30S ribosomal subunit (Carter et al., 2001) where physically it prevents tRNA binding and prepares the P site for fMet-tRNA<sup>fMet</sup> binding. The formation of the “30S initiation complex” (30SIC) takes place at the Ribosome Binding Site (RBS) of the mRNA. The mRNA stably binds the 30S *via* its Shine-Dalgarno sequence (SD) complementary to the anti SD (aSD) sequence at the 3’ end of the 16S rRNA (Hui and de Boer, 1987; Jacob et al., 1987; Shine and Dalgarno, 1974). For many bacterial mRNAs, the selection of the correct start codon (usually AUG) depends largely on the formation of this short SD-aSD helix. Once the 30SIC complex is formed, the translational reading frame is set and protein synthesis can start. During the following steps of translation initiation, the joining of the large ribosomal subunit (50S) to the 30SIC leads to the formation of the “70S initiation complex” (70SIC; made by the small and the large ribosomal subunits, the fMet-tRNA<sup>fMet</sup> and the mRNA), ready for the first peptide bond formation. During this transition, the adjustment of fMet-tRNA<sup>fMet</sup> in the ribosomal P-site and the release of all factors are coupled with the hydrolysis of the GTP molecule bound to IF2 (Allen et al., 2005; Myasnikov et al., 2005; Simonetti et al., 2008). The formation of the correct 70SIC marks the irreversible transition to the elongation phase.

The binding of the mRNA to the 30S subunit is one of the most critical steps of translation initiation and the target of the majority of known translation regulations. It takes place into two sequential steps, first the localization on the platform of the 30S to form the 30S pre-initiation complex, and then the accommodation into the mRNA channel (Figure 6).

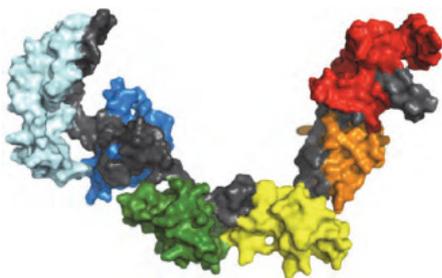


**Figure 6. A schematic view of the initiation process of translation and its regulation in *Escherichia coli*.** Three initiation factors (IF3 in light blue, IF1 in blue and IF2 in green) bind to the 30S subunit (in orange). The platform binding center is in red with the anti-SD (aSD) sequence in cyan. Structured mRNA binds to 30S in two distinct steps: the docking of the mRNA on the platform of the 30S subunit is followed by the accommodation of the mRNA into the normal path to promote the codon-anticodon interaction in the P site. These two steps are submitted to tight control, through the alternative competition and entrapment mechanisms. Numbers refer to: 1) the cellular pool of initiating 30S subunits bound to IF3; 2) 30S pre-Initiation Complex refers to the docking step of the structured mRNA; 3) 30S Initiation Complex refers to the active complex in which the mRNA is accommodated into the normal path forming the codon-anticodon interaction; 4) 70S Initiation Complex, the 50S subunit (in brown) joins the 30S Initiation Complex to proceed protein synthesis. The scheme is derived from (Marzi et al., 2008b). At the bottom, the different domains of the 30S subunit, the mRNA channel and the aSD sequence are given.

### V.2.1. *E. coli* r-protein S1, an RNA chaperone associated with the ribosome for structured mRNA translation

*E. coli* S1 is an atypical ribosomal protein: S1 is the largest (61KDa) protein of the 30S subunit, is among the ribosomal proteins with documented high affinity for numerous mRNAs (Draper et al., 1977), is the most acidic (pI= 4.7) r-protein (Kaltschmidt and Wittmann, 1970), it is the last protein to associate on the 30S and its interaction with the 30S is weak and reversible. *EcoS1* is essential for growth and its functional relevance is related to its ability to simultaneously bind mRNAs and the ribosome. Indeed, S1 mediates initiation of translation by binding the 5' UTR of mRNAs, thus increasing their affinity for the ribosome up to 5000 fold (Draper and von Hippel, 1979; Katunin et al., 1980). This is especially evident for weak SD-containing mRNAs, which necessitate S1 to be stabilized on the 30S (Komarova et al., 2005). In *E. coli*, S1 is also responsible of the accommodation of the mRNA in the 30S decoding channel, a slow process essential for the translation of structured mRNAs (Duval et al., 2013b).

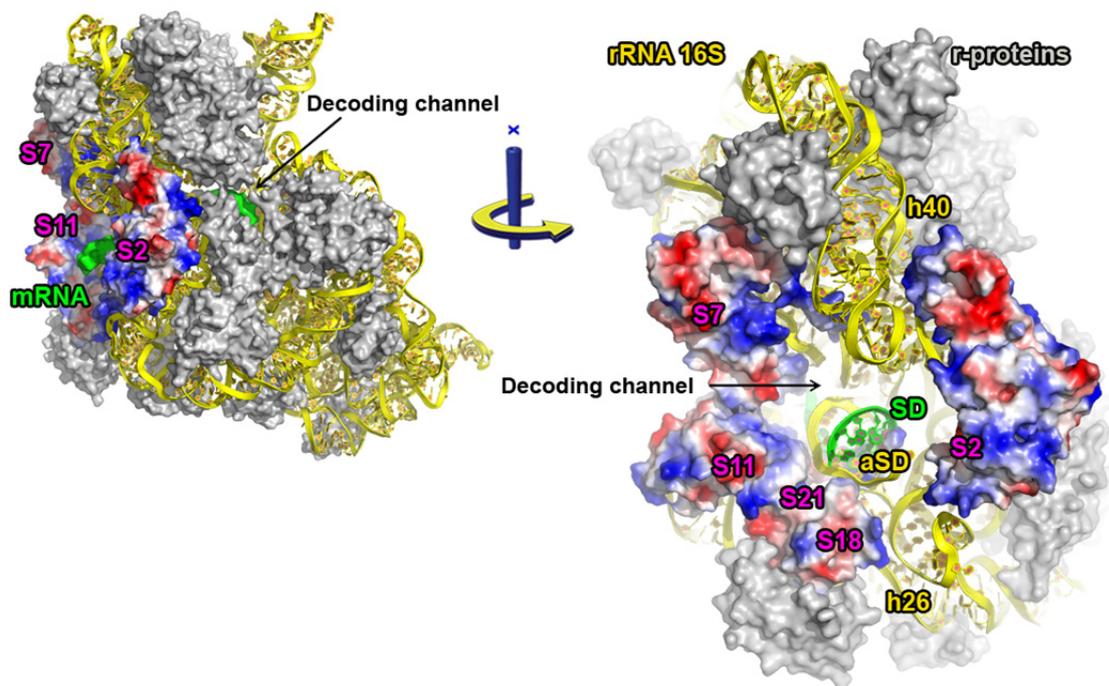
*EcoS1* is composed of 6 OB fold domains (**Figure 7**): the first two domains, d1 and d2, are essential for 30S subunit binding, while the domains d3 and d4 together with d1 and d2 form the minimal protein that recognize mRNAs and allow their accommodation into the decoding channel (Duval et al., 2013b; Duval et al., 2017). The last two C-terminal domains are dispensable and have regulatory functions related to RNA maturation/degradation (unpublished data from the laboratory).



**Figure 7. Modelled structure of *E. coli* S1.** Domain 1 in light blue, domain 2 in dark blue, domain 3 in green, domain 4 in yellow, domain 5 in orange and domain 6 in red. Model realized by Stefano Marzi.

To date, X-ray and/or Cryo-EM ribosomal structures of Gram-negative bacteria such as *E. coli* and *Thermus Thermophilus*, have failed to visualize S1. In this regard, it has to be considered that the purification of ribosomes for X-ray studies involves a step of deliberate removal of S1 in order to increase the homogeneity of the ribosomes and to obtain better diffracting crystals (Schuwirth et al., 2005; Yusupov et al., 2001), while Cryo-EM investigations might be not suitable for a highly dynamic protein due to the averaging of different conformations. Recently, the S1 N-terminal domain (d1) was

nevertheless observed by Cryo-EM interacting *via* a zinc binding pocket to the ribosomal protein S2 on the 30S platform (Byrgazov et al., 2015). Crosslinking experiments in combination with high resolution mass spectrometry analysis have also placed S1 in the same region on the ribosome (Lauber et al., 2012). The platform between the head and the body of the 30S (**Figure 8**) is thus formed by S1 and several other r-proteins (S2, S7, S11, S18 and S21) together with two rRNA helices (h26 and h40) that are surrounding the aSD close to one extremity of the decoding channel. Since both r-protein S1 and the aSD are on the platform, we can imagine that several mRNAs would transit on this site before getting into the mRNA decoding channel. A systematic structure and sequence analysis revealed that conserved residues of the r-proteins of the platform form patches of positive charges on its surface that could localize folded mRNA regardless of their specific structure or sequence (Marzi et al., 2007). Interestingly, it has been observed that even sequences that have low propensity to form structures, like poly(A) or poly(U), get folded into compact structures similar to hairpins close to r-protein S2 (Yusupova et al., 2006) (**Figure 8**).

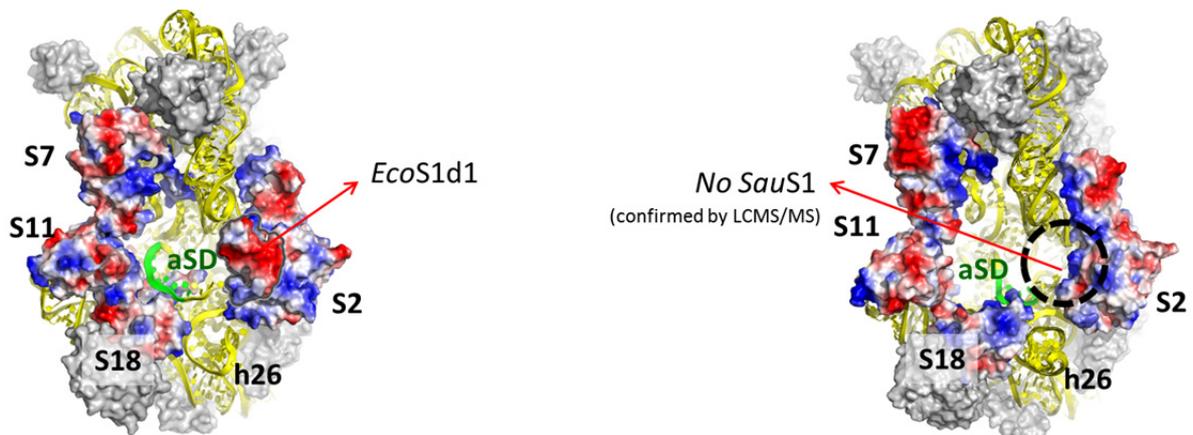


**Figure 8. The platform of the 30S.** On the left, view of the 30S subunit showing the localization of the platform. On the right, enlarged and rotated view of the platform showing the charge distribution of the r-proteins composing it. S2, S7, S11, S18 and S21 are forming a nest of positive charges (blue surfaces) around the aSD. Negatively charged amino acids are represented in red. The mRNA (green) is shown accommodated into the decoding channel. The model in the figure has been obtained using the coordinates of *E. coli* ribosome (pdb file 4TP8) which contains all the r-proteins of the platform but S1 and the mRNA pathway described in the *T. thermophilus* translation initiation complex (pdb file 4HGR).

### V.3. *S. aureus* ribosome structure and specific features

The structures of the bacterial 70S ribosomes have been extensively studied in Gram-negative bacteria such as *E. coli* (e.g. (Schuwirth et al., 2005; Noeske et al., 2015)) and *T. thermophilus* (e.g. (Yusupov et al., 2001; Yusupova et al., 2001)), but have received little attention in Gram-positive bacteria (Beckert et al., 2015; Eyal et al., 2015; Sohmen et al., 2015). Recently, structures of *S. aureus* large ribosomal 50S subunit bound to specific antibiotics (Eyal et al., 2015) and of the whole 70S (Khusainov et al., 2017) have shed light on some features specific to *S. aureus*.

Concerning the 30S platform, two main differences could be spotted. The most evident is the absence of ribosomal protein S1 (**Figure 9**), which slightly changes the charge distribution of the two platforms, making more positive the one from *S. aureus* in the region which is close to the 3' end of the 16S rRNA (aSD) and the h26. The second difference is in the length of h26, which is incremental when going from *T. thermophilus* (19 nt) through *E. coli* (25 nt), *B. subtilis* (26 nt) to *S. aureus* (27 nt). Such variations at a strategic ribosomal region could reflect species-specific involvement of h26 in translation initiation and regulation.



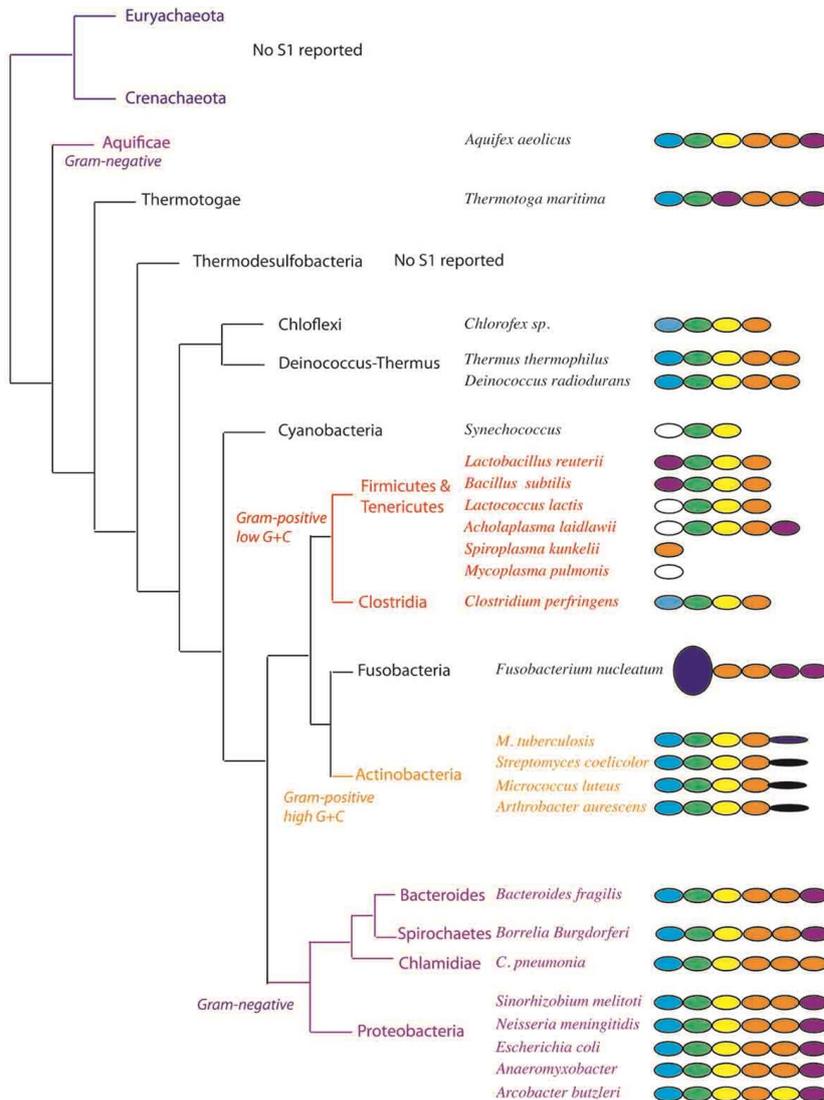
Byrgazov et al., NAR 2015

Khusainov et al., NAR 2017

**Figure 9. *E. coli* and *S. aureus* 30S platform.** On the left, *E. coli* platform as in figure 10, with the exception that the structure used here contains also d1 (Byrgazov et al., 2015). On the right, charge distribution of the r-proteins from *S. aureus* 70S structure (Khusainov et al., 2017). The charge distribution is similar in the two structures, but the absence of S1 increases even more the positively rich environment surrounding the aSD, which also appears shifted toward h26 in *S. aureus* structure.

### V.3.1. *S. aureus* protein S1, a shorter protein not associated with the ribosome

S1 is found in almost all Gram-negative and in several Gram-positive bacteria. A shorter form (45 kDa) exists in chloroplasts, but is absent in eukaryotic cells. Phylogenetic studies have indicated that S1 from low G+C Gram-positive bacteria (Firmicutes), such as *B. subtilis* and *S. aureus*, would not contain the ribosome binding domain d1 (Salah et al., 2009) (**Figure 10**).



**Figure 10. Schematic representation of the organization of protein S1 in different bacteria.** Colors have been attributed for conserved domains: domain 1 in blue, domain 2 in green, domain 3 in yellow, as domains 4/5 in orange and domain 6 in magenta. The OB fold domains, which could not be assigned to any *E. coli* domain are in white. The domains not identified as OB fold domains are in deep blue. The figure is adapted from (Salah et al., 2009).

Indeed, after purification of *S. aureus* 30S and 70S ribosomes (Fechter et al., 2009), only traces of r-protein S1 were observed by mass spectrometry analysis, suggesting that the protein is not tightly associated with the ribosome (Khusainov et al., 2016). It was

previously demonstrated that *B. subtilis* S1 plays no major role in translation and is not an essential protein (Farwell et al., 1992; Juhas et al., 2014; Vellanoweth and Rabinowitz, 1992). It was thus proposed that Firmicutes obviate the need of S1 acting on the 30S because the majority of mRNAs carry short 5' UTRs with strong SD sequences (Omotajo et al., 2015). However in *S. aureus*, numerous mRNAs carrying long 5' or 3' UTRs have been reported (Anderson et al., 2006; Lasa et al., 2011; Ruiz de los Mozos et al., 2013). They include mRNAs encoding virulence determinants, various transcriptional regulators, and metabolic enzymes. How does *S. aureus* ribosome translate them is one of the key question that I have addressed during my PhD project.

In the following review paper, using toe-printing assays, we have monitored the behavior of isolated *S. aureus* 30S and 70S ribosomes and compared them with *E. coli* ribosomes containing or not S1. Different mRNAs have been used in which the SDs were exposed in loops or shaded into hairpins, demonstrating that *S. aureus* 30S, as the *E. coli* S1-depleted 30S, could not efficiently bind to structured mRNAs.

#### ***V.4. A glimpse on Staphylococcus aureus translation machinery and its control***

Khusainov Iskander, Marena Alessandra, Cerciati Marie, Fechter Pierre, Hashem Yaser, Marzi Stefano, Romby Pascale, Yusupova Gulnara & Yusupov Marat

My contribution to the work presented in this review was related to the characterization of *S. aureus* ribosome. Mass-spectrometry analysis of purified 70S has shown the absence of *SauS1*. By gel filtration assay I have demonstrated the inability of the protein to assemble on the 30S ribosomal subunit.

I also contributed to the discussion of the results with all the authors and I have participated to the final writing of the manuscript.



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REVIEWS

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UDC 577.217.34

## A Glimpse on *Staphylococcus aureus* Translation Machinery and Its Control<sup>1</sup>

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**Abstract**—*Staphylococcus aureus* is a major opportunistic and versatile pathogen. Because the bacteria rapidly evolve multi-resistances towards antibiotics, there is an urgent need to find novel targets and alternative strategies to cure bacterial infections. Here, we provide a brief overview on the knowledge acquired on *S. aureus* ribosomes, which is one of the major antibiotic targets. We will show that subtle differences exist between the translation at the initiation step of Gram-negative and Gram-positive bacteria although their ribosomes display a remarkable degree of resemblance. In addition, we will illustrate using specific examples the diversity of mechanisms controlling translation initiation in *S. aureus* that contribute to shape the expression of the virulence factors in a temporal and dynamic manner.

**Keywords:** *Staphylococcus aureus*, quorum sensing, regulatory RNAs, post-transcriptional regulation

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### INTRODUCTION

Protein synthesis is an essential process across all domains of life, which is carried out by the ribosome. Although the ribosome is universally conserved machinery, significant differences were found in the composition of the ribosomal proteins, in extension/reduction of rRNA regions, and in the associated factors between eubacteria, archaea, eukaryotes and their organelles [1–6]. Due to its essential function, the ribosome represents one of the main antibiotic targets [7, 8]. Extensive biochemical and structural studies have now revealed the molecular mechanisms of numerous antibacterial drugs acting directly on the ribosome functioning [8]. Intriguingly, the vast majority of antibiotics act to interfere with the elongation step of protein synthesis while only a few of them interfere with the initiation step [8]. Because initiation of translation and primarily the mRNA recruitment diverge significantly in the three kingdoms of life (reviewed in [9, 10]), these differences could be potentially exploited for drug design against major human pathogens.

The structures of the bacterial 70S ribosomes have been extensively studied in Gram-negative bacteria such as *Escherichia coli* [11, 12] and *Thermus thermophilus* [13, 14], but have received little attention in Gram-positive bacteria [15–17]. *Staphylococcus aureus* is a Gram-positive bacterium that is recognized as a major human opportunistic and versatile pathogen, which causes a large spectrum of infections [18]. Importantly, methicillin-resistant strains (MRSA) are now widespread and are responsible for severe infections in the community as well as in the hospital [19], and strains resistant to vancomycin have also emerged [20, 21]. A recent study shows that MRSA isolates spontaneously diversify into distinct strains that evolve new antibiotic resistance via competition between bacterial cells within a monoclonal population [22]. A monoclonal population of cells is thus heterogeneous due to the fact that the bacteria should constantly compete for space and acquisition of nutrients [23]. Hence, there is an urgent need to develop alternative anti-bacterial strategies [24–26] but also to better understand the mechanisms leading to the bacterial genetic variation [27].

The recent structure of *S. aureus* large ribosomal 50S subunit bound to specific antibiotics shed light on their mechanism of action and highlight peripheral

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motifs specific to *S. aureus* [16]. Furthermore, significant differences in ribosome functioning have been described between low GC content Gram-positive and Gram-negative bacteria [28–32]. The present review will be focus on specific characteristics of the translation initiation in *S. aureus*. In addition, we will illustrate the diversity of mechanisms regulating the recruitment of the ribosomes on the mRNAs that have a direct functional impact on bacterial pathogenesis.

## REGULATION OF *S. aureus* TRANSLATION INITIATION

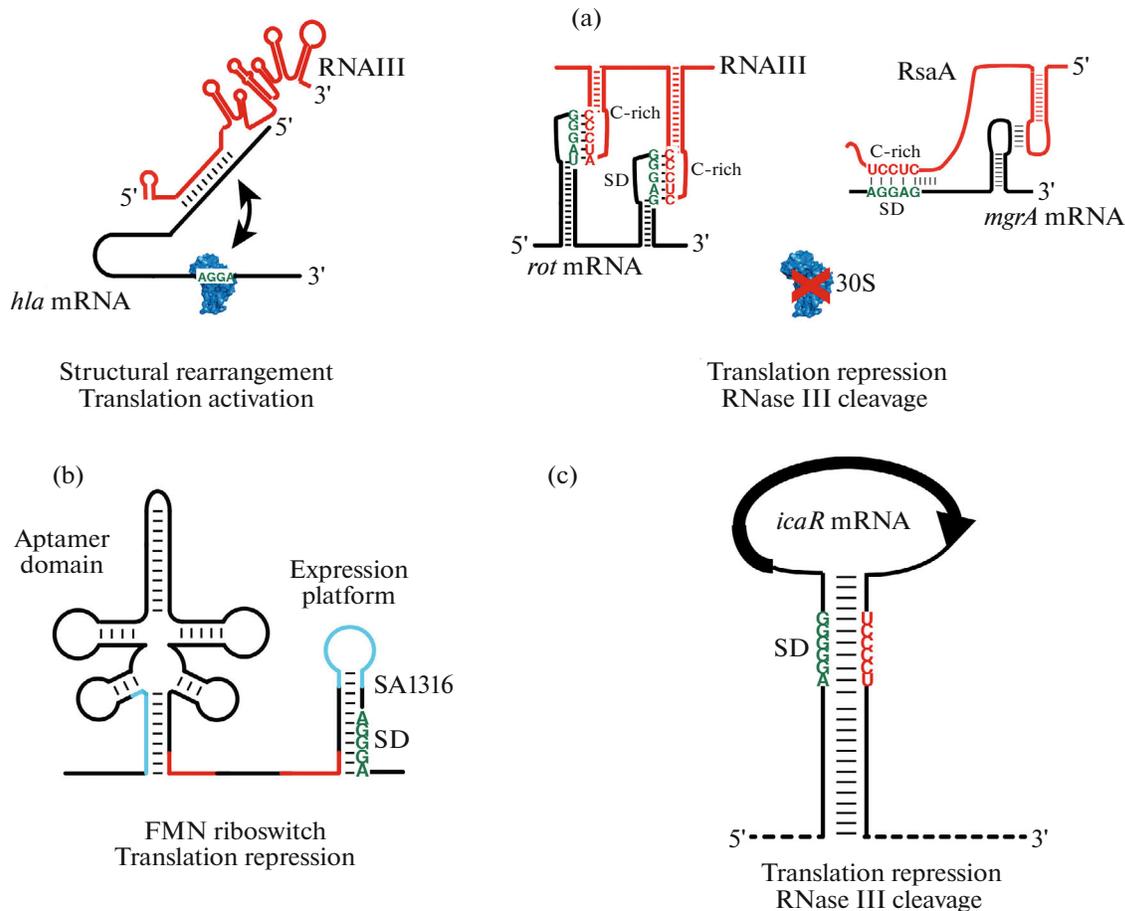
*S. aureus* has developed numerous mechanisms to respond to changing environments and to colonize nearly all niches within a host. Such amazing adaptation is accompanied by a significant remodeling of gene expression and particularly of the virulence determinants mainly driven by transcriptional regulatory proteins [33]. Besides, it is now well admitted that protein-mediated transcriptional control and RNA/protein-mediated translational control are intertwined. Genome-wide studies have discovered a high number of large and small RNAs, which selectively bind to mRNA targets to regulate primarily translation initiation (for reviews [34, 35]). In addition, the discovery of overlapping operons led to the hypothesis that widespread antisense transcription would significantly impact gene regulation at the translational and post-transcriptional levels [36]. Other mRNAs are characterized by large 5' and 3' untranslated regions (UTR) which contain specific structures or sequence signatures that modify the genetic response at the translational level. Hence, a high diversity of mechanisms at the translational level has been evidenced as illustrated by several examples below.

**Trans-acting regulatory RNAs.** The first *S. aureus* regulatory RNA has been discovered by Novick et al. [37] as the main intracellular effectors of the quorum sensing *agr* system. *S. aureus* produces a battery of virulence factors that are responsible for defense against the host immune response, adhesion, invasion, acquisition of novel nutrients, and dissemination in host tissues [38, 39]. The *agr* system is pivotal for the temporal regulation of two sets of virulence factors, adhesins and exotoxins. It is composed of two divergent transcripts, RNAII encodes a quorum sensing cassette (AgrBD) and a two-component system (AgrAC) while RNAIII is the multifunctional RNA, which encodes a PSM  $\delta$ -hemolysin (*hld*). Besides its coding capacity, RNAIII promotes the switch between the expression of surface proteins and the synthesis of excreted toxins [35]. These regulatory properties are endowed within the non-coding parts of RNAIII, which primarily act as antisense RNA to activate or repress the translation of target mRNAs. On one hand, its 5'UTR binds to the leader region of *hla* mRNA encoding  $\alpha$ -hemolysin to prevent the formation of an inhibitory structure in order to facilitate ribosome recruitment [40] (Fig. 1a).

In addition, the 5' and 3' ends of RNAIII both interact with the leader of *mgrA* mRNA to enhance the mRNA stability [41]. On the other hand, its large 3'UTR is primarily acting as a repressor domain (Fig. 1a). This domain is the most highly conserved domain of RNAIII, which contains four C-rich sequence repeats located in unpaired regions [42]. This sequence motif acts as a seed sequence, which initiates basepairing interactions with the Shine and Dalgarno (SD) sequence of target mRNAs (Fig. 1a). The initial GC-rich pairings confer a fast association rate constant for the formation of the complex, and in general are subsequently propagated either to form long duplexes or are stabilized by additional distant pairings according to the mRNA signals (Fig. 1a) [43]. However, in all cases binding of RNAIII prevents the loading of the ribosome and the formation of the initiation complex. These mRNAs encode virulence factors expressed at the surface of the cell (protein A, coagulase, SA1000, Sbi), and the transcriptional repressor of toxins, Rot [43–47]. Through the inhibition of Rot translation, RNAIII indirectly activates the transcription of exotoxins. Such regulatory circuits create a temporal delay between the repression of adhesin synthesis and the induction of exotoxin production enabling an effective transition of the pathogen for spreading and dissemination [48].

Later on, the search for small non-coding RNAs (sRNAs) has revealed the existence of a class of sRNAs carrying unpaired C-rich sequence motif similar to RNAIII [50, 52, 53]. Determination of the functions of several sRNAs demonstrated that this sequence motif is indeed a characteristic signature for translational repressors. For instance, the SigB-dependent RsaA sRNA represses translation initiation of the global transcriptional regulator MgrA through a sequestration of the SD sequence in a manner similar to RNAIII [49] (Fig. 1a). Through MgrA regulation, RsaA attenuates the severity of systemic infections and enhances chronic infection [49]. Another example is *S. aureus* RsaE which represses at the translational level mRNAs involved in the TCA cycle under specific conditions of stresses, NO induction, and when carbon sources are decreasing [50, 53, 54]. In both cases, an unpaired C-rich motif binds to the SD sequence of the mRNAs. Because many mRNAs in Gram-positive bacteria have a strong SD sequence (AGGAGG) and a rather short 5'UTR, targeting the SD is a rapid way to prevent the recruitment of the ribosome and to arrest translation even during the transcription process. However, these examples also demonstrate that additional basepairing interactions outside the SD region are required to ensure a specific response.

**Cis-acting regulatory RNAs.** In *S. aureus*, UTRs of mRNAs are generally of small size, but exceptions of mRNAs carrying large 5' or 3'UTRs have been reported [51, 55, 56]. They include mRNAs encoding virulence determinants, various transcriptional regulators, and metabolic enzymes. Large 5'UTRs are



**Fig. 1.** Mechanisms regulating translation initiation. (a) Examples of *trans*-acting RNAs acting as antisense RNAs. *S. aureus* RNAIII binds to the 5'UTR of *hla* mRNA to prevent the formation of basepairings sequestering the Shine and Dalgarno (SD) sequence, and to favor the ribosome recruitment [40]. Two C-rich motifs of RNAIII bind to two hairpin loops of *rot* mRNA; one of them includes the SD sequence, to form two loop-loop interactions that prevent ribosome binding [43, 45]. The small non coding RNA, RsaA, binds to the SD sequence via its C-rich motif to repress the translation of *mgrA* mRNA. In both cases, the repression of translation is subsequently followed by RNase III degradation [49]. (b) Example of a *cis*-acting regulatory riboswitch responding to flavin mononucleotide (FMN) intracellular concentration. Binding of FMN in the left formed by the compact structure of the aptamer domain led to the stabilization of a hairpin structure sequestering the SD sequence to repress translation initiation of the downstream gene SA1316 [50]. (c) Example of a long-distance interaction affecting in *cis* the translation of *icaR* mRNA. A C-rich motif in the 3'UTR of *icaR* mRNA binds to the SD sequence to prevent translation, and creates a structure suitable for RNase III cleavages [51].

often the reservoirs of regulatory signals such as the riboswitches, which are direct sensors for intracellular metabolite concentrations [57]. Riboswitch consists of a sensor/aptamer domain containing a binding pocket specific for a dedicated metabolite and of an expression platform controlling the downstream transcripts. At least seven *S. aureus* operons are under the control of riboswitches that respond to the intracellular concentrations of S-adenosylmethionine (SAM), thiamine pyrophosphate (TPP), flavin mononucleotide (FMN), lysine, glycine, guanine, 7-aminomethyl-7-deazaguanine (preQ1), and glucosamine-6-phosphate (Glc-6P) [50, 58]. Most of them are regulated through a premature transcription arrest [52], while only one FMN-sensing riboswitch is expected to control the translation of a hypothetical protein. Binding

of FMN to the aptamer domain is predicted to stabilize the formation of a hairpin structure sequestering the SD sequence (Fig. 1b). Because many of these riboswitches regulate the synthesis of essential proteins involved in amino acid biosynthesis, as well as co-factors and nucleotide biosynthesis, they were used as targets for the design of drugs that could constitutively repress the synthesis of essential enzymes [59, 60]. For instance, a pyrimidine derivative compound PC1 binds to the *S. aureus* guanine riboswitch to constitutively repress the expression of *guaA*. This compound showed bactericidal activity against *S. aureus* and significantly reduced mammary gland infection in mice [61]. This compound was more recently assayed to treat bovine intra-mammary infections [62]. Although a significant reduction in bacterial concen-

trations was observed in the milk after PC1 treatment, the clearance of the bacterial was not completely achieved. However, these studies show that ribo-switches are still promising drug targets for the search of novel classes of antibiotics.

In contrast to the 5'UTRs, large 3'UTRs have been underestimated [51, 56, 63]. Recent findings have revealed the importance of a long 3'UTR in *icaR* mRNA, which encodes a transcriptional repressor of biofilm formation. IcaR controls the transcription of the operon *icaADBC* required for the synthesis of the main exopolysaccharidic polymer PIA-PNAG, a major component of the biofilm matrix. Surprisingly, the 390 nt-long 3'UTR of *icaR* appears to be a negative determinant for the translation initiation of its own mRNA (Fig. 1c). The 3'UTR contains a C-rich sequence motif that binds to the SD sequence to prevent ribosome binding, and subsequently recruits RNase III for cleavage [51]. Disrupting this interaction resulted in the accumulation of IcaR and inhibited biofilm formation. This study shows that the 3'UTR can act in *cis* to block the ribosome binding site in the 5'UTR by a mechanism that is reminiscent of RNAIII and other *trans*-acting sRNAs from *S. aureus* (Fig. 1c). A significant number of mRNAs carrying large 3'UTRs have been recently mapped suggesting that the circularization of mRNA is not so uncommon in bacteria. In addition, these regions might also contain specific binding sites for *trans*-acting factors modifying the mRNA stability or translatability, or can also be the reservoir of small regulatory RNAs as recently demonstrated in *Salmonella typhimurium* [64].

These examples demonstrate the importance of the translation regulation for the physiology of *S. aureus*. Analyzing the translational control on a global scale in *S. aureus* in response to the host and *vice versa* [65] will certainly open new horizons and insights into the pathogenesis, persistence of the bacteria within the host, and evolution of the strains.

#### SPECIFIC FEATURES OF *S. aureus* TRANSLATION INITIATION

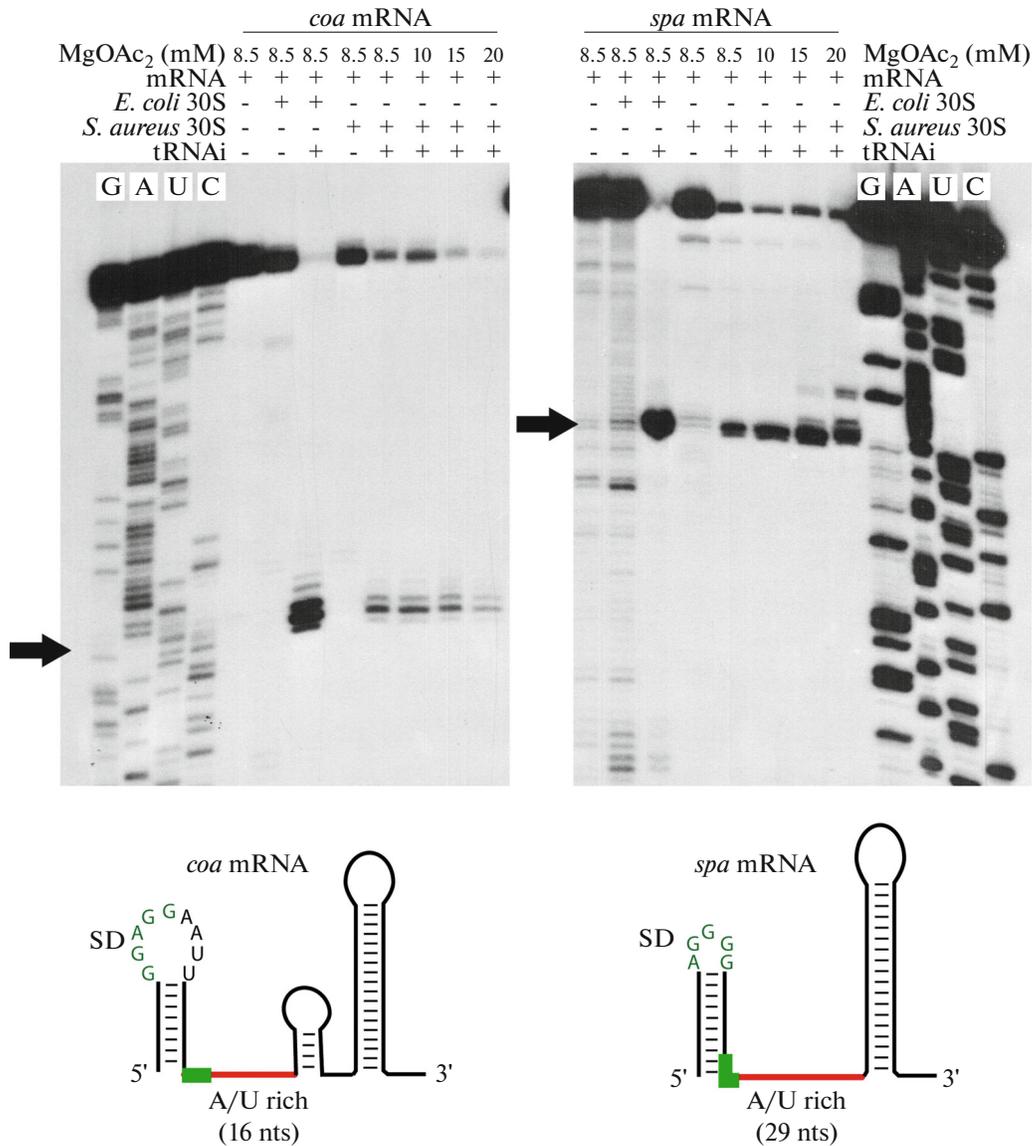
Translation initiation is the rate-limiting step of protein synthesis [66]. This is probably why many regulatory events modulate the mRNA binding to the 30S ribosomal subunit [67–69] (Fig. 1). The initiation process of bacteria requires three initiation factors, IF1, IF2 and IF3, which in addition of the mRNA, and the initiator fMet-tRNA<sup>fMet</sup>, assembled in a multi-step process on 30S to form an active initiation complex [66]. The efficiency of mRNA binding to 30S is independent of the initiation factors and relies solely on specific signatures present on the mRNAs such as the presence of SD sequence (GGAGG), the nature of the initiation codon, the distance between the initiation codon and the SD, the presence or not of structured motif within the mRNA track [70, 71]. Although this process is conserved among bacteria, significant

differences have been observed between Gram-negative bacteria (*E. coli*) and low GC content Gram-positive bacteria such as *Bacillus subtilis* and *S. aureus*. Some of these features are described below.

**Different mRNA binding properties of the 30S from low GC content Gram-positive and Gram-negative bacteria.** In bacteria, the mRNA is recruited at a strategic position at the exit site of the 30S subunit surrounded by several key ribosomal proteins (r-proteins): S1, S2, S7, S11, S18 and S21 [66, 69]. Among these r-proteins, the largest r-protein S1 in *E. coli* with its six OB-fold (oligonucleotide/oligosaccharide-binding fold) domains confers to the 30S the ability to recognize any type of mRNAs [72–75]. Particularly, S1 has been shown to compensate the lack of a strong SD in many mRNAs, and to confer an RNA chaperone activity to the 30S that is essential for the unfolding of structured mRNAs in order to accommodate the initiation codon into the P-site. In *E. coli*, the essential activity of S1 is linked to its association with the 30S via its N-terminal OB-fold domain [75–77]. A phylogenetic study revealed that S1 from Gram-negative bacteria and high GC content Gram-positive bacteria (*Micrococcus*) contained at least the first four OB-fold domains that retained full 30S and RNA binding capacity, and the RNA chaperone activity [29, 75]. Interestingly, it has been shown that *Micrococcus luteus* S1 was able to substitute *E. coli* S1 on the 30S [28]. In contrast, S1 from low GC content Gram-positive bacteria, which contained only four OB-fold domains, has lost the N-terminal domain that was shown to promote specific binding with S2 on *E. coli* 30S [78]. This probably explained why *B. subtilis* S1 plays no major role in translation [28, 79]. Indeed, *B. subtilis* ribosomes have similar properties than S1-depleted *E. coli* ribosomes which have lost their abilities to translate mRNAs with no or weak SD sequence. Addition of S1 from *E. coli* or from *M. luteus* to the S1-depleted *E. coli* ribosomes restored the 30S properties to translate mRNAs bearing weak SD but these proteins had no effect on *B. subtilis* ribosomes [28, 79]. Another characteristic of *B. subtilis* ribosomes is the greater tolerance for the non-AUG initiation codons than *E. coli* ribosome but the presence of a strong SD significantly enhanced the capacity of *E. coli* ribosomes to utilize non-AUG codons [79]. It was thus proposed that firmicutes obviate the need of S1 acting on the 30S because the majority of mRNAs carry a strong SD sequence [80].

Because S1 is very similar in *B. subtilis* and *S. aureus* [29], we have analyzed the ability of *S. aureus* 30S subunits to form initiation complexes using various *S. aureus* mRNA substrates (Figs. 2, 3).

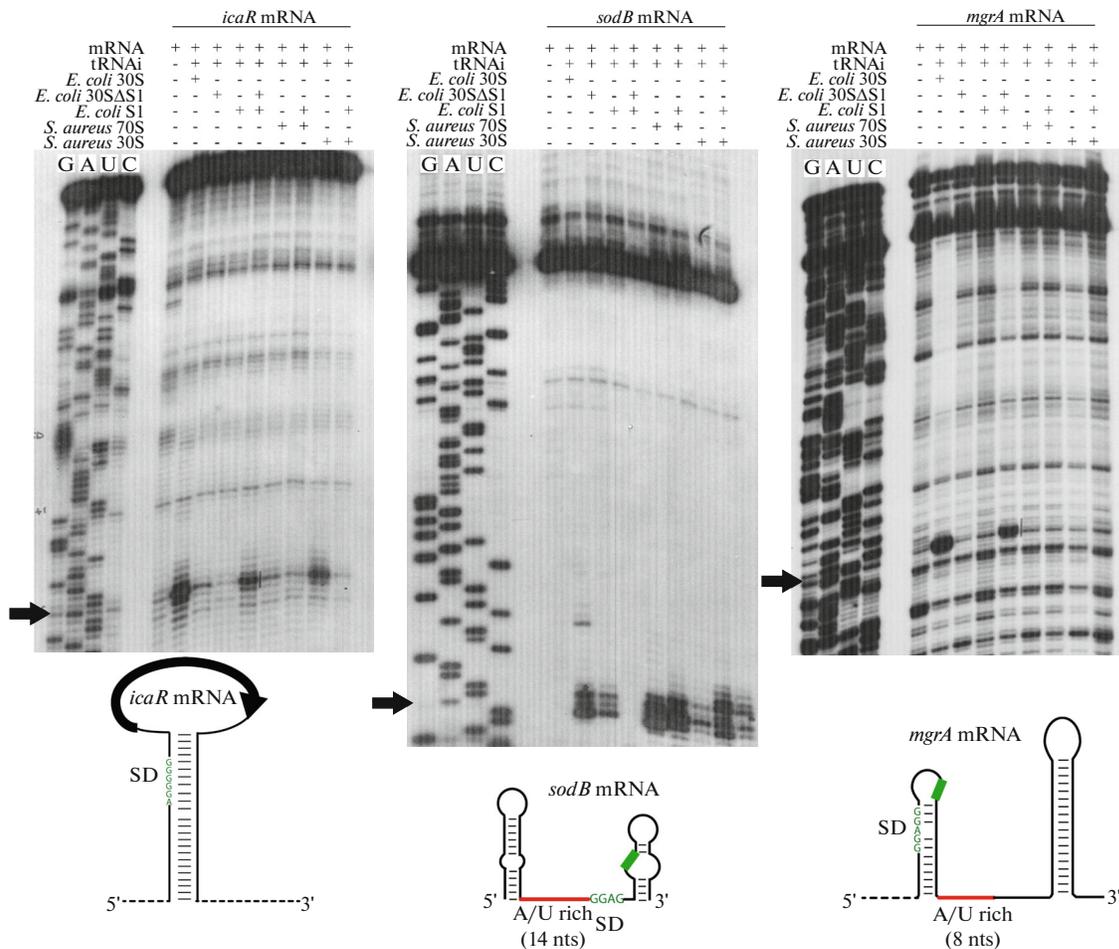
After purification of the 30S and 70S ribosomes [81], only traces of r-protein S1 were observed by mass spectrometry analysis suggesting that the protein is not tightly associated with the ribosome (results not shown). We then monitored the formation of initiation complexes formed by mRNA, 30S subunit and the ini-



**Fig. 2.** Formation of simplified initiation ribosomal complex by toeprinting assays involving *coa* mRNA and *spa* mRNA encoding two virulence factors. The mRNAs (12 nM) were incubated with either *E. coli* 30S or *S. aureus* 30S (300 nM), and the initiator tRNA<sup>fMet</sup> (1 μM). The effect of increasing concentrations of magnesium (8.5 mM to 20 mM) was monitored on the formation of the initiation complex. Lanes G, A, U, C are sequencing ladders. Secondary structure models of *coa* and *spa* mRNAs are shown. SD is for the Shine and Dalgarno sequence (nucleotides in green). The initiation triplet is schematized by a green rectangle. The toeprint at position +16 is shown by an arrow. Experimental conditions were described in Fechter et al. [81]. nts is for nucleotides.

tiator tRNA using toeprinting assays [81, 82]. This approach is based on a premature reverse transcription arrest (called toeprint) caused by the formation of the simplified initiation complex, giving the exact position of the 3' edge of the 30S location on the mRNA. The signature of an active initiation complex is given by the position of the toeprint at position +16 from the AUG codon [82]. We first show that *S. aureus* 30S binds efficiently to *spa* and *coa* mRNAs, which encode two major virulence factors, protein A and coagulase, respectively. These two mRNAs harbor the same structure and sequence signatures, i.e. short 5'UTR,

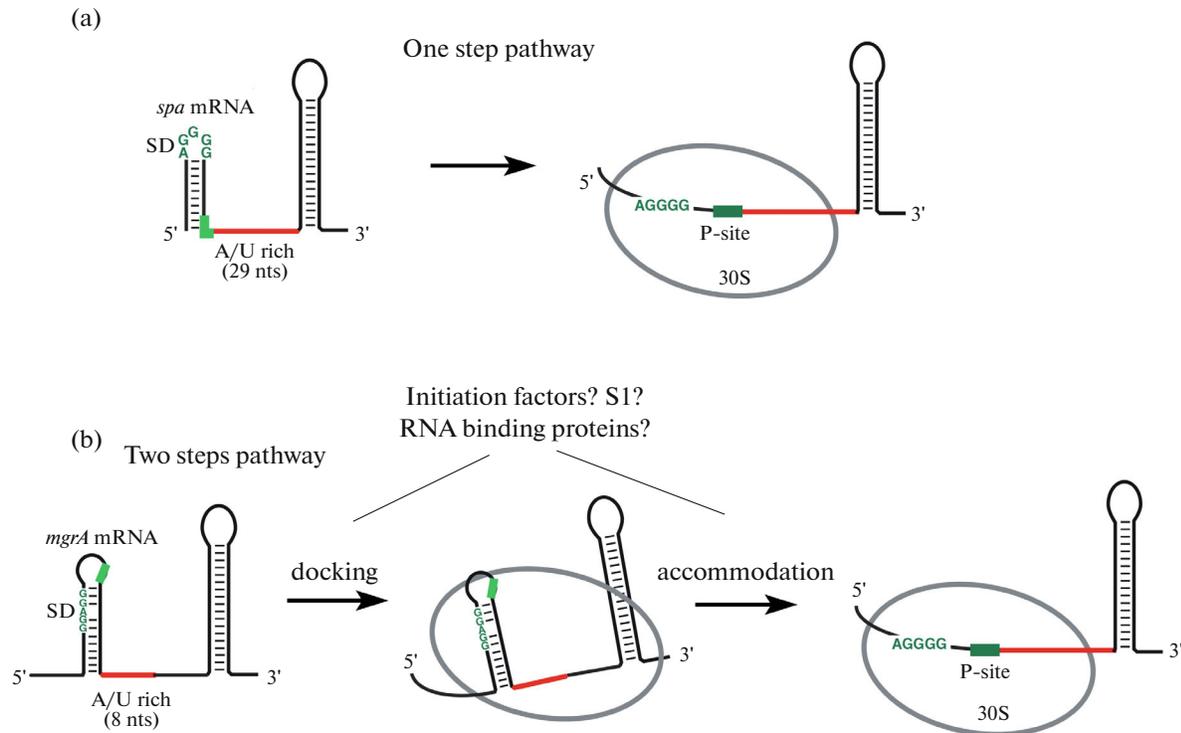
strong SD sequence present in a hairpin loop, and a rather long unpaired AU rich motif just downstream the initiation codon [44, 46] (Fig. 2). Conversely, two other mRNAs *mgrA* and *icaR*, which encode major transcriptional regulatory proteins, were poorly recognized by *S. aureus* 30S (Fig. 3). These two mRNAs contain a large and structured 5'UTR, in which the SD is embedded into a secondary structure [41, 49, 51]. It remains to be addressed whether these two mRNAs are less well translated in vivo than the virulence factors (protein A and coagulase). However, a comparative analysis performed with the *E. coli* ribo-



**Fig. 3.** Effect of *E. coli* ribosomal protein S1 on the formation of various initiation complexes involving *S. aureus icaR* and *mgrA* mRNAs and *E. coli sodB* mRNA. The mRNAs (12 nM) were incubated with *E. coli* 30S (300 nM), or *E. coli* S1-depleted 30S (as prepared in Duval *et al.* [75]), or with *E. coli* S1-depleted 30S pre-incubated with purified r-protein S1 (350 nM) in the presence of the initiator tRNA<sup>Met</sup> (1 μM) (tRNA<sup>i</sup>). The same experiments were carried out with either *S. aureus* 70S or 30S (300 nM) either in the absence or in the presence of *E. coli* r-protein S1 (350 nM), and in the presence of the initiator tRNA<sup>Met</sup> (1 μM). The toeprint at position +16 is shown by an arrow. The experiments show that the addition of *E. coli* S1 to the *E. coli* S1-depleted ribosome restored the formation of the initiation complex as evidenced by the toeprint at +16 while addition of *E. coli* S1 to the *S. aureus* ribosome inhibits the formation of the ternary initiation complex. Secondary structure models of *S. aureus icaR* and *mgrA* mRNAs, and of *E. coli sodB* mRNA are shown below the autoradiographies. SD is for the Shine and Dalgarno sequence (nucleotides in green). The initiation triplet is schematized by a green rectangle. The toeprint at position +16 is shown by an arrow. Experimental conditions were described in Fechter *et al.* [81].

somes containing S1, showed that the 30S are able to form initiation complexes with both *mgrA* and *icaR* mRNAs (Fig. 3). This efficient recognition was linked to the presence of S1 because S1-depleted *E. coli* 30S were not able to recognize the two structured mRNAs while mRNA binding was restored using S1-depleted 30S saturated with purified *E. coli* S1 added in *trans* (Fig. 3). Hence, these experiments indicate that the *S. aureus* 30S behaves as the *E. coli* S1-depleted 30S for the recognition of structured mRNAs. We finally compared the formation of the initiation complex formed with *E. coli sodB* mRNA encoding a superoxide dismutase and the ribosomes of either *E. coli* or of *S. aureus*. This mRNA is recognized by *E. coli* ribosomes in a manner independent of S1 [75]. Indeed,

the toeprinting assays revealed that both *E. coli* and *S. aureus* ribosomes are able to form initiation complexes with *sodB* mRNA in a similar manner (Fig. 3). Surprisingly, the toeprint was strongly decreased if *E. coli* S1 was added to *S. aureus* ribosomes. The same result was also obtained with *icaR* mRNA (Fig. 3). We propose that *E. coli* S1 does not efficiently bind to *S. aureus* ribosome although most of the key residues in *E. coli* S1 are also conserved in *S. aureus* S2 [78]. This probably suggests that the S1 binding site in *E. coli* is more complex than expected [75]. The observed inhibition of the toeprint might result from a direct interaction of S1 with mRNAs outside of the ribosome. For instance *sodB* mRNA presents a large unpaired AU-rich



**Fig. 4.** Possible mechanisms for the formation of initiation complexes in *S. aureus*. (a) In the one-step pathway, the weakly structured mRNA (such as *spa* mRNA) is directly recruited on the 30S ribosomal subunit through its unpaired Shine and Dalgarno (SD) sequence. Due to the strong SD-antiSD interaction, the unpaired initiation codon will be located directly in the P-site. (b) For mRNAs carrying structures sequestering the SD (such as *mgrA* mRNA), it is expected that the recognition will involve at least two steps pathway where docking will be followed by the accommodation process to promote the correct positioning of the initiation codon. It is not yet known *in vivo* whether the unfolding process of the mRNA structure in *S. aureus* occurs on the ribosome through the action of the initiation factors or of an unknown RNA-binding protein or outside the ribosome possibly through the binding of protein S1. In these particular examples, the ribosome might also recognize the SD during the transcription process of the mRNA, i.e. before the formation of the inhibitory structure.

sequence just upstream the SD, known to be a typical binding site for S1 [75] (Fig. 3).

