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Le facteur de terminaison de la transcription Rho, un acteur de la virulence et de l'adaptation aux stress chez *Staphylococcus aureus*

Transcription termination factor Rho, a peculiar player in virulence and stress adaptation in *Staphylococcus aureus*

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List of abbreviations

aa	Amino acid
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil
ADP	Adenosine diphosphate
ATP	Adenosine triphosphae
UDP	Uridine diphosphate
asRNA	Antisense RNA
cDNA	Copy DNA
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
eDNA	Extracellular DNA
mRNA	Messenger RNA
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ssRNA	Single-stranded RNA
tRNA	Transfer RNA
agr	Accessory genes regulator
AIP	Auto-inducing peptide
AMPs	Antimicrobial peptides
CcpA	Carbon catabolite control protein A
CHIPS	Chemotaxis inhibitory protein of Staphylococcus
Clf	Clumping factor
Coa	Coagulase
Efb	Extracellular fibrinogen-binding protein
EHEC	Enterohaemorrhagic *E. coli*
FNBP	Fibronectin binding proteins
Hla	α-Hemolysin
ica	intracellular adhesion
ICU	Intensive care unit
MSCRAAMS	Microbial surface components recognizing adhesive matrix molecules

PBP	Penicillin binding protein
PIA	Polysaccharide of intracellular adhesion
PNAG	β-1,6-N-acetyl-glucosamine
PSM	Phenol soluble modulins
PVL	Panton Valentine Leukocidin
ROS	Reactive oxygen species
Rsa	RNA of *S. aureus*
SA	Super antigen
SaPI	Staphylococcal pathogenicity island
Sbi	Staphylococcal binder of IgG
SCIN	Staphylococcal complement inhibitor
SE	Staphylococcal enterotoxins
SODs	Superoxide dismutases
Spa	Staphyloccocale protein A
SrrAB	Staphyloccocal respiratory response AB
TCS	*Two components system*
ssl	Super antigen like protein
TSST	Syndrome du choc toxique
VISA	Vancomycin intermediate S. aureus
VRSA	Vancomycin resistant S. aureus
vWbp	Willebrand factor binding protein
°C	Celsius degrees
2C	*Complex capture*
ANOVA	*Analysis of variance*
bp	Base pair
CLASH	*Cross-linking, ligation and sequencing of hybrids*
CLIP	*Cross-linking and immunoprecipitation*
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
GO	Gene ontology
GradR	Glycerol gradient sedimentation with RNase treatment and mass spectrometry
h	Hours
iRIL	Intracellular RNA interaction by ligation
J	Joule
kb	Kilobase
l	Liter
M	Molar
m	Mole
MAPS	*MS2-affinity purification coupled with RNA-seq*

Mbp	Mega base pair
nt	Nucleotide
OD	Optical density
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PTex	*Phenol-toluol extraction*
RBPome	*RNA binding proteome*
RIC	*RNA interactome capture*
RIL-seq	*RNA interaction by ligation and sequencing*
RIP	*RNA immunoprecipitation*
rpm	Revolutions per minute
SEC	*Size exclusion chromatography*
SELEX	*Systematic evolution of ligands by exponential enrichments*
seq	Sequencing
UV	Ultraviolet
V	Volt
X	Times

Ca	Calcium
CO	Carbon monoxide
DIG	Digoxygenine
DTT	Dithiothreitol
EDTA	eEhylenediaminetetra-acetic acid
FMN	Flavine mononucleotide
Mg	Magnesium
NO	Nitric oxide
PCI	Phenol, chloroform isomalylic acid
SDS	Sodium dodecyl sulfate

CDS	Coding sequence
ORF	Cpen reading frame
RBS	Ribosome binding site
SD	Shine and Dalgarno
UTR	Untranslated region

Amp	Ampicilline
Cam	Chloramphénicol
CCR	Carbon catabolite repression
DNase	Desoxyribonuclease
Ery	Erythromycin

GFP	*Green fluorescent protein*
Hfq	*Host factor Qβ*
RNase	Ribonuclease
CSD	Cold shock domain
CSP	Cold shock protein
CTD	C-temrinal domain
dsRBD	Double-stranded RNA-binding domain
EC	Elongation complex
KH	K homology domain
NTD	N-terminal domain
OB	Oligonucleotide/oligosaccharide binding
PBS	Primary RNA binding site
RBD	RNA binding domain
RBP	RNA binding protein
RNAP	RNA polymerase
RNP	Ribonucleoprotein
RRM	RNA-recognition motif
rut	Rho utilisation site
SBS	Secondary RNA binding site
TEC	Transcription elongation complex

Table of contents

Chapter I

Thesis summary in French

Résumé de thèse

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Titre du sujet de thèse : Le facteur de terminaison de la transcription Rho, un acteur de la virulence et de l'adaptation aux stress chez *Staphylococcus aureus*

Introduction

Chez les bactéries à Gram-positif, tel que le pathogène opportuniste *Staphylococcus aureus*, la régulation post-transcriptionnelle des gènes est principalement assurée par les ARN régulateurs ou sARN. Chez *S. aureus*, la régulation médiée par les sARN n'a jusqu'à présent pas été associée à l'action de protéines liant l'ARN, telle que Hfq nécessaires à ces réseaux de régulation chez les bactéries à Gram-négatif.[1] Néanmoins, le facteur de terminaison de la transcription Rho, conservé chez la grande majorité des bactéries a été impliqué dans la répression de la « transcription pervasive ».[2,3] Ce phénomène, répandu chez de nombreux organismes, résulte d'une transcription à partir de promoteurs cryptiques ou d'une translecture transcriptionnelle. Les transcrits générés sont majoritairement antisense et peuvent entrainer des perturbations non négligeables de la physiologie bactérienne.[4] Rho participe à d'autres fonctions régulatrices tel que la régulation de riboswitchs ou la terminaison prématurée de certains transcrits dans un phénomène connu comme polarité transcriptionnelle.[5,6] Même si cette protéine est bien conservée chez les bactéries, ses fonctions ont évolué en fonction de l'organisme. Ce facteur de terminaison a été amplement étudié chez *Escherichia coli* du fait de son caractère essentiel dans ce modèle.[2] Chez *Bacillus subtilis*, Rho est impliqué dans la régulation de la motilité, la sporulation et l'adaptation à la phase stationnaire, caractérisée par d'importants remaniements physiologiques.[7,8] Enfin, chez *S. aureus*, Rho qui est non-essentiel, a récemment été décrit en tant qu'atténuateur de la virulence.[9] Il s'avère que la délétion ou l'inactivation de Rho ont pour effet d'augmenter la virulence de la bactérie en sur-exprimant le régulon du système à deux composantes SaeRS par un mécanisme encore inconnu.[9]

La régulation de la virulence chez *S. aureus* est composé de réseaux complexes faisant intervenir de nombreux acteurs tels que les systèmes à deux composantes comme SaeRS mentionné précédemment ou le système Agr, ainsi que les sARN. Notamment, le système Agr

répondant à la densité cellulaire ou *quorum sensing* est un régulateur et activateur global de la virulence. Ce système active l'expression de son effecteur principal, l'ARN régulateur ARNIII.[10] Il s'agit d'un ARN fortement structuré de 514