Taken together, these data show that the presence of a strong and unpaired SD in mRNA is necessary to be recognized by *S. aureus* 30S (Fig. 4a). How do *S. aureus* ribosomes initiate the translation of structured mRNAs is still an open question (Fig. 4b). Do structured mRNAs bind first to the platform of the 30S? Clearly, the melting and chaperone properties of *E. coli* S1 were not able to help *S. aureus* ribosome to unfold mRNA structure and liberate the SD sequence. Many questions remain to be explored. Does the shorter protein S1 in *S. aureus* contribute to the translation of structured mRNAs through direct mRNA binding outside of the ribosome? Do other *trans*-acting factors (RNA chaperone proteins, RNA helicases) act on the ribosome to promote translation of specific structured mRNAs? Does the coupling between transcription and translation provide a window allowing the ribosome to load on mRNA before the formation of structures within the ribosome binding site?

**Ribosome hibernation, another mechanism to regulate translation initiation during growth.** The formation of inactive ribosomal dimers, also referred to as 100S

ribosomes or as hibernating ribosomes, repress protein synthesis in bacteria [83]. Inactive ribosome dimers are usually formed as a response of bacterial cells to unfavorable conditions and can be rapidly rescued after normalization of environment [84]. Formation of dimers is supposed to increase the survival rate under stress conditions [85]. In *B. subtilis*, a recent study showed that the dimers are formed during the early stationary phase and these dimers are required to facilitate rapid regrowth of cells if they are facing better nutrient conditions [86]. However, unlike in *E. coli* the 100S ribosomes in *S. aureus* were found in all growth phases [30] questioning on their exact roles under rich medium conditions during cell growth.

The mechanism of 100S ribosomes formation also varies between bacterial species. Many  $\gamma$ -proteobacteria (including *E. coli*) express three proteins: hibernation promoting factor (HPF, former name YhbH) [87], ribosome modulation factor (RMF) [85, 88] and protein YfiA (former names RaiA or protein Y) [89]. The formation of 100S ribosomes is driven by the concomitant binding of RMF and HPF proteins [90] while YfiA prevents the recycling of ribosomes and favors the formation of translationally inactive 70S

[89]. Despite of high similarity with HPF (~40%), YfiA occupies partially the RMF binding site [90, 91]. Instead in firmicutes (such as *S. aureus*), only one protein called long HPF has been identified [92]. Its N-terminal domain shares high similarity with short HPF and YfiA, but not with RMF protein (reviewed in [93]). This unique HPF protein can both promote the formation of 100S dimers and of non-functional 70S [30, 92].

Phenotypic analysis revealed that the depletion of RMF in *E. coli* leads to reduced long-term viability and stress tolerance during stationary phase while depletion of short HPF or YfiA do not cause strong cellular defects [93]. In contrast, depletion of the long HPF protein leads to decreased survival of *Lactococcus lactis* under stress conditions [94], decreased pathogenesis in *Listeria monocytogenes* [95], and enhanced sensitivity to prolonged antibiotic exposure [96]. Crystal structure of *E. coli* ribosome bound to YfiA showed that the protein is located close to the region where mRNA, tRNAs and initiation factors bind during protein synthesis [97]. Heterologous crystal structures of *T. thermophilus* 70S ribosome with *E. coli* hibernation factors showed that HPF shares the binding site with YfiA, whereas RMF binds next to the anti-SD region and prevents interaction of 16S rRNA and mRNA during the initiation process [91]. Single particle cryo-electron microscopy and cryo-electron tomography confirmed the presence of *E. coli* ribosome dimers in situ [98]. Ribosomal proteins S2, S9, S10 and helix 39 of 16S rRNA make contacts between two 70S particles but the hibernation factors were not directly involved in the contacts [91, 98]. The authors suggested that the dimerization occurs as a result of structural rearrangement of the head of 30S upon binding of RMF. However, it is still unclear whether the same mechanism and contacts are involved in ribosome dimerization in the long HPF in *S. aureus*. Due to high similarity between the short HPF of *E. coli* and the N-terminal domain of long HPF in *S. aureus*, we propose that these two proteins would share the same binding site on the ribosome, and that the C-terminal domain of long HPF might be directly involved in contacts between the two ribosomes in *S. aureus*. Hence, solving the high-resolution structure of the ribosome dimers from *S. aureus* will shed light on one biological mechanism that might promote successful persistence of this severe pathogen in response to stress conditions or antibiotic treatments.

#### TOWARDS THE HIGH RESOLUTION OF THE TRANSLATION MACHINERY IN *S. aureus*

Although the core of the bacterial ribosome is very conserved, certain peculiarities existed between the ribosomes of different bacterial species. These differences may determine some specifications for mRNA recognition, regulation of translation, and susceptibil-

ity to antibiotics. Two structures of low GC content Gram-positive ribosomes have been recently solved. The MifM-stalled 70S ribosome from *B. subtilis* was solved at a resolution of 3.5–3.9 Å by cryoelectron microscopy [15, 17] and the crystal structure of *S. aureus* 50S subunit was elucidated at a resolution of 3.5 Å [16]. The majority of the rRNAs of the core of *S. aureus* 50S subunit is structurally highly conserved. However, several helices, primarily located on the periphery of the ribosome, show sequence and length variability among different bacteria [16]. In contrast to the rRNA, more differences were observed for ribosomal proteins. Although the globular domains as well as the structural elements interacting with rRNAs are rather conserved, many of the r-proteins contained specific extensions. This is the case of universal r-protein uL3 and bacteria-specific r-protein bL17, which both carry a loop motif unique to staphylococci extending toward the solvent side at the periphery of the ribosome. Also the extension at the C-terminus of uL16 is located on the solvent side, while the protein is at the interface close to the tRNA binding sites. Interestingly, bL27 also carries an extended N-terminus that should be cleaved by a specific protease before the assembly of the 70S ribosome [32]. Although the N-terminal part of bL27 was only traced from residue 19 due to its high flexibility in the crystal structure [16], this domain is expected to be located in the proximity to the peptidyl-transferase center (PTC) and of the 5'-end of the P-site tRNA [16]. Nevertheless, these structural features alone could neither explain the different activities in protein synthesis and translation initiation as compared to *E. coli* ribosomes, nor the requirement of higher concentration of magnesium to avoid subunit dissociation as reported in earlier studies [99]. The crystal structure of *S. aureus* 50S was also solved in the presence of several antibiotics such as linezolid, telithromycin, and pleuromutilin revealing unexpected features in the orientation of some of the antibiotics specific for *S. aureus* [16].

Clearly, one of the important challenges for the future will be to determine the structure of the full ribosome under physiological conditions and/or trapped by antibiotics. It should better highlight the peculiarities located at strategic positions of the ribosome, such as the PTC, the decoding center, the tRNA binding sites, and the mRNA channel. Furthermore, more knowledge is required for the *trans*-acting factors (initiation factors, S1, RNA-binding proteins, RNA helicases, etc.) that could modulate the functioning of *S. aureus* ribosome at the initiation step. Time is certainly arrived to better understand at the molecular level, the differences observed in protein synthesis and control between Gram-negative bacteria and the low GC content Gram-positive bacteria, which comprise many human pathogens, such as *S. aureus*. These studies should pave the way to identify specific strategies to selectively inhibit *S. aureus* pathogenesis and/or growth with less effect on the

human microbiome and less selective pressure on resistant population.

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## **Objectives and experimental strategies**



## I. How *S. aureus* ribosome initiates translation of structured mRNAs?

### I.1. Biological questions and objectives

As I have mentioned in the introduction, *S. aureus* has evolved sophisticated means to tune the efficiency of translation of different genes in response to the host, stress and various environmental changes. Gene expression regulation is often exerted at the earliest step of protein biosynthesis, the initiation process, when the mRNA is recruited on the 30S ribosomal subunit and accommodated in the decoding channel for the interaction with the initiator tRNA at the P site. Specific features of mRNAs such as the length and the position of the SD sequence (Chen et al., 1994; Vimberg et al., 2007), the «stand-by » site acting as an enhancer sequence preceding the SD (Salis et al., 2009), the type of initiation codon and the secondary structures at the 5' UTR, are all key determinants for translation efficiency. Transcriptomic analysis of *S. aureus* has revealed that numerous mRNAs encoding for virulence factors, stress responses and metabolism, carry large structured 5' and 3' UTR where regulatory domains are potentially embedded (Ruiz de los Mozos et al., 2013). If Gram-negative ribosomes have the ability to deal with a wealth of different structures and different SD strengths thanks to their protein S1 (Duval et al., 2013a), *S. aureus* ribosomes do not carry S1 and did not show the same plasticity in recognizing structured mRNAs (Khusainov et al., 2016). Nevertheless, those structured mRNAs have to be translated, at least in some specific conditions, and translational regulators are expected to compensate for the loss of the associated S1. Alternatively, *SauS1* would still induce the translation of specific mRNAs outside of the 30S, acting as a translational activator. The first aim of my thesis was to understand the impact of *SauS1* on translation initiation of structured mRNAs and on cell physiology.

### I.2. Main experimental strategies

In order to decipher the impact that *SauS1* could have on the initiation of translation of specific mRNAs, I have used different experimental approaches. I also have benefit from the expertise of the team members: Isabelle Caldelari designed and performed some experiments, Anne-Catherine Helfer and Lucas Herrgott gave their technical support and

conceptual advice, Melodie Duval, Delphine Bronesky and Emma Desgranges gave me intellectual contribution and have also stimulated scientific discussions.

- First I have followed the expression profiles of the *rpsA* gene (*SauS1*) by Northern blot and Western blot analysis, to understand in which conditions the protein accumulated. Its expression profile has been compared with other r-proteins giving some insights on its regulatory functions.
- Mutagenesis of the *rpsA* gene (complete deletion or intron insertion) has been carried out to better understand its functions. Indeed, I have used the *rpsA* mutant strains to look for phenotypic alteration using different stress conditions. Moreover, comparative transcriptomic and quantitative proteomic analyses of the wild type and mutant  $\Delta rpsA$  strains have revealed the genes for which the expression is regulated by *SauS1*, providing some hints on the mRNA targets which would require S1 for their translation.
- Using comparative ( $\Delta rpsA$ /WT) polysome profiles coupled with Northern blot analysis, we have monitored the effect of *SauS1* *in vivo* on the level of translation of selected target mRNAs.
- The mRNAs issued from the OMICS analysis and confirmed by the polysome profiles, have been then validated *in vitro* by toe-printing assays to monitor the effect of purified *SauS1* on the formation of initiation complexes using isolated 30S ribosomal subunit. In parallel, I have analyzed the ability of *SauS1* to directly bind the mRNAs and to present them to the ribosomes using gel retardation assays (EMSA) and gel filtration chromatographies, respectively.

In collaboration with F. Vandenesch (Centre International de Recherche en Infectiologie, CIRI, Lyon), the  $\Delta rpsA$  mutant strain will be used to monitor the effect of *SauS1* on virulence. Cellular and animal models to check host interaction and infectivity are indeed available in Lyon and can be used to specifically analyze tissue colonization, immune system evasion and dissemination.

## II. Could *SauS1* be involved in regulatory mechanisms mediated by small non-coding RNAs?

### II.1. Biological questions and Objectives

In Gram-negative bacteria, most if not all of the identified sRNAs, requires the Sm-like protein Hfq for their stability and functional activation (Wagner and Romby, 2015). Hfq forms a ring-shaped homohexamer that binds the U-rich terminator at the 3' end of most sRNAs (Otaka et al., 2011) protecting the Hfq-associated sRNAs from cellular ribonucleases (Brennan and Link, 2007; Valentin-Hansen et al., 2004) and helping them to recognize their target mRNAs (Storz et al., 2011; Updegrove et al., 2016). Several mechanisms of regulation mediated by Hfq have also been described in *E. coli*. In association with sRNAs, Hfq can directly repress or activate the translation of target mRNAs by sequestering/liberating their RBS. Deletion of *E. coli hfq* leads to pleiotropic effects such as growth defect, sensitivity to UV light and increased cell length (Tsui et al., 1994). Mutations in *hfq* also decreased virulence in several pathogens (for review, see Vogel and Papenfort 2006). In contrast to this, the function of Hfq in low G+C Gram-positive bacteria is still unclear and controversial. In *S. aureus*, Hfq has been shown to interact with RNAPIII, but it neither enhance its recognition with target mRNAs (Zheng et al., 2016) and nor affects their stability (Boisset et al., 2007; Preis et al., 2009). Furthermore, it is not involved in antibiotic resistance and stress responses, it is dispensable for metabolic pathway regulations and no phenotypes were linked to its deletion (Bohn et al., 2007; Geisinger et al., 2006; Huntzinger et al., 2005). Even if Hfq seems to be dispensable for riboregulation by sRNAs in low G+C Gram-positive bacteria (e.g. Firmicutes) (Jousselin et al., 2009), other aspects have to be taken into account. First, it has been shown that in methicillin-resistant staphylococcal strain (MRSA), Hfq largely contributes to stress resistance and pathogenicity (Liu *et al.*, 2010), suggesting a strain-dependent function of this protein. Second, Hfq homologues are found in several species belonging to Firmicutes division (Sun et al., 2002; Valentin-Hansen et al., 2004) with high conservation of the amino acids of the proximal and distal faces, which bind to sRNA and mRNA, respectively (Sun et al., 2002). Interestingly, another region of Hfq has been shown to be essential for the annealing activity, *i.e.* the Arginine-rich rim region (Panja et al., 2013). The ability of increasing the rate of RNA base-pairings and of

stabilizing the sRNA–mRNA complex, depend on the number of Arginine residues in this region (Zheng et al., 2016). In *E. coli*, Hfq rim contains three arginines, in *B. subtilis* only one and in *S. aureus* none, explaining why the protein does not mediate the annealing.

*EcoS1* is an RNA chaperone protein which is able to promote strand displacement (Rajkowitsch and Schroeder, 2007). It binds numerous mRNAs to induce structural rearrangements and to facilitate the binding of the ribosome (Duval et al., 2013b). This activity is primarily carried out by its first four domains (d1 to d4) (Duval et al., 2017). Except d1, all the other domains are present in *SauS1*. Is *SauS1* able to bind RNAs outside the ribosome context? Which are the RNA partners of *SauS1*? Does *SauS1* interact with other protein partners? Could *SauS1* also participate to the sRNA-dependent regulation? Does *SauS1* protect sRNA from degradation or help them to promote annealing to the target RNAs? The second aim of my thesis was to address the *SauS1* regulatory functions expanding our investigation beyond its possible translation roles. Its impact on sRNA-dependent regulations has been particularly studied.

## II.2. Main experimental strategies

The transcriptomic analysis obtained from the first part of my thesis has provided some indications on different roles of *SauS1* in *S. aureus* RNA metabolism. In order to understand which complexes involve *SauS1* and which RNAs are direct targets, different *in vivo* and *in vitro* approaches have been used.

- A strain carrying a flag-tagged version of the *rpsA* gene constructed by Isabelle Caldelari has been used to perform co-immunoprecipitation (Co-IP) assays. RNA-seq and LC/MSMS analyses of the purified complexes identified the *in vivo* targets of *SauS1*.
- Several RNAs specifically co-immunoprecipitated with *SauS1* have been validated *in vitro* using gel retardation assays.
- The possible effect of *SauS1* on sRNA-target RNAs interaction has been monitored using gel-retardation assays, and the annealing activity of *SauS1* has been followed by FRET analysis.
- The localization of *SauS1* on several sRNA targets has been checked by footprinting experiments. However, taken into account the transient and dynamic interaction occurring between the chaperone protein and its target

RNAs, we have introduced a crosslinking step to stabilize the ribonucleoprotein particule (RNP).

### III. Summary of the main experimental strategies

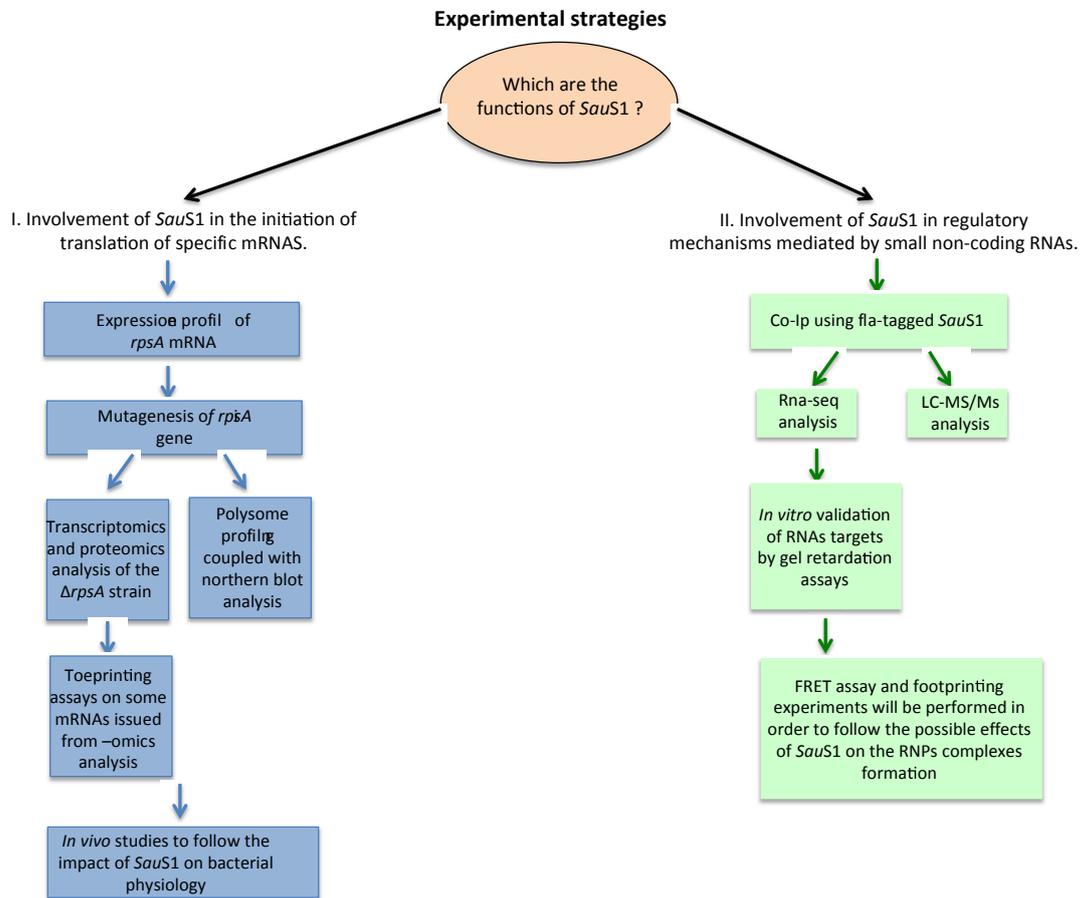


Figure 11 Schematic representation of the experimental approaches that have been used to address the roles of *SauS1* in *S. aureus*.



# Results



## I. Result I: translation functions of *SauS1*

The first aim of my PhD project deals with the characterization of the functional impact of *SauS1* on the initiation of translation of specific structured mRNAs.

Because I showed that *SauS1* is not strictly a ribosomal protein (review in (Khusainov et al., 2016), see introduction), our approach for understanding its biological functions was first based on the characterization of its *in vivo* partners. Rip-Seq assays using chromosomally flag tagged *SauS1* were carried out to co-purify the RNA targets that were further identified by sequencing analysis. Interestingly, among the enriched RNAs, we found several RNA classes including mRNAs, regulatory RNAs (sRNAs, riboswitches) and some tRNAs. Using gel retardation assays, I have validated the specificity of the complex formation. To gain information about the molecular mechanism of action of *SauS1* on its target RNAs, I have also performed footprinting assays, and FRET experiments to monitor its chaperone activity on a model system.

Among the mRNAs that were co-IP with *SauS1*, we identified the operon *apsm1-4* encoding four PSM, which is particularly well structured. Comparative transcriptomic analysis of the wild-type and  $\Delta rpsA$  strains shows down-regulation of several mRNAs, and of the operon *apsm1-4*. We made the hypothesis that the deletion of *SauS1* caused defects in the translation of the mRNA followed by degradation. Interestingly, many of the identified target mRNAs are involved in virulence and they adopt structures in their 5' untranslated regions that could modulate the accessibility of the 30S ribosomal subunit. In this manuscript, we have analyzed in details the mechanism of action of S1 on the operon *apsm1-4*. We first demonstrate that *SauS1* has a major positive impact on the formation of the simplified 30SIC (toeprintings assays) involving the *psm* mRNA, the initiator tRNA and the 30S subunits. Moreover, *in vivo* experiments using polysome profiles coupled with Northern blot experiment have demonstrated the central role of *SauS1* in the initial step of translation of these peptides.

In this work, I have also benefited from the expertise and the collaboration of several team members. I. Caldelari made the two introns insertion mutants of *rpsA* gene and a strain in which *SauS1* carries a flag-tag at its C-terminal domain and performed the purification of the RNAs for the CoIP experiment. Moreover, she has performed the Northern blot to monitor the expression profile of *rpsA* mRNA in various staphylococcal

strains, and to measure the half-life of the *psm* mRNA in the wild-type and mutant  $\Delta rpsA$  strains. Lucas Herrgott has monitored the *in vivo* translation of the *psm* and *hu* mRNAs using polysome profiles followed by Northern blot analysis. Iskander Khusainov has prepared the *S. aureus* 30S ribosomal subunit, which was used for structural studies.

## **I. Result I: translation functions of *SauS1* (manuscript ready for submission)**

### ***Staphylococcus aureus* S1 activates translation initiation of PSM $\alpha$ toxins and stimulates the production of several other secreted virulence factors**

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22 **ABSTRACT (155 words)**

23 *Staphylococcus aureus* is a formidable human pathogen that uses secreted cytolytic factors  
24 to injure immune cells and promote infection of its host. Of these proteins, the PSM family of  
25 pore-forming toxins play critical roles in *S. aureus* pathogenesis. The regulatory mechanisms  
26 governing the expression of these toxins are incompletely defined. Whole-genome  
27 transcriptomics, *S. aureus* exoprotein proteomics, and translation analyses revealed that  
28 ribosomal protein S1 (*SauS1*), which is not associated with the ribosome, influences the  
29 expression and production of exotoxins (PSMs,  $\alpha$ -haemolysin,  $\delta$ -haemolysin and  $\gamma$ -  
30 haemolysins) and exoenzymes (proteases and lipases). We could demonstrate that *SauS1*  
31 specifically promotes translation initiation of the *apsm* 1-4 operon by binding its highly  
32 structured mRNA. We propose that the presence of structures at the RBS of different toxins  
33 requires additional translation activators, which could be either sRNA or S1 protein. *SauS1*  
34 belongs to a new class of RNA chaperones that play key roles in the regulation of translation  
35 in *S. aureus*.

36

37

## 38 INTRODUCTION

39 *Staphylococcus aureus* is a low G+C content Gram-positive bacterium, which is a major  
40 human opportunistic pathogen, causing a large spectrum of infections (e.g.(Lowy, 1998).  
41 Persistent colonization of human nasals with *S. aureus* has been observed for approximately  
42 30% of the population, which are also more susceptible to develop an infection (Wertheim et  
43 al., 2005). During the colonization and infection processes, *S. aureus* often reprograms its  
44 lifestyle in response to many environmental variations including amino acid and carbon  
45 source limitation, iron depletion, decreased pH, and oxidative stress. Many studies have  
46 shown that the bacteria sequentially synchronize the expression of genes encoding surface  
47 proteins involved in adhesion and defense against the host immune system and, later,  
48 numerous toxins ( $\alpha$ -,  $\beta$ - and  $\delta$ - hemolysins, P-V leukocidins, enterotoxins, exfoliative toxins,  
49 Toxic Shock Syndrome toxin and PSM peptides) and enzymes (coagulases, lipases,  
50 hyaluronidases, staphylokinases, nucleases) are secreted to disrupt host cells and tissues,  
51 facilitating the spread of the infection (Thammavongsa et al., 2015). The production of these  
52 virulence factors requires physiological adjustments for energy conservation and a fine  
53 coordination with its metabolism (Somerville and Proctor, 2009). Responsible for these  
54 adaptive responses are multiple interconnected regulatory networks, built on Two  
55 Component Systems, sigma factors, transcriptional regulatory proteins (Ibarra et al., 2013)  
56 and small non-coding RNAs (sRNAs) (e.g., for reviews (Felden et al., 2011; Tomasini et al.,  
57 2014)).

58 A recognized master regulator of *S. aureus* virulence is the regulatory RNAIII encoded by  
59 the *agr* system (Janzon and Arvidson, 1990; Novick et al., 1993). The *agr* system senses the  
60 cellular density and triggers the transition from production of surface-bound proteins  
61 (adhesion mode) to the secretion of soluble exotoxins and degradative enzymes  
62 (dissemination mode) (Geisinger et al., 2009; Novick and Geisinger, 2008; Tomasini et al.,  
63 2014). The switch is mainly promoted by the ability of RNAIII to enhance or repress the  
64 translation of target mRNAs through the formation of basepairing interactions. Both  
65 activation and repression influence the structural context of the Ribosome Binding Sites  
66 (RBS) of the target mRNAs and their ability to be efficiently recognized by *S. aureus* 30S  
67 ribosomal subunits. For instance, the 5' region of RNAIII binds to the leader region of *hla*  
68 mRNA encoding  $\alpha$ -haemolysin, to prevent the formation of an inhibitory structure involving  
69 the Shine and Dalgarno (SD) sequence (Morfeldt et al., 1995). On the other hand, the 3'  
70 domain of RNAIII contains four C-rich sequence motifs located in unpaired regions (Benito et  
71 al., 2000), which directly base-pairs with the SD sequences of target mRNAs preventing  
72 ribosome binding and the formation of the initiation complexes. These mRNAs encode for  
73 virulence factors expressed at the cell surface (protein A, coagulase, SA1000, Sbi), and the

74 transcriptional repressor of toxins, Rot (Boisset et al., 2007; Chabelskaya et al., 2014;  
75 Chevalier et al., 2010; Geisinger et al., 2006; Huntzinger et al., 2005). Through the inhibition  
76 of Rot translation, RNAIII indirectly activates the transcription of exotoxins.

77 Besides RNAIII, the response regulatory protein AgrA also contributes to the *agr* regulon by  
78 affecting the expression of numerous metabolic enzymes and by activating the membrane-  
79 injuring toxins Phenol Soluble Modulines, PSM $\alpha$  and PSM $\beta$ , as well as *hld* a third PSM  
80 encoded by RNAIII (Queck et al., 2008). The translation of PSM $\alpha$  and  $\delta$ -haemolysin (Hld) is  
81 nevertheless delayed. Even if the onset of PSM $\alpha$  production is anticipated by 2 h compared  
82 to  $\delta$ -haemolysin (Vuong et al., 2004), only a small amount of peptide is produced and a real  
83 activation takes place later, at the same time as  $\delta$ -haemolysin,  $\alpha$ -haemolysin and several  
84 exoprotein production (Balaban and Novick, 1995; Li et al., 2010; Vandenesch et al., 1991;  
85 Vuong et al., 2004). Thus, an exceptional temporal control correlates the expression of *hla*,  
86 *psm-a* and *hld*, which could not be simply explained by the intervention of RNAIII and AgrA,  
87 the two effectors of the quorum sensing system. It has been proposed that an unknown  
88 translational factor should be responsible for the delay in  $\delta$ -haemolysin and PSM $\alpha$   
89 production (Balaban and Novick, 1995; Li et al., 2010; Vandenesch et al., 1991; Vuong et al.,  
90 2004).

91 In bacteria, changes in translation efficiency are correlated with specific mRNA features,  
92 including structures responding to the intracellular concentration of metabolites  
93 (riboswitches), to pH changes (fermentation...), temperature, or to the binding of regulatory  
94 proteins or sRNAs (Duval et al., 2015; Romby, 2007; Wagner and Romby, 2015). Opening of  
95 mRNA structures on the 30S ribosomal subunit is a slow process operated in Gram-negative  
96 and in high G+C content Gram-positive bacteria, by ribosomal protein S1 (Duval et al., 2013).  
97 S1 is an RNA chaperone composed by six OB-fold domains bearing distinct functions.  
98 Domain 1 was shown to be responsible for ribosome anchoring through specific binding with  
99 r-protein S2 (Byrgazov et al., 2015; Byrgazov et al., 2012; Duval et al., 2013). Phylogenetic  
100 studies have indicated that S1 from low G+C Gram-positive bacteria, such as *B. subtilis* and  
101 *S. aureus*, would not contain the ribosome binding domain (Salah et al., 2009). Indeed, after  
102 purification of *S. aureus* 30S and 70S ribosomes, only traces of r-protein S1 were observed  
103 by mass spectrometry analysis, suggesting that the protein is not tightly associated with the  
104 ribosome (Khusainov et al., 2016; Khusainov et al., 2017). It was previously demonstrated  
105 that *B. subtilis* S1 plays no major role in translation and is not an essential protein (Farwell et  
106 al., 1992; Juhas et al., 2014; Vellanoweth and Rabinowitz, 1992). It was thus proposed that  
107 firmicutes obviate the need of S1 acting on the 30S because the majority of mRNAs carry  
108 short 5' UTRs with strong SD sequences (Omotajo et al., 2015). However, in *S. aureus*,

109 mRNAs carrying long 5' or 3' UTRs have been reported (Anderson et al., 2006; Lasa et al.,  
110 2011; Ruiz de los Mozos et al., 2013). They include mRNAs encoding virulence factors,  
111 various transcriptional regulators, and metabolic enzymes. We have recently analyzed *in*  
112 *vitro* the ability of *S. aureus* 30S subunits to form initiation complexes using various *S.*  
113 *aureus* mRNA substrates, in which the SD was either located in unpaired and flexible  
114 regions, or sequestered into hairpins. *S. aureus* 30S, as the *E. coli* S1-depleted 30S, could  
115 not recognize structured mRNAs (Khusainov et al., 2016) suggesting that a translation  
116 activator would be necessary to translate structured mRNAs.

117 Here, we show that *S. aureus* S1 (*SauS1*) which is expressed in late-exponential phase, is  
118 not a ribosomal protein. *SauS1* is not essential for the growth in rich medium *in vitro* but is  
119 required for the correct coordination of virulence factors, by affecting the production of  
120 exotoxins (PSMs,  $\alpha$ -haemolysin,  $\delta$ -haemolysin and  $\gamma$ -haemolysins) and exoenzymes  
121 (proteases and lipases). Moreover, we demonstrate that *SauS1* can directly bind the highly  
122 structured  *$\alpha$ psm1-4* mRNA operon, promoting translation initiation of its four peptides. We  
123 propose that the presence of structures at the RBS of different toxins requires additional  
124 translation activators, which could be either sRNA or S1 protein. The translational functions  
125 of *SauS1* are not broad, but rather specific to structured mRNAs in order to facilitate their  
126 recruitment on the ribosome.

127

## 128 MATERIAL AND METHODS

### 129 Strains, plasmids and growth conditions

130 *S. aureus* strains, plasmids and PCR primers used in this study are listed in **Table S1**. *E. coli*  
131 *strain* DC10B (Monk et al., 2012) was used as a host strain for plasmid construction.  
132 Plasmids extracted from *E. coli* DC10B can be used directly for *S. aureus* electroporation. *E.*  
133 *coli* strain was cultivated in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, 1%  
134 NaCl) supplemented with ampicillin (100  $\mu$ g/ml) when necessary. LB-agar plates (with or  
135 without ampicillin) were also used for growth on solid medium. *S. aureus* strains were grown  
136 in Brain-Heart Infusion (BHI) medium (Sigma-Aldrich) supplemented with erythromycin (10  
137  $\mu$ g/ml) when necessary. Blood-agar (VWR Chemicals) and BHI plates (with or without  
138 erythromycin) were used for growth on solid medium.

139 Plasmids were prepared from transformed *E. coli* pellets using the Nucleospin Plasmid kit  
140 (Macherey-Nagel). Transformation of both *E. coli* and *S. aureus* strains was performed by  
141 electroporation (Bio-Rad Gene Pulser). The plasmid for *rpsA* complementation was prepared  
142 using pCN51 as template vector (Charpentier et al., 2004). Synthesis of PCR products was  
143 performed using Phusion Polymerase (Thermoscientific). To remove the cadmium inducible

144 promoter, pCN51 was digested by *SphI/PstI*. The P1-*rpsA* promoter region was amplified by  
145 PCR and cloned into pCN51 following *SphI/PstI* digestion, forming pCN51::*rpsA*.

#### 146 **Northern blot**

147 Total RNAs were prepared from different volumes of *S. aureus* HG001, HG001, RN6390 and  
148 Mu50 cultures taken at 2, 4 and 6 h of growth. After centrifugation, bacterial pellets were  
149 resuspended in RNA Pro Solution (MP Biomedicals). Lysis was performed with FastPrep  
150 and the RNA purification followed strictly the procedure described for the FastRNA Pro Blue  
151 Kit (MP Biomedicals). Electrophoresis of total RNAs (10 µg) was performed on 1% agarose  
152 gel containing 20 mM guanidium thiocyanate. After migration, RNAs were vacuum  
153 transferred on nitrocellulose membrane. Hybridization with specific digoxigenin (DIG)-  
154 labelled probes complementary to *rpsA* sequence followed by luminescent detection was  
155 carried out as described previously (Boisset et al., 2007). Hybridization with DNA  
156 radioactively labelled probes complementary to *psm* and 5S sequences have been detected  
157 by autoradiography film exposition.

#### 158 **Western blot**

159 *S. aureus* BCJ100-*SauS1*-flag culture (100 ml) have been growth in BHI at 37°C and  
160 samples were taken at 2, 3, 4, 5 and 6 h. A total of 1 OD for each of them was pelleted by  
161 centrifugation and suspended in Laemmli SDS PAGE loading buffer. Total proteins have  
162 been separated on 12% SDS-PAGE polyacrylamide gel and transferred on western blot  
163 PVDF membranes (Biorad) using trans-blot turbo transfer system (Biorad) setted on low  
164 molecular weight proteins for 5 min. The membrane was incubated for 1 h (or overnight) in  
165 blocking solution (4,8% of milk in TBS-Tween20 (Sigma Aldrich)). The membrane was  
166 washed and incubated with anti-flag antibody (Sigma) at a 1:2500 dilution in TBS-Tween20  
167 for 1 h at 20°C under continuous agitation. The membrane was then washed 3 times for 10  
168 min with TBS-Tween20 and further incubated with goat anti-mouse IgG (H+C) HRP antibody  
169 (Biorad) diluted 1:2500 in TBS-Tween20 for 1 h at 20°C under continuous agitation. The  
170 membrane was then washed and the result detected using detection reagent GE Healthcare  
171 (Amersham, western blotting detection reagents).

#### 172 **Mutagenesis of the *rspA* gene**

173 Three different mutants have been constructed to abolish *SauS1* production in the HG001  
174 strain. A complete deletion mutant ( $\Delta rpsA$ ) has been obtained by allelic replacement  
175 according to Boisset et al. (2007). Alternatively, transposon introns containing several stop  
176 codons were inserted at position 111 after the AUG start codon (*rpsA111::LtrB*) or at position  
177 1029 close to the last domain of S1 (*rpsA1029::LtrB*) according to (Kiedrowski et al., 2011)  
178 using the primers described in **Table S1**. The deletion of *rpsA* gene and the insertion of the  
179 intron were followed by Northern blot analysis (data do not shown). Mass-spectrometry  
180 analysis has confirmed the absence of *SauS1* in all the three strains.

## 181 **RNA preparation and transcriptomics analysis**

182 *S. aureus* HG001 wild-type (WT) and  $\Delta rpsA$  mutant strains were grown in 50 ml BHI medium  
183 to an OD<sub>600nm</sub> of 5 (6h of culture at 37°C), immediately chilled on ice, and then pelleted by  
184 centrifugation (3750 rpm, 15 min, 4°C). Lysis was performed with FastPrep and the RNA  
185 purification followed strictly the procedure described for the FastRNA Pro Blue Kit (MP  
186 Biomedicals). DNase I (0.1 U/ $\mu$ l) treatment was performed 1h at 37°C. The reactions  
187 mixtures were then purified by phenol/chloroform/isoamylalcohol and subsequent ethanol  
188 precipitation. RNA pellets were re-suspended in sterile milliQ water. RNA quality and  
189 quantity assessments were performed on Agilent Nano Chip on the Bioanalyzer 2100. The  
190 RNAs for total transcriptomics were then treated to deplete abundant rRNAs, and the cDNA  
191 libraries were performed using the Random Hexamer approach and sequenced with Illumina  
192 Mi-seq using a V4 chemistry sequencing kit (Illumina). Each RNA-seq was performed in  
193 duplicates. The standard protocol used is the “TruSeq Stranded mRNA” which is based on  
194 the TruSeq Illumina kit. It preserves the information about the orientation of the transcripts  
195 and produces reads of 150 nts, which map on the complementary strand. The reads were  
196 then processed to remove adapter sequences and poor quality reads by Trimmomatic  
197 (Bolger et al., 2014), then they were converted to the FASTQ format with FASTQ Groomer  
198 (Blankenberg et al., 2010), and were aligned on the HG001 genome (Caldelari et al., 2017)  
199 using BOWTIE2 (Langmead et al., 2009). Finally, the number of reads mapping to each  
200 annotated feature has been counted with HTSeq (Anders et al., 2015) using the interception  
201 non-empty protocol. All processing steps were performed using the Galaxy platform (Afgan  
202 et al., 2016). To estimate the enrichment values for the differential expression analysis for  
203 the transcriptomic experiment, we used DEseq2 (Varet et al., 2016). The statistical analysis  
204 process includes data normalization, graphical exploration of raw and normalized data, test  
205 for differential expression for each feature between the conditions, raw p-value adjustment,  
206 and export of lists of features having a significant differential expression (threshold p-  
207 value=0.05; fold change threshold=2) between the conditions.

## 208 **Preparation of RNAs for *in vitro* experiments**

209 Transcription of full *psm* operon, *spa*, *mgrA* and RNAIII was performed using linearized  
210 pUC18 vectors (Romilly et al., 2014) or PCR fragments containing the T7 promoter (See  
211 **Table S1**). The RNAs were *in vitro* transcribed using T7 RNA polymerase, and purified using  
212 a 6% polyacrylamide-8 M urea gel electrophoresis. After elution with 0.5 M ammonium  
213 acetate pH 6.5 containing 1 mM EDTA, the RNAs were precipitated in cold absolute ethanol,  
214 washed with 85% ethanol and vacuum-dried. The labelling of the 5' end of dephosphorylated  
215 RNAs (*psm*, RNAIII) and DNA oligonucleotides were performed with T4 polynucleotide  
216 kinase (Fermentas) and [ $\gamma$ <sup>32</sup>P] ATP as previously described (Boisset et al., 2007). Before  
217 use, cold or labelled RNAs were renatured by incubation at 90°C for 1 min in 20 mM Tris-

218 HCl pH 7.5, cooled 1 min on ice, and incubated 10 min at 20°C in ToeP+ buffer (20 mM Tris-  
219 HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 60 mM KCl, 1 mM DTT).

### 220 **SauS1 cloning and purification**

221 *SauS1* coding sequence with an His(6)-tag and a TEV cleavage site at the N-terminus  
222 (Table S1) was cloned into the pQE30 vector (Quiagen), then transformed into *E. coli* M15.  
223 Expression and purification of *SauS1* was done as described in (Duval et al., 2013) with the  
224 following modifications. After the first Ni-NTA chromatography, the fractions containing  
225 *SauS1*, were dialyzed in Buffer Q (20 mM Tris-HCl pH 7.5, 2 mM MgCl<sub>2</sub>, 40 mM NH<sub>4</sub>Cl, 1  
226 mM EDTA, 6 mM β-mercaptoethanol), concentrated to 25 mg/ml and the N-terminal His-tag  
227 enzymatically removed using Tev protease (Protean) digestion following the manufacture  
228 protocol. The cleaved tag and the His-tagged Tev have been then removed by a second Ni-  
229 NTA chromatography to isolate not retained *SauS1*. Finally, *SauS1* was purified on a mono  
230 Q column. The protein was dialyzed and stored at -20°C in the storage buffer (20 mM Tris  
231 HCl pH 7.5, 2 mM MgCl<sub>2</sub>, 100 mM NH<sub>4</sub>Cl, 1 mM EDTA, 1 mM DTT, 10% glycerol).

### 232 **Differential proteomics for cytoplasmic and secreted proteins**

233 Triplicate protein extracts from supernatant or cytoplasm of HG001 (WT) and  $\Delta rpsA$  mutant  
234 strain both transformed with the empty pCN51 plasmid, and the  $\Delta rpsA$  mutant strain  
235 complemented with a plasmid expressing *SauS1* (pCN51::*rpsA*), were analyzed in separate  
236 LC/MS experiments. MS/MS spectra numbers were compared for each protein. Total protein  
237 extracts were prepared as follows: 1.5 ml of a *S. aureus* culture (OD<sub>600nm</sub> = 5) was  
238 centrifuged and the pellet resuspended in 150 μl of Lysis buffer P (10 mM Tris pH 7.5, 20  
239 mM NaCl, 1 mM EDTA, 5 mM MgCl<sub>2</sub>) in the presence of 50 μg/ml lysostaphin, 15 μl of  
240 protease inhibitor cocktail (Thermo Fischer Scientific), 2 μl DNase 10 U/μl (Roche), 2 μl  
241 RNase 500 μg/ml (Roche) and incubated for 30 min at 37°C. Then, 1 ml Trizol Reagent (Life  
242 Technologies) was used according to the manufacturer's protocol. The final protein phases  
243 were then precipitated in ice-cold acetone at least for 2h at -20°C. Secreted proteins were  
244 prepared as follows: supernatants of cultures were filtered through a 0.22 μm membrane  
245 and precipitated with 5 volumes of 0.1 M ammonium acetate in methanol. To quantify protein  
246 extracts by Bradford assay, air-dried protein pellets were resuspended in 2D buffer (7 M  
247 urea, 2 M thiourea, 4% Chaps, 25 mM Tris-HCl pH 8) for total extracts or Triton buffer (1%  
248 triton x100, 50 mM NaCl, 50 mM Tris-HCl pH 8) for secreted proteins. Proteins (5 μg) were  
249 precipitated with methanol/0.1 M ammonium acetate, reduced and alkylated (5 mM DTT, 10  
250 mM iodoacetamide), and digested overnight with 1/25 (W/W) of trypsin. The peptide  
251 mixtures (1 μg /sample) were analyzed using a NanoLC-2DPlus system coupled to a  
252 TripleTOF 5600 mass spectrometer (ABSciex), as previously described (Tomasini et al.,  
253 2017). Protein identifications were assigned using Mascot algorithm (version 2.5, Matrix

254 Science, London, UK) through ProlineStudio 1.2 package (<http://proline.profiroteomic.fr/>).  
255 Data were searched against the *S. aureus* HG001 genome (Caldelari et al., 2017). Peptide  
256 modifications allowed during the search were: N-acetyl (protein), carbamidomethylation (C)  
257 and oxidation (M). Mass tolerances in MS and MS/MS were set to 20 ppm and 0.5 Da,  
258 respectively. Two trypsin missed cleavages sites were allowed. After the import of the  
259 Mascot data files, proteins were validated on Mascot pretty rank equal to 1.1% FDR (False  
260 Discovery Rate), on peptide spectrum matches (PSM) based on PSM score, and 1% FDR  
261 on protein sets on protein set score. A Spectral Counting quantitative strategy was applied  
262 on the Mascot identification summaries. To evaluate the reproducibility, a statistical Student  
263 t-test was applied to this experiment.

#### 264 ***psm* operon half-life determination**

265 Bacterial cultures were grown to an OD<sub>600</sub> of 3. They were then treated with rifampicin (final  
266 concentration of 500 µg/ml) to abrogate transcription. RNA samples were collected at  
267 indicated time points and quantified by northern blot analysis with ImageQuant TL software  
268 (GE Healthcare Life Sciences).

#### 269 **Polysome profiling coupled to Northern blot analysis**

270 WT and  $\Delta rpsA$  mutant strains (transformed with the empty pCN51 plasmid for control) and  
271 the same mutant strain complemented with the pCN51::*rpsA* plasmid expressing S1 (**Table**  
272 **S1**) were cultured in BHI medium at 37°C for 4h (OD<sub>600</sub> of 4). Chloramphenicol was added to  
273 the cultures to have 5 mM of final concentration. After two minutes, the cells have been  
274 pelleted by centrifugation (15 minutes at 4°C), resuspended in 500 µl of Lysis buffer R (20  
275 mM Tris pH 7.5, 100 mM NH<sub>4</sub>Cl, 10 mM MgCl<sub>2</sub>, 0.1 % Nonidet p-40, 0.4 % Triton X-100, 1  
276 mM Chloramphenicol, 100 U/ml DNaseI) and disrupted with the FastPrep apparatus (MP  
277 Biomedicals). 40 K OD<sub>260</sub> of cell lysates were loaded on sucrose gradient (5% - 50%) and  
278 separated on Biocomp instrument. The RNA was extracted from the fractions using acid  
279 phenol at 65°C and then precipitated. Northern blot analysis was performed using 1 µg of  
280 RNA.

#### 281 **Toe-printing assays**

282 The preparation of *S. aureus* 30S subunits, the formation of a simplified translational  
283 initiation complex with mRNA, and the extension inhibition conditions were performed as  
284 previously described (Fechter et al., 2009) with slight modifications in the buffer used to  
285 dissociate *Sau*70S into subunits (10 mM HEPES-KOH pH 7.5, 100 mM NH<sub>4</sub>Cl, 1 mM  
286 Mg(OAc)<sub>2</sub>, 1 mM DTT). Increasing concentrations of either *Sau*S1 were used to monitor its  
287 effects on the formation of the initiation complex with *psm* operon, *spa*, *mgrA* and RNAIII.  
288 Prior to toeprinting assay, *Sau*30S subunits were chilled on ice for 10 min then incubated at  
289 37°C for 15 min in ToeP+ buffer. In parallel, mRNA (0,5 pmol) was annealed to a 5' end-

290 labeled oligonucleotide (50000 cps), heated at 90°C for 1 min, cooled on ice for 1 min and  
291 incubated at RT°C for 10 min in ToeP+ buffer. *SauS1* was pre-incubated in ToeP+ buffer for  
292 15 min at 37°C prior to use. 30SIC were constituted at 37°C for 15 min in the presence of  
293 the mRNA annealed to the labelled primer and *Sau30S* (0,25; 0,5; 0,75; 1 pmole) pre-  
294 incubated or not with 1.5 excess of *SauS1*. The tRNAi (20 pmoles) was then added and the  
295 complexes were formed for 5 min at 37°C. Primer extension reactions were subsequently  
296 performed by adding 2 units of AMV-RT at 37°C for 30 min. Reactions were stopped by  
297 phenol extraction followed with ethanol precipitation, and samples were loaded on 10%  
298 urea-PAGE. Quantification of the toe-printing signals present on the autoradiography was  
299 done with ImageQuant TL software (GE Healthcare Life Sciences).

### 300 **Gel filtration**

301 *Sau30S* were reactivated at 37°C for 10 min and incubated in Buffer G (50 mM KCl, 10 mM  
302 NH<sub>4</sub>Cl, 10 mM MgCl<sub>2</sub>, 10 mM Tris HCl pH 7.5, 1 mM DTT) with *SauS1* with or without *psm*  
303 mRNA. Previously, *SauS1* was centrifuged for 1h at 4°C at 13000 rpm in order to remove  
304 aggregates, new concentration was measured and the protein re-activated in Buffer G at  
305 37°C for 10 min. 250 pmoles of *SauS1* were incubated with 25 pmoles of *Sau30S* ribosomal  
306 subunit, for 10 min at 37°C in Buffer G, in a total volume of 50 µl. The mix was then  
307 centrifuged at 4°C for 10 min at 4200 rpm and the supernatant was loaded on size exclusion  
308 column (GE Healthcare Superose TM 6 Increase 3.2 /300). The eluted peaks for *Sau30S*  
309 and the free protein have been then analysed by quantitative LC/MSMS to determine  
310 stoichiometric ratios between *SauS1* and other ribosomal proteins.

### 311 **Gel retardation assays**

312 Radiolabelled purified *psm* operon and RNAIII (50000 cps/sample, concentration < 1 pM)  
313 were renaturated as described above. For each experiment, increasing concentrations of  
314 purified *SauS1* (100-900 nM) were added to the 5' end labelled *psm* or RNAIII in a total  
315 volume of 10 µl containing the ToeP+ buffer. Complex formation was performed at 37°C  
316 during 15 min. After incubation, 10 µl of glycerol blue was added and the samples were  
317 loaded on a 10% PAGE under non denaturing conditions (1h, 300 V, 4°C).

318

## 319 **RESULTS**

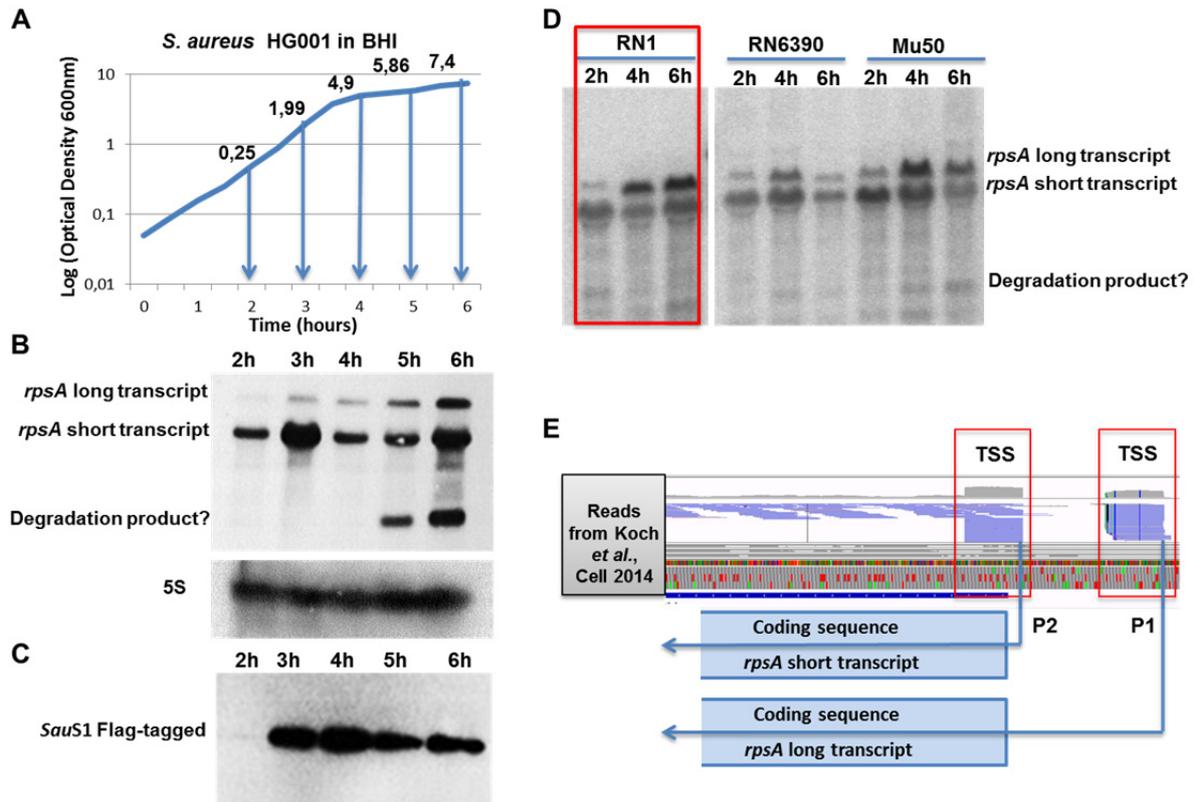
### 320 ***SauS1* has an atypical expression profile if compared to other r-proteins**

321 In bacteria, the synthesis of the r-proteins is coordinated to the transcription of the ribosomal  
322 RNA, which is regulated according to environmental changes and to the different phases of  
323 bacterial growth (Kaczanowska and Ryden-Aulin, 2007; Kjeldgaard et al., 1958; Nomura,  
324 1999; Nomura et al., 1984; Wagner, 1994). Ribosomes need to be quickly assembled, thus  
325 r-RNA and r-proteins accumulate already in the lag and early exponential growth phases

326 (Condon et al., 1995; Rolfe et al., 2012). New ribosomes are also rapidly synthesized to react  
327 to ribosome-directed antibiotics challenges (Wenzel and Bandow, 2011). *SauS1*, encoded  
328 by *rpsA* gene, has nevertheless a peculiar expression profile. For instance, in response to  
329 linezolid stress, *SauS1* is not produced when the other r-proteins are rapidly upregulated  
330 (Bonn et al., 2016). We have first monitored the levels of *rpsA* transcript at different stages of  
331 bacterial growth in BHI medium by Northern blot (**Figure 12**). The experiment was carried  
332 out with *S. aureus* HG001 strain, a derivative of RN1 (NCT8325) strain with restored *rbsU*  
333 (Herbert et al., 2010). The data showed that the *rpsA* mRNA has two distinct isoforms with a  
334 different pattern of expression. The shorter transcript appears to be constitutively expressed,  
335 while the long transcript starts to be expressed at the late exponential phase of growth after  
336 3 h ( $OD_{600} \sim 2$ ) and accumulates at the stationary phase (**Figure 12B**). A similar transcription  
337 pattern could be observed in other *S. aureus* strains, indicating a conserved mechanism of  
338 transcription regulation (**Figure 12D**). The transcripts possibly originate from two different  
339 Transcription Start Sites (TSS), as evidenced by aligning raw data from (Koch et al., 2014)  
340 on our genome (**Figure 12E**).

341 We have also monitored in parallel the levels of the protein *SauS1* using Western  
342 blot analysis (**Figure 12C**). In this experiment, we have introduced into the chromosome of *S.*  
343 *aureus* HG001 a 3xflag tag peptide sequence at the C-terminus of *SauS1*. After purification  
344 of the total protein extracts, the *SauS1* protein was detected using an anti-flag antibody.  
345 Surprisingly, the data showed that the protein levels do not correspond to the pattern of the  
346 mRNA since the protein can only be detected after 3h of growth.

347 Taken together these data showed that the expression of *SauS1* is regulated during  
348 the growth phase of the bacteria.



349

350 **Figure 12: *S. aureus rpsA* expression profile.** A. Growth curve of HG001 strain in BHI  
 351 medium and time points at which cells were harvested for Northern blot and Western blot  
 352 analyses. B. Northern blot analysis of *rpsA* mRNA detected at different time points of the  
 353 growth (2h, 3h, 4h, 5h, and 6h). The two transcripts (short and long) are noted on the side of  
 354 the gel. A short product has been visualized after 5h of growth, which most likely  
 355 corresponded to a degradation product. C. Western blot analysis on *SauS1* detected at  
 356 different time points of the growth (2h, 3h, 4h, 5h, and 6h). D. Northern blot analysis of *rpsA*  
 357 mRNA in different *S. aureus* strains (RN1, RN6390 and Mu50). E. Organization of *rpsA*  
 358 locus and possible transcription units (dataset SRR949025 obtained by (Koch et al., 2014)  
 359 realigned on the HG001 genome and visualized by IGV (Thorvaldsdottir et al., 2013)). P1  
 360 and P2 indicated putative Transcription Start Sites (TSS) from different promoters.  
 361

362 ***SauS1* has significant effect on the virulon of *S. aureus* as revealed by comparative**  
 363 **transcriptomics and proteomics**

364 We have then investigated the impact of *SauS1* on *S. aureus* total transcriptome (Table S1)  
 365 by comparing the RNAs expressed from HG001 (WT) and the isogenic  $\Delta rpsA$  mutant strains.  
 366 Total RNAs were extracted from WT and  $\Delta rpsA$  strains grown to  $OD_{600} \sim 5$  (6h) in BHI at  
 367 37°C. Under these conditions, *SauS1* is abundant in the WT (Figure 12C). The extracted  
 368 RNAs were then used for library preparation and sequencing. The data were analyzed and  
 369 visualized using Galaxy (Afgan et al., 2016) and the Integrative Genomics Viewer (IGV)  
 370 browser, respectively (Thorvaldsdottir et al., 2013). A detailed protocol for the bioinformatics  
 371 analysis is provided in Material and Methods. Briefly, we aligned the sequencing reads onto

372 HG001 genome (Caldelari et al., 2017), counted per feature and normalized. The data were  
373 reproduced in two independent experiments.

374 Rather unexpectedly, the differential expression analysis revealed changes in a small  
375 number of mRNAs. Among the 2565 annotated mRNAs, only 47 genes are up- and 55 are  
376 down-regulated by at least a factor of 2 (**Tables S2 and S3**). Interestingly, more than 35% of  
377 down-regulated mRNAs encoded virulence factors, with the *sspABC* and *apsm1-4* operons  
378 being the less abundant mRNAs in  $\Delta$ *rsaA* strain. The non-coding transcriptome showed  
379 more extensive variations (**Supplementary excel file S1**). Indeed, the majority of tRNAs  
380 and half of the annotated sRNAs showed significant decreased levels in the  $\Delta$ *rpsA* strain  
381 such as the quorum-sensing induced RNAIII and 6S RNA (**Table S4**). In addition, the yields  
382 of cis-acting regulatory elements such as riboswitches, and T-boxes were also diminished in  
383  $\Delta$ *rsaA* strain (**Table S4**).

384 Quantitative differential proteomic and transcriptomic analyses were carried out on  
385 total RNA and cytosolic and secreted proteins, prepared from the WT and  $\Delta$ *rpsA* mutant  
386 strains and the same mutant strain complemented with a plasmid expressing S1. The WT  
387 and  $\Delta$ *rpsA* mutant strains were also transformed with the pCN51 plasmid for control (**Table**  
388 **S1**). Bacterial growth was performed in BHI medium for 6h. Triplicates experiments have  
389 been analyzed by LC/MSMS (**Tables S5, S6**). The differential spectral count analysis nicely  
390 correlates with the observed effects on the mRNAs encoding virulence factors (**Table 2**). Of  
391 the 79 classified virulence factors, 23 of them are less abundant in the  $\Delta$ *rpsA* strain. A  
392 classification analysis of these factors further evidenced a clear perturbation in the proteins  
393 and/or mRNA levels for exoenzymes (serine and cysteine proteases and lipases) and of  
394 membrane pore forming toxins, including the four  $\alpha$ PMS peptides and the  $\alpha$ -  $\delta$ - and  $\gamma$ -  
395 hemolysins (**Table 2**).

396 These data strongly suggested that S1 altered significantly the virulon. Because the  
397 levels of several sRNAs and of RNAIII were enhanced in strain expressing S1, some of the  
398 effects primarily on proteases might be indirect. However, this is not the case of  $\alpha$ -*psm* and  
399  $\beta$ -*psm* operons, which are transcribed by AgrA (Queck et al., 2008), whose level is not  
400 affected by SauS1 (**Supplementary excel file S1**). Therefore, we proposed that S1 might  
401 regulate the translation of the *psm* operon, and belongs to the class of post-transcriptional  
402 regulatory protein.

id	gene	product	classification	Transcriptomics $\Delta rpsA$ /WT		Proteomics $\Delta rpsA$ /WT		Secretomics $\Delta rpsA$ /WT		Secretomics $\Delta rpsA$ /comp	
				FoldChange	pvalue	FoldChange	pvalue	FoldChange	pvalue	FoldChange	pvalue
HG001_00899	sspA	Glutaryl endopeptidase	Exoenzyme, Serine protease	0.10	2,48E-55	too few peptides		0.17	0.16390	0.03	1,44E-06
HG001_03280	psm-a4	4 psm peptide	Toxin, Pore-forming	0.17	2,20E-32	n.d.		n.d.		n.d.	
HG001_03282	psm-a2	2 psm peptide	Toxin, Pore-forming	0.29	1,68E-16	n.d.		n.d.		n.d.	
HG001_01009	hly	Alpha-hemolysin	Toxin, Pore-forming	0.31	2,80E-07	0.23	0.02739	0.39	0.02508	0.12	1,90E-19
HG001_00898	sspB	Staphopain B	Exoenzyme, Cysteine protease	0.32	1,28E-11	0.36	0.03388	SI only	3,32E-03	COMET only	3,19E-14
HG001_02345	65 kDa membrane protein Eap		Exoenzyme, C (G0386)	0.33	8,93E-13	SI only	0.00309	0.29	0.02929	2.00	
HG001_00897	sspC	Staphostatin B	Exoenzyme, Cysteine protease	0.33	3,16E-07	n.d.		n.d.		n.d.	
HG001_02058	hld	Delta-hemolysin	Toxin, Pore-forming	0.35	9,33E-10	n.d.		n.d.		n.d.	
HG001_01006	scn_2	Staphylococcal complement inhibitor	Immune evasion	0.39	1,47E-04	n.d.		n.d.		n.d.	
HG001_02456	sbi	Immunoglobulin-binding protein	Immune evasion	0.39	3,02E-06	n.d.		0.77	0.61590	1.00	0.39520
HG001_01023	psm-b2	2 beta pm peptide	Adherence	0.39	2,92E-10	SI only	0.23790	n.d.		n.d.	
HG001_03283	psm-a1	1 alpha pm peptide	Toxin, Pore-forming	0.41	4,79E-05	n.d.		n.d.		n.d.	
HG001_00264	lip2	Lipase 2	Exoenzyme, Lipase	0.43	2,06E-09	0.25	1,74E-09	0.47	0.05042	0.18	3,06E-19
HG001_02459	HlgB	Gamma-hemolysin component B	Toxin, Pore-forming	0.46	8,94E-05	n.d.		0.11	0.04432	0.02	5,34E-09
HG001_01971	-	65 kDa membrane protein Eap	Adherence	0.46	5,37E-04	n.d.		n.d.		n.d.	
HG001_01943	sspP	Staphopain A	Exoenzyme, Cysteine protease	0.47	1,95E-04	0.43	0.19580	0.42	0.44860	0.04	2,35E-20
HG001_01005	fib_2	Fibrinogen-binding protein	Immune evasion, Adherence	0.47	1,23E-03	n.d.		1.37	0.01020	2.17	7,70E-04
HG001_02458	HlgC	Gamma-hemolysin component C	Toxin, Pore-forming	0.48	3,27E-04	n.d.		1.10	0.19490	0.37	0.07451
HG001_02709	lipA_2	Lipase 1	Exoenzyme, Lipase	0.56	3,18E-03	0.38	0.59470	0.75	0.33850	0.52	0.04707
HG001_03281	psm-a3	3 alpha pm peptide	Toxin, Pore-forming	0.57	8,89E-05	n.d.		n.d.		n.d.	
HG001_00978	isdA	Iron-regulated surface determinant protein A	Iron uptake, Heme uptake	0.57	0.00757	n.d.		0.76	0.34240	0.71	0.76880
HG001_02044	-	putative leukocidin-like protein 2	Toxin, Pore-forming	0.60	0.01068	n.d.		n.d.		n.d.	
HG001_01340	ebp5	Elastin-binding protein Ebp5	Adherence	0.63	0.00097	0.79	0.34790	1.00	0.21290	1.27	0.12920
HG001_02538	fibA_1	Fibrinectin-binding protein A	Adherence	0.63	0.45381	n.d.		1.00	0.63420	2.00	0.36280
HG001_01974	scn_3	Staphylococcal complement inhibitor	Immune evasion	0.68	0.03382	SI only	0.04091	0.63	0.98870	0.63	0.89640
HG001_02043	-	putative leukocidin-like protein 1	Toxin, Pore-forming	0.69	0.05932	0.70	0.64810	0.43	0.58700	0.21	0.04609
HG001_01978	sak	Staphylokinase	Plasminogen activator	0.69	0.05998	n.d.		n.d.		n.d.	
HG001_01976	chp	Chemotaxis inhibitory protein	Immune evasion	0.71	0.22727	n.d.		1.00	0.41380	0.86	0.68490
HG001_02707	icaB	Poly-beta-1,2C6-N-acetyl-D-glucosamine N-deacetylase	Adherence	0.71	0.23161	n.d.		n.d.		n.d.	
HG001_02539	fibA_2	Fibrinectin-binding protein A	Adherence	0.71	0.06276	n.d.		n.d.		n.d.	
HG001_02559	cifB	Clumping factor B	Immune evasion	0.71	0.03383	n.d.		1.63	0.03239	0.85	0.87440
HG001_00172	scn_1	Staphylococcal complement inhibitor	Immune evasion	0.72	0.22543	n.d.		n.d.		n.d.	
HG001_01972	esp	65 kDa membrane protein Eap	Adherence	0.72	0.06984	n.d.		n.d.		n.d.	
HG001_00728	cifA	Clumping factor A	Immune evasion	0.73	0.02679	0.83	0.65790	0.79	0.56450	1.57	0.09078
HG001_00977	isdB	Iron-regulated surface determinant protein B	Iron uptake, Heme uptake	0.74	0.27830	n.d.		n.d.		n.d.	
HG001_01752	lukDv	Leucotoxin LukDv	Toxin, Pore-forming	0.74	0.26637	n.d.		n.d.		n.d.	
HG001_02708	icaC	poly-beta-1,2C6-N-acetyl-D-glucosamine export protein	Adherence	0.74	0.30381	n.d.		n.d.		n.d.	
HG001_01002	fib_1	Fibrinogen-binding protein	Immune evasion, Adherence	0.74	0.27907	ArpsA only	0.24020	0.38	0.35330	1.25	0.37010
HG001_00981	isdE	High-affinity heme uptake system protein	Iron uptake, Heme uptake	0.75	0.29359	n.d.		n.d.		n.d.	
HG001_02706	icaD	Poly-beta-1,2C6-N-acetyl-D-glucosamine synthesis protein	Adherence	0.78	0.39029	n.d.		n.d.		n.d.	
HG001_00767	thyC	Hemolysin C	Allows resistance to high Mg++	0.79	0.17686	n.d.		n.d.		n.d.	
HG001_00234	esxB	Virulence factor EsxB	Type VII secretion system	0.79	0.34392	n.d.		n.d.		n.d.	
HG001_01753	lukE	Leucotoxin LukE	Toxin, Pore-forming	0.79	0.41746	n.d.		n.d.		n.d.	
HG001_00979	isdC	Iron-regulated surface determinant protein C	Iron uptake, Heme uptake	0.80	0.43470	n.d.		n.d.		n.d.	
HG001_01973	hly_1	Phospholipase C	Toxin, Hydrolase	0.82	0.47461	n.d.		n.d.		n.d.	
HG001_00233	esxA	hypothetical protein	Type VII secretion system	0.83	0.40875	n.d.		n.d.		n.d.	
HG001_00980	isdD	hypothetical protein	Iron uptake, Heme uptake	0.84	0.51617	n.d.		n.d.		n.d.	
HG001_00231	essB	putative membrane protein essB	Type VII secretion system	0.84	0.43664	n.d.		n.d.		n.d.	
HG001_00984	isdG	Heme oxygenase (staphylobilin-producing) 1	Iron uptake, Heme uptake	0.85	0.57256	n.d.		n.d.		n.d.	
HG001_02705	icaA	Poly-beta-1,2C6-N-acetyl-D-glucosamine synthase	Adherence	0.85	0.58631	n.d.		n.d.		n.d.	
HG001_02576	aur	Zinc metalloproteinase aureolysin	Exoenzyme, Zinc metalloprotease	0.87	0.43983	n.d.		n.d.		n.d.	
HG001_00217	esxA	Virulence factor EsxA	Type VII secretion system	0.89	0.50433	1.37	0.49980	n.d.		n.d.	
HG001_00102	ywqE_1	Tyrosine-protein phosphatase YwqE	Antiphagocytosis	0.90	0.50249	SI only	0.09507	n.d.		n.d.	
HG001_01022	psm-b1	1 beta pm peptide	Adherence	0.905	0.50906	1.00	0.99450	n.d.		n.d.	
HG001_00719	VWbp	Staphylocoagulase	Exoenzyme	0.92	0.76399	n.d.		n.d.		n.d.	
HG001_00230	yukD	YukD	Type VII secretion system	0.92	0.77518	n.d.		n.d.		n.d.	
HG001_02242	hlyA	Hyaluronate lyase	Exoenzyme, Spreading factor	0.92	0.73210	n.d.		n.d.		n.d.	
HG001_00101	ywqD_1	Tyrosine-protein kinase YwqD	Antiphagocytosis	0.95	0.72346	0.84	0.40500	n.d.		n.d.	
HG001_00112	epsL	putative sugar transferase EpsL	Antiphagocytosis	0.95	0.72866	n.d.		n.d.		n.d.	
HG001_00983	srtB	Sortase family protein	Iron uptake, Heme uptake	0.95	0.85382	n.d.		n.d.		n.d.	
HG001_00219	essA	Firmicute eSAT-6 protein secretion system EssA	Type VII secretion system	0.97	0.98057	n.d.		n.d.		n.d.	
HG001_00982	isdF	heme-iron transport system permease	Iron uptake, Heme uptake	0.98	0.93990	n.d.		n.d.		n.d.	
HG001_00113	gpfE	UDP-glucose 4-epimerase	Antiphagocytosis	0.99	0.94326	n.d.		n.d.		n.d.	
HG001_00232	eccCa1	ESK-1 secretion system protein EccCa1	Type VII secretion system	0.99	0.95368	too few peptides		n.d.		n.d.	
HG001_02042	hly_2	Phospholipase C	Toxin, Hydrolase	0.99	0.95750	n.d.		n.d.		n.d.	
HG001_02457	HlgA	Gamma-hemolysin component A	Toxin, Pore-forming	1.01	0.98451	n.d.		n.d.		n.d.	
HG001_00493	isdI	Serine-aspartate repeat-containing protein D	Adherence, MSCRAMMs	1.03	0.89302	too few peptides		0.73	0.45960	3.27	1,70E-04
HG001_00115	mnaA_1	UDP-N-acetylglucosamine 2-epimerase	Antiphagocytosis	1.07	0.86886	1.76	0.28740	n.d.		n.d.	
HG001_00104	capD	UDP-glucose 4-epimerase	Antiphagocytosis	1.08	0.68071	Del-S1 only	0.09371	n.d.		n.d.	
HG001_00105	fdl	GDP-L-Fucose synthase	Antiphagocytosis	1.11	0.48166	Del-S1 only	0.00667	n.d.		n.d.	
HG001_02704	icaR	Biofilm operon icaADBC HFH-type negative transcriptional regulator	Adherence	1.17	0.39326	0.76	0.73090	n.d.		n.d.	
HG001_00228	esaA	Secretion accessory protein EsaA/YueB @ Bacteriophage SPP1 receptor	Type VII secretion system	1.18	0.36029	n.d.		n.d.		n.d.	
HG001_00100	cap8A_1	Capsular polysaccharide type 8 biosynthesis	Antiphagocytosis	1.19	0.25569	0.43	0.55600	n.d.		n.d.	
HG001_00106	wbpI	UDP-2,2C3-diacetamido-2,2C3-dideoxy-D-glucuronate 2-epimerase	Antiphagocytosis	1.19	0.27180	1.00	0.98980	n.d.		n.d.	
HG001_00027	yfjM_1	Trifunctional nucleotide phosphoesterase	Immune evasion	1.25	0.15295	SI only	0.10650	1.25	0.29720	1.67	0.21470
HG001_00114	wbpA	UDP-N-acetyl-D-glucosamine 6-dehydratase	Antiphagocytosis	1.27	0.14709	0.65	0.51870	n.d.		n.d.	
HG001_00111	cap5L	putative glycosyl transferase	Antiphagocytosis	1.29	0.10239	n.d.		n.d.		n.d.	
HG001_00103	pgfI	UDP-N-acetyl-alpha-D-glucosamine C6 dehydratase	Antiphagocytosis	1.33	0.16077	n.d.		n.d.		n.d.	
HG001_00060	spa	Immunoglobulin G-binding protein A	Immune evasion, Adherence	1.35	0.07927	n.d.		0.86	0.05719	5.00	2,02E-11

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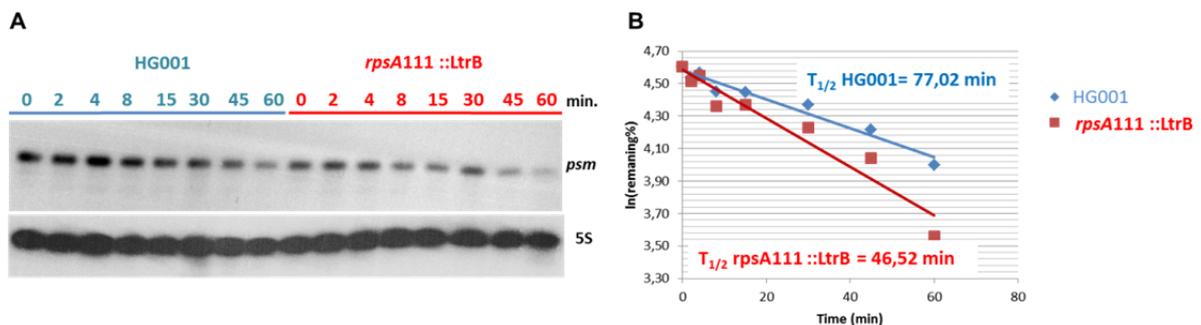
**Table 2: Differential transcriptomics and proteomics analysis of virulence factors expression in  $\Delta rpsA$ .** Fold change ( $\Delta rpsA$ /WT) and p-values were calculated for the transcriptomics analysis by DESeq2 using shrinkage estimation for dispersions and fold changes (Varet et al., 2016), for the proteomics by the R-Studio software. Fold change estimations and p-values have p-values<0.005 (high significance), when highlighted by dark-grey boxes they have p-values>0.001; light-grey boxes indicate 0.005>p-values>0.001.

**SauS1 protects *psm* mRNA operon against *in vivo* degradation.**

Due to the structural resemblance of *SauS1* to the ribosomal protein *EcoS1*, we make the hypothesis that the protein would regulate the translation initiation process of the *psm* operon. Indeed, in Gram-positive bacteria, stabilization of mRNAs can be due to the binding

414 of the ribosome or of key factors close to their 5' ends (Condon, 2003). To test whether the  
 415 decreased level of *psm* mRNA (0.17; **Table 2**) in the  $\Delta rpsA$  strain could be due to a more  
 416 rapid turnover, we have analysed its stability by measuring the kinetics of its degradation  
 417 after rifampicin treatment, an antibiotic which prevents initiation of new transcripts by binding  
 418 to the  $\beta$  subunit of RNA polymerase (Campbell et al., 2001). Rifampicin was added to WT  
 419 and the mutant *rpsA111::LtrB* strains grown in BHI to  $OD_{600}=3$  (4h), when *psm* started to  
 420 accumulate (**Figure S1**). The *psm* mRNA was detected by Northern blot using total RNAs  
 421 extracted after 2, 4, 8, 15, 30, 45 and 60 min (**Figure 13A**). Quantification, normalization and  
 422 interpolation of the data by linear regression in logarithmic scale, showed that the *psm*  
 423 transcript is highly stable, with a half-life of 77.02 min in the WT strain. In contrast to the  
 424 majority of the transcripts (90%), which have half-lives shorter than 5 min (Roberts et al.,  
 425 2006), this operon is one of the most stable transcript. In the mutant strain, the *psm* half-life  
 426 appears to be significantly shorter and was decreased to 46.52 min (**Figure 13B**).

427 Therefore, these data showed that SauS1 has a significant effect on the stabilization  
 428 of the *psm* operon, in agreement with the transcriptomics and proteomics analysis.



429  
 430 **Figure 13. *psm* mRNA stability.** A. Northern blot analysis of the *psm* transcript in WT and  
 431 *rpsA111::LtrB*. Cells were growth at 37°C in BHI and treated with rifampicin at 4h. Total RNA  
 432 was extracted after 2, 4, 8, 15, 30, 45 and 60 min. *psm* mRNA and 5S rRNA were probed  
 433 with specific oligonucleotides (**Table S1**). B. After quantification of the Northern blot signals  
 434 and normalization of each point to the corresponding 5S signals, the % of remaining *psm*  
 435 mRNA has been plotted to calculate *psm* half-lives in the two strains.  $T_{1/2}$ HG001 (WT) is  
 436 77.02 min, while in absence of *SauS1*,  $T_{1/2}$ *rpsA111::LtrB* decreases to 46.52 min.

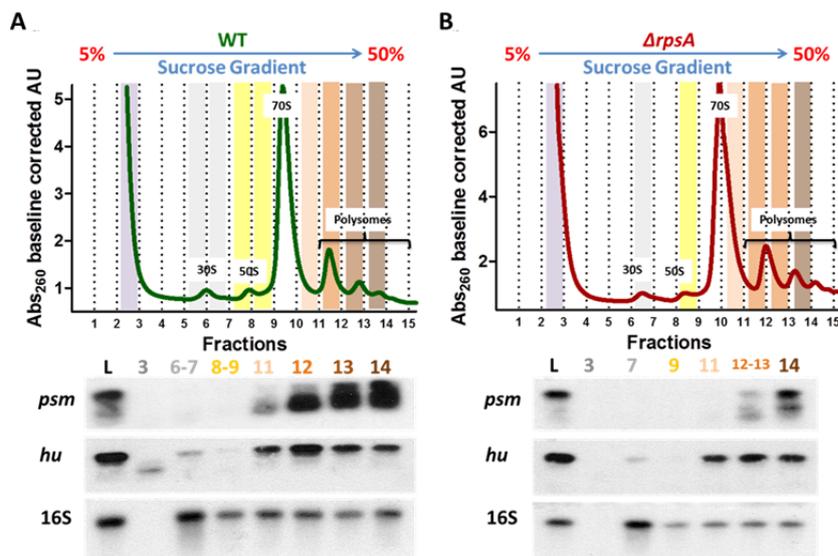
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### 438 Polysome occupancy of *psm* mRNA is higher in presence of *SauS1*

439 Because the effect of S1 on the mRNA stability can be the result of an enhanced translation,  
 440 we have analyzed the mRNA distribution using polysome profile analysis coupled with  
 441 Northern blot experiments. The experiments were done on WT and mutant  $\Delta rpsA$  strains  
 442 grown in BHI at 37°C until mid-exponential phase ( $OD_{600}=4$ ). Translation was then stopped  
 443 by adding chloramphenicol to the cultures and the cells were rapidly harvested by  
 444 centrifugation. After cell lysis, polysomes have been separated *via* ultracentrifugation on a 5-  
 445 50% sucrose gradient. The RNA was extracted from each fraction and Northern blot was  
 446 done using specific oligonucleotides to detect *psm*, *hu* mRNAs and 16S rRNA. Quantification

447 and normalization on the 16S rRNA level have revealed huge differences on the amount of  
 448 *psm* mRNA recruited in the polysomes in the two strains (**Figure 14AB**). In the absence of  
 449 *SauS1*, much less mRNA (0.23 fold change  $\Delta rpsA$ /WT) is engaged on the ribosomes to be  
 450 translated compared to WT strain. Moreover, we could show that translation activation by  
 451 *SauS1* is specific for *psm* mRNA. Indeed, the translation of *hu* mRNA does not depend on  
 452 *SauS1* and its polysome occupancy does not significantly vary (1.27 fold). Because in both  
 453 WT and mutant  $\Delta rpsA$  strain, we did not detect the free mRNA in the fractions of low density  
 454 (**Figure 14AB**), we could not exclude that the observed differences in the polysome fractions  
 455 reflect the variation in *psm* mRNA levels. However, one cannot exclude that the free *psm*  
 456 transcript, which is not protected by the ribosomes, is also rapidly degraded.

457 These data showed that the S1-dependent stabilization of the *psm* mRNA might  
 458 result from an enhanced translatability.



459

460 **Figure 14. Effects of *SauS1* on the translation of *psm* and *hu* mRNAs by polysome**  
 461 **profile coupled with Northern blot analysis.** WT and  $\Delta rpsA$  strains (A and B, respectively)  
 462 were cultured in BHI at 37°C until OD<sub>600</sub>=4, translation was stopped with chloramphenicol  
 463 and polysomes fractionated on a 5-50% sucrose gradient. The RNA was extracted from  
 464 each fraction and Northern blot was done using specific oligonucleotides to detect *psm*, and  
 465 *hu* mRNAs, and 16S rRNA. L= cellular lysate. The 16S rRNA in the L line was used for the  
 466 normalization.

467

#### 468 ***SauS1* specifically promotes ribosome binding on *psm* mRNA**

469 Toe-printing assays (Hartz et al., 1988) were used to decipher the effect of *SauS1* on the  
 470 formation of the ternary initiation complex formed in the presence of *psm* mRNA, the initiator  
 471 tRNA and the *Sau30S* subunit (**Figure 15**). We have verified that *SauS1* was not bound to  
 472 the 30S. A toe-print is observed at position +16 (+1 is the adenine of the start codon) if the



493 **Figure 15. Toeprinting assays to monitor the effect of *SauS1* on the translation**  
494 **initiation complex formation with *psm* mRNA.** (A) Effect of *SauS1* on the formation of  
495 initiation complex using *psm* mRNA and an oligonucleotide that anneals at the 3' end of the  
496 mRNA. When present, *SauS1* was pre-incubated with the ribosome at a constant 1.6 molar  
497 ratio. Lane 1: incubation control of mRNA ; Lane 2: incubation control of mRNA with 30S  
498 subunits ; Lanes 3, 4, 5 and 6: formation of the initiation complex containing mRNA,  
499 increasing concentration of 30S (25, 50, 100, 200 nM) and fMet-tRNA. Lane7: incubation  
500 control of mRNA with purified *SauS1*. Lane 8: incubation control of mRNA, 30S and *SauS1*.  
501 Lanes 9, 10, 11 and 12: formation of initiation complex in presence of *SauS1* and increasing  
502 concentrations of 30S (25, 50, 100, 200 nM). Lanes U, A, G, C: sequencing ladders. The  
503 toe-printing signals at position +16 are indicated by arrows. (B) Toe-printing done with an  
504 oligonucleotide annealing close to the 5' end to better visualize the 1<sup>st</sup> RBS of the *psm*  
505 mRNA. Same legend as in the panel A. (C) Quantification of the toe-printing using  
506 ImageQuanTL software (GE Healthcare). Signals were normalized according to the total  
507 amount of radioactivity (full-length extension and +16 product bands).

508 In order to assess if the activity of *SauS1* is specifically linked to the alleviation of translation  
509 repression mediated by cis-acting mRNA structures, we tested by toe-printing one more  
510 natural *S. aureus. spa* mRNA, which harbours distinct structural features. A short 5' UTR  
511 containing a strong SD sequence exposed in a hairpin loop, and an unpaired AU rich  
512 sequence downstream the start codon which has low propensity of forming stable structures  
513 (predicted  $\Delta G = -2.7$  kcal/mol) (**Figure S3 and S4**). We have previously shown that *E. coli*  
514 ribosomes, containing or not S1, are able to form initiation complexes with *spa* mRNA  
515 (Khusainov et al., 2016). As shown in **Figure S3**, *SauS1* enhances only very weakly the  
516 formation of the translation initiation complex on *spa* mRNA.

517

### 518 ***SauS1* stimulates *psm* translation initiation by binding directly to the mRNA**

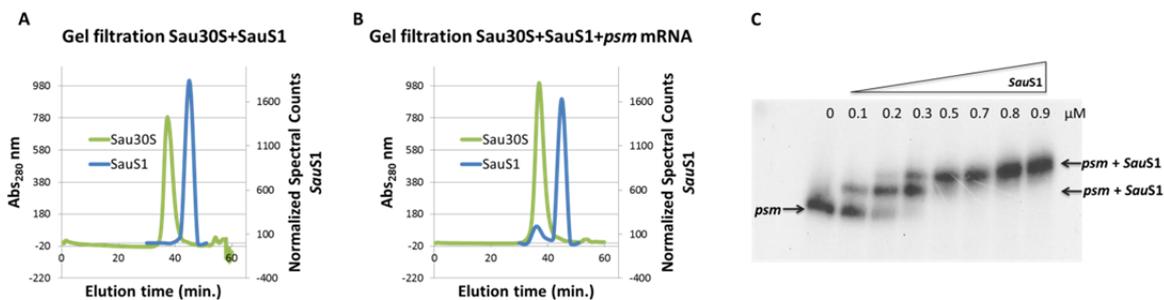
519 To further investigate the mechanism by which *SauS1* stimulates translation of the *psm*  
520 mRNA, we checked its ability to interact with the 30S ribosome. Mass spectrometry analyses  
521 of purified *S. aureus* 70S and 30S have shown only traces of *SauS1* (Khusainov et al., 2016;  
522 Khusainov et al., 2017). This could have been resulted from dissociation occurring during  
523 ribosome purification or could be due to its inability to bind the ribosome. To address this  
524 question we forced a possible interaction by incubating large excess of *SauS1* with *Sau30S*.  
525 Gel filtration chromatography (GFC) was used to purify the complex, which has been  
526 analyzed by LC/MSMS to determine the protein content (**Figure 16A**). The chromatogram,  
527 following the absorbance of the 30S at 280 nm, shows a single peak where all 30S r-proteins  
528 have been found except *SauS1* (**Table S7**), which was eluted at a later time. This data  
529 indicates that *SauS1* does not interact with the 30S. The same experiment was then  
530 performed in the presence of *psm* mRNA (**Figure 16B**). The obtained absorbance profile  
531 resembles strictly the previous one, while the spectral count analysis of the fractions

532 indicates the presence of *SauS1* on the 30S at a level compatible with the other r-proteins  
533 (Table S7).

534 We then analyzed whether *SauS1* binds directly to *psm* mRNA using gel retardation  
535 assays (Figure 16C). *In vitro* 5' end-labelled *psm* was incubated with increasing  
536 concentrations of *SauS1*. The data showed that *SauS1* is able to form two distinct  
537 complexes with *psm* mRNA possibly indicating that two *SauS1* molecules are able to bind to  
538 the mRNA with different affinity (between 100-200 nM and around 300 nM). To finally prove  
539 that the activation mechanism relies on the formation of *SauS1*-*psm* complex, a new toe-  
540 printing assay was done on *psm* mRNA pre-incubated with *SauS1* before the addition of the  
541 30S and the initiator tRNA (Figure S5). A strong enhancement of the initiation complex  
542 formation on the 1<sup>st</sup> CDS could be observed even at a low concentration of *SauS1* (100 nM).

543 Taken together, these data revealed that *SauS1* is not a ribosomal protein but  
544 activates *psm* translation through a direct binding with *psm* mRNA, possibly at multiple sites.  
545 The presence of inhibitory structures on *psm* RBSs would suggest a possible remodeling of  
546 the RNA structure upon *SauS1* interaction, which would liberate their SD sequences to  
547 facilitate the 30S recruitment.

548



549

550 **Figure 16. *SauS1* activates translation of *psm* by direct binding to the mRNA.** A.  
551 Chromatogram of the gel filtration (Superose TM 6 Increase 3.2/300) for the *Sau30S*+*SauS1*  
552 complex (green profile) indicating the peak for the *Sau30S* and the peak for *SauS1* (blue  
553 profile) observed by spectral counts analysis of the fractions. B. As for panel A. with the sole  
554 exception that *psm* mRNA has been added to the complex. *SauS1* spectral counts in the  
555 different fractions have been normalized to the total *SauS1* counts obtained in each of the  
556 two experiments. Peaks in panel A and B could be directly compared. C. Gel retardation  
557 assays to monitor *SauS1* binding to *psm* mRNA. The 5' end-labelled *psm* mRNA was  
558 incubated with increasing concentrations (nM) of *SauS1*. Two complexes could be detected.  
559 The positions of the complexes and of the free *psm* are indicated by arrows.

560

561

## 562 DISCUSSION

563 In bacteria, the main determinant for mRNA recruitment on the 30S is the SD sequence  
564 which base-pairs with the 3' end of the 16S rRNA in the center of the 30S platform, a  
565 positively charged ring shape environment made by several key r-proteins (S1, S2, S7, S11,

566 S18 and S21)(Duval et al., 2015; Simonetti et al., 2009). Among these r-proteins, the largest  
567 r-protein S1 confers to the *E. coli* 30S the ability to recognize any type of mRNAs (e.g.,(Boni  
568 et al., 1991; Duval et al., 2013; Sorensen et al., 1998; Tzareva et al., 1994)). Particularly, S1  
569 has been shown to increase the affinity of weak SD containing mRNAs, and to confer an  
570 RNA chaperone activity to the 30S that is essential to unfold different structures promoting  
571 mRNA accommodation into the decoding channel. In *E. coli*, the essential activity of S1 is  
572 linked to its N-terminal OB-fold domain, which directly interacts with the 30S (Byrgazov et al.,  
573 2015; Byrgazov et al., 2012; Duval et al., 2013). A phylogenetic study revealed that S1 from  
574 Gram-negative bacteria and high G+C content Gram-positive bacteria (i.e., actinobacteria)  
575 share similar domain organization containing at least the first four OB-fold domains that  
576 retained full 30S and RNA binding capacity, and the RNA chaperone activity (Duval et al.,  
577 2013; Duval et al., 2017; Salah et al., 2009). In contrast, S1 from low G+C content Gram-  
578 positive bacteria (firmicutes), contained only four OB-fold domains (Salah et al., 2009). A  
579 specific domain alignment analysis, strongly suggested that *SauS1* organization is most  
580 likely d3-d2-d3-d4, and does not carry the domain required for ribosome binding  
581 **(Supplementary Figure S6).**

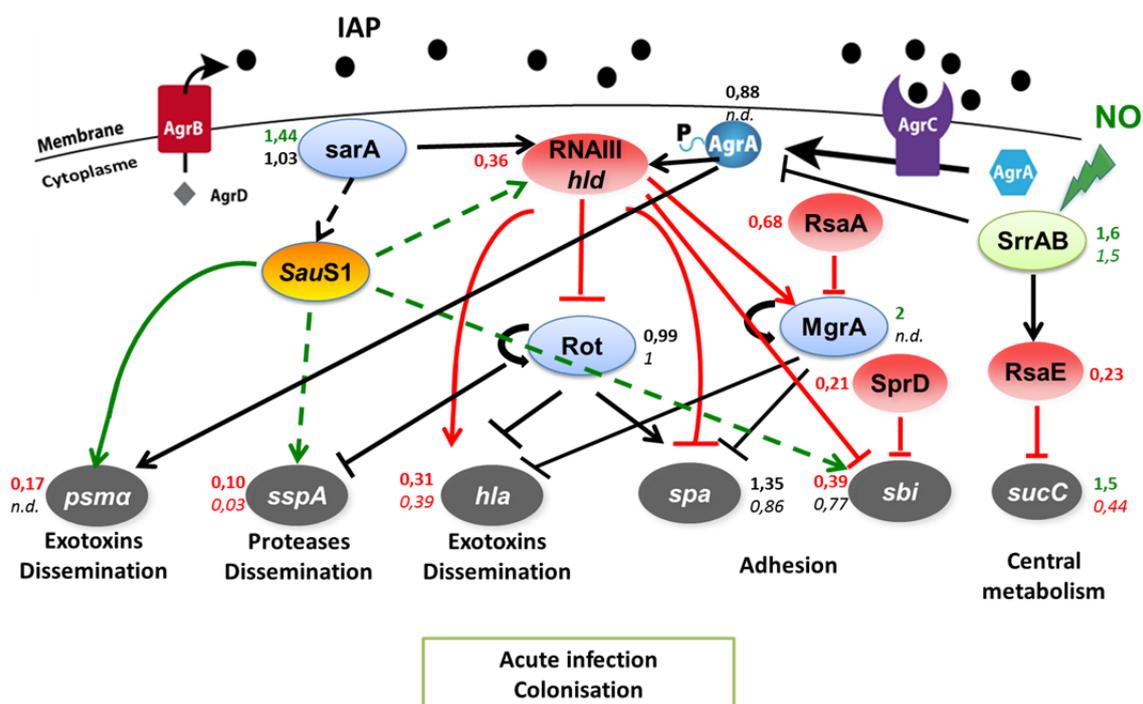
582 Here, we show that *SauS1* is not an essential protein as it was demonstrated for  
583 other Gram-positive bacteria (e.g. in *B. subtilis* (Kobayashi et al., 2003), and in *S.*  
584 *pneumoniae* (Song et al., 2005)), and is not a ribosomal protein since we did not observe any  
585 detectable interaction with the 30S and the 70S ribosomes. Despite its non-ribosomal  
586 localization, we provide the first example of translation activation involving S1 in the major  
587 bacterial pathogen *S. aureus*. Specifically, we show that *SauS1* strongly and specifically  
588 activates the translation of the structured *psm* operon mRNA. Our data suggest that *SauS1*  
589 plays multiple functions in gene regulation: (i) the protein facilitates the ribosome binding to  
590 *psm* mRNA operon at the initiation step of protein synthesis; (ii) *SauS1* is present on  
591 polysomes only through its interaction with *psm* mRNA; (ii) transcriptomics and proteomics  
592 analysis revealed that *SauS1* is also an important regulator of exotoxin production, and  
593 might be a partner of sRNA-mediated regulation.

594 Although previous experiments suggested that S1 homologues had different  
595 properties and functions in Enterobacteriaceae and in low G-C Gram-positive bacteria, we  
596 show here that *SauS1* still exerts an important function in translation disconnected from the  
597 ribosome. The lack of the N-terminal domain most likely coincides with the fact that many  
598 mRNAs in low G-C Gram-positive bacteria contains strong SD sequences. Although  
599 additional experiments will be required to assess the molecular mechanism of S1-dependent  
600 activation of *psm* translation, we propose that *SauS1* has evolved as a translational regulator  
601 and RNA chaperone protein, in a way reminiscent to the cold shock protein CspA in *E. coli*  
602 (Giuliodori et al., 2010). Indeed, the *psm* operon is predicted to be a highly structured RNA in

603 which the four SD and the coding sequences are sequestered into hairpin motifs. These  
604 hairpins are connected through unpaired A/U rich regions (**Figure S2**), which are known to  
605 be ideal binding sites for *E. coli* S1 (Boni et al., 1991; Duval et al., 2013). Band shift  
606 experiments revealed that several proteins bind to *psm* transcript with low or medium  
607 affinities (from 100 to 300 nM). . These data suggested that *SauS1* forms dynamic  
608 complexes with RNA and most probably acts as an RNA chaperone to remodel the RNA  
609 structure, which becomes competent for translation. Another example of a structured RNA  
610 encoded *psm* is RNAIII, which contained several long-range interactions bringing in close  
611 proximity its 5' and 3' non coding regions (Benito et al., 2000). Noteworthy, it has been  
612 shown that the translation of the PSM delta-hemolysin and the activation of many exotoxins  
613 are delayed after the transcription of RNAIII, and this delay was abolished if the 3' non  
614 coding region of RNAIII is deleted (Balaban and Novick, 1995). It is tempting to propose that  
615 *SauS1* might help to promote the RNAIII conformational switch allowing the recruitment of  
616 the ribosome on *hld* RBS.

617         What could be the rationale of the *SauS1* regulation? The transcription of *rpsA* is  
618 regulated upon cell growth and its synthesis enhances strongly at the late exponential level.  
619 The functional significance for the existence of the two transcripts awaits for more  
620 experimental data. Nevertheless, the accumulation of S1 at the late exponential level  
621 corresponded to the expression pattern of the longest of the two transcripts (**Figure 12**).  
622 Intriguingly, this expression pattern follows the synthesis of the quorum sensing system  
623 RNAIII and of many exotoxins, for which the expression was found downregulated in the  
624 mutant *rpsA* (**Table 2**). These exotoxins include the PSMs ( $\alpha$ -PSM,  $\beta$ -PSM, Hld), the  
625 endopeptidases SspAB, the fibronectin binding proteins (FnbA and FnbB), and hemolysins  
626 (Hla, Hld, HlgB and HlgC). The PSMs are short, amphipathic  $\alpha$ -helical peptides, which play  
627 key roles in virulence by promoting lysis of neutrophils, and contributing to the dissemination  
628 of biofilm-associated infection (reviewed in Otto, 2013). In contrast to many exotoxins, the  
629 transcription of PSM is strictly dependent on the *agr* quorum sensing system. A recent study  
630 revealed that SarA is required for the PSM synthesis as well as other exotoxins contributing  
631 to the acute phase of *S. aureus* osteomyelitis (Loughran et al., 2016) but this regulatory  
632 event could be the result of the SarA-dependent activation of AgrA transcription (Loughran et  
633 al., 2016; Queck et al., 2008). Interestingly, the expression of *rpsA* is also drastically reduced  
634 in different *sarA* mutant strains (Loughran et al., 2016; Roberts et al., 2006). Because SarA  
635 is strongly induced during the transition from late exponential phase to stationary phase  
636 (Manna et al., 1998), it might be responsible for the coordination of *rpsA* transcription with  
637 that of *psm*. The strict regulation of PSMs expression under both the quorum sensing control  
638 and *SauS1* might be necessary for the concerted action during acute infection, when they

639 are produced to promote dissemination and tissue lysis. Since they contribute to biofilm  
640 detachment/dissemination (Kong et al., 2006; Periasamy et al., 2012; Tsompanidou et al.,  
641 2011), an early induction could expose *S. aureus* to the host immune system before a critical  
642 mass could have been attained. In that regard, it is worth to notice that *SauS1* could also  
643 regulate the translation of the *sspABC* operon, coding endopeptidases important for immune  
644 suppression and infection dissemination (Imamura et al., 2005; Jusko et al., 2014;  
645 Ohbayashi et al., 2011). Their expression is *agr*-dependent, probably mediated by Rot, as  
646 inactivation of *rot* in an *agr* mutant resulted in upregulation of *sspABC* mRNA levels (Said-  
647 Salim et al., 2003). Rot levels are not changed in our  $\Delta rpsA$  mutant (**Supplementary excel**  
648 **file S1**), leaving open the possibility that *SauS1* would also enhance their translation.  
649 Nevertheless, many other regulators have been described, like  $\sigma^B$ , SarA, MgrA, SaeRS,  
650 SarV, SarR, SarS, SrrAB and ArlRS (Bischoff et al., 2004; Cheung et al., 2001; Fournier et  
651 al., 2001; Luong et al., 2003; Manna et al., 2004; Novick and Jiang, 2003). Given the  
652 complexity of *sspABC* regulation, assessing the impact of *SauS1* would require individual  
653 analysis on the different pathways. Noteworthy, the RBSs of *sspA* and *sspB* are predicted to  
654 be embedded into inhibitory structures that would require the chaperone activity of *SauS1* for  
655 active translation (**Figure S4**). A similar situation could be shared by other virulence genes,  
656 such as the immune evasion protein *sbi*, which shows structured RBS (**Figure S4**) and a  
657 decreased level despite the strong *SauS1*-dependent reduction of the levels of the two  
658 translational repressor RNAs, SprD and RNAIII (Chabelskaya et al., 2014). Taken together,  
659 S1 adds another layer of regulation to modulate the expression of virulence factors (**Figure**  
660 **17**).



661

662 **Figure 17. Impact of *SauS1* on the regulatory circuits involved in virulence gene**  
663 **expression.** Only parts of the regulatory circuits taken from the literature have been  
664 represented. SarA might induce *rpsA* transcription coordinating *SauS1* expression with the  
665 transcription of RNAIII which is also under the control of the quorum sensing system (*agr*)  
666 via the activation of the transcription factor AgrA. *SauS1* activates the translation of PSM $\alpha$   
667 peptides and possibly also Ssp proteins, Sbi and  $\delta$ -hemolysin (*hld*). The feedforward loop  
668 motif involving RNAIII, the transcriptional regulator Rot and the circuits controlled by RsaA,  
669 SprD and RsaE are also represented. The transcriptional regulatory proteins are in blue, the  
670 regulatory RNAs are in red and the target proteins are in purple, *SauS1* is green. Values  
671 reported on the sides of each gene represent their fold changes in the  $\Delta rpsA$  mutant, as  
672 transcripts (upper values) or proteins (lower values). The transcriptional regulation is shown  
673 by black line, sRNA regulation is shown by red line while *SauS1* translation regulation is  
674 shown with green lines. Arrows correspond to activation while bars correspond to repression.  
675 Regulations shown by dotted lines await experimental validation.

676

677 Interestingly, the production of toxins and exoenzymes was reported to be  
678 specifically perturbed by sub-inhibitory concentration of linezolid antibiotic (Coyle et al., 2003;  
679 Diep et al., 2012; Dumitrescu et al., 2007; Gemmell and Ford, 2002; Otto et al., 2013).  
680 Linezolid targets the A site of the ribosome and blocks peptide bond formation (Wilson et al.,  
681 2008). At sub-inhibitory concentration, it promotes the synthesis of new ribosomal proteins to  
682 make more ribosomes (Bonn et al., 2016). However, under these conditions, *SauS1* is no  
683 more synthesized, thus producing a situation similar to the mutant  $\Delta rpsA$  strain. It is possible  
684 that the specific effect of linezolid on the synthesis of virulence factors is linked to the  
685 incapacity of the newly synthesized ribosome to recognize the structured mRNAs encoded  
686 these toxins and exoenzymes.

687 Finally, the yields of several sRNAs appear to be significantly reduced in  $\Delta rpsA$  strain.  
688 Such a phenotype has been largely demonstrated in Enterobacteriaceae for the RNA  
689 chaperone Hfq (Cui et al., 2013; Sonnleitner et al., 2006; Updegrove et al., 2016; Vogel and  
690 Luisi, 2011) and for ProQ (Smirnov et al., 2016; Smirnov et al., 2017). These two proteins  
691 are key co-factors helping the sRNAs to regulate the expression of mRNAs at the post-  
692 transcriptional level. Mutations in *hfq* also decrease virulence in several pathogens (for  
693 review, see (Vogel and Papenfort, 2006)). In contrast to this, the function of Hfq in low G+C  
694 Gram-positive bacteria is still unclear and controversial (Bouloc and Repoila, 2016), and  
695 there is no ProQ equivalent in *S. aureus* (Attaiech et al., 2017; Olejniczak and Storz, 2017).  
696 In *S. aureus*, Hfq binds to RNAs, but it neither enhances the recognition between antisense  
697 RNAs and their target mRNAs (Zheng et al., 2016) nor the stability of sRNAs (Boisset et al.,  
698 2007; Preis et al., 2009). Furthermore, no major phenotypes were linked to its deletion in *S.*

699 *aureus* (Bohn et al., 2007). By acting on the stability of several sRNAs, such as RNAIII,  
700 RsaE, RsaA, and SprD, known to bind mRNAs and to affect their translation (reviewed in  
701 (Felden et al., 2011; Mandin and Guillier, 2013; Tomasini et al., 2014), *SauS1* might be a  
702 key partner of sRNA regulation. Some of the observed deregulations in the mutant  $\Delta rpsA$   
703 strain might be indirect. For instance, the *hla* reduced expression could be explained by less  
704 amount of the translational activator RNAIII (Morfeldt et al., 1995), and the enhanced levels  
705 of the pleiotropic regulatory protein MgrA might be due to a decreased level of its main  
706 repressor RsaA (Romilly et al., 2014).

707 This work shows that *SauS1* belongs to a new class of RNA chaperones that play  
708 key roles in the regulation of translation in *S. aureus* and most probably in Gram-positive  
709 bacteria. Our current study provides some hints for further investigation of the molecular  
710 functions and mechanisms of *SauS1* in gene regulation.

711

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716

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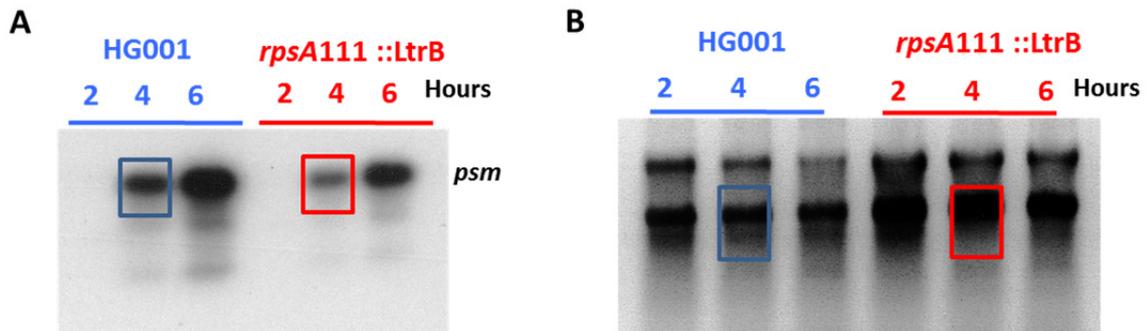
1 **SUPPLEMENTARY MATERIALS AND METHODS**

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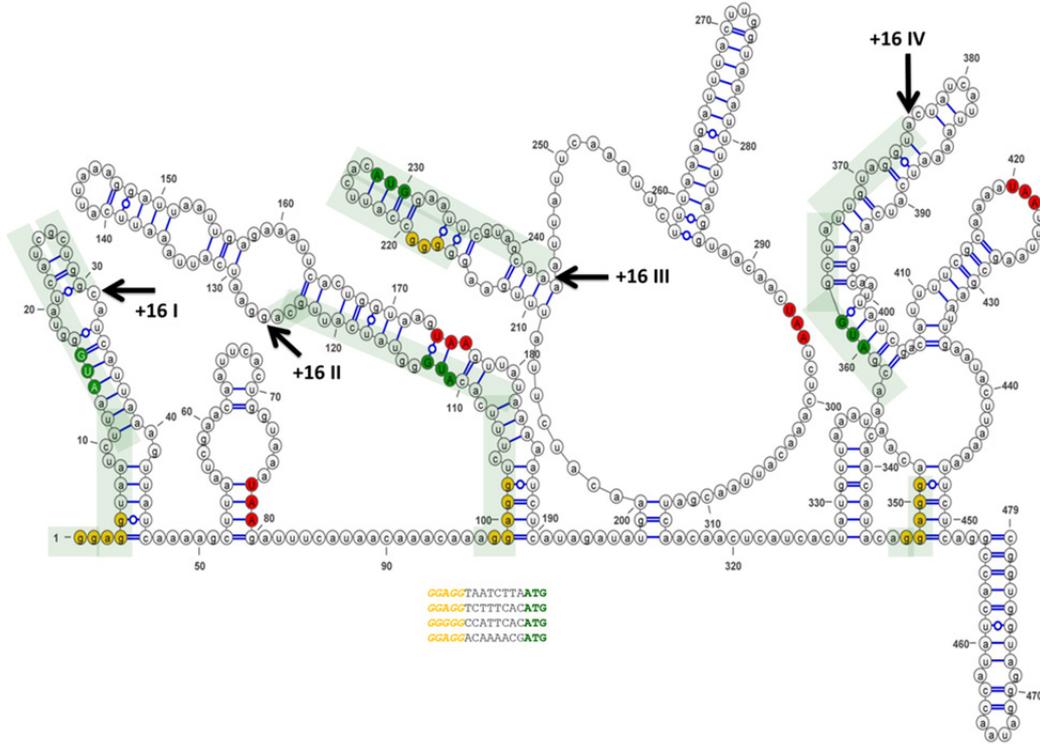
3 **SUPPLEMENTARY FIGURES**

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5 **Figure S1: Northern blot analysis of *psm* expression.** (A) HG001 and *rpsA111::LtrB*  
6 strains were growth in BHI medium at 37°C, cells were harvested and RNA extracted at pre-  
7 (2h), mid- (4h) and post- exponential (6h) phases. The Northern blot was performed with  
8 specific oligonucleotide complementary to *psm* mRNA. In the *SauS1* mutant strain, much  
9 less *psm* signal could be detected. (B) The membrane was colored using ethidium bromide  
10 to visualize the rRNAs. Quantification and normalization against total RNAs revealed that the  
11 *psm* operon is 4.6 fold less abundant in the mutant than in the WT strain.

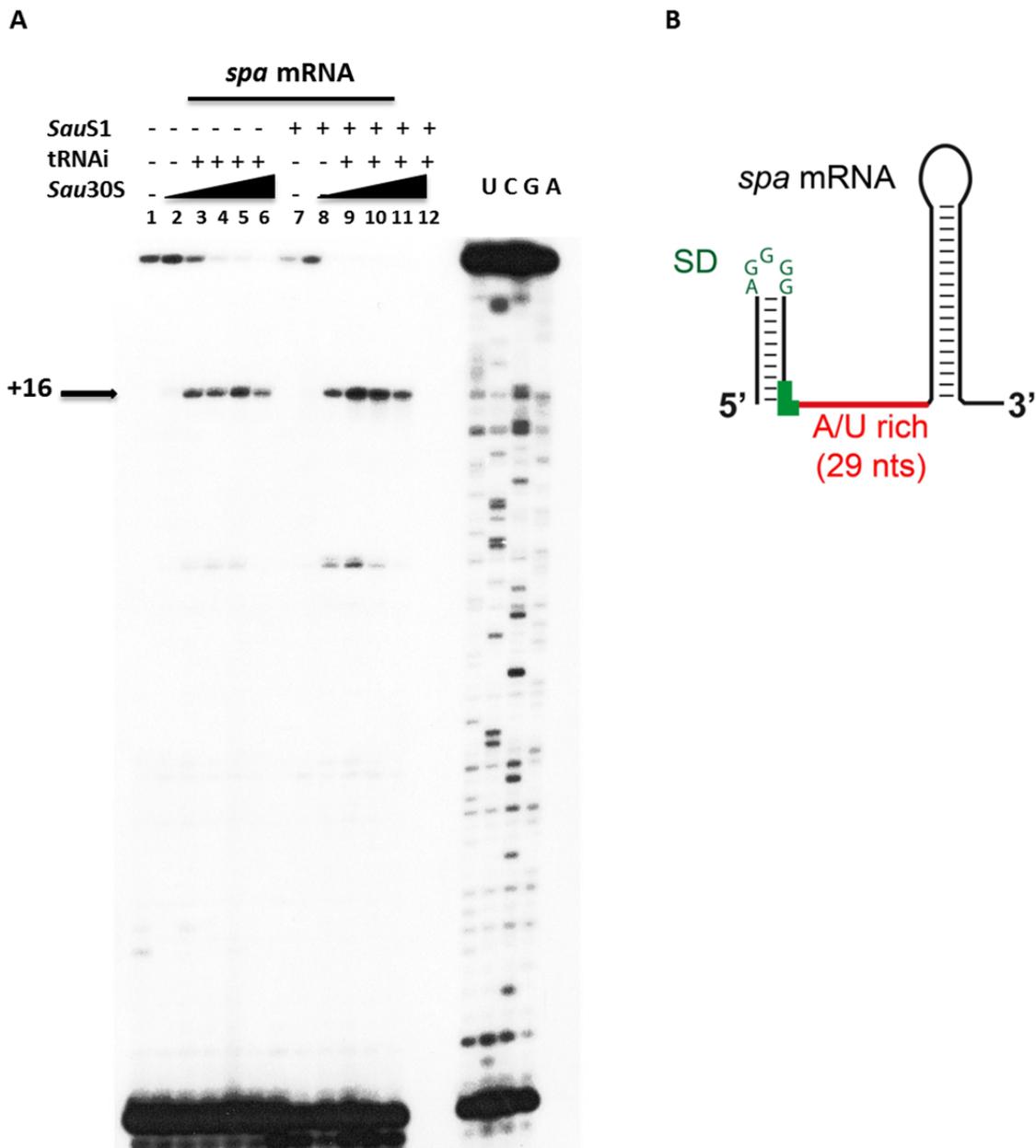


30 **Figure S2: Secondary structure of *psm* mRNA.** The secondary structure has been  
31 predicted with the Mfold server (Zuker, 2003). The *psm* operon contained four open reading  
32 frames encoding PSM1-4. The Ribosome Binding Site are shaded in light-blue. The four toe-  
33 printing signals are marked at +16 position by arrows.



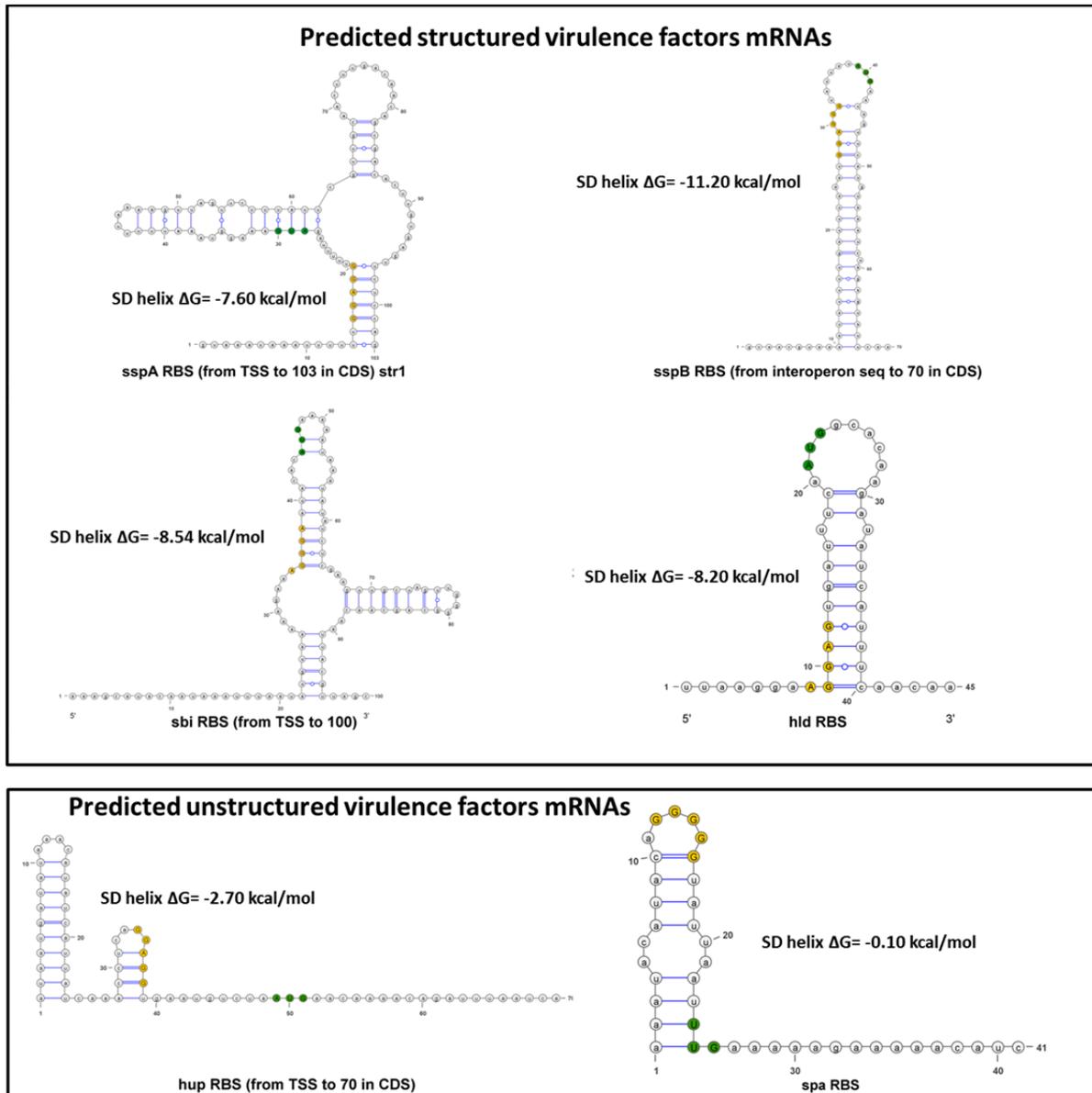
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52 **Figure S3. Toeprinting assays to monitor the effect of *SauS1* on the translation**  
 53 **initiation complex formation with *spa* mRNA.** (A) Effect of *SauS1* on the formation of  
 54 initiation complex using *spa* mRNA. When present, *SauS1* was pre-incubated with the  
 55 ribosome at a constant 1.6 molar ratio. Lane 1: incubation control of mRNA ; Lane 2:  
 56 incubation control of mRNA with 30S subunits ; Lanes 3, 4, 5 and 6: formation of the  
 57 initiation complex containing mRNA, increasing concentration of 30S (25, 50, 100, 200 nM)  
 58 and fMet-tRNA. Lane 7: incubation control of mRNA with purified *SauS1*. Lane 8: incubation  
 59 control of mRNA, 30S and *SauS1*. Lanes 9, 10, 11 and 12: formation of initiation complex in  
 60 presence of *SauS1* and increasing concentrations of 30S (25, 50, 100, 200 nM). Lanes U, A,  
 61 G, C: sequencing ladders. The toe-printing signals at position +16 are indicated by arrows.  
 62 (B) Scheme for the secondary structure of the RBS and beginning of coding region of *spa*  
 63 mRNA..  
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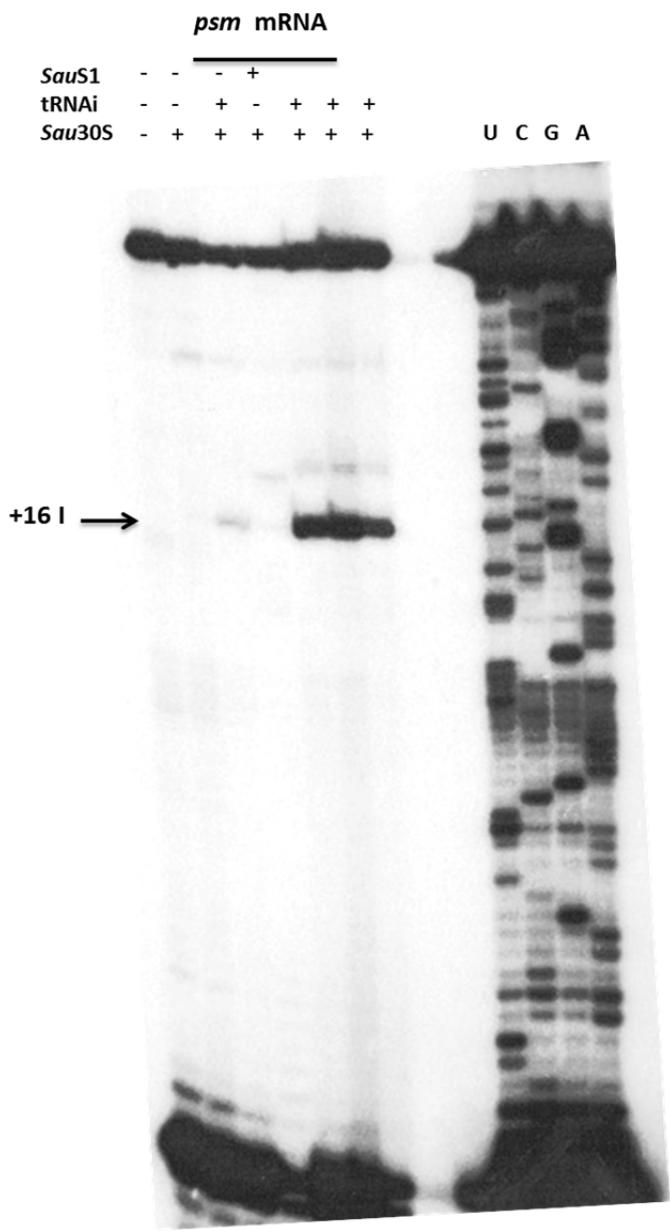
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67 **Figure S4: Predicted secondary structures for different Ribosome Binding Sites (RBS)**  
 68 **of mRNAs encoding virulence factors.** Secondary structures have been predicted with the  
 69 Mfold server (Zuker, 2003). For *sspA*, *sspB*, *sbi*, *hld* mRNAs, RBSs show structures  
 70 sequestering their SDs.  
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81 **Figure S5: Toe-print with *psm* mRNA pre-incubated with *SauS1*.** Effect of *SauS1* on the  
 82 formation of initiation ribosomal complex on *psm* mRNA. *SauS1-psm* complex has been  
 83 formed with increasing concentrations of *SauS1*. Lane 1 : control incubation of mRNA ; lane  
 84 2 : mRNA in the presence of 30S ribosomal subunits; lane 3 : formation of the ribosomal  
 85 initiation complex containing mRNA, the 30S subunits, and tRNA<sub>i</sub> ; lane 4: mRNA incubated  
 86 with 30S and *SauS1* (400 nM); lanes 5 to 8 : formation of the ribosomal initiation complex in  
 87 the presence of increasing concentrations of *SauS1* : 100, 200, 400 nM. Lanes U, A, G, C :  
 88 sequencing ladders. The toe-printing signal at the position +16 is indicated by an arrow.  
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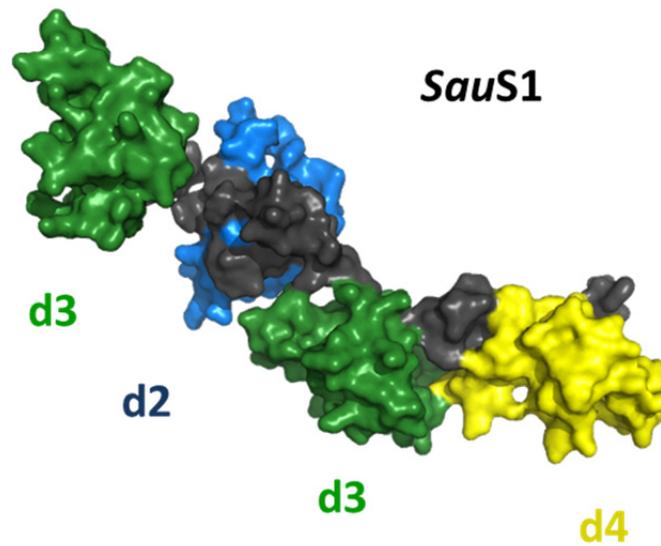


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94 **Figure S6: Domains alignment score matrix on *EcoS1* domains to determine *SauS1***  
95 **domain organization.**

### Alignment scores matrix for domain similarity

Alignments	EcoS1_d1	EcoS1_d2	EcoS1_d3	EcoS1_d4	EcoS1_d5	EcoS1_d6
SauS1_d1	Score: 58.5	Score: 47.5	Score: 94.5	Score: 72.5	Score: 79.0	Score: 80.0
SauS1_d2	Score: 37.0	Score: 129.0	Score: 49.5	Score: 40.0	Score: 51.5	Score: 73.0
SauS1_d3	Score: 32.0	Score: 32.0	Score: 193.0	Score: 142.5	Score: 139.5	Score: 108.5
SauS1_d4	Score: 32.0	Score: 57.0	Score: 147.5	Score: 153.5	Score: 116.0	Score: 110.0



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110 **SUPPLEMENTARY TABLES**

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112 **Table S1: Plasmids, strains and oligos**

List of oligos		
NAME	USAGE	Sequences are 5' end to 3' end
Oligo spa	Toeprinting spa	TTT GCA GGT GTT ACG CC
Oligo PSM 3' 4 CDS	Toeprinting PSM	CTG AGA ATT TTA AGT ATT CAA TTC GC
Oligo PSM 5' only 1' CDS	Toeprinting PSM	CIT TGT TTG TTA TGA AAT CIT ATT TACC
Oligo T7 FW <i>psm</i>	PCR and in vitro transcription	ATT ACG AAT TCA ATA CGA CTC ACT ATA GGG ACT CAT AAG CAA AGG AGG TAA TCT TAA
Oligo RW <i>psm</i>	PCR and in vitro transcription	TAG AGG ATC CAG TAC TGC CAC CAT CCC TAT TGG TAT AGT GGC C
<i>hu</i>	Northern blot	Tomasini et al., 2017
<i>psm</i>	Northern Blot	CCC ATG TGA AAG ACC TCC TTT GTT TGT TAT GAA ATC TTA TTT AC
5S	Northern Blot	Tomasini A. et al., 2017
16S	Northern Blot	CTGTGCGGGTCCCCGTC
<i>rpsA</i> PCR T7 FW	Northern Blot	TAA TAC GAC TCA CTA TAG GGG TCT ATC AAT AGA TTT AAT
<i>rpsA</i> PCR RW	Northern Blot	AAA GCA GTT GAA CAA GAA GAA
RNAlII	Northern Blot	Benito et al., 2000
Upstream <i>rpsA</i> FW	$\Delta$ rpsA	CTG ATC GGA TCC AGC AGA TGA TGC AGT GAC AT
Upstream <i>rpsA</i> RW	$\Delta$ rpsA	CAT CIT GTT TGC CTC CIT ATA CA
Downstream <i>rpsA</i> FW	$\Delta$ rpsA	TGT ATA AGG AGG CAA ACA AGA TGT TTA ATA TTT AAT AGT CAA CT
Downstream <i>rpsA</i> RW	$\Delta$ rpsA	GAG TCA GGA TCC GAA TAG AAA TCA TAC ACG TCT
Deletion <i>rpsA</i> FW	Verification $\Delta$ rpsA	CTG ATC GGA TCC AGC AGA TGA TGC AGT GAC AT
Deletion <i>rpsA</i> RW	Verification $\Delta$ rpsA	GAG TCA TTA TAG TTT AAG A TTTTT AAG
Complementation of $\Delta$ rpsA FW	$\Delta$ rpsA	TTT GCA TGC ATT CCA AAA ATA ATT CAT
Complementation of $\Delta$ rpsA RW	$\Delta$ rpsA	AAC TGC AGT TAT AGT TTA AGA TTT TT
SauS1 cloning His(6x) TEV FW	Cloning	AAA AAA GCA TGC GAA AAC CTG TAT TTT TCA GAT GACTGA AGA ATT CAA TGA ATC A
SauS1 cloning RW	Cloning	GCT AAT TAA GCT TTT ATT ATA GTT TAA G
SauS1 intron mutant at position 1089 RW	Mutation <i>rpsA</i>	AAA AAA GCT TAT AAT TAT CCT TAA ATG ACA GAG TAG TGC GCC CAG ATA GGG TG
SauS1 intron mutant at position 1089 FW	Mutation <i>rpsA</i>	CAG ATT GTA CAA ATG TGG TGA TAA CAG ATA AGT CAG AGT ATC TAA CIT ACC TTT CIT TGT
SauS1 intron mutant at position 404 FW	Mutation <i>rpsA</i>	TGA ACG CAA GTT TCT AAT TTC GAT TTC ATT TCG ATA GAG GAA AGT GTC T
SauS1 intron mutant at position 404 RW	Mutation <i>rpsA</i>	AAA AAAA GCT TAT AAT TAT CCT TAA CAA TCG GCG ATG TGC GCC CAG ATA GGG TG

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Strains	Description
<b><i>E. coli</i> strains</b>	
<i>E. coli</i> DC10B	Used for cloning of plasmids which can be directly transformed in <i>S. aureus</i> cells
<b><i>S. aureus</i> strains</b>	
HG001	Derivative of 8325-4, <i>rsbU</i> -restored RN1, <i>agr</i> positive
RN6390	Mutated in <i>rsbU</i> that results in a phenotype resembling that of <i>sigB</i> mutant
Mu50	Strain with vancomycin-intermediate resistance
BCJ100	Flag-tag at the C-terminus of <i>rpsA</i> gene
HG001 $\Delta$ rpsA	Deletion of <i>rpsA</i> gene for allelic replacement using pMAD vector
HG001 pCN51:: <i>rpsA</i>	$\Delta$ rpsA complemented strain
HG001 <i>rpsA</i> 111:: <i>LtrB</i>	<i>rpsA</i> insertion mutant 1
RN1 <i>rpsA</i> 1029:: <i>LtrB</i>	<i>rpsA</i> insertion mutant 2
<b>Plasmids</b>	
pUCN51	Shuttle vector
pUC18	Shuttle vector
pMAD	Thermosensitive origine of replication, constitutively expressed <i>bgaB</i> gene

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117 **Table S2: mRNAs UP regulated in *ΔrpsA***

mRNAs UP regulated in <i>ΔrpsA</i>					
id	gene	uniprotKB	product	FoldChange	pvalue
HG001_02434	<i>narG</i>	P09152	Respiratory nitrate reductase 1 alpha chain	7,907	6,47E-17
HG001_02438	<i>sirB</i>	O34632	Sirohydrochlorin ferrochelata	5,676	3,13E-13
HG001_02437	<i>nasD</i>	P42435	Nitrite reductase %5BNAD%28P%29H%5D	4,982	5,93E-11
HG001_00607	<i>sarX</i>	Q2G0D1	HTH-type transcriptional regulator SarX	4,959	2,55E-10
HG001_01334	<i>gpsA</i>	P64191	Glycerol-3-phosphate dehydrogenase %5BNAD%28P%29%2B%5D	4,438	4,83E-23
HG001_01691	<i>ribE</i>	P16440	Riboflavin synthase	4,379	7,96E-23
HG001_01335	<i>der_2</i>	P64060	GTPase Der	3,102	3,97E-13
HG001_02295	-	-	hypothetical protein	3,084	5,73E-06
HG001_01152	<i>glpD</i>	Q7A5V7	Aerobic glycerol-3-phosphate dehydrogenase	2,994	2,33E-13
HG001_01690	<i>ribBA</i>	Q99TA0	Riboflavin biosynthesis protein RibBA	2,927	5,99E-11
HG001_02649	<i>betA</i>	P60337	Oxygen-dependent choline dehydrogenase	2,758	9,79E-09
HG001_01689	<i>ribH</i>	P99141	6%2C7-dimethyl-8-ribityllumazine synthase	2,573	4,93E-10
HG001_02424	<i>narT</i>	O33854	putative nitrate transporter NarT	2,51	0,001146
HG001_00927	<i>ykoE</i>	O34738	Putative HMP/thiamine permease protein YkoE	2,509	4,75E-07
HG001_01571	-	-	hypothetical protein	2,468	1,24E-05
HG001_02436	<i>nasE</i>	P42436	Assimilatory nitrite reductase %5BNAD%28P%29H%5D small subunit	2,462	0,001334
HG001_00780	<i>patA_1</i>	O25526	Peptidoglycan O-acetyltransferase	2,423	1,92E-08
HG001_01692	<i>ribD</i>	P17618	Riboflavin biosynthesis protein RibD	2,352	9,84E-07
HG001_01142	<i>korB</i>	O53181	2-oxoglutarate oxidoreductase subunit KorB	2,347	8,19E-06
HG001_02290	<i>pbuG</i>	O34987	Guanine/hypoxanthine permease PbuG	2,316	9,80E-07
HG001_00527	-	-	hypothetical protein	2,295	1,20E-05
HG001_01306	-	-	hypothetical protein	2,274	1,71E-08
HG001_02433	<i>narH</i>	P11349	Respiratory nitrate reductase 1 beta chain	2,258	0,000768
HG001_01670	-	-	Phosphotransferase enzyme family protein	2,243	1,38E-05
HG001_02112	-	-	hypothetical protein	2,235	1,42E-05
HG001_01705	-	-	hypothetical protein	2,227	0,00216
HG001_02268	<i>rplO</i>	P0A0F6	50S ribosomal protein L15	2,217	2,11E-05
HG001_01501	<i>mtaB_2</i>	P54462	Threonylcarbamoyladenosine tRNA methyltransferase MtaB	2,182	0,000671
HG001_02097	<i>mazE</i>	P0C7B4	Antitoxin MazE	2,178	1,78E-05
HG001_02520	-	-	hypothetical protein	2,176	1,07E-05
HG001_01018	-	-	hypothetical protein	2,15	0,000593
HG001_01654	<i>acsA_1</i>	P39062	Acetyl-coenzyme A synthetase	2,145	7,02E-08
HG001_00739	-	-	hypothetical protein	2,145	0,000662
HG001_01212	<i>plsY</i>	Q45064	Glycerol-3-phosphate acyltransferase	2,14	0,001328
HG001_01633	<i>tpx</i>	P99146	putative thiol peroxidase	2,135	2,06E-06
HG001_01082	<i>fabG</i>	P99093	3-oxoacyl-%5Bacyl-carrier-protein%5D reductase FabG	2,097	4,03E-06
HG001_01218	<i>mprF</i>	Q2G2M2	Phosphatidylglycerol lysyltransferase	2,095	3,33E-07
HG001_00741	-	-	hypothetical protein	2,095	5,84E-05
HG001_01141	<i>korA</i>	O53182	2-oxoglutarate oxidoreductase subunit KorA	2,084	4,87E-05
HG001_00604	<i>sle1_2</i>	Q7A7E0	N-acetylmuramoyl-L-alanine amidase sle1	2,066	2,63E-06
HG001_00946	<i>pdhD</i>	P99084	Dihydrolipoyl dehydrogenase	2,049	7,24E-06
HG001_01150	<i>glpF</i>	P18156	Glycerol uptake facilitator protein	2,039	9,83E-05
HG001_02048	<i>ktrB_2</i>	O32081	Ktr system potassium uptake protein B	2,037	0,000973
HG001_02435	<i>nasF</i>	P42437	Uroporphyrinogen-III C-methyltransferase	2,037	0,003747
HG001_00625	<i>mgrA</i>	P0C1S0	HTH-type transcriptional regulator MgrA	2,035	6,72E-06
HG001_00411	-	-	hypothetical protein	2,024	0,0012
HG001_02325	<i>ureC</i>	P67404	Urease subunit alpha	2,014	6,68E-06

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120 **Table S3: mRNAs DOWN regulated in *ΔrpsA***

mRNAs DOWN regulated in <i>ΔrpsA</i>					
id	gene	uniprotKB	product	FoldChange	pvalue
HG001_01336	<i>rpsA_1</i>	Q7A5J0	30S ribosomal protein S1	0,042	5,20E-82
HG001_00899	<i>sspA</i>	POC1U8	Glutamyl endopeptidase	0,099	2,48E-55
HG001_03280	<i>psm-4</i>	-	4 psm peptide	0,166	2,20E-32
HG001_01548	-	-	hypothetical protein	0,224	1,24E-25
HG001_03282	<i>psm-2</i>	-	2 psm peptide	0,286	1,68E-16
HG001_01009	<i>hly</i>	Q2G1X0	Alpha-hemolysin	0,312	2,80E-07
HG001_00711	-	-	hypothetical protein	0,317	6,43E-15
HG001_02124	<i>ssb_2</i>	P37455	Single-stranded DNA-binding protein <i>ssb</i>	0,319	2,05E-10
HG001_00898	<i>sspB</i>	Q2FZL3	Staphopain B	0,324	1,26E-11
HG001_02245	-	A6QIG2	65 kDa membrane protein <i>Eap</i>	0,326	8,93E-13
HG001_00897	<i>sspC</i>	Q7A189	Staphostatin B	0,333	3,16E-07
HG001_01874	-	-	hypothetical protein	0,341	9,05E-05
HG001_01463	<i>aroK_2</i>	O67925	Shikimate kinase	0,35	2,01E-10
HG001_02058	<i>hld</i>	POC1V1	Delta-hemolysin	0,35	9,33E-10
HG001_00735	-	-	hypothetical protein	0,367	5,38E-07
HG001_01006	<i>scn_2</i>	Q6GFB4	Staphylococcal complement inhibitor	0,386	0,000147
HG001_02456	<i>sbi</i>	Q2FVK5	Immunoglobulin-binding protein <i>sbi</i>	0,389	3,02E-06
HG001_01023	-	P11699	Antibacterial protein 3	0,39	2,92E-10
HG001_02683	-	Q2G222	N-acetylmuramoyl-L-alanine amidase domain-containing protein	0,396	2,54E-10
HG001_01877	-	-	hypothetical protein	0,409	0,001013
HG001_03283	<i>psm-1</i>	-	1 psm peptide	0,412	4,78E-06
HG001_00326	<i>tcyP</i>	P54596	L-cystine uptake protein <i>TcyP</i>	0,415	2,63E-08
HG001_00958	-	-	hypothetical protein	0,415	2,94E-06
HG001_02584	-	-	hypothetical protein	0,416	0,000209
HG001_02745	<i>cspLA</i>	P0A355	Cold shock-like protein <i>CspLA</i>	0,417	1,08E-06
HG001_00647	-	-	Electron transfer DM13	0,424	1,91E-06
HG001_00866	-	-	hypothetical protein	0,425	0,00016
HG001_02279	<i>rpmC</i>	P66173	50S ribosomal protein L29	0,426	5,55E-05
HG001_01065	-	-	TM2 domain protein	0,428	3,04E-05
HG001_00264	<i>lip2</i>	P10335	Lipase 2	0,431	2,00E-09
HG001_02492	<i>pnbA</i>	P37967	Para-nitrobenzyl esterase	0,431	5,31E-09
HG001_02454	<i>gpmA_2</i>	P99153	2%2C3-bisphosphoglycerate-dependent phosphoglycerate mutase	0,435	5,35E-08
HG001_01595	<i>rplT</i>	P66108	50S ribosomal protein L20	0,45	6,19E-06
HG001_01890	-	-	hypothetical protein	0,452	0,000418
HG001_01261	<i>cspA_2</i>	Q7A2R8	Cold shock protein <i>CspA</i>	0,453	1,57E-07
HG001_00258	<i>sglT</i>	P96169	Sodium/glucose cotransporter	0,453	1,03E-05
HG001_01456	-	-	hypothetical protein	0,453	0,004245
HG001_02459	<i>hlgB</i>	P0A077	Gamma-hemolysin component B	0,455	8,94E-05
HG001_00609	-	-	hypothetical protein	0,458	3,35E-05
HG001_01899	-	-	hypothetical protein	0,459	0,000744
HG001_02626	-	-	hypothetical protein	0,46	0,000162
HG001_01057	-	-	hypothetical protein	0,46	0,000181
HG001_01971	-	A6QIG2	65 kDa membrane protein <i>Eap</i>	0,462	0,000537
HG001_02001	-	-	HNH endonuclease	0,465	0,004861
HG001_02664	-	-	hypothetical protein	0,466	2,45E-05
HG001_01943	<i>sspP</i>	Q2G2R8	Staphopain A	0,468	0,000195
HG001_01005	<i>fib_2</i>	A6QG59	Fibrinogen-binding protein	0,469	0,001234
HG001_00976	<i>rpmF</i>	P66210	50S ribosomal protein L32	0,473	6,83E-06
HG001_02635	<i>panD</i>	Q2FV23	Aspartate 1-decarboxylase	0,476	0,001569
HG001_02458	<i>hlgC</i>	Q2FVK2	Gamma-hemolysin component C	0,478	0,000327
HG001_00415	<i>spoVG</i>	Q7A7B5	Putative septation protein <i>SpoVG</i>	0,483	2,51E-07
HG001_02678	-	-	hypothetical protein	0,491	0,000331
HG001_01897	-	-	hypothetical protein	0,491	0,003621
HG001_01884	-	-	Helix-turn-helix domain protein	0,493	0,004261

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Table S4: non coding RNAs variations in *ΔrpsA*

non coding RNAs variations in <i>ΔrpsA</i>							
tRNAs variations in <i>ΔrpsA</i>				sRNAs variations in <i>ΔrpsA</i>			
id	product	F.C.	pvalue	id	product	F.C.	pvalue
HG001_00441	tRNA-Arg(acg)	<b>0,024</b>	1,08E-39	HG001_00584	RsaD	<b>0,115</b>	1,61E-13
HG001_01760	tRNA-His(gtg)	<b>0,025</b>	1,74E-61	HG001_01553	6S	<b>0,143</b>	1,77E-12
HG001_01802	tRNA-Tyr(gta)	<b>0,037</b>	3,23E-94	HG001_02756	RsaG	<b>0,16</b>	2,20E-11
HG001_01814	tRNA-Arg(acg)	<b>0,038</b>	2,54E-29	HG001_02347	RsaOG	<b>0,204</b>	1,30E-31
HG001_01815	tRNA-Leu(taa)	<b>0,04</b>	1,30E-35	HG001_01975	SprD	<b>0,215</b>	7,11E-09
HG001_00440	tRNA-Leu(taa)	<b>0,041</b>	2,53E-29	HG001_00847	RsaE	<b>0,234</b>	4,80E-05
HG001_02195	tRNA-Tyr(gta)	<b>0,041</b>	3,71E-88	HG001_02057	RNAIII	<b>0,363</b>	5,71E-09
HG001_01817	tRNA-Leu(tag)	<b>0,042</b>	2,60E-53	HG001_01981	rli28	<b>0,418</b>	0,00018
HG001_01800	tRNA-His(gtg)	<b>0,043</b>	4,49E-30	HG001_00880	rli28	<b>0,458</b>	0,00244
HG001_01809	tRNA-Ser(tga)	<b>0,043</b>	6,97E-31	HG001_02466	RsaJ	0,558	0,10163
HG001_02089	tRNA-Leu(gag)	<b>0,044</b>	3,99E-32	HG001_02470	RsaJ	0,623	0,13983
HG001_01021	tRNA-Arg(tct)	<b>0,048</b>	4,63E-39	HG001_02554	fstAT	0,658	0,24125
HG001_02197	tRNA-Glu(ttc)	<b>0,048</b>	9,96E-23	HG001_00516	RsaA	0,676	0,09411
HG001_01757	tRNA-Glu(ttc)	<b>0,049</b>	1,10E-21	HG001_01652	RsaB	0,703	0,30954
HG001_02201	tRNA-Ile(gat)	<b>0,05</b>	1,35E-31	HG001_01125	rliD	0,732	0,38281
HG001_01813	tRNA-Pro(tgg)	<b>0,062</b>	4,62E-18	HG001_02471	fstAT	0,768	0,41632
HG001_01818	tRNA-Lys(ttt)	<b>0,071</b>	3,49E-18	HG001_00710	RsaH	0,832	0,59711
HG001_00442	tRNA-Pro(tgg)	<b>0,071</b>	5,38E-16	HG001_02469	RsaJ	0,931	0,77697
HG001_02193	tRNA-Lys(ttt)	<b>0,076</b>	1,82E-15	HG001_01716	STnc490k	0,977	0,90181
HG001_00020	tRNA-Glu(ttc)	<b>0,08</b>	2,43E-14	HG001_00848	RsaF	0,989	0,96155
HG001_00438	tRNA-Lys(ttt)	<b>0,08</b>	3,46E-14	HG001_00569	RsaC	1,037	0,89814
HG001_00385	tRNA-Ser(tga)	<b>0,084</b>	1,85E-18	HG001_02176	rli28	1,092	0,59565
HG001_01807	tRNA-Ser(tga)	<b>0,09</b>	1,28E-16	HG001_01732	fstAT	1,265	0,51022
HG001_01823	tRNA-Ala(tgc)	<b>0,093</b>	4,79E-17				
HG001_01763	tRNA-Met(cat)	<b>0,098</b>	6,82E-15				
HG001_01806	tRNA-Met(cat)	<b>0,104</b>	1,44E-13				
HG001_01798	tRNA-Cys(gca)	<b>0,11</b>	3,47E-12				
HG001_02088	tRNA-Gly(tcc)	<b>0,12</b>	3,00E-10				
HG001_01797	tRNA-Gly(tcc)	<b>0,158</b>	9,56E-08				
HG001_01756	tRNA-Ser(gga)	<b>0,171</b>	3,25E-07				
HG001_00437	tRNA-Thr(tgt)	<b>0,187</b>	2,79E-06				
HG001_02194	tRNA-Gln(ttg)	<b>0,197</b>	5,57E-06				
HG001_01810	tRNA-Met(cat)	<b>0,197</b>	4,53E-06				
HG001_01803	tRNA-Thr(tgt)	<b>0,232</b>	4,02E-05				
HG001_02196	tRNA-Val(ttc)	<b>0,247</b>	9,06E-05				
HG001_01799	tRNA-Gln(ttg)	<b>0,265</b>	0,000182				
HG001_01819	tRNA-Thr(tgt)	<b>0,285</b>	0,000359				
HG001_01804	tRNA-Phe(gaa)	<b>0,291</b>	0,000442				
HG001_01796	tRNA-Gly(tcc)	<b>0,298</b>	0,000695				
HG001_00436	tRNA-Val(ttc)	<b>0,304</b>	0,000723				
HG001_01808	tRNA-Asp(gtc)	<b>0,325</b>	0,0013				
HG001_00443	tRNA-Ala(tgc)	<b>0,341</b>	0,002103				
HG001_01805	tRNA-Asp(gtc)	<b>0,344</b>	0,002053				
HG001_01820	tRNA-Val(tac)	<b>0,356</b>	0,002877				
HG001_01812	tRNA-Ala(tgc)	<b>0,364</b>	0,003576				
HG001_01762	tRNA-Asp(gtc)	<b>0,411</b>	0,008131				
HG001_00021	tRNA-Asp(gtc)	<b>0,416</b>	0,008908				
HG001_01761	tRNA-Phe(gaa)	<b>0,438</b>	0,012357				
HG001_01811	tRNA-Met(cat)	<b>0,441</b>	0,013319				
HG001_01758	tRNA-Asn(gtt)	<b>0,445</b>	0,016241				
HG001_00874	tRNA-Asn(gtt)	<b>0,458</b>	0,019652				
HG001_02198	tRNA-Asn(gtt)	<b>0,469</b>	0,022848				
HG001_00873	tRNA-Ser(gct)	<b>0,478</b>	0,020962				
HG001_01824	tRNA-Ile(gat)	<b>0,485</b>	0,023908				
HG001_01816	tRNA-Gly(gcc)	0,523	0,042309				
HG001_01795	tRNA-Gly(tcc)	0,568	0,071387				
HG001_00439	tRNA-Gly(gcc)	0,576	0,070428				
HG001_01801	tRNA-Trp(cca)	0,62	0,102456				
HG001_00707	tRNA-Arg(ccg)	0,652	0,127072				
HG001_01794	tRNA-Leu(caa)	0,655	0,131339				
HG001_01759	tRNA-Gly(tcc)	0,747	0,236529				
HG001_02310	tRNA-Gln(ttg)	<b>3,493</b>	4,00E-06				
riboswitches variations in <i>ΔrpsA</i>							
id	product	F.C.	pvalue				
HG001_00755	SAM <i>metN2</i>	<b>0,238</b>	8,98E-18				
HG001_01043	T-box <i>ileS</i>	<b>0,333</b>	3,54E-06				
HG001_01345	FMN <i>ribU</i>	<b>0,338</b>	1,09E-13				
HG001_01598	L20 <i>leader</i>	<b>0,343</b>	7,03E-05				
HG001_01680	T-box <i>leuS</i>	<b>0,414</b>	1,81E-07				
HG001_01225	T-box <i>trpE</i>	<b>0,436</b>	0,0012				
HG001_01581	T-box <i>vsIS</i>	0,555	0,01325				
HG001_01462	Glycine <i>gcvT</i>	0,62	0,10996				
HG001_01600	Lysine <i>lysP_2</i>	0,645	0,14845				
HG001_00303	T-box <i>metI</i>	0,669	0,02351				
HG001_02363	SAM <i>mleN_2</i>	0,71	0,03617				
HG001_00458	T-box <i>cysE</i>	0,714	0,05277				
HG001_01542	T-box <i>alaS</i>	0,728	0,28617				
HG001_00986	T-box <i>pheS</i>	0,78	0,20049				
HG001_01713	SAM <i>metK</i>	0,815	0,31446				
HG001_01251	Lysine <i>lysC</i>	0,831	0,29448				
HG001_01226	T-box <i>trpE</i>	0,874	0,68588				
HG001_00013	SAM <i>metX</i>	0,894	0,64935				
HG001_02122	TPP <i>tenA</i>	1,019	0,90794				
HG001_00009	T-box <i>serS</i>	1,163	0,59618				
HG001_01649	T-box <i>tyrS</i>	1,219	0,45625				
HG001_02182	<i>glmS</i>	<b>1,575</b>	0,09555				
HG001_01602	T-box <i>thrS</i>	<b>1,813</b>	0,03484				
HG001_00469	L10 <i>leader</i>	<b>1,904</b>	0,01479				
HG001_02255	L13 <i>leader</i>	<b>2,137</b>	0,00139				
HG001_01556	T-box <i>hisS</i>	<b>2,192</b>	0,00063				
HG001_01693	FMN <i>ribDEBAH</i>	<b>2,476</b>	4,48E-09				
HG001_00330	Purine	<b>4,508</b>	9,44E-09				
HG001_00928	TPP <i>ykoE</i>	<b>4,66</b>	2,94E-07				
other ncRNAs variations in <i>ΔrpsA</i>							
id	product	F.C.	pvalue				
HG001_00723	tmRNA SsrA	0,971	0,81765				
HG001_00389	SRP	<b>1,538</b>	0,00063				
HG001_01304	RNaseP	<b>5,95</b>	5,06E-14				



HG001_01769	-	-	Bacterial ABC transporter protein EcsB	0,24020	Del-S1 only
HG001_00513	yhdG_1	O07576	putative amino acid permease YhdG	0,24020	Del-S1 only
HG001_02600	dapL	A0LEA5	LL-diaminopimelate aminotransferase	0,24020	Del-S1 only
HG001_02362	mleN_2	P54571	Malate-2H(+)/Na(+)-lactate antiporter	0,24020	Del-S1 only
HG001_00684	dosC	P0AA89	Diguanylate cyclase DosC	0,24020	Del-S1 only
HG001_01066	def1	Q819U0	Peptide deformylase 1	0,24020	Del-S1 only
HG001_00424	divIC	P37471	Cell division protein DivIC	0,24020	Del-S1 only
HG001_02634	cocE	Q9L9D7	Cocaine esterase	0,24020	Del-S1 only
HG001_00256	psuG	P33025	Pseudouridine-5'-phosphate glycosidase	0,24020	Del-S1 only
HG001_00052	yxeP_1	P54955	putative hydrolase YxeP	0,24020	Del-S1 only
HG001_02116	-	-	putative hydrolase	0,24020	Del-S1 only
HG001_02157	coaW	Q6G7I0	Type II pantothenate kinase	0,24020	Del-S1 only
HG001_01625	-	Q8XIQ9	Cobalt-dependent inorganic pyrophosphatase	0,24020	Del-S1 only
HG001_01721	menE	P63526	2-succinylbenzoate--CoA ligase	0,24020	Del-S1 only
HG001_00047	-	Q2G1Q1	putative lipoprotein precursor	0,24020	Del-S1 only
HG001_01218	mprF	Q2G2M2	Phosphatidylglycerol lysyltransferase	0,24020	Del-S1 only
HG001_00305	ykuT	O34897	putative MscS family protein YkuT	0,24020	Del-S1 only
HG001_00575	tagA	Q7A714	Putative N-acetylmannosaminyltransferase	0,24020	Del-S1 only
HG001_01220	msrR	Q99Q02	Regulatory protein MsrR	0,24020	Del-S1 only
HG001_00361	-	-	hypothetical protein	0,24020	Del-S1 only
HG001_01593	-	-	hypothetical protein	0,24020	Del-S1 only
HG001_00592	-	-	hypothetical protein	0,24020	Del-S1 only
HG001_01668	ytnP	O34760	putative quorum-quenching lactonase YtnP	0,24020	Del-S1 only
HG001_01149	-	-	Glycerol-3-phosphate responsive antiterminator	0,24020	Del-S1 only
HG001_00406	rsml	P67087	Ribosomal RNA small subunit methyltransferase I	0,24020	Del-S1 only
HG001_00706	whiA	A0QWV9	Putative sporulation transcription regulator WhiA	0,24020	Del-S1 only
HG001_02090	-	-	Protein SprT-like protein	0,24020	Del-S1 only
HG001_02560	catE_2	P54721	Catechol-2%2C3-dioxygenase	0,24020	Del-S1 only
HG001_00802	yug_2	P80870	General stress protein 13	0,24020	Del-S1 only
HG001_01227	trpE	P20580	Anthranilate synthase component 1	0,24020	Del-S1 only
HG001_02216	iucA_2	Q47316	N(2)-citryl-N(6)-acetyl-N(6)-hydroxylysine synthase	0,24020	Del-S1 only
HG001_02075	tsaE	O05515	tRNA threonylcarbamoyladenine biosynthesis protein TsaE	0,24020	Del-S1 only
HG001_01433	malR	P0A4T2	HTH-type transcriptional regulator MalR	0,24020	Del-S1 only
HG001_00204	lytR_1	P60611	Sensory transduction protein LytR	0,24020	Del-S1 only
HG001_01770	ecsA	P55339	ABC-type transporter ATP-binding protein EcsA	0,24020	Del-S1 only
HG001_02440	nirC	P0AC26	Nitrite transporter NirC	0,24020	Del-S1 only
HG001_01002	fib_1	A6QG59	Fibrinogen-binding protein precursor	0,24020	Del-S1 only
HG001_01289	-	Q931R7	hypothetical protein	0,24020	Del-S1 only
HG001_00827	-	A6QIG2	65 kDa membrane protein precursor	0,24020	Del-S1 only
HG001_02498	relJ	P65067	Antitoxin RelJ	0,25280	2,43
HG001_01912	-	P44886	putative acyl-CoA thioester hydrolase	0,30950	3,33
HG001_01078	recG	P64325	ATP-dependent DNA helicase RecG	0,30950	3,33
HG001_00630	phrB	P00914	Deoxyribodipyrimidine photo-lyase	0,30950	3,33
HG001_01011	-	-	hypothetical protein	0,30950	3,33
HG001_02428	nreC	Q7WZY4	Oxygen regulatory protein NreC	0,30950	3,33
HG001_02323	ureA	Q4A0J3	Urease subunit gamma	0,30950	3,33
HG001_01697	arsC2	P0DKS7	Arsenate-myocthiol transferase ArsC2	0,30950	3,33
HG001_01640	ugpQ_2	P10908	Glycerophosphoryl diester phosphodiesterase	0,56390	2,33
HG001_01341	recQ_2	P15043	ATP-dependent DNA helicase RecQ	0,56390	2,33
HG001_00806	glpQ1_1	P96236	putative glycerophosphoryl diester phosphodiesterase 1	0,56390	2,33
HG001_01190	thrB_2	Q8Y4A6	Homoserine kinase	0,56390	2,33
HG001_02521	-	Q99RE8	putative lipoprotein precursor	0,56390	2,33
HG001_00026	yycJ	C0SP91	Putative metallo-hydrolase YycJ	0,56390	2,33
HG001_01831	-	Q99T13	Putative multidrug export ATP-binding/permease protein	0,56390	2,33
HG001_01599	lysP_2	P25737	Lysine-specific permease	0,56390	2,33
HG001_02404	lctP_2	P55910	L-lactate permease	0,56390	2,33
HG001_00769	-	-	hypothetical protein	0,56390	2,33
HG001_01145	-	-	hypothetical protein	0,56390	2,33
HG001_00724	-	-	hypothetical protein	0,56390	2,33
HG001_00402	-	-	hypothetical protein	0,56390	2,33
HG001_02597	copA	Q2FV64	Copper-exporting P-type ATPase A	0,56390	2,33
HG001_01275	arlR	P0C000	Response regulator ArlR	0,56390	2,33

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HG001_02338	ssaA2_3	Q7A423	Staphylococcal secretory antigen ssaA2 precursor	0,56390	2,33
HG001_02429	nreB	Q7WZY5	Oxygen sensor histidine kinase NreB	0,56390	2,33
HG001_02423	-	P80485	Acid shock protein	0,56390	2,33
HG001_00036	dus_2	P67717	putative tRNA-dihydrouridine synthase	0,56390	2,33
HG001_00041	-	-	hypothetical protein	0,56390	2,33
HG001_00555	pip	P46541	Proline iminopeptidase	0,56390	2,33
HG001_00319	gpmA_1	Q1MMY4	2%2C3-bisphosphoglycerate-dependent phosphoglycerate mutase	0,24850	2,08
HG001_02125	-	-	hypothetical protein	0,24850	2,08
HG001_02732	yfIS	O34726	Putative malate transporter YfIS	0,31760	2,00

### DelS1-enriched proteins (same global trend):

accession	gene	uniprotKB	product
HG001_00783	nfuA	P63020	Fe/S biogenesis protein NfuA
HG001_01439	-	-	hypothetical protein
HG001_00865	murE_1	Q2FZP6	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--L-lysine ligase
HG001_01237	-	-	hypothetical protein
HG001_01199	lexA_1	P31080	LexA repressor
HG001_01140	-	-	Calcineurin-like phosphoesterase
HG001_02210	-	-	hypothetical protein
HG001_01075	rpmB	P23374	50S ribosomal protein L28
HG001_02658	cysJ	O32214	Sulfite reductase [NADPH] flavoprotein alpha-component
HG001_02179	ywpJ_2	P94592	Putative phosphatase YwpJ
HG001_02537	gtaB	Q7A3J9	UTP--glucose-1-phosphate uridylyltransferase
HG001_01457	lipM	P54511	Octanoyltransferase LipM
HG001_02519	-	-	hypothetical protein
HG001_01946	-	-	hypothetical protein
HG001_01552	tcdA	Q46927	tRNA threonylcarbamoyladenosine dehydratase
HG001_01444	recN	P05824	DNA repair protein RecN
HG001_00975	-	-	hypothetical protein
HG001_01284	msrA2	P0A086	Peptide methionine sulfoxide reductase MsrA 2
HG001_02325	ureC	P67404	Urease subunit alpha
HG001_01213	parE	P66939	DNA topoisomerase 4 subunit B
HG001_00543	-	-	YwhD family protein
HG001_00453	-	P65205	Putative ATP:guanido phosphotransferase
HG001_01071	prkC	A6QGCO	Serine/threonine-protein kinase PrkC
HG001_00640	nagA	O34450	N-acetylglucosamine-6-phosphate deacetylase
HG001_01565	queA	P65951	S-adenosylmethionine:tRNA ribosyltransferase-isomerase
HG001_00375	metQ_1	P31728	putative D-methionine-binding lipoprotein MetQ precursor
HG001_01561	recJ	P21893	Single-stranded-DNA-specific exonuclease RecJ
HG001_01094	rbgA	O31743	Ribosome biogenesis GTPase A
HG001_00317	-	-	hypothetical protein
HG001_01632	-	-	N-6 DNA Methylase
HG001_02372	lyrA	Q7A3Z2	Lysostaphin resistance protein A
HG001_01280	-	Q7A5M9	putative CtpA-like serine protease
HG001_00926	ykoD_1	O34362	Putative HMP/thiamine import ATP-binding protein YkoD
HG001_00211	rbsK	P0A9J6	Ribokinase
HG001_02736	nhoA	Q00267	N-hydroxyarylamine O-acetyltransferase
HG001_01788	trmL	P0AGJ7	tRNA (cytidine(34)-2'-O)-methyltransferase
HG001_00472	rsmC	P39406	Ribosomal RNA small subunit methyltransferase C
HG001_00520	galK_1	-	Galactokinase
HG001_01827	ghrB_2	P37666	Glyoxylate/hydroxypyruvate reductase B
HG001_01104	hslV	P65797	ATP-dependent protease subunit HslV
HG001_02449	tcyC_2	P39456	L-cystine import ATP-binding protein TcyC
HG001_01300	der_1	-	GTPase Der
HG001_01641	-	-	OsmC-like protein
HG001_02629	pyrD	P0A7E1	Dihydroorotate dehydrogenase (quinone)

TT5600+		Q-Exactive+	
p.value	ArpsA /S1	p.value	ArpsA/S1
0,00060	Del-S1 only	0,41020	1,59
0,00296	Del-S1 only	0,51770	1,26
0,00003	Del-S1 only	0,45480	1,20
0,00060	Del-S1 only	0,98660	1,00
0,00296	Del-S1 only	0,84250	0,94
0,00012	Del-S1 only	0,72340	0,90
0,00000	2,08	0,00000	1,68
0,00195	2,73	0,51230	1,14
0,00031	5,15	0,91510	1,04
0,00323	4,70	0,60280	0,88
0,00323	4,70	0,63810	0,88
0,00667	Del-S1 only	0,23040	1,27
0,00667	Del-S1 only	0,80640	1,17
0,00667	Del-S1 only	0,98610	1,00
0,00667	Del-S1 only	0,98910	1,00
0,00667	Del-S1 only	0,53960	0,84
0,01525	Del-S1 only	0,36860	1,77
0,01525	Del-S1 only	0,48320	1,70
0,01525	Del-S1 only	0,25690	1,39
0,01525	Del-S1 only	0,69860	1,12
0,01525	Del-S1 only	0,83010	1,10
0,01525	Del-S1 only	0,48110	0,73
0,03561	Del-S1 only	0,50490	1,26
0,03561	Del-S1 only	0,70910	0,88
0,03561	Del-S1 only	0,77190	0,87
0,08621	Del-S1 only	0,41020	1,59
0,08621	Del-S1 only	0,74640	1,31
0,08621	Del-S1 only	0,76830	1,11
0,08621	Del-S1 only	0,86720	1,06
0,08621	Del-S1 only	0,88170	1,05
0,08621	Del-S1 only	0,99450	1,00
0,08621	Del-S1 only	0,82810	0,93
0,08621	Del-S1 only	0,62670	0,82
0,08621	Del-S1 only	0,29300	0,70
0,22510	Del-S1 only	0,41450	1,86
0,22510	Del-S1 only	0,25470	1,74
0,22510	Del-S1 only	0,41020	1,59
0,22510	Del-S1 only	0,69530	1,16
0,22510	Del-S1 only	0,98720	1,00
0,22510	Del-S1 only	0,99610	1,00
0,22510	Del-S1 only	0,82810	0,93
0,22510	Del-S1 only	0,84640	0,93
0,22510	Del-S1 only	0,82200	0,93
0,22510	Del-S1 only	0,70910	0,88

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HG001_01144	miaB	Q7A5W3	(Dimethylallyl)adenosine tRNA methylthiotransferase MiaB	0,22510	Del-S1 only	0,57660	0,82
HG001_02226	-	P99173	Zinc-type alcohol dehydrogenase-like protein	0,22510	Del-S1 only	0,33100	0,74
HG001_02649	betA	P60337	Oxygen-dependent choline dehydrogenase	0,22510	Del-S1 only	0,64810	0,70
HG001_02499	yehR	P33354	putative lipoprotein YehR precursor	0,22510	Del-S1 only	0,27880	0,68
HG001_01343	-	P10245	Ferredoxin	0,22510	Del-S1 only	0,51870	0,65
HG001_00433	folK	P43777	2-amino-4-hydroxy-6-hydroxymethylidihydropteridine pyrophosphokinase	0,22510	Del-S1 only	0,47120	0,59
HG001_01606	gapA2	P99067	Glyceraldehyde-3-phosphate dehydrogenase 2	0,00663	2,13	0,16050	1,32
HG001_00539	yhdN_1	P80874	General stress protein 69	0,01041	4,00	0,58980	1,23
HG001_01915	ptpA	P0C5D2	Low molecular weight protein-tyrosine-phosphatase PtpA	0,01584	2,08	0,42150	0,78
HG001_01123	ribF	P0AG40	Riboflavin biosynthesis protein RibF	0,03224	3,30	0,98070	1,00
HG001_01050	pyrP	P39766	Uracil permease	0,03607	6,67	0,69830	0,77
HG001_01129	phnF	P16684	putative transcriptional regulator PhnF	0,03647	3,86	0,83010	1,10
HG001_01666	pepA_2	Q48677	Glutamyl aminopeptidase	0,04946	2,00	0,66670	0,92
HG001_01331	ubiE	P67062	Demethylmenaquinone methyltransferase	0,07097	2,15	0,55140	0,80
HG001_00619	yvdD	O06986	LOG family protein YvdD	0,07113	5,67	0,52230	0,82
HG001_02518	-	Q99RF5	putative oxidoreductase	0,07437	2,54	0,70360	0,92
HG001_02371	hutG	P99158	Formimidoylglutamase	0,08317	2,04	0,63040	1,13
HG001_01115	polC_1	P63982	DNA polymerase III PolC-type	0,11810	2,86	0,85860	0,96
HG001_00291	-	-	hypothetical protein	0,13910	4,33	0,71170	1,30
HG001_01646	plsC	O07584	1-acyl-sn-glycerol-3-phosphate acyltransferase	0,13910	4,33	0,98980	1,00
HG001_01485	sigA	P0A018	RNA polymerase sigma factor SigA	0,15750	2,30	0,97530	1,00
HG001_00854	ppnK	P65777	putative inorganic polyphosphate/ATP-NAD kinase	0,19030	2,08	0,30750	0,66
HG001_02598	copZ	Q7A3E5	Copper chaperone CopZ	0,51260	2,33	0,35120	1,61
HG001_01321	-	-	Putative neutral zinc metallopeptidase	0,51260	2,33	0,85490	1,08
HG001_02146	tdk	P65231	Thymidine kinase	0,78870	0,77	0,01676	4,00
HG001_01432	malL	P29094	Oligo-1%2C6-glucosidase	0,55480	1,14	0,10580	2,54
HG001_00927	ykoE	O34738	Putative HMP/thiamine permease protein YkoE	0,72230	0,70	0,25280	2,43
HG001_01130	-	-	Peptidase M16 inactive domain protein	0,83510	0,76	0,15750	2,00

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Table S6: Differential Proteomics analysis of  $\Delta rpsA$  and WT HG001Proteomics Analysis: DOWN in  $\Delta rpsA$ **S1-enriched proteins (2 MS instruments):**

accession	gene	uniprotKB	product	TT5600+		Q-Exactive+	
				p.value	$\Delta rpsA$ /S1	p.value	$\Delta rpsA$ /S1
HG001_01336	rpsA_1	Q7A5J0	30S ribosomal protein S1	0,00000	S1 only	0,00000	S1 only
HG001_02674	arcA	P63554	Arginine deiminase	0,00000	S1 only	0,00004	S1 only
HG001_02245	-	A6QIG2	65 kDa membrane protein precursor Eap	0,00061	S1 only	0,00009	S1 only
HG001_00423	hslR	P0ACG8	Heat shock protein 15	0,00252	S1 only	0,04840	0,26
HG001_02673	arcB	P65602	Ornithine carbamoyltransferase%2C catabolic	0,00252	S1 only	0,02019	0,40
HG001_00175	fadN	O32178	putative 3-hydroxyacyl-CoA dehydrogenase	0,00000	0,11	0,00104	0,50
HG001_02492	pnbA	P37967	Para-nitrobenzyl esterase	0,00515	S1 only	0,14530	0,35
HG001_00177	lcfB	O07610	Long-chain-fatty-acid--CoA ligase	0,02237	S1 only	0,00153	0,43
HG001_01918	vraR	Q7A4R9	Response regulator protein VraR	0,10320	0,43	0,02070	0,43
HG001_02514	ahpD	-	Alkyl hydroperoxide reductase AhpD	0,34660	0,30	0,03668	0,40
HG001_02045	dapE	Q99SN6	putative succinyl-diaminopimelate desuccinylase	0,05977	0,33	0,06735	0,43
HG001_00016	nrnA_1	Q5SM25	Bifunctional oligoribonuclease and PAP phosphatase NrnA	0,10630	S1 only	0,14530	0,35
HG001_02159	-	-	hypothetical protein	0,19430	0,23	0,14920	0,50

**S1-enriched proteins (1 MS instrument):**

accession	gene	uniprotKB	product	TT5600+		Q-Exactive+	
				p.value	$\Delta rpsA$ /S1	p.value	$\Delta rpsA$ /S1
HG001_00398	speA	P21885	Arginine decarboxylase	0,01066	S1 only		
HG001_00027	yfkN_1	O34313	Trifunctional nucleotide phosphoesterase protein YfkN precursor	0,10630	S1 only		
HG001_02685	-	-	hypothetical protein	0,25340	S1 only		
HG001_01939	pcrA	Q53727	ATP-dependent DNA helicase PcrA			0,00004	S1 only
HG001_02544	ywaC	P39583	GTP pyrophosphokinase YwaC			0,00001	0,10
HG001_00277	slyA_1	-	Transcriptional regulator SlyA			0,00084	S1 only
HG001_00636	ybaK	P0AAR3	Cys-tRNA(Pro)/Cys-tRNA(Cys) deacylase YbaK			0,00179	S1 only
HG001_00140	-	-	hypothetical protein			0,00384	S1 only
HG001_01192	-	-	hypothetical protein			0,00831	S1 only
HG001_02103	kdpC	P94606	Potassium-transporting ATPase C chain			0,01824	S1 only
HG001_00949	potA	Q7A679	Spermidine/putrescine import ATP-binding protein PotA			0,01824	S1 only
HG001_02436	nasE	P42436	Assimilatory nitrite reductase [NAD(P)H] small subunit			0,01824	S1 only
HG001_01466	-	-	hypothetical protein			0,01824	S1 only
HG001_01557	lytC	Q02114	N-acetylmuramoyl-L-alanine amidase LytC precursor			0,04092	S1 only
HG001_01974	scn_3	Q2FWV6	Staphylococcal complement inhibitor precursor			0,04092	S1 only
HG001_02671	arcC2	P99069	Carbamate kinase 2			0,04092	S1 only
HG001_01723	yokF	O32001	SPBc2 prophage-derived endonuclease YokF precursor			0,04092	S1 only
HG001_02694	-	-	Flavin reductase like domain protein			0,04092	S1 only
HG001_02558	mhqR	O31672	HTH-type transcriptional regulator MhqR			0,04092	S1 only
HG001_00141	murQ	Q45582	N-acetylmuramic acid 6-phosphate etherase			0,09507	S1 only
HG001_00793	mnhG1	P60698	Na(+)/H(+) antiporter subunit G1			0,09507	S1 only
HG001_00127	-	P33160	Formate dehydrogenase			0,09507	S1 only
HG001_01670	-	-	Phosphotransferase enzyme family protein			0,09507	S1 only
HG001_02258	ecfA2	Q7A471	Energy-coupling factor transporter ATP-binding protein EcfA2			0,09507	S1 only
HG001_01642	-	P16421	Soluble hydrogenase 42 kDa subunit			0,09507	S1 only
HG001_00048	-	Q2G1Q0	putative lipoprotein precursor			0,09507	S1 only
HG001_02305	mobA	P65405	putative molybdenum cofactor guanylyltransferase			0,09507	S1 only
HG001_00262	nanE	P65517	Putative N-acetylmannosamine-6-phosphate 2-epimerase			0,09507	S1 only
HG001_00094	yfkN_2	O34313	Trifunctional nucleotide phosphoesterase protein YfkN precursor			0,09507	S1 only
HG001_00102	ywqE_1	P96717	Tyrosine-protein phosphatase YwqE			0,09507	S1 only
HG001_00403	-	Q99WB7	Initiation-control protein YabA			0,09507	S1 only
HG001_00968	-	-	hypothetical protein			0,09507	S1 only
HG001_01664	-	-	hypothetical protein			0,09507	S1 only
HG001_00126	-	-	hypothetical protein			0,09507	S1 only
HG001_01832	-	-	hypothetical protein			0,09507	S1 only

HG001_00378	nudC	-	NADH pyrophosphatase	0,09507	S1 only
HG001_02079	ilvC	P37253	Ketol-acid reductoisomerase	0,09507	S1 only
HG001_02583	ssaA2_4	Q7A423	Staphylococcal secretory antigen ssaA2 precursor	0,09507	S1 only
HG001_01614	citZ	P39120	Citrate synthase 2	0,09507	S1 only
HG001_02150	yodB	O34844	HTH-type transcriptional regulator YodB	0,09507	S1 only
HG001_01968	ytrA	O34712	HTH-type transcriptional repressor YtrA PmtR	0,09507	S1 only
HG001_01239	oppD_3	P24136	Oligopeptide transport ATP-binding protein OppD	0,09507	S1 only
HG001_02661	yxdL	P42423	ABC transporter ATP-binding protein YxdL	0,09507	S1 only
HG001_01494	ybeY	O67367	Endoribonuclease YbeY	0,09507	S1 only
HG001_00494	tagE_1	P13484	putative poly(glycerol-phosphate) alpha-glucosyltransferase	0,09507	S1 only
HG001_01944	-	-	Staphostatin A	0,23790	S1 only
HG001_00178	ydiF	Q8X5X6	Acetate CoA-transferase YdiF	0,23790	S1 only
HG001_01520	aroE	Q5HNV1	Shikimate dehydrogenase	0,23790	S1 only
HG001_01643	serA	P0A544	D-3-phosphoglycerate dehydrogenase	0,23790	S1 only
HG001_02614	-	-	Glyoxalase-like domain protein	0,23790	S1 only
HG001_01133	-	-	ACT domain protein	0,23790	S1 only
HG001_01318	cca	Q7SIB1	CCA-adding enzyme	0,23790	S1 only
HG001_01079	fapR	O34835	Transcription factor FapR	0,23790	S1 only
HG001_01964	-	-	ABC-2 family transporter protein PmtD	0,23790	S1 only
HG001_01782	liaS	O32198	Sensor histidine kinase LiaS	0,23790	S1 only
HG001_00163	-	-	Xylose isomerase-like TIM barrel	0,23790	S1 only
HG001_01153	-	-	lysophospholipase L2	0,23790	S1 only
HG001_00259	nanA	Q2G160	N-acetylneuraminatase lyase	0,23790	S1 only
HG001_00299	metE	P65342	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	0,23790	S1 only
HG001_00282	luxA	P07740	Alkanal monooxygenase alpha chain	0,23790	S1 only
HG001_01949	nos	P0A004	Nitric oxide synthase oxygenase	0,23790	S1 only
HG001_00952	potD	P0AFK9	Spermidine/putrescine-binding periplasmic protein precursor	0,23790	S1 only
HG001_02236	lacR	P67744	Lactose phosphotransferase system repressor	0,23790	S1 only
HG001_01486	dnaG	P63964	DNA primase	0,23790	S1 only
HG001_01473	gluP	P54493	Rhomboid protease GluP	0,23790	S1 only
HG001_01023	-	P11699	Antibacterial protein 3	0,23790	S1 only
HG001_01919	vraS	Q99S27	Sensor protein VraS	0,23790	S1 only
HG001_02228	-	-	hypothetical protein	0,23790	S1 only
HG001_01837	-	-	hypothetical protein	0,23790	S1 only
HG001_00075	-	-	hypothetical protein	0,23790	S1 only
HG001_00223	-	-	hypothetical protein	0,23790	S1 only
HG001_00651	queC	O31675	7-cyano-7-deazaguanine synthase	0,23790	S1 only
HG001_01967	ytrB_2	O34641	ABC transporter ATP-binding protein YtrB PmtA	0,23790	S1 only
HG001_00336	-	-	PemK-like protein	0,23790	S1 only
HG001_00129	grsB	P0C064	Gramicidin S synthase 2	0,00221	0,44
HG001_02693	sraP	Q2FUW1	Serine-rich adhesin for platelets precursor	0,00251	0,39
HG001_00142	-	Q7A804	PTS system EIIBC component	0,01570	0,37
HG001_02607	oatA_2	Q7A3D6	O-acetyltransferase OatA	0,02422	0,30
HG001_01009	hly	Q2G1X0	Alpha-hemolysin precursor	0,02739	0,23
HG001_00898	sspB	Q2FZL3	Staphopain B precursor	0,03388	0,36
HG001_00463	-	-	YacP-like NYN domain protein	0,04484	0,30
HG001_02691	-	-	hypothetical protein	0,04484	0,30
HG001_00579	tagX	Q7A711	Putative glycosyltransferase TagX	0,04840	0,26
HG001_01551	rarA	P0AAZ4	Replication-associated recombination protein A	0,06735	0,43
HG001_02442	-	-	hypothetical protein	0,08449	0,30
HG001_01118	-	-	hypothetical protein	0,08449	0,30
HG001_00368	est_1	Q06174	Carboxylesterase	0,08449	0,30
HG001_02405	-	-	CDP-Glycerol:Poly(glycerophosphate) glycerophosphotransferase	0,08649	0,18
HG001_01590	engB	P64071	putative GTP-binding protein EngB	0,08649	0,18
HG001_01860	-	-	Phage major tail protein 2	0,08649	0,18
HG001_00326	tcyP	P54596	L-cystine uptake protein TcyP	0,10110	0,47
HG001_00650	queD	O31676	6-carboxy-5%2C6%2C7%2C8-tetrahydropterin synthase	0,12190	0,37
HG001_00161	ycjS_1	P77503	putative oxidoreductase YcjS	0,14530	0,35
HG001_01724	-	-	Telomeric repeat-binding factor 2	0,14530	0,35
HG001_02592	ydfJ	P96687	Membrane protein YdfJ	0,15650	0,43
HG001_00594	aes	Q8ZRA1	Acetyl esterase	0,16280	0,23

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HG001_01943	sspP	Q2G2R8	Staphopain A precursor	0,19580	0,43
HG001_00542	-	-	HD domain protein	0,19580	0,43
HG001_01608	mutM	O50606	Formamidopyrimidine-DNA glycosylase	0,19580	0,43
HG001_02507	nikA	P33590	Nickel-binding periplasmic protein precursor	0,19580	0,43
HG001_00044	recD	-	RecBCD enzyme subunit RecD	0,24550	0,41
HG001_01764	ydeN	P96671	Putative hydrolase YdeN	0,24550	0,41
HG001_00179	-	-	hypothetical protein	0,24550	0,41
HG001_01488	ccpN	O34994	Transcriptional repressor CcpN	0,24550	0,41
HG001_00024	yycH	Q794W0	Two-component system YycF/YycG regulatory protein YycH	0,24550	0,41
HG001_00298	kynB	-	Kynurenine formamidase	0,30320	0,30
HG001_00412	ispE	P65178	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	0,30320	0,30
HG001_00904	yjcF	O31628	putative N-acetyltransferase YjcF	0,30320	0,30
HG001_00742	pspB	D3DFP8	Putative phosphoserine phosphatase 2	0,30320	0,30
HG001_01916	-	-	hypothetical protein	0,30320	0,30
HG001_01605	nrdR	P0A8D0	Transcriptional repressor NrdR	0,30320	0,30
HG001_00028	rlmH	P0C1V0	Ribosomal RNA large subunit methyltransferase H	0,30320	0,30
HG001_02173	czrA	O31844	HTH-type transcriptional repressor CzrA	0,30320	0,30
HG001_00656	kipA_1	Q7WY77	KipI antagonist	0,23900	0,48
HG001_00008	hutH	P64416	Histidine ammonia-lyase	0,30780	0,50
HG001_02601	crtN	Q7A3E2	Dehydrosqualene desaturase	0,30780	0,50
HG001_02581	cynR_2	P27111	HTH-type transcriptional regulator CynR	0,30780	0,50
HG001_00973	coaD	P63820	Phosphopantetheine adenylyltransferase	0,55600	0,43
HG001_00787	erpA	P45344	Iron-sulfur cluster insertion protein ErpA	0,55600	0,43
HG001_01330	hepT	P31114	Heptaprenyl diphosphate synthase component 2	0,55600	0,43
HG001_00712	cggR	O32253	Central glycolytic genes regulator	0,55600	0,43
HG001_00730	ssp	A6QF98	Extracellular matrix protein-binding protein emp precursor	0,55600	0,43
HG001_00363	yicC_1	P94400	Putative metal chaperone YicC	0,55600	0,43
HG001_02185	mtlF	P17876	Mannitol-specific phosphotransferase enzyme IIA component	0,55600	0,43
HG001_00100	cap8A_1	P72367	Capsular polysaccharide type 8 biosynthesis protein cap8A	0,55600	0,43
HG001_01309	-	-	hypothetical protein	0,55600	0,43
HG001_01302	-	-	hypothetical protein	0,55600	0,43
HG001_00037	-	-	hypothetical protein	0,55600	0,43
HG001_02300	-	-	hypothetical protein	0,55600	0,43
HG001_00819	-	-	hypothetical protein	0,55600	0,43
HG001_00511	-	-	hypothetical protein	0,55600	0,43
HG001_00029	-	-	hypothetical protein	0,55600	0,43
HG001_00247	-	-	hypothetical protein	0,55600	0,43
HG001_00492	sdrC	Q7A781	Serine-aspartate repeat-containing protein C precursor	0,55600	0,43
HG001_00285	ydaF	P96579	Putative ribosomal N-acetyltransferase YdaF	0,55600	0,43
HG001_02575	-	-	Thioredoxin	0,55600	0,43
HG001_01911	queE_2	-	7-carboxy-7-deazaguanine synthase	0,55600	0,43

### S1-enriched proteins (same global trend):

accession	gene	uniprotKB	product	TT5600+		Q-Exactiv+	
				p.value	$\Delta$ rpsA/S1	p.value	$\Delta$ rpsA/S1
HG001_00118	aldA	Q7A825	Putative aldehyde dehydrogenase AldA	0,00124	S1 only	0,08007	0,57
HG001_01498	-	-	hypothetical protein	0,00015	S1 only	0,75420	0,85
HG001_00501	hxlB	P42404	3-hexulose-6-phosphate isomerase	0,00002	S1 only	0,98910	1,00
HG001_00449	pdxT	Q7A7A1	Glutamine amidotransferase subunit PdxT	0,00061	S1 only	0,25210	0,69
HG001_01496	-	-	hypothetical protein	0,00061	S1 only	0,31900	0,73
HG001_01332	-	-	Heptaprenyl diphosphate synthase (HEPPP synthase) subunit 1	0,00252	S1 only	0,60700	0,77
HG001_01662	sftA	C0SP86	DNA translocase SftA	0,00252	S1 only	0,51090	0,86
HG001_00758	metQ_2	O32167	Methionine-binding lipoprotein MetQ precursor	0,00252	S1 only	0,86930	0,95
HG001_00264	lip2	P10335	Lipase 2 precursor	0,00000	0,25	0,00448	0,71
HG001_02315	modA	P45323	Molybdate-binding periplasmic protein precursor	0,00001	0,11	0,07118	0,70
HG001_00924	purD	P65896	Phosphoribosylamine-glycine ligase	0,00004	0,38	0,00286	0,61
HG001_00789	pepA_1	O86436	Cytosol aminopeptidase	0,00006	0,25	0,21480	0,76

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HG001_02298	femX	Q2FVZ4	Lipid II:glycine glycytransferase	0,00063	0,16	0,13970	0,77
HG001_00139	ptsG_1	Q7A807	PTS system glucose-specific EIICBA component	0,00148	0,26	0,63630	0,91
HG001_02330	sarR	Q7A425	HTH-type transcriptional regulator SarR	0,00149	0,35	0,33180	0,78
HG001_00582	bmrA	O06967	Multidrug resistance ABC transporter ATP-binding/permease protein BmrA	0,00186	0,18	0,29630	0,77
HG001_00274	-	-	PTS system ascorbate-specific transporter subunits IICB	0,00186	0,18	0,17150	0,53
HG001_01285	-	P67370	DegV domain-containing protein	0,00215	0,21	0,79540	0,95
HG001_02186	mtlD	P99140	Mannitol-1-phosphate 5-dehydrogenase	0,00278	0,41	0,76220	1,08
HG001_00697	uvrA	P63383	UvrABC system protein A	0,00515	S1 only	0,70990	0,92
HG001_02752	mnmE	Q8YN91	tRNA modification GTPase MnmE	0,02237	S1 only	0,68190	0,85
HG001_00774	-	-	hypothetical protein	0,01066	S1 only	0,51870	0,65
HG001_01495	ybeZ	P0A9K3	PhoH-like protein	0,02237	S1 only	0,13690	0,54
HG001_00704	aroK_1	-	Shikimate kinase	0,02237	S1 only	0,98780	1,00
HG001_01178	cls_1	P63801	Cardiolipin synthase	0,02237	S1 only	0,30820	0,61
HG001_02615	azoB	Q8KU07	NAD(P)H azoreductase	0,04792	S1 only	0,19250	0,57
HG001_01624	nrnA_2	O34600	Bifunctional oligoribonuclease and PAP phosphatase NrnA	0,04792	S1 only	0,10910	0,63
HG001_00061	sarS	Q7A872	HTH-type transcriptional regulator SarS	0,04792	S1 only	0,38600	0,75
HG001_01411	-	-	hypothetical protein	0,04792	S1 only	0,53670	0,79
HG001_01445	argR_1	P63580	Arginine repressor	0,04792	S1 only	0,42270	0,82
HG001_01673	-	-	hypothetical protein	0,04792	S1 only	0,82810	0,93
HG001_01203	-	-	hypothetical protein	0,04792	S1 only	0,84250	0,94
HG001_01278	murG	P65482	UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	0,04792	S1 only	0,86550	0,96
HG001_01059	-	-	hypothetical protein	0,04792	S1 only	0,88170	1,05
HG001_00413	purR	P37551	Pur operon repressor	0,10630	S1 only	0,13670	0,59
HG001_01579	fgs	P15925	Folypolyglutamate synthase	0,10630	S1 only	0,47120	0,59
HG001_02574	lipR_2	O53301	Putative acetyl-hydrolase LipR precursor	0,10630	S1 only	0,60700	0,77
HG001_02529	pgcA	Q2FVC1	Phosphoglucomutase	0,10630	S1 only	0,56460	0,81
HG001_01412	-	-	hypothetical protein	0,10630	S1 only	0,75420	0,85
HG001_01265	-	P60108	TelA-like protein	0,10630	S1 only	0,70090	0,89
HG001_02356	-	Q9X0Y1	Phosphorylated carbohydrates phosphatase	0,10630	S1 only	0,85300	0,95
HG001_01068	rsmB	P36929	Ribosomal RNA small subunit methyltransferase B	0,10630	S1 only	0,98910	1,00
HG001_01020	-	-	hypothetical protein	0,10630	S1 only	0,99140	1,00
HG001_01408	dnaQ	P03007	DNA polymerase III subunit epsilon	0,10630	S1 only	0,99330	1,00
HG001_02619	-	-	Amidohydrolase	0,10630	S1 only	0,89970	1,05
HG001_00692	prfB	Q7A6R4	Peptide chain release factor 2	0,10630	S1 only	0,80050	1,08
HG001_00886	tagE_3	P13484	putative poly(glycerol-phosphate) alpha-glucosyltransferase	0,25340	S1 only	0,16990	0,58
HG001_01523	mtnN	Q7A5B0	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	0,25340	S1 only	0,68190	0,85
HG001_00721	rnr	P21499	Ribonuclease R	0,25340	S1 only	0,98320	1,00
HG001_01311	ponA	P39793	Penicillin-binding protein 1A/1B	0,00377	0,45	0,09972	0,77
HG001_01249	cvfB	Q2FYF3	Conserved virulence factor B	0,00680	0,46	0,02650	0,54
HG001_01088	ffn	P37105	Signal recognition particle protein	0,01330	0,19	0,38230	0,78
HG001_00486	ppaX	Q9JMQ2	Pyrophosphatase PpaX	0,02233	0,39	0,40170	0,82
HG001_02168	-	-	EVE domain protein	0,02456	0,25	0,27290	0,75
HG001_00491	azoI	Q50H63	FMN-dependent NADPH-azoreductase	0,03968	0,43	0,87110	0,97
HG001_02709	lipA_2	Q2FUU5	Lipase 1 precursor	0,04208	0,38	0,59470	0,90
HG001_01016	arcC1	Q7A627	Carbamate kinase 1	0,05934	0,15	0,67060	0,86
HG001_02098	alr1	Q5HED1	Alanine racemase 1	0,05977	0,33	0,31430	0,75
HG001_02361	-	-	hypothetical protein	0,06469	0,30	0,89320	1,04
HG001_01558	dtd	P0A026	D-tyrosyl-tRNA(Tyr) deacylase	0,07066	0,40	0,98270	1,00
HG001_00553	-	-	hypothetical protein	0,07066	0,40	0,12110	0,59
HG001_00017	rplI	P66318	50S ribosomal protein L9	0,11110	0,30	0,87110	0,97
HG001_01938	ligA	Q9AIU7	DNA ligase	0,11110	0,30	0,61750	0,92
HG001_00155	azoR	Q99X11	FMN-dependent NADH-azoreductase	0,11110	0,30	0,68110	0,91
HG001_02064	scrB	P13394	Sucrose-6-phosphate hydrolase	0,14000	0,39	0,82380	1,07
HG001_01324	-	-	tetrapeptide repeat protein	0,19430	0,23	0,85300	0,95
HG001_01678	glpE_2	-	Thiosulfate sulfurtransferase GlpE	0,24910	0,43	0,69860	1,12
HG001_00064	yfiY	O31567	putative siderophore-binding lipoprotein YfiY precursor	0,24910	0,43	0,55510	0,86
HG001_00197	tagF	P13485	CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase	0,24910	0,43	0,45320	0,75
HG001_02680	manP	O31645	PTS system mannose-specific EIIBC component	0,29660	0,41	0,37250	0,75

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HG001_01259	lysA	P0A5M4	Diaminopimelate decarboxylase	0,34660	0,30	0,83950	1,12
HG001_00914	purE	Q9WYS7	N5-carboxyaminoimidazole ribonucleotide mutase	0,01649	0,45	0,17530	0,73
HG001_02208	-	Q7A4A4	putative uridylyltransferase	0,04776	0,46	0,44240	0,88
HG001_01965	yxjF_3	P94374	putative ABC transporter ATP-binding protein YxjF PmtC	0,00390	0,48	0,08067	0,71
HG001_00176	acdA	P45867	Acyl-CoA dehydrogenase	0,00054	0,48	0,01163	0,63
HG001_00400	-	-	hypothetical protein	0,17600	0,46	0,81500	0,89
HG001_01434	-	-	Glyoxalase-like domain protein	0,07661	0,50	0,43370	0,85
HG001_02585	mvaA	P13702	3-hydroxy-3-methylglutaryl-coenzyme A reductase	0,21050	0,50	0,38960	0,82
HG001_01073	rpe	P74061	Ribulose-phosphate 3-epimerase	0,35440	1,86	0,55600	0,43
HG001_00818	yidA	P0A8Y5	Sugar phosphatase YidA	0,93450	0,90	0,08234	0,43
HG001_01826	perR	Q2G282	Peroxide-responsive repressor PerR	0,62460	1,30	0,08449	0,30
HG001_01491	era	P64085	GTPase Era	0,95280	1,00	0,16280	0,23

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152 **Table S7: Spectral count analysis of gel filtration fraction**

**LEGEND:**

# spectra (BASIC Spectral Count)	= 1 spectrum
# spectra (BASIC Spectral Count)	= 2-5 spectra
# spectra (BASIC Spectral Count)	= 6-10 spectra
# spectra (BASIC Spectral Count)	= 11-30 spectra
# spectra (BASIC Spectral Count)	>= 31 spectra

**Samples 36/39/42/45 min: 1µg injected** (The Spectral Count values are comparable)  
**Samples 48 min: whole sample injected**

**Total number of proteins & protein of interest (S1):**

Elution Time (min.)	BASIC Spectral Count					BASIC Spectral Count				
	36	39	42	45	48	36	39	42	45	48
	22	31	24	25	0	35	35	21	26	14
	BASIC Spectral Count					BASIC Spectral Count				
	36	39	42	45	48	36	39	42	45	48
HG001_01336 gene_rpsA_1 30S ribosomal protein S1		1	76	1475		189	62	50	1633	4
	BASIC Spectral Count					BASIC Spectral Count				
	36	39	42	45	48	36	39	42	45	48
HG001_02281 gene_rpsC 30S ribosomal protein S3	24	82	59	57		37	104	80	51	1
HG001_02283 gene_rpsS 30S ribosomal protein S19	17	49	90	34		33	52	97	37	
HG001_01639 gene_rpsD 30S ribosomal protein S4	14	67	43	39		28	85	48	49	1
HG001_00309 gene_rpsF 30S ribosomal protein S6	11	45	31	39		24	46	44	33	
HG001_00476 gene_rpsL 30S ribosomal protein S12	26	65	63	25		43	73	133	40	3
HG001_01510 gene_rpsT 30S ribosomal protein S20	26	75	94	28		58	74	119	38	
HG001_02270 gene_rpsE 30S ribosomal protein S5	22	54	34	17		34	64	100	28	
HG001_02263 gene_rpsM 30S ribosomal protein S13	26	91	33	14		37	104	57	22	
HG001_00477 gene_rpsG 30S ribosomal protein S7	14	41	51	19		15	57	34	31	
HG001_01107 gene_rpsB 30S ribosomal protein S2	15	64	24	30		24	77	57	36	
HG001_02262 gene_rpsK 30S ribosomal protein S11	25	64	30	23		25	66	57	15	
HG001_02273 gene_rpsH 30S ribosomal protein S8	15	49	23	15		15	44	34	18	
HG001_02288 gene_rpsI 30S ribosomal protein S10	14	55	28	4		10	45	45	5	
HG001_00311 gene_rpsR 30S ribosomal protein S18	12	29	14	8		21	39	28	7	
HG001_02253 gene_rpsJ 30S ribosomal protein S9	9	15	11	14		4	31	24	14	
HG001_02278 gene_rpsQ 30S ribosomal protein S17	5	33	42	7		23	21	52	7	
HG001_01089 gene_rpsP 30S ribosomal protein S16	3	30	5	15		7	32	20	26	2
HG001_02274 gene_rpsN1 30S ribosomal protein S14	5	5	8	1		5	2	18	3	
HG001_01196 gene_rpsN2 Alternate 30S ribosomal protein S14	1	2	2	1		1	2	1	1	
HG001_00830 gene_fabF 3-oxoacyl-[acyl-carrier-protein] synthase 2	1									2
HG001_00717 gene_eno Enolase	1									1
HG001_00713 gene_gapA1 Glyceraldehyde-3-phosphate dehydrogenase 1	1									1
HG001_01124 gene_rpsO 30S ribosomal protein S15		16	11	18		10	26	18	13	1
HG001_02271 gene_rplR 50S ribosomal protein L18		2	1	1		2	1		2	
HG001_00976 gene_rpmF 50S ribosomal protein L32		2	1			1			1	
HG001_02284 gene_rplB 50S ribosomal protein L2		2		1		2	1		2	
HG001_01499 gene_rpsU 30S ribosomal protein S21		1	1				1			
HG001_01075 gene_rpmB 50S ribosomal protein L28		1		1			1			
HG001_01595 gene_rplT 50S ribosomal protein L20		1				5	1			
HG001_02269 gene_rpmD 50S ribosomal protein L30		1				2	1			
HG001_02254 gene_rplM 50S ribosomal protein L13		1				1	1			
HG001_02260 gene_rplQ 50S ribosomal protein L17		1				1				
HG001_01597 gene_infC Translation initiation factor IF-3		1								
HG001_02287 gene_rplC 50S ribosomal protein L3				1						
HG001_02282 gene_rplV 50S ribosomal protein L22						3	3			
HG001_02280 gene_rplP 50S ribosomal protein L16						1	1			
HG001_02286 gene_rplD 50S ribosomal protein L4						1				1
HG001_01092 gene_rplS 50S ribosomal protein L19						1				
HG001_02275 gene_rplE 50S ribosomal protein L5						1				
HG001_02277 gene_rplN 50S ribosomal protein L14						1				
HG001_02285 gene_rplW 50S ribosomal protein L23							1		1	
HG001_02279 gene_rpmC 50S ribosomal protein L29							1			
HG001_00722 gene_smpB SsrA-binding protein							1			
HG001_01572 gene_rplU 50S ribosomal protein L21							1			
HG001_02276 gene_rplX 50S ribosomal protein L24							1			
HG001_00425 gene_yug1_1 General stress protein 13									1	
HG001_00479 gene_tuf Elongation factor Tu										12
HG001_01618 gene_pyk Pyruvate kinase										1
HG001_00473 gene_rpoB DNA-directed RNA polymerase subunit beta										1
HG001_01963 gene_NONE hypothetical protein										1

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155 **References**

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157 Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic*  
158 *Acids Res* 31, 3406-3415.

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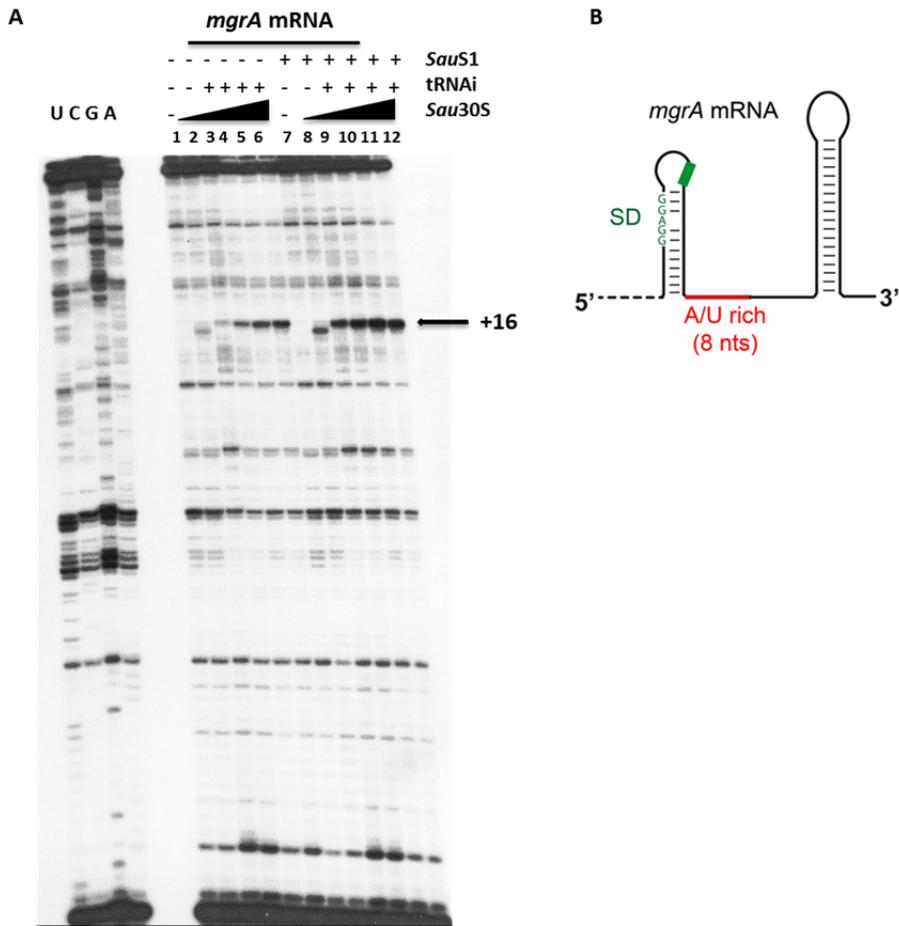


## II. Result II: *SauS1* is an RNA chaperone involved in different steps of sRNA-dependent regulation and of RNA metabolism in *S. aureus*

In the previous section, I have described how *SauS1* affected the production of late expressed virulence factors (exotoxins and exoenzymes) and showed how it promoted the translation initiation of the highly structured *apsm1-4* mRNA. The transcriptomic and proteomics studies have also revealed other possible roles for this protein in stabilizing sRNAs or helping the correct folding of complicated RNA structures, like the riboswitches (see §I. Result I, “DISCUSSION” of the manuscript “*Staphylococcus aureus* S1 activates translation initiation of PSM $\alpha$  toxins and stimulates the production of several other secreted virulence factors”). This second section will focus on study of the possible extra translational functions of *SauS1*.

### II.1. *SauS1* helps translation initiation of structured mRNAs

In order to assess if the activity of *SauS1* is linked to the alleviation of translation repression mediated by cis-acting mRNA structures, besides the *apsm1-4* we tested by toe-printing one more natural *S. aureus* mRNA harbouring distinct structural features. *mgrA* mRNA carries structured 5' UTR in which the SD sequence is hidden in a double-strand region (Romilly et al., 2014; Gupta et al., 2015). We have previously shown that *E. coli* ribosomes, containing S1, are able to form initiation complexes with *mgrA* mRNAs (Khusainov et al., 2016). This efficient recognition, was linked to the presence of *EcoS1* because S1-depleted *E. coli* ribosome, were not able to efficiently recognize *mgrA* mRNA, while mRNA binding was restored using S1-depleted 30S saturated with purified *EcoS1* added in trans (Khusainov et al., 2016). Moreover, we had demonstrated that *Sau30S*, similarly to S1-depleted *Eco30S*, could recognize only the unstructured *spa* mRNA. As shown in **Figure 18**, *SauS1* enhances the formation of the initiation complex at low concentration of 30S.



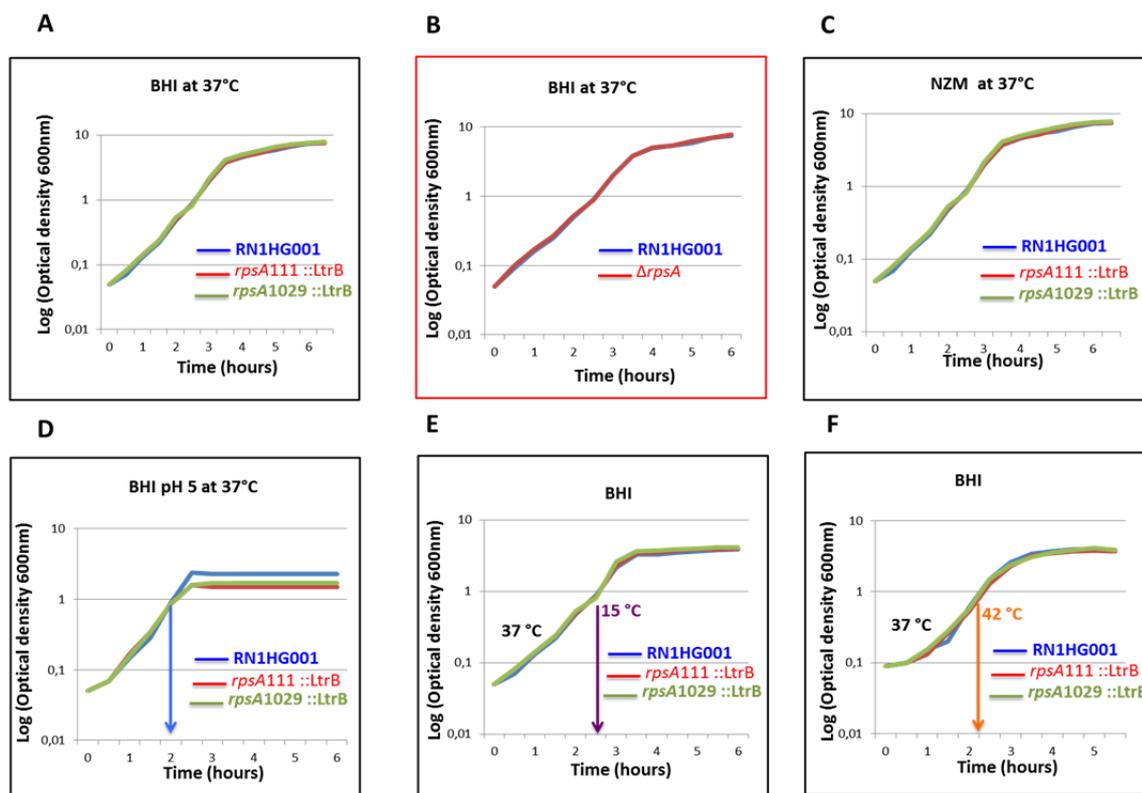
**Figure 18. Toeprinting assays to monitor the effect of *SauS1* on the translation initiation complex formation with *mgrA* mRNA.** (A) Effect of *SauS1* on the formation of initiation complex using *mgrA* mRNA. When present, *SauS1* was pre-incubated with the ribosome at a constant 1.6 molar ratio. Lane 1: incubation control of mRNA ; Lane 2: incubation control of mRNA with 30S subunits ; Lanes 3, 4, 5 and 6: formation of the initiation complex containing mRNA, increasing concentration of 30S (25, 50, 100, 200 nM) and fMet-tRNA. Lane7: incubation control of mRNA with purified *SauS1*. Lane 8: incubation control of mRNA, 30S and *SauS1*. Lanes 9, 10, 11 and 12: formation of initiation complex in presence of *SauS1* and increasing concentrations of 30S (25, 50, 100, 200 nM). Lanes U, A, G, C: sequencing ladders. The toeprinting signals at position +16 are indicated by arrows. (B) Scheme for the secondary structure of the RBS and beginning of coding region of *mgrA* mRNA.

## II.2. Phenotypic characterization of *rpsA* mutants

To elucidate how *SauS1* is impacting the physiology of *S. aureus*, the three previously obtained mutants strains were analyzed for phenotypic alterations under different stress conditions. Two mutant strains resulted from introns insertion, one located immediately after the AUG codon (*rpsA111::LtrB*) and the second close to the stop codon (*rpsA1029::LtrB*) of the *rpsA*, while the third strain corresponded to a deletion of the gene per allelic replacement ( $\Delta rpsA$ ).

I first demonstrated that the three mutant strains grow similarly in normal laboratory conditions (e.g. rich medium at 37°C) in agreement with a previous study showing that *S. aureus rpsA* gene is not essential (Chaudhuri et al., 2009). I have then compared the growth rates of the mutant and WT (HG001) strains under various stresses. Because the expression of the virulence factors is modulated by metabolic changes and stress responses, I have analyzed whether *SauS1* might also be required for other adaptive processes. **Figure 19** shows growth curves in BHI (Brain Heart Infusion) at different pH, during cold and heat shocks, or in NZM minimal medium supplemented with glucose. No differences could be observed between the HG001 and the three mutants strains in BHI at 37°C (**Figures 19A and B**). Nutrient starvation conditions (NZM minimal medium supplemented with glucose) did not affect the specific growth of the WT and the mutant *rpsA111::LtrB* or *rpsA1029::LtrB* strains (**Figure 19C**) although we observed a reduced duplication time when compared with the growth in rich media.

An acid shock (pH 5) was also tested (**Figure 19D**). The main reason why this stress was selected is because in the host body, infecting microorganisms frequently face acidity e.g. in the stomach, the phagolysosomes (Jensen and Bainton, 1973) and in the oral cavity and for the presence of fermentation products from other co-colonizing anaerobic bacteria. It was also shown that mild acidic stress altered the expression of a large set of virulence factors (Weinrick et al., 2004), which most likely illustrate the ability of the bacteria to adapt to particular tissue sites of the host. The acidic stress has been applied when bacteria reached  $OD_{600}=1$ . A drastic arrest of the growth could be observed which was even more sudden for the *rpsA111::LtrB* and *rpsA1029::LtrB* strains than the WT (HG001).



**Figure 19. Bacterial growth under different stress conditions.** Samples were taken each 30 min through the time course of six hours to check the optical density at OD<sub>600nm</sub>. A. BHI growth at 37°C for WT (HG001) and the insertion mutants *rpsA111* ::LtrB and *rpsA1029* ::LtrB. B. BHI growth at 37°C for WT (HG001) and the  $\Delta rpsA$  strain. C. Growth curves in NZM minimum media supplemented with glucose. D. Acidic stress. Growth in BHI 37°C was allowed to proceed until OD<sub>600nm</sub>=1 was reached. E. The bacteria were exposed to pH 5. E. The cells were grown at 37°C in rich media and at OD<sub>600nm</sub>=1 and then they were placed at 15°C. F. The bacteria were exposed to pH 5. E. The cells were grown at 37°C in rich media and at OD<sub>600nm</sub>=1 and then they were placed at 42°C.

Bacterial cold shock response has been largely studied in *E. coli* and *Bacillus subtilis* (Brandi et al., 1994; Graumann and Marahiel, 1996; Graumann et al., 1996; Jones and Inouye, 1994). Exposition to cold induces the synthesis of specific set of cold-shock proteins able to help microorganisms to overcome the damaging effects of rapidly reduced temperatures on transcription and translation (Brandi et al., 1994; Giangrossi et al., 2007; Giuliadori et al., 2007; Giuliadori et al., 2004; Giuliadori et al., 2010; Gualerzi et al., 2003). These proteins are presumed to function as RNA chaperones preventing the formation of secondary structures in RNAs thereby facilitating translation at low temperature (Yamanaka et al., 1998). The common protein fold (cold shock domain CSD) of the cold shock proteins such as *E. coli* CspA is very similar to the OB-fold S1 domain. As I described above (“Introduction”), the domains of *EcoS1* are not functionally equivalent and it has been shown that the deletion of domains 5 and 6 at the C-terminal region of *EcoS1* (*rpsA* $\Delta$ 56) does not affect the general translation, but causes a cold

sensitive phenotype ((Duval et al., 2013b). and unpublished data from the lab). The cold-sensitive phenotype could be due to an impaired ability to unfold RNA structures stabilized at low temperature. The fact that mutations could affect the chaperone activity preferentially at low temperatures is not so surprising. Indeed, *EcoS1* does not need energy like an RNA helicase, and therefore at the permissive temperature, the thermal energy may help the protein to melt RNA secondary structures. Surprisingly, **Figure 19E** shows no difference in the behavior of the different strains subjected to cold shock. It has to be noted though that no slow down or temporary arrest of the growth was observed for any of the strains.

The bacterial heat shock response has been also extensively studied in several Gram-positive and Gram-negative bacteria (Bukau, 1993; Chuang and Blattner, 1993; Cowing et al., 1985). Upon shifts to higher temperature, the cells start to induce the expression of numerous heat-shock proteins (HSPs). Many of them are molecular chaperones including DnaK and GroEL and ATP-dependent proteases such as Lon and ClpAP that are essential to overcome protein denaturation (Barrios et al., 1994; Wild et al., 1996; Yura et al., 1993). In *P. putida*, exposure to high temperatures has been shown to downregulate *rpsA* (Ito et al., 2014). Since the unrestricted synthesis of thermolabile proteins can potentially lead the cell to danger, *P. putida* might arrest the de novo protein synthesis of non-HSPs reducing S1 level upon exposure to high temperatures. However, as evidenced in **Figure 19F**, no significant effects were produced when the WT and mutant strains were exposed to elevated (42°C) temperatures.

Although we did not monitor all the phenotypes with the three mutant strains, our data nevertheless suggested that mutations or the deletion of the gene had very similar effects, and that S1 had little effect on cell growth. These data suggest that S1 is not a ribosomal component and support the idea that *SauS1* is a regulatory protein.

### II.3. *SauS1* and its constellation of RNAs. RIP-seq (co-immunoprecipitation and RNA-seq) analysis

As mentioned above, *SauS1* is able to modulate the translation of PSM $\alpha$  peptides by direct binding to the *apsm1-4* operon. Other mRNAs have been also postulated to be potential target of *SauS1*, which *in vitro* seems to modulate the translation of other

structured mRNAs (e.g. *mgrA*) while not affecting unstructured one (e.g. *spa*). Moreover, several sRNAs have been found to be downregulated in the  $\Delta rpsA$  strain. To characterize the repertoire of RNA targets, we have performed RIP-seq analysis (co-immunoprecipitation and RNA-seq) using the *SauS1*-3X flag-tagged strain, which allow us to detect the synthesis of *SauS1* during bacterial growth (Figure 1 of the manuscript). We also performed RIP-seq on the WT (HG001) strain as the negative control. The experiments were done in triplicates. Bacterial growth was performed in BHI medium for 6 h where *SauS1* is sufficiently abundant. After immunoprecipitation with the anti-flag agarose beads and washing to remove unspecific binders, the sample was extracted with acidic phenol and then by chloroform-isoamyl alcohol. RNA was precipitated with ethanol, treated with DNase I, extracted with phenol, precipitated and prepared for sequencing. In parallel, we have performed a transcriptomic analysis from total RNA extracts prepared from the wild-type strain and the *SauS1* flagged strain to gain some indication on the expression levels of the mRNAs. This analysis has revealed no major changes in the corresponding transcriptomes including *rpsA* levels in the two strains (1.26 fold). The data were analyzed and visualized using Galaxy (Afgan et al., 2016) and the Integrative Genomics Viewer (IGV) browser, respectively (Thorvaldsdottir et al., 2013). A detailed protocol for the bioinformatics analysis is provided in Material and Methods. Briefly, we aligned the sequencing reads onto HG001 genome (Caldelari et al., 2017), counted per feature, and normalized. We have estimated the enrichment of putative targets by comparing the number of reads obtained from the RNA immunoprecipitated with the flag-tagged S1 and the non tagged (WT) S1 as control. **Table 3** and **Table 4** show the best hits divided into mRNAs and other classes of RNAs (sRNAs, riboswitches, tRNAs...), respectively.

RIP-seq of *Sau* S1. Co-immuno precipitated mRNAs

Virulence factors				
id	gene	product	Enrichment	pvalue
HG001_02059	agrB	Accessory gene regulator protein B	14,108	1,03E-08
HG001_02755	psm_locus	psm $\alpha$ operon	13,228	2,09E-08
HG001_02245	-	65 kDa membrane protein precursor Eap	12,192	9,09E-08
HG001_00728	clfA	Clumping factor A precursor	5,556	0,00011
HG001_00113	galE	UDP-glucose 4-epimerase	5,301	0,00021
HG001_00100	cap8A_1	Capsular polysaccharide type 8 biosynthesis protein cap8A	3,998	0,0012
HG001_01022	-	psm $\beta$ 1	3,98	0,00206
HG001_00899	sspA	Glutamyl endopeptidase precursor	3,974	0,00139
HG001_00227	esxA	Virulence factor EsxA	3,787	0,00184
HG001_00556	sarA	Transcriptional regulator SarA	3,372	0,00457
Other mRNAs				
id	gene	product	Enrichment	pvalue
HG001_01747	-	Thermophilic serine proteinase	20,53	4,20E-12
HG001_02647	hmoB	Heme-degrading monooxygenase HmoB	9,433	3,30E-07
HG001_00560	xerD_2	Tyrosine recombinase XerD	8,288	3,32E-06
HG001_01665	ytpP	Thioredoxin-like protein YtpP	8,243	1,48E-06
HG001_02406	-	putative lipoprotein precursor	7,815	1,83E-05
HG001_00733	-	cspA	7,447	2,40E-06
HG001_02161	luxS	S-ribosylhomocysteine lyase	6,734	1,38E-05
HG001_02543	zntR	HTH-type transcriptional regulator ZntR	6,58	8,53E-05
HG001_02609	isaA	putative transglycosylase IsaA precursor	6,375	1,10E-05
HG001_01549	-	CsbD-like protein	6,189	6,98E-05
HG001_02222	-	Alkaline shock protein 23	6,095	6,76E-05
HG001_02418	ydaG	General stress protein 26	5,946	0,00011
HG001_01065	-	TM2 domain protein	5,924	0,00017
HG001_00759	-	CsbD-like protein	5,663	0,00062
HG001_00087	deoC1	Deoxyribose-phosphate aldolase 1	5,502	0,00012
HG001_02655	nrdD	Anaerobic ribonucleoside-triphosphate reductase	5,237	8,47E-05
HG001_00214	degA	HTH-type transcriptional regulator DegA	5,222	0,00023
HG001_02644	fda	Fructose-bisphosphate aldolase class 1	5,124	0,00047
HG001_02628	-	Fructosamine kinase	5,111	0,00021
HG001_00131	yagU	Inner membrane protein YagU	5,066	0,00013
HG001_01336	rpsA_1	30S ribosomal protein S1	5,018	0,0005
HG001_01127	-	Ribonuclease J 2	4,93	0,00019

**Table 3: List of mRNAs sequenced by RIP-seq using *Sau*S1-flag immunoprecipitation.** Fold change (IP-S1flag/IP-S1) correspond to enrichment values and together with p-values were calculated for by DESeq2 using shrinkage estimation for dispersions and fold changes (Varet et al., 2016).

Interestingly, among the best enrichments obtained for mRNAs, we have found the  $\alpha$  *psm* operon, which was previously experimentally validated (Figure 5C of the manuscript). *Sau*S1 protein was also co-purified with other mRNAs encoding virulence factors and of two main regulators of virulence (*agr*, *sarA*; **Table 3**).

RIP-seq of *SauS1*. Co-immuno precipitated RNAs

sRNAs								
id	gene	product	Enrichment	pvalue	$\Delta rpsA/WT$	pvalue	Binding (Kd <sup>a</sup> )	
HG001_02347	-	RsaI	70,443	6,16E-19	0,204	1,30E-31	~ 500 nM	
HG001_01553	-	6S	63,163	4,87E-22	0,143	1,77E-12	n.d.	
HG001_00710	-	RsaH	20,371	2,88E-09	0,832	0,59711	~ 100 nM	
HG001_00584	-	RsaD	19,601	4,32E-09	0,115	1,61E-13	n.d.	
HG001_00848	-	RsaE	9,727	9,48E-06	0,234	4,80E-05	> 1 $\mu$ M	
HG001_02057	-	RNAIII	3,885	0,00438	0,363	5,71E-09	~ 200 nM	
HG001_00516	-	RsaA	3,846	0,00175	0,676	0,09411	no binding	
HG001_02756	-	RsaG	2,556	0,04221	0,16	2,20E-11	no binding	
New sRNAs <sup>b</sup>								
id	gene	product	Enrichment	pvalue	$\Delta rpsA/WT$	pvalue	Binding (Kd <sup>a</sup> )	
HG001_03138	-	RsaOT	18,167	3,71E-09				
HG001_03067	-	SprX	6,258	0,00045				
HG001_03009	-	SprX2	6,016	0,00069				
HG001_02954	-	RsaOF	4,606	0,00119				
tRNAs								
id	gene	product	Enrichment	pvalue	$\Delta rpsA/WT$	pvalue	Binding (Kd <sup>a</sup> )	
HG001_00443	-	tRNA-Ala(tgc)	22,261	7,49E-11	0,341	0,0021	n.d.	
HG001_01823	-	tRNA-Ala(tgc)	7,036	9,03E-05	0,093	4,79E-17	n.d.	
HG001_02201	-	tRNA-Ile(gat)	5,841	8,04E-05	0,05	1,35E-31	n.d.	
HG001_02088	-	tRNA-Gly(tcc)	3,67	0,01249	0,12	3,00E-10	n.d.	
HG001_01816	-	tRNA-Gly(gcc)	3,439	0,0506	0,523	0,04231	n.d.	
HG001_01812	-	tRNA-Ala(tgc)	3,36	0,03646	0,364	0,00358	n.d.	
Riboswitches								
id	gene	product	Enrichment	pvalue	$\Delta rpsA/WT$	pvalue	Binding (Kd <sup>a</sup> )	
HG001_01602	-	T-box	5,569	7,82E-05	1,813	0,03484	n.d.	
HG001_01693	-	FMN Riboswitch	5,544	7,12E-05	2,476	4,48E-09	n.d.	
HG001_01462	-	Glycine Riboswitch	5,121	0,00079	0,62	0,10996	n.d.	
HG001_00986	-	T-box	4,22	0,00162	0,78	0,20049	n.d.	
HG001_02363	-	SAM Riboswitch	3,894	0,00756	0,71	0,03617	n.d.	
HG001_01542	-	T-box	3,554	0,00509	0,728	0,28617	n.d.	

**Table 4: List of sRNAs, tRNAs and other *cis*-acting non coding RNAs (riboswitches) sequenced by RIP-seq using *SauS1*-flag immunoprecipitation.** Fold change (IP-S1flag/IP-S1) correspond to enrichment values and together with p-values were calculated for by DESeq2 using shrinkage estimation for dispersions and fold changes (Varet et al., 2016). For the sake of simplicity also the corresponding differences in RNA levels observed by the transcriptomic analysis (see § Result I) is reported with the corresponding p-values. <sup>a</sup>The last column refers to apparent Kds obtained by the gel-shift experiments (§ Result II; II.4.). <sup>b</sup>New sRNAs are sRNAs newly incorporated into the annotation file (Caldelari et al., 2017).

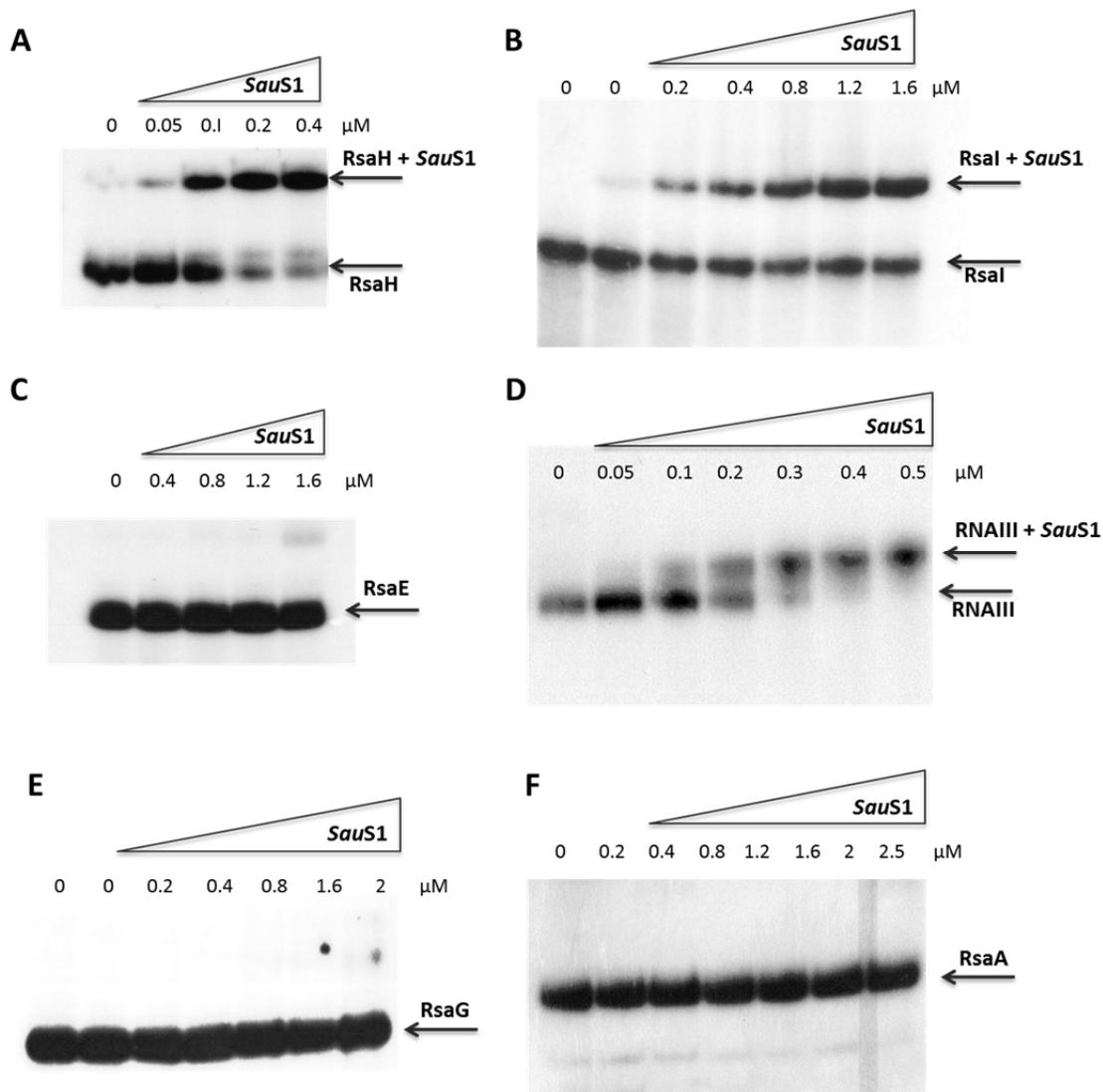
Several sRNAs have been found together with *SauS1* (**Table 4**). The most enriched is the sRNA RsaI (70,4 enrichment). RsaI level was also strongly decreased in the  $\Delta rpsA$  mutant strain (0,2 **Table 4**). Interestingly, the yields of many co-IP sRNAs were also found less abundant in  $\Delta rpsA$  mutant strain. Like RsaI, RsaD (0,11), RsaG (0,16), RsaE (0,23), RNAIII (0,36) and RsaA (0,68) might be stabilized by the binding with *SauS1*. Such a correlation was also found for the co-IP tRNAs (**Table 4**).

These data suggested that *SauS1* binds to many of the co-IP RNAs, and by doing so, might activate the translation of some mRNAs and stabilize other RNAs.

#### II.4. *SauS1* forms stable complexes with various sRNAs but does not interact with all of them

Based on the RIP-seq data, we first analyzed whether *SauS1* directly binds to different sRNA candidates using gel retardation assays (**Figure 20**). In vitro 5' end-labeled RsaI, RsaH, RsaG, RsaA, RsaE, and RNAIII were incubated with increasing concentrations of purified *SauS1*. The data show that the protein directly binds to RsaH (**Fig. 20A**,  $K_d$  100 nM), RsaI (**Fig. 20B**,  $K_d$  500 nM), RNAIII (**Fig. 20D**,  $K_d$  200 nM). In contrast, no significant interaction was observed for RsaE (**Fig. 20C**,  $> 1 \mu\text{M}$ ), RsaG (**Fig. 20E**), and RsaA (**Fig. 20F**). The apparent  $K_d$  for the different sRNAs is also included in **Table 4**.

Taken together, the gel retardation assays showed that many of the co-IP sRNAs are direct binders of *SauS1*. For those, which do not directly interact, their presence in the list could be explained by the formation of multi-partner complexes. *SauS1* could thus interact with a specific RNA or protein which in turn could establish other interaction with other RNAs.

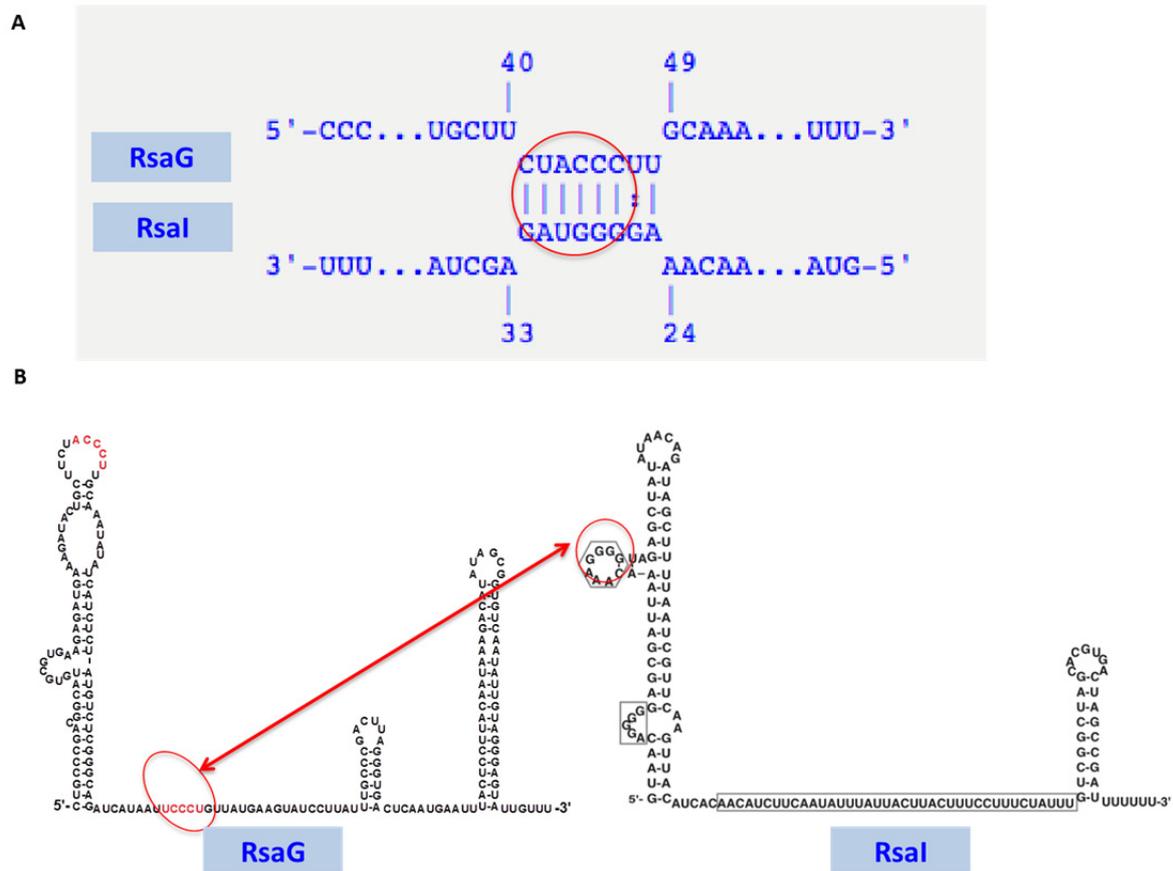


**Figure 20. Gel retardation assays to monitor *SauS1* binding to several sRNAs.** Experiments were performed on complexes formed with the 5' end-labeled RsaH (A), RsaI (B), RsaE (C), RNAIII (D), RsaG (E) and RsaA (F) in presence of increasing concentrations of *SauS1* as marked on the figure.

## II.5. *SauS1* forms a ternary complex with RsaI and RsaG

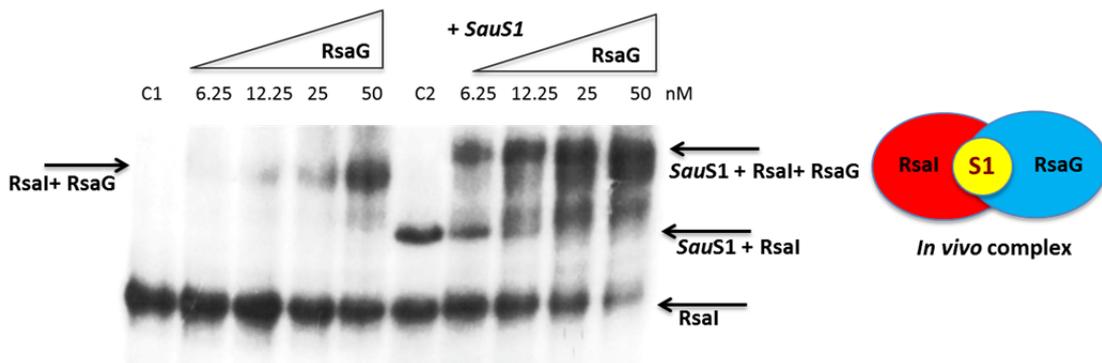
The gel retardation analysis has shown a direct binding of *SauS1* to RsaI while no detectable interaction was observed with RsaG, even with high concentrations of protein (up to 3.5  $\mu\text{M}$ , data do not shown). RsaG belongs to the class of sRNA, which contain a C-rich sequence motif (UCCC) as the seed sequence to interact with the SD sequence of target mRNAs (Geissmann et al., 2009a). RsaI is characterized by several conserved stretches of nucleotides including a long unpaired region rich in uridines and adenines and two G-rich tracts (**Figure 21**). MS2-affinity purification approach coupled to RNA sequencing (MAPS) (Lalaouna and Masse, 2015; Tomasini et al., 2017), used to identify

the targetomes of both RsaI and RsaG, have shown that the two sRNAs are interacting *in vivo* (Delphine Bronesky and Emma Desgranges unpublished results). The predicted interaction site would involve one of the C-rich motif of RsaG and one G-rich region of RsaI (Figure 21).



**Figure 21 Predicted interaction between RsaG and RsaI.** A. Predicted base-parings between C-rich motif of RsaG and G-rich tract of RsaI carried out using IntaRNA program (Mann et al., 2017). B. Secondary structures of RsaG and RsaI. The sequences involved in their interaction are highlighted by red circles and connected by an arrow. (DG= -9,5 kcal/mol).

I then checked the possibility that a ternary complex could form between *SauS1*, RsaI and RsaG by gel-retardation assays. Increasing concentrations of cold RsaG were incubated with constant amount of 5' end-labeled RsaI in the presence and in the absence of *SauS1* (1  $\mu$ M). The results showed that, although RsaI and RsaG are able to interact (apparent  $K_d$ = 50 nM), the addition of *SauS1* drastically enhances their binding affinity as shown in **Figure 22** (apparent  $K_d$ = 6,25 nM). *SauS1* is thus able to interact with RsaI to facilitate its binding to RsaG leading to the formation of a highly stable ternary complex both *in vitro* and *in vivo*.

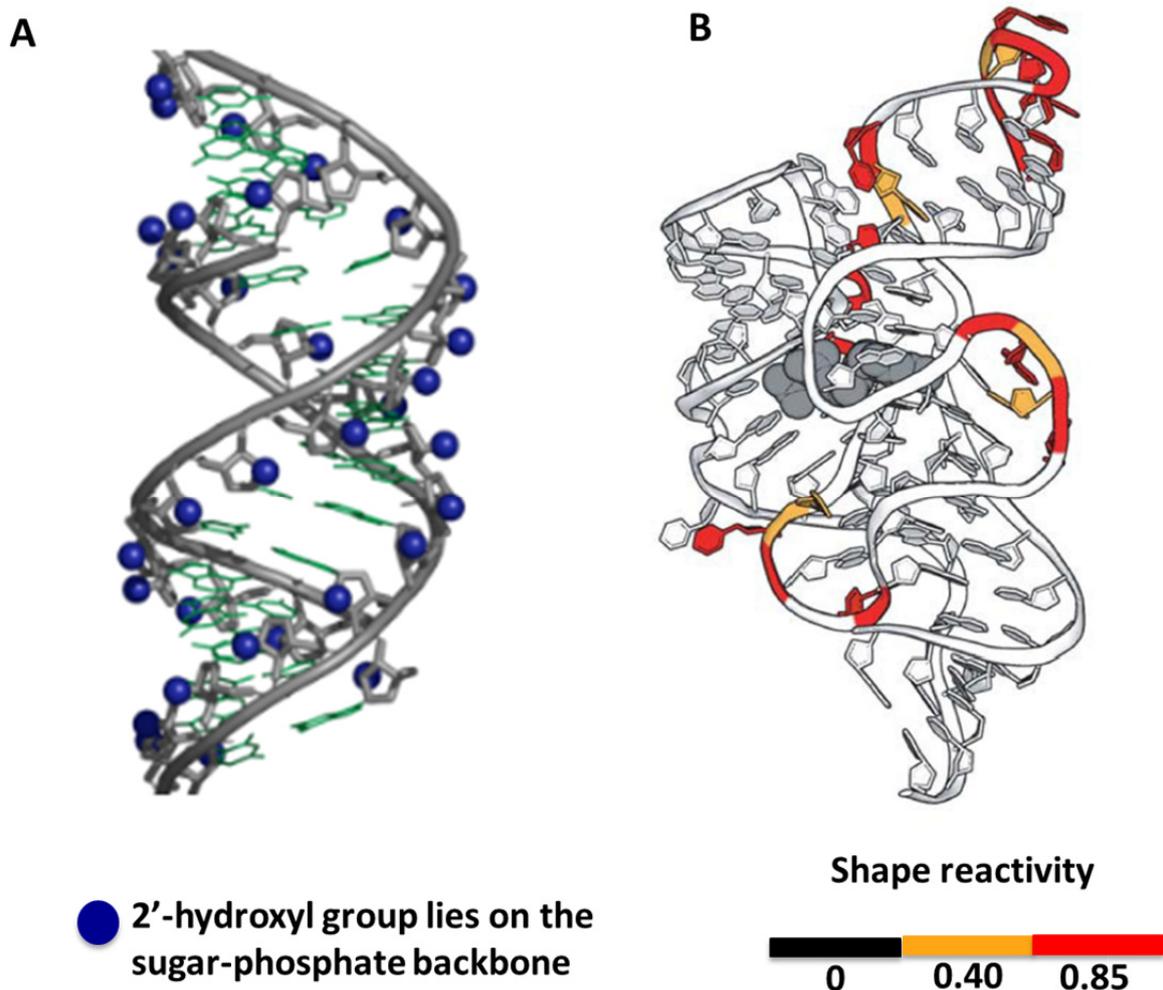


**Figure 22: Gel retardation assays to follow the formation of ternary complex SauS1-RsaI-RsaG.** The assay was performed on complexes formed with the 5' end-labeled RsaI, increasing concentration of cold RsaG (marked on the figure) in the presence and in the absence of SauS1 (1  $\mu$ M). Lanes C1 and C2 are control lanes with RsaI and RsaI-SauS1, respectively. The binary (RsaI-RsaG and RsaI-SauS1) and ternary (SauS1-RsaI-RsaG) complexes are indicated by arrows. The observed SauS1-RsaI-RsaG complex could explain the RIP-seq data.

## II.6. RsaI binding site for RsaG is hindered into a pseudoknot structure

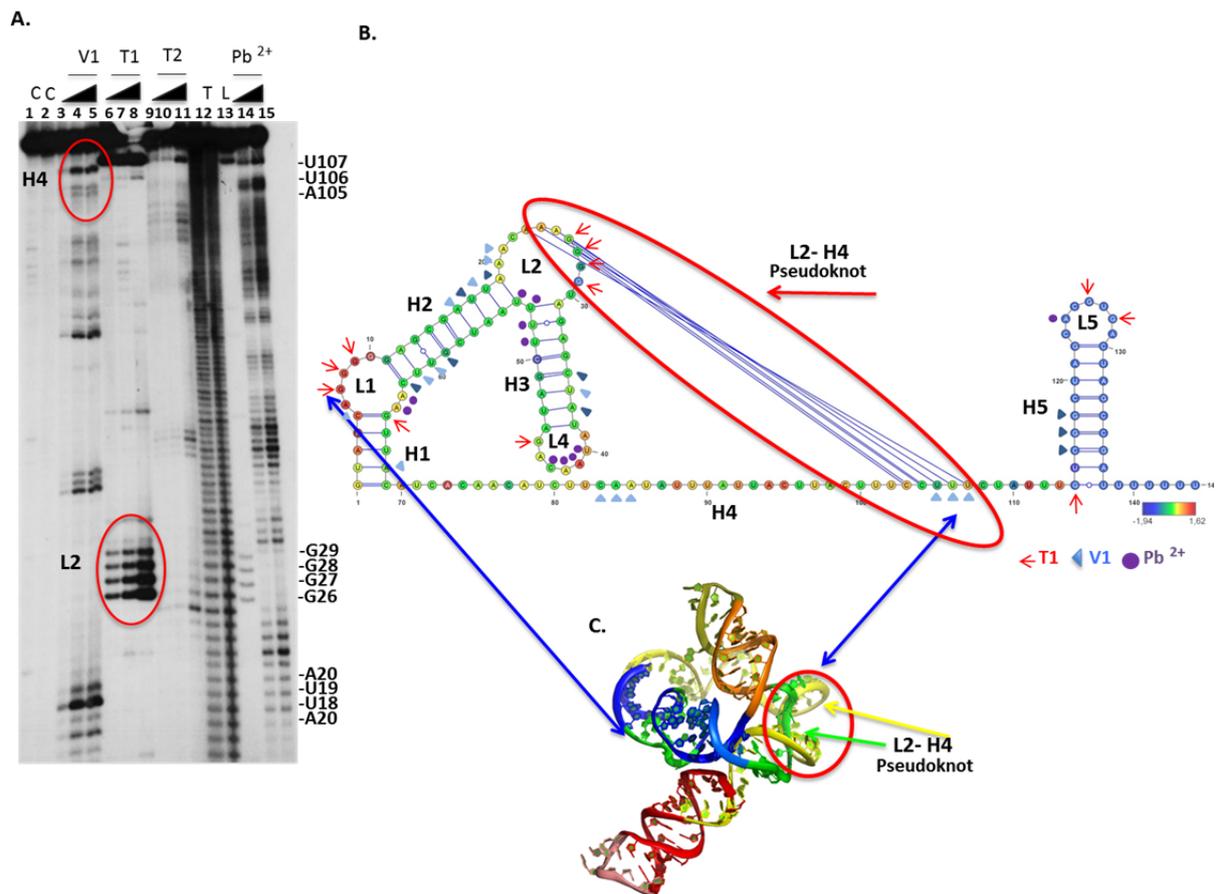
RsaI has been proposed to fold in a stable pseudoknot structure involving its highly conserved regulatory regions, the G-rich tract and the long single-stranded region (Marchais et al., 2010). This G-rich motif is supposed to bind to RsaG, and recent works have shown that the two conserved regions of RsaI are required for the recognition of target RNAs (Delphine Bronesky unpublished results). The pseudoknot structure would be thus incompatible with the regulatory functions of RsaI. This opens the following question: Is SauS1 able to help the RsaI targeting process by promoting pseudoknot remodeling?

In order to probe RsaI structure, I have performed SHAPE (Rice et al., 2014) and structure mapping using different RNases and lead (II) (Fechter et al., 2016). As an RNA folds into a defined tertiary structure, specific set of nucleotides are expected to be constrained in base-pairing interactions, while unpaired nucleotides remains exposed and flexible. SHAPE is based on the chemical modification of the ribose 2'-hydroxyl position which appears to be strongly dependent on the nucleotide flexibility (Steen et al., 2011). Hence, flexible nucleotides preferentially adopt conformations that react with a hydroxyl-selective chemical to form a 2'-O-adduct while basepaired nucleotides are unreactive. This method was particularly well appropriate to map long-range interactions such as the pseudoknot structure motif (Figure 23).



**Figure 23: SHAPE reactivity.** In panel A is represented a generic RNA helix where the 2'-hydroxyl groups, accessible for chemical modification, are represented as blue spheres, while the aromatic bases are in green. All the nucleotides have the same level of reactivity to chemical compound. In panel B, is shown the reactivity of nucleotides in a pseudoknot structure. The increasing reactivity of 2'-OH is indicated by different colors, from constrained and unreactive (black), to not very reactive (orange) and completely accessible nucleotides (red). The figure was adapted from (Weeks, 2015).

Double-stranded or stacked regions were tested with RNase V1, and unpaired guanine residues with RNase T1. I also used lead (II) that cleaves preferentially interhelical and loop regions, with high sensitivity for flexible regions (**Figure 24A**). Modified sites or cleaved nucleotides were detected as stops of primer extension reactions using reverse transcriptase. Our data were in agreement with the secondary structure model (**Figure 24B**) and is compatible with the presence of a pseudoknot interaction. Based on the data, a 3D structure has been built with RNAComposer (Popenda et al., 2012) (**Figure 24C**).



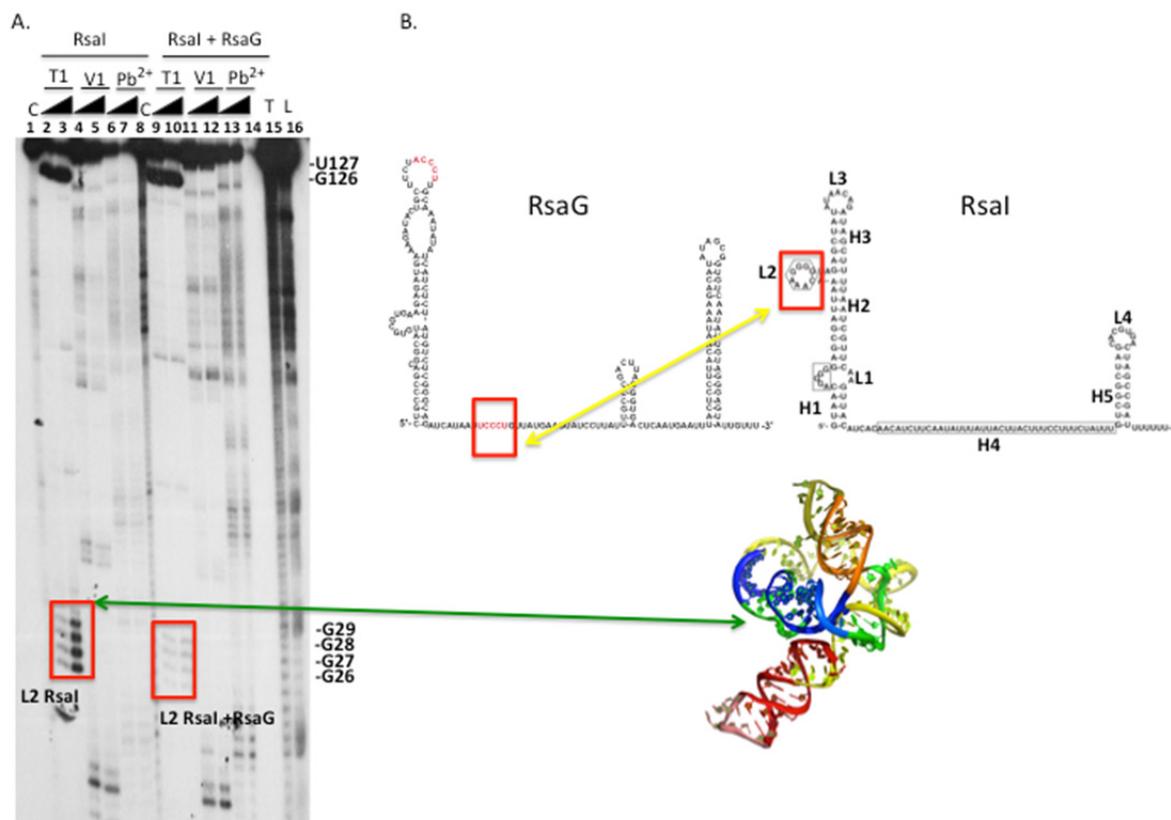
**Figure 24: Results of the probing experiments.** (A). Autoradiographs of enzymatic hydrolysis. Lanes 1 and 2: incubations controls. Lanes 3, 4 and 5: increasing concentration of RNase V1. The red square show the regions involved in the pseudoknot formation; Lanes 6, 7 and 8: increasing concentration of RNase T1; Lanes 9, 10 and 11, increasing concentration of RNase T2. Lanes T and L: RNase T1 in denaturing condition and formamide ladders, respectively. Lanes 15 and 16: Lead (II)-induced cleavage (B). Summary of the probing experiments reported on the secondary structural model of RsaI pseudoknot. T1 induced cuts are represented by red arrows. The reactivity of guanine residues to the V1 cuts are represented by blue triangles. The intensity of the cuts is given from weak to strong cleavages. Lead (II) induced cleavages are represented by purple circles. (C). 3D structure of RsaI pseudoknot obtained by RNAComposer. The interaction occurring between the G-rich track and the U-rich motifs, involved in the pseudoknot folding are highlighted by red circles on the structures in B and C.

## II.7. Probing the interaction between RsaI and RsaG by footprinting experiments

In order to identify the sequence-specific contacts of RsaG and RsaI and to better clarify the mechanisms of action of *SauS1*, I have performed footprinting experiments using RNases V1 and T1, and lead (II)-induced cleavages. The results, summarized in **Figure 25**, support the prediction that the G-rich tract of RsaI, which is engaged in the pseudoknot structure, interacts with RsaG. Indeed the major RsaG-induced protections were located at the G-tract motif which is not exposed to RNase T1 cuts.

We thus propose that *SauS1* would bind to RsaI to promote the melting of the pseudoknot and to facilitate its interaction with RsaG. I have tried to localize the

footprint of *SauS1* on *RsaI* using enzymes and lead II). However, no signature for *SauS1* could be detected most probably due to the dynamic nature of the RNP. This is a typical RNA chaperone behavior (Duval et al., 2017).

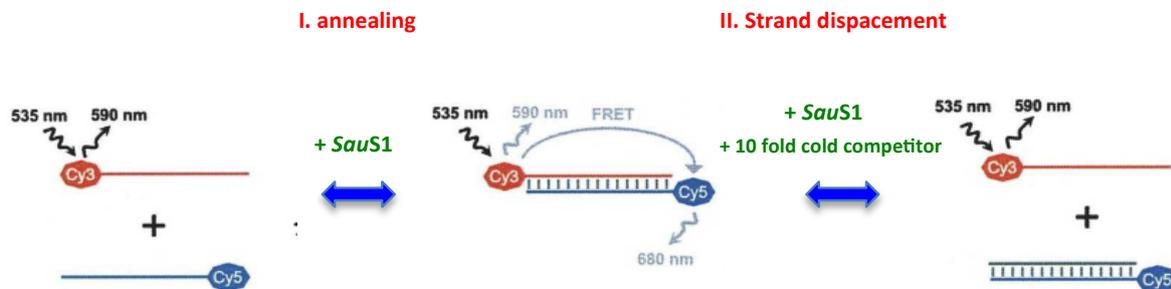


**Figure 25. Footprinting assays for RsaG on RsaI.** 3'-end labeled *RsaI* alone (first part of the autography) or in the presence of cold *RsaG*, was treated with RNases T1, V1 and lead (II). Lane 1: incubation control (*RsaI*); Lanes 2 and 3: *RsaI* with and without RNase T1, respectively; Lanes 4 and 5: *RsaI* with and without RNase T1, respectively; Lanes 6 and 7: *RsaI* with and without lead (II), respectively. Lane 8: incubation control (*RsaI* + *RsaG*). Lane 9 and 10: *RsaI*+ *RsaG* with and without RNase T1, respectively; Lanes 11 and 12: *RsaI* + *RsaG* with and without RNase V1, respectively; lanes 13 and 14: *RsaI* + *RsaG* with and without lead (II), respectively. Sequence ladder identifying position of guanines, is shown in the last line.

## II.8. Characterization of RNA annealing and strand displacement activities of S1 by FRET experiments

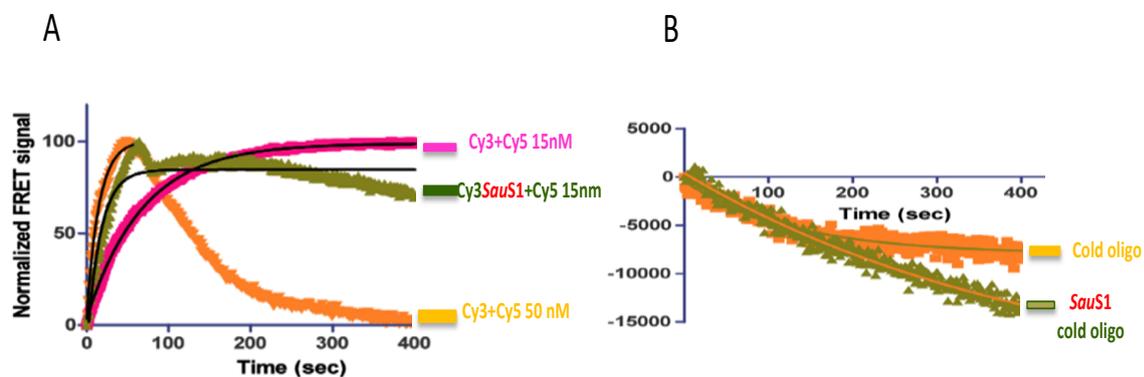
Chaperone proteins could act in two reactions: the RNA annealing and the dissociation of RNA duplexes (Rajkowitsch and Schroeder, 2007). In order to dissect the chaperone activity of *SauS1*, we used Fluorescence Resonance Energy Transfer (FRET) assay using the protocol proposed by Rajkowitsch and Schroeder in 2007 (Rajkowitsch and Schroeder, 2007). Briefly, for the experiment, we used two different fluorophore-labeled RNA oligonucleotides that are fully complementary. The assay was divided in two main phases (**Figure 26**). During the first step, we have followed the kinetic of annealing of

the two complementary oligonucleotides in the presence or in the absence of *SauS1*. In the second step, we have injected an excess of a cold competitor oligonucleotide to monitor the strand displacement (**Figure 20**).



**Figure 26: RNA chaperone proteins are basically active in two reactions.** RNA annealing and strand displacement. The chaperone activity could be followed by FRET assay. (I.) Annealing of two fluorophore-labeled RNAs completely self-complementary gives a FRET signal that is reduced upon RNA chaperone facilitating strand displacement with a cold competitor RNA (II).

Our data shown that in contrast to *E. coli* S1, *SauS1* enhances the rate of annealing five fold (**Figure 27A**) while it is not involved in the strand displacement reaction (**Figure 27B**).



**Figure 27: We used Fluorescence-based assays to monitor the chaperone activity of *SauS1* in two reactions, annealing and strand displacement.** In the phase I. the two fluorophore-labeled oligonucleotides were mixed in a microplate reader in the absence /presence of S1. The donor (Cy3) and acceptor (Cy5) fluorescence emission, were registered each second. The FRET index was estimated and normalized at t 180 s. The annealing of two oligonucleotides, is enhanced five times by the presence of S1 (A) while no effect of S1 was monitored on the strand displacement during the phase II (B).

## Discussions and Perspectives

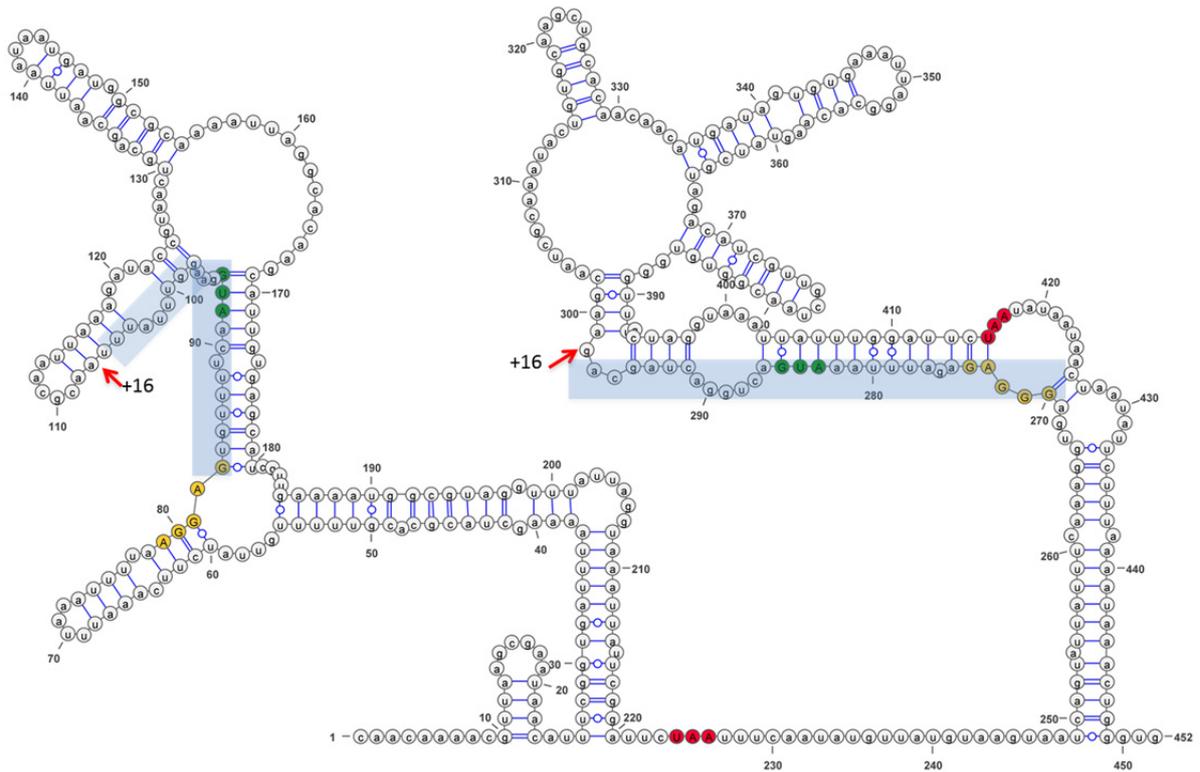


## I. *Staphylococcus aureus* S1 as translational activator. Further considerations

### I.1. General discussion

In the manuscript (Results, §I) “*Staphylococcus aureus* S1 activates translation initiation of PSM $\alpha$  toxins and stimulates the production of several other secreted virulence factors”, we have demonstrated that *SauS1* is not associated to the ribosome, but is required for translation initiation of  $\alpha$ -*psm* mRNA coding for Phenol Soluble Modulines of type  $\alpha$ . Moreover, it affects the production of many other exotoxins ( $\alpha$ -haemolysin,  $\delta$ -haemolysin and  $\gamma$ -haemolysins) and exoenzymes (proteases and lipases). We have proposed that, by direct binding to the mRNAs, it could remove inhibitory structures at the RBS to allow the correct 30S-mRNA interactions to take place. Our differential transcriptomics analysis has provided indications on the mRNAs which would require *SauS1* to be translated and protected from degradation. With the RIP-seq experiments (Results, §II.3; **Table 3** and **Table 4**), we could detect *in vivo* the whole set of mRNAs interacting with *SauS1*. The two datasets are well correlated, providing a detailed picture of the regulatory network coordinated by *SauS1*.

First,  $\alpha$ -*psm* operon was found as one of the best target of *SauS1* (Results, §II.3; **Table 3**). In addition, the RNAlII transcript coding for another PSM peptide (*hld*) with stable structure at its RBS (Benito et al., 2000) is also downregulated in the  $\Delta$ *rpsA* strain, is enriched in the RIP-seq (Results, §II.3; **Table 4**), and directly binds to *SauS1* (Results, **Figure 20D**). The third *psm* locus coding for two PSM $\beta$  peptides is also downregulated in  $\Delta$ *rpsA* (**Table 2**, Results, §I). PSM $\beta$ 1, co-immunoprecipitated with *SauS1* (Results, §II.3; **Table 3**) has strong and inaccessible Shine and Dalgarno sequence, suggesting possible needed of *SauS1* to be expressed, while the second peptides PSM $\beta$ 2, results to be less structured (**Figure 28**). Although experimental validation is needed to demonstrate the *hld* and *psm* $\beta$  translation activation by *SauS1*, it is tempting to propose that the three PSM coding genes would be coordinated both at transcriptional level by AgrA (Queck et al., 2008) and at translational level by *SauS1*. Because their mRNAs are highly structured, there is a risk that the efficiency of translation might be different from one operon to the other.



**Figure 28 Secondary structure model of PSM $\beta$  operon.** The PSM $\beta$  operon codes for two peptides 44 amino acids long. PSM $\beta$ 1 has strong SD sequence constrained in a stable double strand region while the SD of PSM $\beta$ 2 is more accessible. Yellow and green nucleotides correspond to the SD sequences and the initiation codons, respectively. In light blue the ribosome binding sites. The position +16 is indicated by red arrows

Hence, the role of *SauS1* would add another layer of regulation allowing an efficient synthesis of PSM peptides at a very similar level. In favor of this hypothesis is the fact that the expression pattern of *SauS1* follows the expression of RNAlII and of the *psm* mRNAs. This fine coordination of the PSMs under both the quorum sensing control and *SauS1* might be necessary for the concerted action during acute infection, when they are produced to promote dissemination and tissue lysis. Since they contribute to biofilm detachment/dissemination (Kong et al., 2006; Periasamy et al., 2012; Tsompanidou et al., 2011), an early induction could expose *S. aureus* to the host immune system before a critical mass could have been attained. In that regard, it is worth to notice that *SauS1* could also regulate the translation of the *sspABC* operon, coding endopeptidases important for immune suppression and infection dissemination (Imamura et al., 2005; Jusko et al., 2014; Ohbayashi et al., 2011). Indeed, the expression of the *sspABC* operon is strongly affected by the absence of S1 and which the RNA is among the best enriched RIP-seq targets (**Table 3**).

The differential transcriptomics and RIP-seq experiments also revealed other virulence factors as candidates for translation activation by *SauS1*. Among them the gene HG001\_02245 coding for the Extracellular Adherence Protein (Eap) involved in adherence and internalization (Hagggar et al., 2003; Palma et al., 1999). Its mRNA interacts with *SauS1* (enrichment 12,2), and both the Eap protein (0,29 in the secretome) and its mRNA (0,33) levels are highly perturbed in the  $\Delta rpsA$  strain. Also the Clumping factor *clfA*, which binds to fibrinogen to inhibit phagocytosis (Higgins et al., 2006), shares the same situation, although milder effects on the mRNA and protein levels have been detected.

Finally, two regulatory genes directly linked to virulence were found in the RIP-seq list, *agrB* and *sarA* mRNAs. *AgrB* is the membrane protease responsible for the release of the autoinducing peptide AIP, which is the quorum sensing signal. Its mRNA level does not change in the  $\Delta rpsA$  strain. The significance of the possible interaction with *SauS1* is not clear, but since this membrane protein is detected with difficulty in *S. aureus* proteomics analyses, we cannot rule out the possibility that S1 could play a role in its translation. In that respect, the mRNA presented a short 5'UTR with a potential large hairpin motif where the SD is engaged in base-pairings. The other gene, *sarA*, is the transcription factor potentially responsible for the simultaneous expression of both *rpsA* and *psm* transcripts (see "DISCUSSION" of the manuscript). Its level is slightly increased in the  $\Delta rpsA$  strain (1,44). The functional significance of this potential *sarA-SauS1* interaction awaits further experimental data.

## 1.2. Perspectives

To have a complete picture of the mRNAs, which are directly recruited by *SauS1* on the ribosome to be translated, ribosome profiling experiments would be an appropriate and sensitive method. Ribosome profiling approach is based on deep sequencing of ribosome-protected mRNA fragments (RPFs, usually around 30 nt). The distribution and abundance of RPF reads mapped on a given mRNA transcript reveal the locations and densities of ribosome occupation (Ingolia et al., 2009). This approach bridges the gap between global measurements of steady state mRNA and protein levels, providing a snapshot of active ribosomes in the bacteria at a specific time and under specific conditions of growth. Statistical analysis on the differential ribosome occupancy

between WT (HG001) and  $\Delta rpsA$  would provide the extent of *SauS1*-dependent translational control at the genome-wide scale. Furthermore, by applying different stresses encountered during the infection (*i.e.*, oxidative and NO), it would be possible to define the contribution of the *SauS1*-mediated translational control in the regulatory circuits taking place in response to stress. Pilot experiments have been already performed (by Lucas Herrgott) to set up the conditions for optimizing cell lysis, chloramphenicol treatment to stall the elongated ribosomes on the mRNAs, nuclease digestion to recover RNA fragments protected by the ribosomes, separation of the ribosome bound mRNA fragments using sedimentation by centrifugation with a sucrose gradient, and purification of specific 25-35 nucleotides long RNA fragments before the analysis by high throughput sequencing.

Our data suggested that the *SauS1*-mRNA complexes are dynamic explaining why I did not manage to get the footprint of *SauS1* on *psm* mRNA using the classical enzymatic and chemical mapping. Therefore, it would be better to adapt the CLIP-seq (cross-linking immunoprecipitation and RNA-seq) approach (Jensen and Darnell, 2008) to *S. aureus*. In addition to the RIP-seq, the CLIP-seq involves a pre-treatment of the cells with UV irradiation to generate a covalent bond between RNA-protein complexes prior to the purification of RNP complexes by immunoprecipitation. RT arrest at crosslink sites during cDNA library preparation can then be used as a means of mapping the interaction sites. By combining the ribosome profiling data with the CliP-Seq, we will be able to define the interaction sites, and to gain knowledge on the regulatory mechanism at the molecular level. Our preliminary data showed that many of the mRNAs that are downregulated by *SauS1* carry hairpin structures with base-paired SD, and just downstream or upstream the hairpin is often present an unstructured AU rich sequence that could be appropriate for the recognition by the OB-fold domain.

Finally, to get mechanistic details on the translation activation by *SauS1* we could also try a more direct structural approach by analyzing ribosomal complexes using cryo-electro microscopy (cryo-EM). The gel filtration experiment (**Figure 16**; Results, §I) indicated that *SauS1* does not interact directly with the ribosome but together with *psm* mRNA can form a ternary complex. This complex is stable and pure (**Table S9**; Results, §I). Thus, it would be possible to get its structure by cryo-EM. In collaboration with Yaser Hashem (IBMC, Strasbourg), the laboratory contributed to the structure of the 70S

ribosome from *S. aureus* (Khusainov et al., 2017) and more ribosomal complexes are currently under investigation.

## II. Involvement of *SauS1* in sRNAs stabilization

### II.1. General discussion

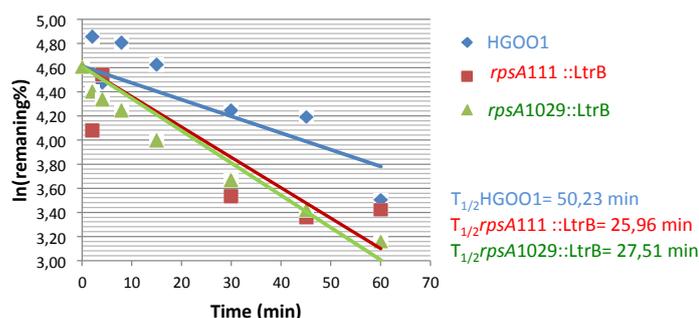
*SauS1* was co-immunoprecipitated with numerous sRNAs (6S RNA, RsaI, RsaH, RsaE, RsaD, RNAIII, RsaA and RsaG). With the sole exception of RsaH and RsaA, their levels of expression are severely affected in the  $\Delta rspA$  strain (**Table 4**; Results, §II.3). The observed down-regulation of these genes in the mutant strain could be due a defect of their transcription or a more rapid degradation. In Enterobacteriaceae, different classes of sRNAs are stabilized through the binding of the chaperone protein Hfq (Cui et al., 2013; Gottesman, 2004; Masse et al., 2003; Sonnleitner et al., 2006; Updegrove et al., 2016; Vogel and Luisi, 2011) and for ProQ (Smirnov et al., 2016; Smirnov et al., 2017), and more recently of another class of RNA chaperone called ProQ (Attaiech et al., 2017; Smirnov et al., 2017). It has been proposed that Hfq binding stabilizes sRNAs through a variety of mechanisms, e.g., blocking the attack of RNase E in many sRNAs (Masse et al., 2003), protect the sRNAs from a 3'-exoribonuclease attack by binding to the polyU tails of their Rho-independent terminators (Kovach et al., 2014);(Otaka et al., 2011). In contrast to this behavior, in *S. aureus* Hfq has no effect on sRNA stability (Boisset et al., 2007; Preis et al., 2009) and there is no ProQ equivalent (Attaiech et al., 2017; Olejniczak and Storz, 2017). Furthermore, in *S. aureus*, Hfq does not seem to be important for the recognition between sRNAs acting through base-pairings with target mRNAs (Zheng et al., 2016) and no major phenotypes were linked to its deletion (Bohn et al., 2007). In fact, little is known on the function of RNA-binding protein in regulation and clearly the machineries associated with RNAs have evolved differently in Gram-positive and Gram-negative bacteria. However, several ribonucleases contributed to RNA regulation such as RNase III (Boisset et al., 2007; Lasa et al., 2011; Lioliou et al., 2012), RNase J1 (Linder et al., 2014), and RNase Y (Khemici et al., 2015; Marincola et al., 2012). Besides, it was shown that the CshA DEAD-Box helicase is important to control the degradation of the *agr* operon (Oun et al., 2013) most likely through the recruitment of the degradosome (Giraud et al., 2015). Finally, SarA was unexpectedly found as an important factor that controlled mRNA stability but the binding to RNA seems to be not specific (Morrison et

al., 2012). Up to now, no major success was obtained to identify specifically RNP involving regulatory RNAs (Zhang et al., 2015).

In our study, we could demonstrate that *SauS1* would be responsible for a protective effect on several sRNAs. What could be the mechanism? At this regard, almost of the sRNAs strongly enriched with *SauS1* carry U-rich tails at their 3', which could be an appropriate binding site for S1. In these lines, *RsaA*, which carries a weak Rho-independent terminator (Geissmann et al., 2009a), does not bind to *SauS1* (**Figure 20F**). Another possibility is that *SauS1* would recognize other regions on the sRNAs. Many identified sRNAs in *S. aureus*, carry C-rich regions (CRRs) predicted to interact with G-rich sequences such as SD elements in their mRNA targets. It was proposed that besides this role, the CRR could also be recognized as binding site for specific proteins, as it was demonstrated for CRR found in the 3' UTR of some mRNAs in eukaryotes (Durand et al., 2015; Makeyev and Liebhaber, 2002). The CLIP approach will be useful to map the *SauS1* binding site while further experiments would be necessary to clarify the stabilizing role of *SauS1*.

## II.2. Perspectives

The stability of sRNAs in the *rpsA* mutant could be determined by measuring the kinetics of their degradation after rifampicin treatment (Campbell et al., 2001). Preliminary data have been already obtained by Delphine Bronesky on the stability of *RsaI*, the main target of S1. The experiment was done comparing WT (HG001) with the two intron insertion mutants of *rpsA* (*rpsA1111::LtrB* and *rpsA1029::LtrB*). Rifampicin was added to WT and the mutant strains grown in BHI to  $OD_{600}=3$  (4h), when both *SauS1* and *RsaI* are present. *RsaI* was detected by Northern blot using total RNAs extracted after 2, 4, 8, 15, 30, 45 and 60 min (**Figure 29**). Quantification, normalization and interpolation of the data by linear regression in logarithmic scale, show an effect of *SauS1* on *RsaI* stability by factor of two, as shown in the **Figure 29**.



**Figure 29: Stability of RsaI in different strains.** After quantification of the Northern blot signals and normalization of each point to the corresponding 5S signals, the % of remaining *psm* mRNA has been plotted to calculate RsaI half-life in the three strains.  $T_{1/2}$ HG001 (WT) is 50.23 min, while in absence of *SauS1*,  $T_{1/2}$ *rpsA111::LtrB* and  $T_{1/2}$ *rpsA1029::LtrB* decreased to 25.96 min and 27.51 min, respectively.

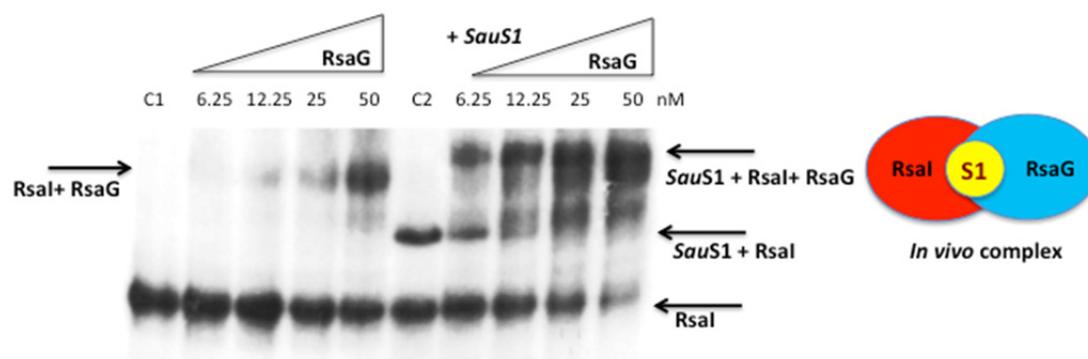
To determine the mechanism by which *SauS1* recognizes sRNAs to protect them from degradation, the CLIP-seq experiment proposed above could be used to get sRNA interaction sites. Moreover, a mutagenesis analysis of those sites would be necessary to confirm their importance in *SauS1* recognition. At this regard, two mutants of RsaI have been obtained by Delphine Bronesky in the laboratory. RsaI regulatory regions (G-rich tract and UC-rich sequence, **Figure 24**) have been deleted separately or in combination and these RsaI variants could be used to analyze their ability to bind *SauS1* using gel retardation assays.

### III. Involvement of *SauS1* in sRNA-target recognition

#### III.1. General discussion

Among the sRNAs isolated in complex with the protein, RsaI is the most enriched RNA. We have demonstrated that RsaI directly interacts with *SauS1* using gel retardation assays (**Figure 20B**; Results, §II). The works of Delphine Bronesky and Emma Desgranges in our laboratory have clarified the role of RsaI. Briefly, MS2-affinity purification approach coupled to RNA sequencing (MAPS) (Lalaouna and Masse, 2015; Tomasini et al., 2017) has allowed the characterization of its targetome. RsaG is its major sRNA target and it was demonstrated to synergically work with RsaI for the regulation of glucose metabolism. The two non-coding RNAs are expressed during the late exponential phase of bacterial growth, when *SauS1* is well expressed too. Both of them negatively respond to the presence of free glucose in the media (D glucose) while the expression of RsaG is enhanced by glucose 6 phosphate (G 6P). RsaI is a bifunctional

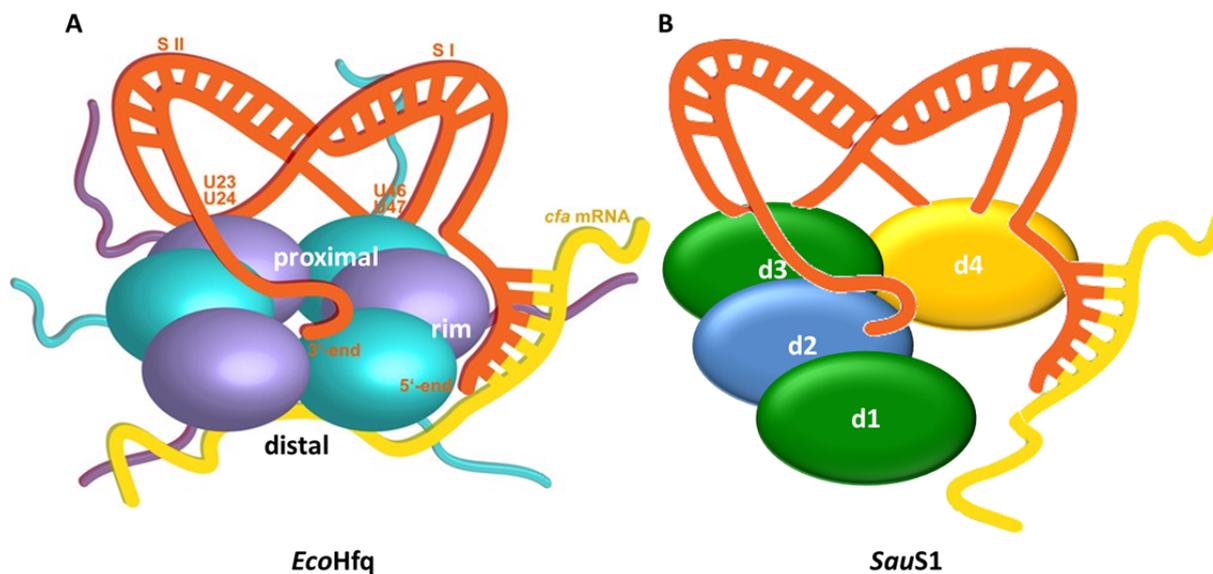
molecule able to simultaneously bind more than one target RNA throughout two different conserved regions: a G-rich tract and UC rich sequences. By footprinting experiments, I could show that the G-rich region of RsaI is responsible for the recognition of RsaG (**Figure 25**; Results, §II) while the UC rich sequence is required for the binding of many mRNA targets (D. Bronesky and E. Desgranges). Nevertheless, the predicted secondary model of RsaI (Marchais et al., 2010) and structure probing experiments (**Figure 24**; Results, §II), have evidenced that this G-rich could be constrained in a pseudoknot structure. Indeed, our preliminary probing experiments have shown that on one hand, the nucleotides of the UC rich region of RsaI are highly reactive to SHAPE, indicating a single strand conformation, but at the same time these nucleotides are also subjected to RNase V1 cuts, specific for double stranded region. On the other hand, the G rich motif is cleaved by RNase T1 (specific for unpaired G), while these nucleotides are poorly cleaved by Pb(II) induced cleavages and less reactive to SHAPE. Such mixed behavior is typical of the co-existence of multiple structures at the equilibrium and might be the signature of the presence of a pseudoknot. In fact, thermodynamic analysis has shown that an equilibrium between pseudoknot structure and alternative hairpin loop conformation existed (Philippe et al., 1990). The inaccessibility of the RsaI regulatory sequences makes necessary the action of a trans-acting factor able to unfold and remodel the secondary structure of RsaI, thus allowing target binding. RsaI and RsaG are able to interact even in the absence of *SauS1* (apparent  $K_d= 50$  nM), while the addition of *SauS1* largely promotes the ability of RsaI to recognize RsaG (apparent  $K_d= 6,25$  nM). Even if *SauS1* interacts only with RsaI, a ternary complex could be evidenced (**Figure30**).



**Figure 30** : Gel retardation assays to follow the formation of ternary complex *SauS1*-RsaI-RsaG. 5' end-labeled RsaI was incubated with increasing concentration of cold RsaG in the presence and in the absence of *SauS1* used at 1  $\mu$ M. Lanes C1 and C2 are control lanes with RsaI and RsaI-*SauS1*, respectively. The binary (RsaI-RsaG and RsaI-*SauS1*) and ternary (*SauS1*-RsaI-RsaG) complexes are indicated by arrows.

In Enterobacteria, the Sm protein Hfq binds to sRNAs and facilitates their base-pairing with mRNA targets. The mechanism by which it stimulates the annealing has been demonstrated by several structural works and mutagenesis analyses. Hfq from *Escherichia coli* and other Enterobacteriaceae form a compact hexamer that presents two structurally non-equivalent surfaces for RNA recognition: the proximal face, which interacts preferentially with uridine-rich sequences of sRNA and the distal face, favoring the binding of the target RNAs (Schumacher et al., 2002; Link et al., 2009; Sauer and Weichenrieder, 2011) (**Figure 31A**). In addition to the distal and proximal faces, the torus-shaped (or donut) of the Hfq hexamer bears an arginine patch sequence motif called rim that has recently been identified as a surface contributing to the annealing of both RNAs (Sauer et al., 2012; Zhang et al., 2013; (Zheng et al., 2016)). In *S. aureus*, Hfq does not accomplish this function because of the absence of this conserved rim motif of the hexamer (Panja S. et al, 2013(Zheng et al., 2016)). For instance, studies carried out on *S. aureus* RNAlII/spa mRNA model system have shown that Hfq neither form a ternary complex nor promote their annealing although it specifically binds to RNAlII and spa mRNA *in vitro*. Furthermore, Hfq did not affect the stability of RNAlII and spa mRNA *in vivo* (Eric Huntzinger et al., 2005). Therefore Hfq has probably evolved other functions in Gram-positive bacteria that are not yet determined.

Instead, in this work, we have demonstrated the ability of *SauS1* to promote base-pairing between short RNA molecules while the protein is not able to perform the strand displacement reaction (Figure 13). Differently from Hfq, *SauS1* does not interact with both RNAs (only RsaI, not RsaG) but is able to promote the formation of ternary complexes and stimulate RsaI-RsaG base pairings (**Figure 31B**). Based on the probing experiment, we proposed that this annealing activity is indirect and resulted from the remodeling of the RsaI secondary structure, i.e. melting of the pseudoknot, which would render accessible the regulatory regions of RsaI. A model is presented in **Figure 31**.



**Figure 31. Hypothetical models of the sRNA/mRNA interacting with EcoHfq or SauS1.** Figure adapted from (Dimastrogiovanni et al., 2014). A. EcoHfq model represented by six spheres, from which the disordered C-terminal tails extend radially. sRNA (RydC pseudoknot model; orange) sits on the proximal face of Hfq, with the 3' end U-rich tail interacting with the central channel. The two double strands conferring the pseudoknot structure to RydC are indicated as S I and S II. The target mRNA (*cfa*, depicted in yellow) associates with the distal face of Hfq, and it is proposed to form a duplex with the 5' end seed region of RydC that is recognized by the circumferential rim of Hfq. B. *SauS1* model represented by 4 spheres for its four domains colored according to Supplementary Figure S5 of the manuscript. The sRNA (orange) binds directly to *SauS1* and prepare it for interaction with its target (mRNA or another sRNA as for the RsaI-RsaG case; yellow). The ternary complex thus forms via RNA-RNA base pairing

### III.2. Perspectives

The annealing activity of *SauS1* could be further investigated by *in vitro* biophysical experiments. The thermodynamics of *SauS1*-RsaI-RsaG complex formation could be obtained by ITC (Isothermal Titration Calorimetry). ITC is a quantitative technique that can determine accurately the binding affinity ( $K_a$ ), the enthalpy ( $\Delta H$ ) and entropy ( $\Delta S$ ) changes, and binding stoichiometry ( $n$ ) of the interaction between two or more molecules in solution. In this way, we could for example understand whether RsaI pseudoknot is melted by *SauS1* before RsaG binding. Another approach could be the use of the SwitchSense apparatus. In this technique, binding kinetics and conformational changes can be monitored by the real time tracking of hydrodynamic friction difference in the motion of short DNA nanolevers upon ligand binding. RsaI could be linked to the DNA levers and RsaG binding in the presence of in the absence of *SauS1* could be monitored. Both ITC and SwitchSense are available in our Unit.

The effect of *SauS1* on the kinetics of sRNA target recognition could also be monitored *in vivo*. Vanderpool and Ha laboratories have recently developed a technique to visualize

by super-resolution imaging *in vivo* fluorescently labelled sRNA (SgrS) and mRNA (*ptsG*) in *E. coli* and to determine base-pairing kinetics using mathematical modeling (Fei et al., 2015). They could quantitatively examine the effect of Hfq, showing that in the  $\Delta hfq$  strain, the degradation rate of SgrS increased 20-fold, while the SgrS-*ptsG* mRNA association rate decreased only slightly. Similar experiments could be done to assess sRNA-target RNA couples in *S. aureus* WT or  $\Delta rpsA$ . In collaboration with Michaël Ryckelynck (IBMC, Strasbourg), we are developing single-cell analyses on *S. aureus* RNA regulations, using microfluidics lab-on Chip and the recently developed bright aptamer probes to visualize the sRNA (Autour et al., 2016).

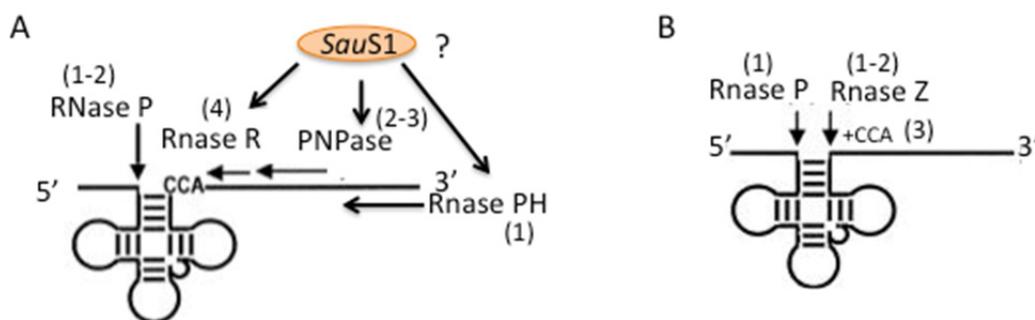
## IV. Involvement of *SauS1* in tRNA maturation

### IV.I. General statement

Several tRNAs were isolated from the RIP-seq analysis. They all belong to a special class of tRNAs with chromosomally encoded CCA 3'-end. The secondary structure of the tRNAs is composed of three stem-loops (D, anticodon and T) and its 5'- and 3'-ends pair to form a fourth terminal stem, where the amino acid is attached. At the extremity of this stem a single strand region, the CCA sequence, mediates amino acid attachment occurring to the 2' or 3'-hydroxyl group of the 3' terminal A in the CCA-motif by the aminoacyl-tRNA synthetases (Meinzel *et al*, 1995; Giegé and Springer, 2016).

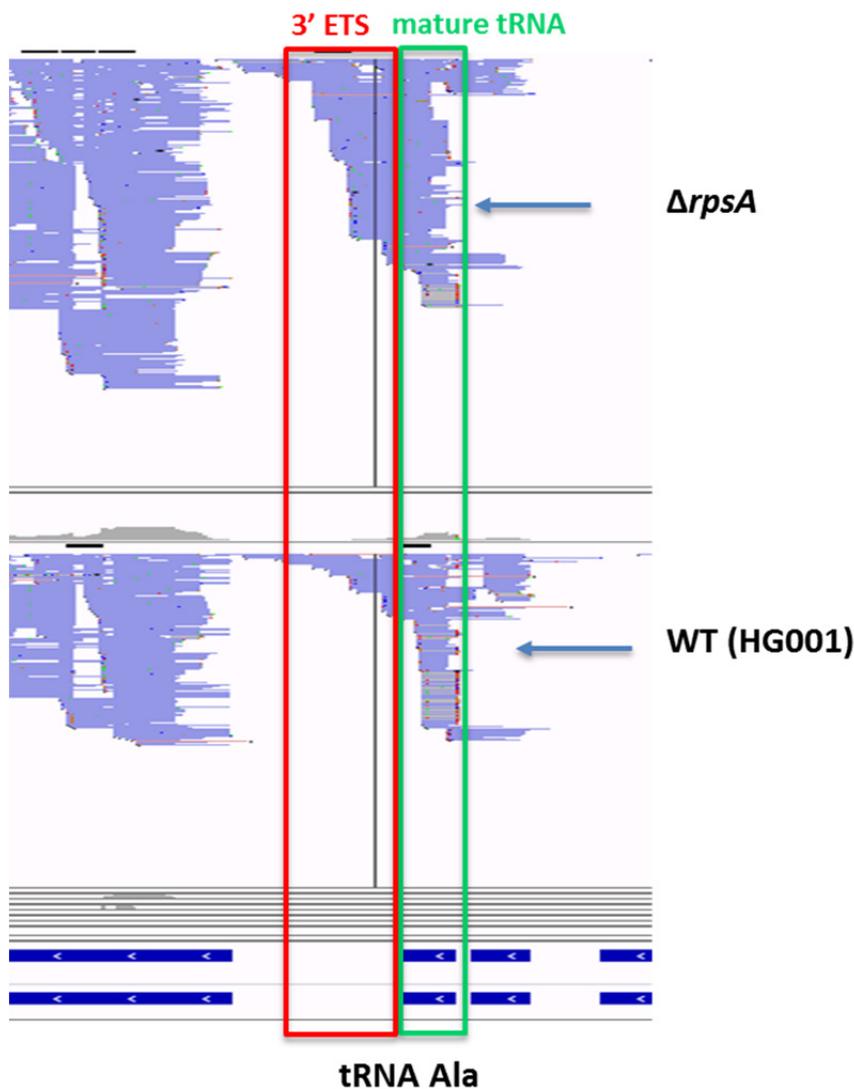
The presence of chromosomally encoded 3'-CCA, is not a universally conserved feature of tRNA genes. Indeed some bacteria like *E. coli*, have this sequence encoded in all tRNAs (Hartmann, 2009; Blattner, 1997), while other bacteria, such as *S. aureus*, lack the 3'-CCA in some of its tRNA genes. In these cases, the CCA-end is added post-transcriptionally like in eukaryotes by the tRNA-nucleotidyl transferase (Weiner, 2004; Xiong et al., 2006). When directly transcribed as in the *SauS1* interacting tRNAs, the CCA is a crucial signal required for the activation of alternative 3' maturation pathway of the tRNA primary transcript (Kirsebom and Svärd, 1994; Wegscheid and Hartmann, 2007). Indeed while the tRNA 5'-end processing is a largely conserved process catalyzed by the ubiquitous RNase P (Hartmann, 2009; Gopalan, 2007), the maturation of the 3'-end is a more complex mechanism and it varies according to the presence or not of the chromosomally encoded 3'-CCA (Hartmann et al., 2009; Marck et al., 1993). In *E. coli*, the tRNA processing is initiated by the endoribonuclease RNase E few nucleotides

downstream of the 3'-CCA sequence to generate pre-tRNAs with short or long 3' External Transcription Sequences (3'ETS) (Li and Deutcher, 2002). An AU-rich sequence in the proximity of the tRNA 3'-end (Li et al., 2005) is presumably recognized as a signal sequence during the endonucleolytic processing of the 3'-ETS. In contrast, Gram-positive bacteria lack the RNase E and the conserved AU-rich sequence is also not present in this region. In tRNAs, where RNase E cleaves further away from the 3'-CCA sequence, exoribonucleases like RNase II and PNPase initiate 3'-end maturation producing shorter 3' trailers which are then completely matured by the action of RNase PH and RNase T (Li and Deutscher, 1995, 1996). Alternative pre-tRNA 3' end maturation processes were observed in *B. subtilis* (**Figure 32**). Indeed, the tRNAs missing the CCA end, are processed at their 3' end by a single endoribonuclease, the RNase Z. After this primary step, the CCA is added by the tRNA nucleotidyl transferase (Pellegrini et al., 2003), an enzyme also involved in the repairing of damaged 3'CCA ends in mature tRNAs. In contrast, if the 3'-CCA is encoded, the pre-tRNAs are matured by the exonucleolytic action of RNase R, PNPase and RNase PH in a process similar to *E. coli* (Pellegrini et al., 2003; Wen et al., 2005).



**Figure 32 Processing of 3' end CCA- encoded and 3' end CCA- less tRNAs.** Vertical arrows on 5' leader of tRNA precursor represents the endoribonucleolytic cleavage carried out by the RNase P (A-B), while horizontal arrows on the 3' trailer represent the exoribonucleolytic trimming accomplished by RNase PH, PNPase RNase R (A) and RNaseZ (B). *SauS1* could be required for the exonucleolytic activity of one of the enzyme involved in the 3' end processing. Addition of CCA by tRNA nucleotidyl transferase is marked as "+CCA." The order of processing reactions is marked by numbers in parentheses.

By inspecting the reads distribution of the differential transcriptomic analysis at the loci of the immunoprecipitated tRNAs with the IGV browser (Thorvaldsdottir et al., 2013), an higher density could be observed at the 3'ETS in the  $\Delta rpsA$  (**Figure 33**). We speculate a possible role of *SauS1* in assisting the exoribonucleolytic activity of one of the involved enzymes (RNase R, PNPase or RNase PH).



**Figure 33. tRNA maturation defect in the  $\Delta rpsA$  strain.** Comparative analysis of reads accumulation between wild-type HG001 and the  $\Delta rpsA$  strains. Here details of the HG001\_00443 locus for the Ala-tRNA (tgc) that was isolated in complex with *SauS1*. The accumulation of reads at the 3'-ETS could indicate a defect of maturation.

Moreover, defects in tRNAs processing or incorrect folding, are linked to the formation of non-functional molecules that are subjected to quality control mechanisms (Li et al., 2002) and are often rapidly degraded. It has been proposed that this process takes place at the 3'-end via the polyadenylation made by the poly (A) polymerase (PAP) followed by the trimming of the adenines by RNase R and the PNPase (Zhongwei Li et al., 2002). The presence of non-matured tRNA species in the  $\Delta rpsA$  would imply defects in the maturation process but also an unefficient clearance of the unprocessed pre-tRNA.

To understand which nucleases might require *SauS1* action, we have performed an immunoprecipitation experiment under the same conditions for the RIP-seq, but looking at which protein complexes would be co-purified with *SauS1* by LC/MSMS. Among the most enriched proteins, we have identified several ribosomal proteins and RNases, which are known to be part of the *S. aureus* degradosome (Giraud et al., 2015; Roux et al., 2011) (Table 5).

Ribosome								
Name	Gene	Description	References	log2FC	p.value	adjp	-log10(adjp)	
<b>Small subunit</b>								
bS16	<i>rpsP</i>	Ribosomal protein bS16	Khusainov et al., 2016	6,05	0,00005	0,00033	3,48	
uS9	<i>rpsI</i>	Ribosomal protein uS9	Khusainov et al., 2016	5,77	0,00016	0,00092	3,04	
uS4	<i>rpsD</i>	Ribosomal protein uS4	Khusainov et al., 2016	3,78	0,00042	0,00223	2,65	
uS7	<i>rpsG</i>	Ribosomal protein uS7	Khusainov et al., 2016	3,61	0,00018	0,00098	3,01	
uS17	<i>rpsQ</i>	Ribosomal protein uS17	Khusainov et al., 2016	3,57	0,00001	0,00005	4,27	
uS13	<i>rpsM</i>	Ribosomal protein uS13	Khusainov et al., 2016	2,75	0,00122	0,00518	2,29	
bS20	<i>rpsT</i>	Ribosomal protein bS20	Khusainov et al., 2016	2,34	0,00000	0,00000	5,48	
<b>Large subunit</b>								
uL23	<i>rplW</i>	Ribosomal protein uL23	Khusainov et al., 2016	5,05	0,00693	0,02407	1,62	
bL9	<i>rplL</i>	Ribosomal protein uL9	Khusainov et al., 2016	5,04	0,00323	0,01264	1,90	
uL29	<i>rpmC</i>	Ribosomal protein uL29	Khusainov et al., 2016	4,61	0,01046	0,03454	1,46	
bL12	<i>rplL</i>	Ribosomal protein uL12	Khusainov et al., 2016	4,57	0,01313	0,04175	1,38	
uL6	<i>rplF</i>	Ribosomal protein uL6	Khusainov et al., 2016	1,35	0,01614	0,04905	1,31	
<b>Degradosome</b>								
Name	Gene	Description	References	log2FC	p.value	adjp	-log10(adjp)	
RNJ1	<i>rnjA</i>	Ribonuclease J 1	Roux et al., 2011	8,86	0,00000	0,00000	10,84	
RNJ2	<i>rnjB</i>	Ribonuclease J 2	Roux et al., 2011	8,71	0,00000	0,00000	10,31	
PNP	<i>pnp</i>	PNPase	Roux et al., 2011	7,80	0,00001	0,00007	4,18	
RNY	<i>rny</i>	Ribonuclease Y	Roux et al., 2011	5,78	0,00142	0,00597	2,22	
Tex	<i>Tex</i>	Toxin Expression, structurally similar to Eukaryotic Spt6 involved in transcription and RNA maturation		5,75	0,00143	0,00597	2,22	
PNP-like	<i>SAOUHSC_00483</i>	Sequence similarity with PNPase		4,67	0,01026	0,03422	1,47	
CshA	<i>CshA</i>	DEAD-box ATP-dependent RNA helicase	Giraud et al., 2015	3,67	0,06397	0,14370	0,84	

**Table 5. *In vivo* complexes involving *SauS1*.** *SauS1* co-immunoprecipitation was performed under the same conditions as for the RIP-seq experiment (Results). The protein were extracted and analyzed by LC/MSMS. Two main cellular complexes have been found to co-purify with *S1*, the ribosome and the degradosome.

While RNase R and RNase PH do not seem to be associated with *SauS1*, PNPase was found to be the most enriched together with RNases J1 and J2. It is thus possible that *SauS1* helps PNPase in the processing of 3'ETS of tRNAs with encoded CCA. PNPase is arrested by stem-loop structures and it is tempting to propose that the chaperone activity of *SauS1* would help removing them to prepare the pre-tRNA substrate for efficient maturation. RNases J1 and J2 are 5'-3' exoribonucleases (and endonucleases as well (Hausmann et al., 2017)) involved in the 5' maturation of both the 16S rRNA and the RNase P. They could also perform initial cleavages, competing with ribosomes for the stability of *S. aureus* mRNAs (Linder et al., 2014). In our transcriptomic analysis, we have removed the rRNA (RiboZero depletion; Illumina) and therefore we cannot draw any conclusion about 16S rRNA processing. Nevertheless, a slight accumulation of reads

at the 5' of the RNase P RNA could be observed in the  $\Delta rpsA$  strain (data not shown). Is *SauS1* helping exoribonucleases in their maturation activities? More experiment will be necessary to clarify this possible function.

Interestingly, the multiple functions of *SauS1* in translation and tRNA maturation, was also demonstrated for a protein largely conserved in wide variety of eukaryotes, named protein La. As *SauS1*, La protein is an RNA chaperone able to interact with different RNA molecules including precursor tRNAs, 5S rRNA, the signal recognition particle SRP (J.P Hendrick et al., 1981 - J Rinke, J.A Steitz, 1982 - J Rinke, J.A Steitz, 1985). The La proteins recognize their RNAs through a UUU<sub>OH</sub> 3' sequence. Despite its best characterized function is to protect premature exoribonucleolytic digestion of tRNAs during their processing, La protein has been show to bind to several mRNAs affecting their translation (Christopher J Yoo<sup>1</sup>, Sandra L Wolin, 1997). ). A La-related protein LARP7 is also a component of the 7SK RNP assembly and as the La protein binds to a UUU sequence motif at the 3' end of the RNA (Market et al., 2008; Uchikawa et al., 2015).

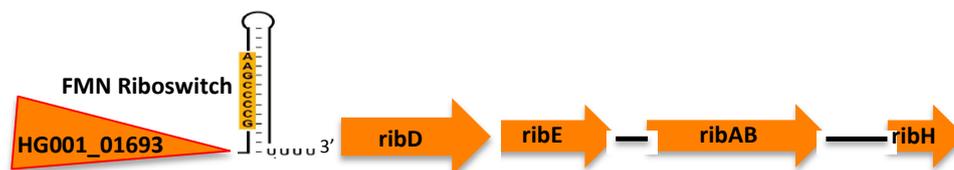
## IV.2. Perspectives

To confirm the 3'ETS maturation defect, Northern blot analysis using specific probes targeting the ETSs will be performed. Alternatively, RT-PCR experiments can be envisaged. Two-dimensional (denaturing and semi-denaturing conditions) electrophoresis on polyacrylamide gels (Suyama, 1986) could also be used for the separation of mature tRNAs from unprocessed transcripts. In the long term, mutant strains at specific genes encoding exoribonucleases will be constructed and studied to define the pre-tRNA processing pathway at the 3' end, and the role of *SauS1* in this pathway.

## V. Involvement of *SauS1* in *cis-acting* regulatory elements

The most widespread example of RNA regulatory elements in bacteria are riboswitches, complex folded RNA domains located in the non-coding regions of mRNAs, that control gene expression by binding specific metabolites (cofactors, vitamins, amino acids, nucleotides, Mg<sup>2+</sup>, second messenger cyclic di-GMP). Simple riboswitches are composed of two regions: an aptamer, responsible for ligand binding and an expression platform,

located in the 5' UTR of the regulated mRNA and/or operon. Upon ligand binding to the aptamer domain, a structural rearrangement is promoted on the nearby platform. Regulation mediated by riboswitches can occur at the transcriptional and/or translational levels. Translational riboswitches act throughout the formation of secondary structures, which sequester the RBS or the SD sequences of target mRNAs. This regulatory mechanism is not widespread in *S. aureus*. The only example in *S. aureus* is given by the flavin mononucleotide (FMN) riboswitch that controls the expression of *ribU* (HG001\_01344 in HG001), encoding a membrane riboflavin transporter. It has been proposed that in the absence of the ligand, the platform has a conformation competent for the mRNA translation, while the binding of FMN induces the formation of a hairpin structure which sequesters the SD sequence of the mRNA thus inhibiting its translation (Geissmann et al. 2009; Marchais et al. 2009; Abu-Qatouseh et al. 2010; Beaume et al. 2010; Bohn et al. 2010; Ten Broeke-Smits et al. 2010). In general, riboswitches work by forming Rho-dependent terminators to prematurely arrest the transcription of the downstream genes (Gusarov and Nudler 1999; Yarnell and Roberts, 1999). *S. aureus* FMN riboswitch (HG001\_01693) which was specifically co-immunoprecipitated with *SauS1*, is a transcriptional riboswitch which controls the expression of genes involved in the biosynthesis and/or transport of riboflavin (vitamin B2). It has been shown that the binding of FMN to the aptamer of the *ribD* operon (*ribDEBAH* genes) induces the formation of an intrinsic transcription terminator thus blocking the synthesis of vitamin when it is not required (Pedrolli D. Biscaro, Suess B, 2015) (Serganov et al., 2009; Wickiser et al., 2005) (**Figure 34**).



**Figure 34: FMN riboswitch controlling *ribD* operon.** In *S. aureus* as well as in *B. subtilis*, the riboflavin genes are organized in a large operon. The 5' UTR of the corresponding mRNA contains a riboswitch (HG001\_01693) that controls the expression of the whole operon. When the levels of FMN are sufficient, it binds to the riboswitch with the formation of a terminator at the platform region. Transcription is then halted and the genes necessary for the riboflavin production and transport are repressed.

Our transcriptomic analysis of the  $\Delta rpsA$  strain has shown an up-regulation of the FMN riboswitch (2.5) as well of the genes that are under its control (*ribD* 2.3; *ribE* 4.4; *ribBA* 2.9; *ribH* 2.6). The whole operon is thus enhanced. This indicates that in the absence of *SauS1*, the Rho-independent terminator did not form efficiently otherwise the downstream gene of the *ribD* operon would have been repressed. It is possible that to form correctly, the terminator needs the action of *SauS1*. By binding specifically to the riboswitch region (RIP-seq data), *SauS1* might act as a helper protein to promote the switch between alternative conformers for efficient regulation.

Interestingly, other riboswitches seem to require *SauS1*. The Glycine riboswitch (HG001\_01462) interacts with *SauS1*, its level is slightly less in the  $\Delta rpsA$  (0.62 but with a bad p-value) while the regulated downstream operon *gcvT* is upregulated. Finally, also the T-box riboswitches for the aminoacyl tRNA synthetases ThrRS and AlaRS are enriched with *SauS1* as shown in the RIP-Seq, and the downstream corresponding genes are upregulated in the  $\Delta rpsA$  mutant strain.

The CLIP-Seq approach and single-round *in vitro* transcription assays would be helpful to precisely define the role of *SauS1* in these processes (Choonee et al., 2007).

## VI. General conclusion

Despite the fact that *SauS1* is not essential, we have demonstrated its crucial role in the correct coordination of different virulence factors.

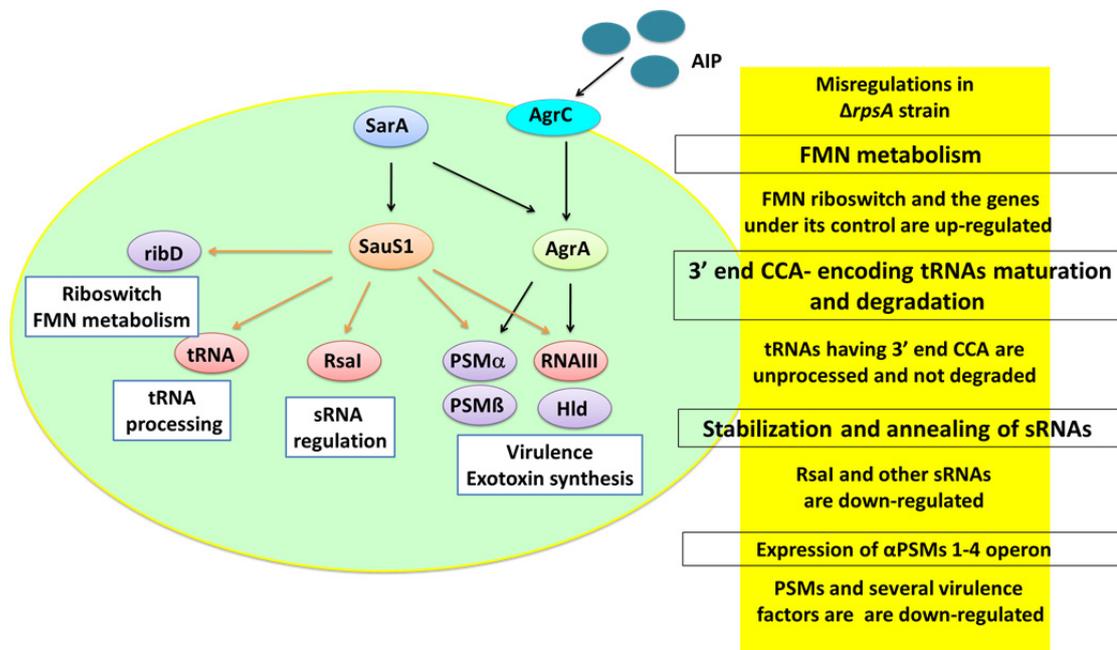
First, we have shown that it acts as translational activator of PSM $\alpha$  peptides, even if not associated on the ribosome (**Figure 35**). *SauS1* could work as an RNA chaperone on its target mRNAs, removing inhibitory structures and increasing their ability to bind the ribosome and to present their AUG start codon for initiator tRNA interaction. We have also shown that the protein is involved in the production of several secreted virulence factors thus globally impacting *Staphylococcal* virulence.

In our study we have proposed the possible involvement of *SauS1* in RNA metabolism. Analysis of isolated S1-*in vivo* complexes has allowed the identification of several classes of RNA targets whose level of expression is affected in the  $\Delta rpsA$  strain. By binding to sRNAs, not only *SauS1* could protect them from rapid degradation but also we have demonstrated, at least in one case, to promote the annealing between a sRNA and its target, probably by helping the RNA to get its functional conformation.

Comparative transcriptomic analysis has been found an up-regulation of the FMN riboswitch, *in vivo* isolated with *SauS1*, and of the genes under its control. We assume that in absence of the protein the formation of the terminator fails, thus allowing the expression of downstream genes.

Moreover we have proposed a link between *SauS1* and the degradosome. Indeed, in absence of the protein a defect of maturation and degradation of 3' end CCA- encoding tRNAs has been observed.

Taken together, my results highlight, a key RNA binding protein involved in gene expression regulation in *S. aureus*. Whether the action of *SauS1* in translation initiation as well as in regulatory mechanisms might be conserved in other Gram positive bacteria remained to be elucidated.



**Figure 35: Summarized picture of regulatory networks coordinated by *SauS1*:** AgrC activates AgrA in response of appropriate level of secreted AIP. The three *PSMs* locus are controlled both at transcriptional level by AgrA and at translational level by *SauS1*. SarA is responsible of the *rpsA* and *psm* transcript expression. Co-immunoprecipitation assay of a flag-tagged version of *SauS1* has allowed the identification of its *in vivo* targets including mRNAs, sRNAs, riboswitches and 3' end encoding CCA- tRNAs. All the identified targets are affected in the  $\Delta rpsA$  strain. Examples of misregulation per each class of molecule are given in the yellow panel.

## Materials and Methods



## Media and growth of bacteria

Pre-cultures were started from glycerol stocks. *S. aureus* was grown in Brain Heart Infusion medium (Fluka, analytical) while *E. coli* in Luria-Bertani Broth Powder microbial growth medium (Sigma-Aldrich) supplemented if needed with antibiotics (100 mg/liter ampicillin and 100 mg/liter erythromycin), at 37 °C over night under constant agitation (300 rpm). The cultures were started at  $OD_{600} = 0,02$  respecting 1:5 flask-culture volume ratio.

The tested stress conditions were applied at  $OD_{600} = 1$  and then optical density was followed each 30 minutes until the end of bacterial growth.

## Purification of SauS1

Single colony of *E. coli* M15, transformed with the pQE30 (Qiagen) plasmid carrying *SauS1*, was grown in LB medium supplemented with 100 mg/liter ampicillin and 100 mg/liter erythromycin at 37°C under constant agitation (300 rpm) over night. The pre-culture was inoculated in 1 L of fresh LB supplemented with appropriated antibiotics and incubated at 37 °C until  $OD_{600nm} = 0.6$ , when overexpression of the protein was induced using IPTG. An aliquot of 1 ml of culture was taken before the induction (BI) as control, centrifuged (2 min at 1000g, 4°C) and resuspended in 100 µL of protein loading buffer. 1, 2 and 3 hours after induction, aliquots of 1 ml each were taken as controls after induction (AI) and treated in the same way as BI. At the end of the growth the cells were pelleted by centrifugation (15 min at 4200rpm, 4 °C), washed with 10 mM Tris-HCl pH 7.5 and then resuspended in buffer A. Before sonication (120 V, 10 sec of sonication followed by 30 sec on ice, repeated at least 15 times), 3 µl of DNase (RNase free, Roche 10 U/ul), protease inhibitor cocktail 1X (Roche) and 35 mg lysozyme (Sigma), were added.

## Sonication

Bacterial lysis was obtained for sonication (120 V, 10 sec of sonication followed by 30 sec on ice, repeated at least 15 times). In order to remove cellular debris, the sample was centrifuged at 4200 rpm for 30 min, 4 °C. The supernatant (S30) was recovered and an aliquot of 15 µl was taken for SDS-page analysis. The SDS-page gel was prepared at 12%.

## First Ni-NTa column purification

The Ni-Nta agarose beads (Agarose, Qiagen) were washed using Qiagen protocol (<http://www.qiagen.com/literature/render.aspx?id=201426>) and then were resuspended in 8 ml of Buffer E. The S30 was incubated with the Ni-Nta beads for 1 hour at 4 °C under agitation. At the end of the incubation, the sample was loaded on Poly-prep chromatography column (Biorad). The flow-through (FT) was recovered in a falcon tube and the column was washed with 8 ml of Buffer E. The elution of *SauS1* fused with 6-Hys was obtained by using increasing concentration of imidazole (10 ml of imidazole at 20 mM, 50 mM and 100 mM). An aliquot from each fraction (10 µl of FT, 30 µl of wash, 20 mM, 50 mM and 100 mM supplemented with ½ of loading proteins buffer) was loaded on 12% SDS-PAGE gel.

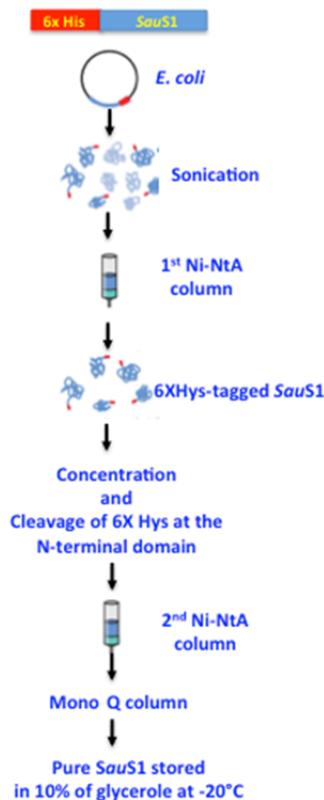


Figure 36 Schematic summary of *SauS1* purification

## Dialysis

The fractions containing *SauS1*, were dialyzed over night at 4 °C under agitation against

buffer Q. (Slide-A-Lyzer dialysis cassette, 3500 MWCO, 15 ml)

### **Protein concentration**

Dialyzed protein was concentrated using M Centrifugal filter units 15 ml, 10K tubes. The centrifugation was performed for 25 minutes at 4200 rpm, 4 °C until the protein reached a final volume of 1 ml (25 mg/ml). *SauS1* was then enzymatically digested using 120 µl of Protean 10U Tev.

### **Second Ni-Nta column purification**

The Ni-Nta agarose beads were washed as described above and then were resuspended in 8 ml of Buffer Q. The cleaved protein was incubated with the Ni-Nta beads for 1 hour at 4 °C under agitation. At the end of the incubation, the sample was loaded on Poly-prep chromatography column (Biorad). The flow-through (FT) was recovered in a falcon tube and then the column was washed with 8 ml of Buffer Q and treated with 10 ml of imidazole at 20 mM, 50 mM and 100 mM. An aliquot from each fraction (10 µl of FT, 30 µl of wash, 20 mM, 50 mM and 100 mM of imidazole, supplemented with ½ of loading proteins buffer) was loaded on 12% SDS-PAGE gel.

### **FPLC using anion exchange column**

The fractions containing the cleaved protein (FT and wash) were centrifuged for 1 hour at 8000 rpm, 4 °C in order to pellet the cellular debris and eventually formed aggregates that could obstruct the anion exchange column (GE Healthcare). Before loading the sample, the column was washed with 5 vol of mQ water, 5 vol of buffer QB and 5 vol of buffer Q (all the buffer were filtered and degassed). Upon the injection of the sample, *SauS1* was eluted by gradient of  $\text{HH}_4\text{Cl}$  (from 40 mM to 1 M, buffer QB). The fraction identified on the MonoQ analysis, having an absorbance peak at 280 nm were analyzed by polyacrylamide-SDS gel 12%. The fraction containing pure protein, were pooled and dialyzed over night at 4 °C using storage buffer. As dialysis cassettes were used the Slide-A-Lyzer dialysis cassette, 3500 MWCO. The concentration of the protein was

estimated using the specific protein extinction coefficient ( $\epsilon = 47565\text{M}^{-1}\text{cm}^{-1}$ , [www.expasy.ch](http://www.expasy.ch)). It has to be taken into account that *SauS1* does not contain any Trp and this could result in more than 10% error in the computed extinction coefficient. The purified protein was finally stored at  $-20\text{ }^{\circ}\text{C}$  in 10% of glycerol.

Buffer A : Tris-HCl pH 7,5 20 mM, MgCl<sub>2</sub> 2 mM, KCl 60 mM, NH<sub>4</sub>Cl 1 M, EDTA 10 mM,  $\beta$  mercaptoethanol 10 mM

Buffer E : Tris HCl pH 7,5 20 mM, MgCl<sub>2</sub> 2 mM, KCl 60 mM, NH<sub>4</sub>Cl 1 M, imidazole 10 mM,  $\beta$  mercaptoethanol 10 mM

Buffer Q : Tris-HCl pH 7,5 20 mM, MgCl<sub>2</sub> 2 mM, NH<sub>4</sub>Cl 40 mM, EDTA 1 mM,  $\beta$  mercaptoethanol 6 mM

Buffer QB : Tris-HCl pH 7,5 20 mM, MgCl<sub>2</sub> 2 mM, NH<sub>4</sub>Cl 1 M, EDTA 1 mM,  $\beta$  mercaptoethanol 6 mM

Storage buffer : Tris-HCl pH 7,5 20 mM, NH<sub>4</sub>Cl 40 mM, KCl 60 mM, TCEP 200  $\mu\text{M}$ , glycerol 7 %

## SDS-PAGE analysis

The SDS-PAGE gel is composed of two gels, the first of resolving and the second of running. Its concentration is depending on the size of the molecules that has to be analyzed (for *SauS1* 10 – 12%). Before loading on the gel, the samples were diluted in 1 vol of protein loading buffer and heated at  $90\text{ }^{\circ}\text{C}$  for 3 min. The migration buffer was TGS 1X and the gel was runned at 80V until the end of the resolving part and then at 150 V until the end of the run.

Resolving gel : Tris HCl pH 6,8 80 mM, SDS 0,1%, 5% acrylamide, APS 0,1%, Temed 1/1000

Running gel : Tris HCl pH 8,8 500 mM, SDS 0,1%, 12% acrylamide, APS 0,1%, Temed 1/1000

Loading protein buffer : Tris-HCl pH 6,8 60 mM, Glycerol 25 %, SDS 2 %,  $\beta$  mercaptoethanol 5 % (0,7 M), bromophenol blue 0,1 %

TGS 1x : Tris 25 mM, glycine 200 mM, SDS 0,1%

## Co-immunoprecipitation assays

Chromosomally flag-tagged *SauS1* and wild type BEJ100 strains were grown in BHI medium at 37°C, under constant agitation at late exponential phase. At the end of the growth, the cells were centrifuged for 10 min at max speed and the pellet was stored at -20 °C. Bacterial cell pellet was suspended in 2 mL of lysis buffer, transferred onto glass beads (provided by FastRNA Pro Blue Kit, Qbiogene) and processed in the FastPrep instrument (3645 s at a setting of 6.0). Samples were centrifuged at 13,000 rpm for 2 min. The supernatants were mixed with Anti-Flag M2 Affinity Gel (Sigma, A2220) and incubated at 4°C for 60 min. Then the beads were washed three times with TBS. Elution was made with 0.2 ml of 3X Flag Peptide (Sigma, F3290) prepared at the concentration recommended by the supplier. The sample was extracted with acidic phenol and then by chloroform-isoamyl alcohol. RNA was precipitated with ethanol, treated with DNase I, extracted with phenol and precipitated. The final RNA samples were dissolved in 50 ml of sterile water and lyophilized.

## Deep-sequencing analysis

cDNA-seq libraries were constructed with RNA samples isolated from Co-IP experiments under late-exponential phase of growth of the Flag-tagged *SauS1* and wild-type BJ100. The resulting cDNA libraries were sequenced on a Roche 454 sequencer using FLX and Titanium chemistry. From the resulting cDNA reads, 5'-linker sequences and polyA-tails were clipped from the sequenced cDNA reads. Only reads of  $\geq 18$  nt were aligned to the reference genome, which was retrieved from the NCBI server (accession number of the chromosome: NC\_002745.2; accession number of the plasmid: NC\_003140.1), using the program segemehl. Based on the resulting mapping data, read coverage files were generated in the GR format representing the number of mapped reads per nucleotide. The GR files were visualized in combination with FASTA and GFF files of the genome using the Integrated Genome Browser (IGB) (Nicol JW *et al.*, 2009). Additionally, overlaps of mapped reads and gene annotation positions were identified and counted. The overlap between mapped read and a gene annotation had to be at least 10 nucleotides long to be taken into account. Each single overlap counting was normalized by the number of positions to which the overlapping read was mapped and the number

of annotations that overlap with the read. For instance, if reads map to multiple regions with exactly the same score (e.g. this is the case for reads that map to the different multiple copies of the rRNA genes), only a relative fraction of one read is counted instead of a count of one read. For example, if a read maps twice, each location gets a score of 0.5 reads. Moreover, if a read overlaps two annotations, each annotation gets a score of 0.5 reads.

Lysis buffer : 20 mM Tris HCl pH8 – 150 mM KCl – 1M MgCl<sub>2</sub> – 1 mM DTT.

### **Bandshift on polyacrylamide gel**

The 5'-end labeled RNAs (50.000 cpm/line) and cold RNAs were denatured at 95 °C for 1 min, cooled on ice for 1 min and then renatured at 20 °C for 10 min in presence of 10 mM of MgCl<sub>2</sub>. The protein was incubated in *SauS1* buffer 1X + for 15 min at 37 °C. The RNAs were incubated with increasing concentration of protein, except for the first tube used as RNA control and complex formation was performed at 37 °C for 15 min. After incubation, 1 vol of glycerol blue was added in each tube before the loading on acrylamide gel containing 1mM of MgCl<sub>2</sub> in TBE 1X. The gels were runned according to the size of the RNAs at 4°C at 300 V.

*SauS1* buffer 10 X + : 200 mM Tris HCl 7.5, 600 mM KCl, 400 mM NH<sub>4</sub>Cl, 30 mM DTT, 100 mM MgCl<sub>2</sub>, 0.2mg/ml BSA.

### **Chemical probing of *S. aureus* RsaI**

Chemical probing with BzCN was performed as in Helfer *et al.* 2013 with the exception that Vic and Ned fluorescent labeled oligonucleotides (**Tab5**) have been used. Separation of RT fragments has been done using ABI PRISM 31 30XI Genetic analyzer.

### **Enzymatic probing of RsaI**

5' end-labeled RsaI (50 .000 cpm/line) was denatured in sterile water at 90°C for 1 min, cooled on ice for 2 min and briefly centrifuged. Native buffer 5X was added and the

samples are incubated at 20°C for 15 min for renaturation. 1µl of total tRNA 1 µg/µl was added to all samples. Enzymatic hydrolysis was performed by addition of 1 mL RNase as follows:

-RNase T1 10 min at 20°C

-RNase T2 10 min at 20°C

-RNase V1 5 min at 25°C

-Lead II 5 min at 20°C

0.3 M Na-acetate of pH 6.0 and cold ethanol were then added to all samples. After a vigorous mix, the samples are transferred in a dry ice-ethanol bath for 10 min. The samples are then precipitated, washed and dried and resuspended in 6 µL of urea blue.

RNase T1 ladder: labeled mRNA (25,000 cpm) is preincubated at 50°C for 5 min in 5 mL of the Buffer ΔT1 containing 1 mg total tRNA. Reaction is then performed at 50°C for 10 min in the presence of 1 mL of RNase T1 (0.5 U).

Alkaline ladder: labeled mRNA (100,000 cpm) is incubated at 90°C for 3 min in the presence of total tRNA (2 mg) in 5 mL of the Ladder Buffer.

Before loading, each sample was adjusted to contains the same amount of radioactivity (except for the ladder that should have twice more radioactivity). The samples were heated (except the RNase T1 and alkaline ladders) for 3 min at 90°C. The 12% acrylamide was prerun at 75 W for 30 min using 1× TBE as running buffer.

The samples were finally loaded on the gel, runned in TBE 1X for 2 hours at 75W.

Native buffer 5X: Hepes Na-OH pH 7.5 100mM – Mg<sup>2+</sup> Acetate 25 mM - KCH<sub>3</sub>COO 250 nM

### Footprinting RsaI\*/RsaG

5' end-labeled RsaI (50.000 cpm/line) and 1.25 µM of cold RsaG were denatured in sterile water at 90°C for 1 min, cooled on ice for 2 min and briefly centrifuged. Native buffer 5X was added and the samples are incubated at 20°C for 10 min for renaturation. The sample containing RsaI and the sample containing RsaG were mixed and incubated at 37 °C for 15 min. 6µl of total tRNA 1 µg/µl were added to all samples. Enzymatic hydrolysis was performed as described above.

0.3 M Na-acetate of pH 6.0 and cold ethanol were then added to all samples. After a vigorous mix, the samples are transferred in a dry ice-ethanol bath for 10 min. The samples are then precipitated, washed and dried and resuspended in 20  $\mu$ L of blue formamide .

RNase T1 ladder: labeled mRNA (25,000 cpm) is preincubated at 50°C for 5 min in 5 mL of the Buffer  $\Delta$ T1 containing 1 mg total tRNA. Reaction is then performed at 50°C for 10 min in the presence of 1 mL of RNase T1 (0.5 U).

Alkaline ladder: labeled mRNA (100,000 cpm) is incubated at 90°C for 3 min in the presence of total tRNA (2 mg) in 5 mL of the Ladder Buffer.

Before loading, each sample was adjusted to contains the same amount of radioactivity (except for the ladder that should have twice more radioactivity). The samples were heated (except the RNase T1 and alkaline ladders) for 3 min at 90°C. The 12% acrylamide was prerun at 75 W for 30 min using 1 $\times$  TBE as running buffer.

The samples were finally loaded on the gel, runned in TBE 1X for 2 hours at 75W.

## FRET

Two fluorophore-tagged and self-complementary RNA 21mers were incubated in FRET buffer at 37°C. Annealing reaction was started by injection of 30 nM Cy3-21R+ into a cuvette containing an equal volume of 30 nM Cy3-21R- and (**Tab6**), where applicable, 6  $\mu$ M (final concentration) of the *SauS1* protein. The reaction was allowed to proceed for 120 sec, and with Cy3 excited, donor and acceptor dye fluorescence emissions were measured once every second at 37°C. Then non labeled competitor RNA (21R-) were injected to yield a 10-fold molar excess over the labeled strands, the mixture was rapidly mixed for 2 sec, and readings were taken for another 120 sec.

Buffer FRET : 50 mM Tris HCl pH 7.5 – 3 mM MgCl<sub>2</sub> – 1 mM DTT

## Northern Blot

### In vitro transcription of RNA-DIG probes

The Digoxigenin (DIG)-labeled RNA antisense probes were *in vitro* transcribed starting

from PCR template of the gene of interest. (The primer, complementary to the 3' end of the strand encoding for the target gene, carries the T7 promoter at its 5'). The transcription was performed using DIG-RNA labelling mix Kit, according to the manufacturer protocol (Roche 11277073910). The transcript was treated with DNase I (04716728001 10 U/ $\mu$ L) for 15 min at 37°C. The reaction was arrested by the addition of 17 mM EDTA and AcNa 0.3M. The RNA-DIG were then treated with phenol/chloroforme and chloroforme/alcool isoamylique, precipitated in absolute ethanol at 20°C for 2 hours, washed, dried and resuspended in 20  $\mu$ L of mQ water. Their size and integrity were verified on 1% agarose gel in presence of guanidium thiocyanate 20 mM and ethidium bromide (5% vol/vol), runned for 30 min at 135 V. The use of DIG-labeled probes was used to follow the expression profile of *rpsA* mRNA in different bacterial strains, while DNA-labeled probes were used for the detection of the *psm* mRNA.

### **Migration, transfer, hybridization and detection**

Total RNA extracted at different point of bacterial growth, was loaded on 1% agarose gel, runned at 4 °C (120 V, 3h). The RNA was then transferred on a positively charged membrane (Hybond Dutscher RPN303B) by capillarity or by vacuum absorption and cross-linked on it. The membrane was pre-hybridized for 45 min at 68°C. 1 to 5  $\mu$ L of the RNA-DIG probe and the hybridation buffer, were heated for 5 min at 75°C, while the membrane hybridization was carried out at 68°C over night. The probe can be stored at -20°C, and could be reused for mounth. The membrane was washed twice for 5 min at room temperature for 5 min using wash buffer n°1 and than using the buffer n°2 for 15 min at 68°C. The membrane was than treated for 30 min with blocking buffer (Roche 11 096 176 001) diluted in 1X maleic acid buffer. 2  $\mu$ L of anti-DIG antibodies (Roche 11 093 274 910) were incubated to the membrane for 30 min at room temperature. The membrane is finally washed twice using maleic/tween solution for 15 min. The addition of bioluminescent CDP-star (Roche 70427821) substrate, has allowed the detection.

Hybridization buffer : Formamide 50%, SSC 5x, NaPO<sub>4</sub> pH 7 50 mM, blocking solution 2% (Roche 11 096 176 001 diluted in maleic acid pH 7,5 VWR 8.00380.1000), N-Lauroyl sarcosine 0,1% (Sigma L7414), SDS 7%

Wash buffer n°1 : 2x SSC, 0,1% SDS

Wash buffer n°2 : 0,2x SSC, 0,1% SDS

SSC 20 X : 3M NaCl, 300 mM Sodium Nitrate

### **30S ribosomal subunits purification**

Purification of the 30S subunits from *S. aureus* was performed by dissociation of the 70S ribosomes. First, full 70S ribosomes were prepared as described in Khusainov et al., 2016b. The 70S ribosomes were diluted with buffer G until final concentration 6-8 mg/mL. The sample was dialyzed against 1 L of dissociation buffer for 2h. The solution was diluted in dissociation buffer until concentration 7 mg/mL, 500  $\mu$ L were layered onto 0 – 30% sucrose density gradients prepared in dissociation buffer and centrifuged at  $58,357 \times g$  for 15 h using a Beckman SW28 rotor (Figure 1B). After fractionation the purity of each peak was analyzed by loading 0.03 AU<sub>260</sub> of each fraction on 1% agarose gel in TAE buffer. The fractions containing mostly 30S ribosomal subunit were pooled together and dialyzed twice for 2 h against 1 L of buffer G. Sample was then concentrated using Centricon MWCO 100K until 150 – 170 AU<sub>260</sub>/mL. Aliquots of 6  $\mu$ L were flash frozen in liquid nitrogen and stored at -80 °C.

Buffer G: 10 mM Hepes-KOH pH 7.5, 50 mM KCl, 10 mM NH<sub>4</sub>Cl, 10 mM Mg(OAc)<sub>2</sub>

Dissociation buffer: 10 mM Hepes-KOH pH 7.5, 100 mM NH<sub>4</sub>Cl, 1 mM Mg(OAc)<sub>2</sub>, 1 mM DTT

Gel in TAE buffer: 40mM Tris, 20mM acetic acid, 1mM EDTA

### **Toeprinting assay**

#### **5' end Labeling of oligonucleotides**

The DNA oligonucleotides were labeled using  $\gamma$  <sup>32</sup>P ATP -370 MBq/ml – 10 mci/ml. 1  $\mu$ L of oligonucleotides at 10  $\mu$ M, was mixed with 1  $\mu$ L of Buffer PNK 10X A (Fermentas), 1  $\mu$ L of T4 PNK 10U/ $\mu$ L enzyme (Fermentas) and 5  $\mu$ L of  $\gamma$  ATP. The reaction was carried out in a total volume of 10  $\mu$ L at 37 °C for 1 hour. The labeled oligonucleotides were then purified on Micro Bio-spin chromatography column (Biorad) according to the manufacture protocol. The level of radioactivity was measured using Multi-purpose Scintillator counter (Beckman).

## Toeprinting

The toeprintings were carried out using radiolabeled oligonucleotides.

The formation of a simplified translational initiation complex (30SIC) was done using published procedure, Fechter *et al.* 2009.

Buffer toe (-) 5x : Tris-HCl pH 7,5 100 mM, NH<sub>4</sub>Cl 300 mM, DTT 5 mM

Buffer toe(+) 5x : buffer toe (-) 5x , MgCl<sub>2</sub> 50 mM

## Sequences preparation

For the preparation of sequences were used ddNTs. In each mix there were 0,3 μM of RNA and 200.000 cpm of radioactivity. The RNA was denatured at 90 °C for 1 min, cooled for 1 min on ice and incubated in AMV 1V commercial buffer. For instance, for the tube « U », 1 mM of dTTP, dCTP, dGTP, 0,25 mM of dATP and 2 mM of ddATP were mixed in presence of 0,8 U AMV (007S-1), in a total volume of 26 μL. The RT was performed at 37 °C for 30 min.

The RNA template is then destroyed with 0,25 M of KOH and 40 μL of destroy buffer. The sample were putted for 1 min at 90°C, followed by an incubation of 15 min at 37°C. 0,4 mM of acetic acid were added in order to adjust the pH, then the sample was precipitated, centrifuged, washed, dried and resuspended in 10 μL of urea blue. Before loading on the gel, the samples were putted 3 min at 90°C.

Destroy buffer : Tris HCl 50mM pH8, SDS 0,5%, EDTA 7,5mM

**Table 6 List of plasmids**

Plasmid	Description
pUC18-RsaE	pUC18 expressing RsaE under T7 promoter
pUC18-RsaH	pUC18 expressing RsaH under T7 promoter
pUT7-RsaG	pUT7 expressing RsaG under T7 promoter
pCN51-RsaA	pCN51 expressing RsaA under T2 promoter
pQE30Xa	6 X Hys-Tag plasmid

**Table 7 List of oligonucleotides**

<b>NAME</b>	<b>USAGE</b>	<b>Sequences are 5' end to 3' end</b>
Rsal Vic	Probing	AAA AAA ATC GGc TAG TCA CG
Rsal Ned	Probing	AAA AAA ATC GGC TAG TCA CG
<i>S. aureus</i> Rsal	PCR Amplification	Fw: TAA TAC GAC TCA CTA TAG TAA CAG GGG GAG CGA TTA
<i>S. aureus</i> Rsal	PCR Amplification	Rw: AAA AAA ATC GGC TAG TCA CG
Cy3 oligonucleotides	FRET	AUG CAC CGU AAA GC
CY5 oligonucleotides	FRET	GCU UUA CGG UGC UA
Cold compeitor	FRET	AUG CAC CGU AAA GC

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# *Staphylococcus aureus* protein S1, an RNA chaperone involved in translation initiation and sRNA regulation

## Résumé

Bien que l'initiation de la traduction soit un processus conservé entre les bactéries, nous avons montré que le mécanisme par lequel les ARNm structurés sont reconnus et adaptés sur le ribosome diffère chez *Staphylococcus aureus*, un micro-organisme avec un bas taux de G+C et chez *Escherichia coli*. Une particularité du ribosome de *S. aureus* est l'absence de la protéine ribosomale S1, qui non seulement est plus courte que celle de *E. coli* mais qui possède également une organisation distincte des domaines. Mes expériences suggèrent que la protéine S1 (*SauS1*) favorise spécifiquement l'initiation de la traduction de l'opéron  $\alpha$ -*psm* 1-4 en liant son ARNm hautement structuré. En outre, il influence aussi l'expression et la production de facteurs de virulence comme les exotoxines ( $\alpha$ -hémolysine,  $\delta$ -hémolysine et  $\gamma$ -hémolysine) et les exoenzymes (protéases et lipases). En plus de son rôle dans la traduction, *SauS1* pourrait être impliquée dans d'autres processus cellulaires tels que le métabolisme de l'ARN et la régulation par des ARN non-codants (ARNnc). Elle forme des complexes *in vivo* avec plusieurs ARNnc dont la stabilité serait affectée dans la souche délétée du gène *rpsA* codant S1. *SauS1* a donc une activité chaperonne favorisant la cinétique d'appariement entre deux molécules d'ARN et au moins dans un cas, elle stimule la reconnaissance entre un ARNnc et son ARN cible.

Ainsi, *SauS1* appartient à une nouvelle classe de chaperons d'ARN qui jouent un rôle clé dans la régulation du virulon de *S. aureus*.

Mots-clés:

*S. aureus* S1, traduction, ARNnc, régulation du virulon

## Résumé en anglais

Even if translation initiation is a conserved process among bacteria, we have recently shown that low G+C content Gram-positive, such as *Staphylococcus aureus*, differ from *E. coli* on the mechanism by which structured mRNAs are recognized and adapted on the ribosome. One peculiarity of the *S. aureus* ribosome is the absence of ribosomal protein S1, which is shorter than *E. coli* S1 and has different domains organization. My work could demonstrate that *S. aureus* S1 (*SauS1*) specifically promotes translation initiation of the  $\alpha$ -*psm* 1-4 operon by binding its highly structured mRNA. Moreover, it influences the expression and production of other exotoxins ( $\alpha$ -haemolysin,  $\delta$ -haemolysin and  $\gamma$ -haemolysins) and exoenzymes (proteases and lipases). Besides its role in translation, *SauS1* could be implicated in other cellular processes such as RNA maturation/degradation and sRNA-mediated regulation. It forms *in vivo* complexes with several sRNAs whose level is affected in a strain deleted of *rpsA* gene, coding for S1. Preliminary results show that *SauS1* has a chaperone activity promoting the kinetic of annealing of two model RNA molecules and at least in one case, we could demonstrate that it stimulates the recognition between a sRNA and its target RNA.

Taken together, *SauS1* belongs to a new class of RNA chaperones that play key roles in the regulation of *S. aureus* virulon.

Key words:

*S. aureus* S1, translation, sRNA, regulation of *S. aureus* virulon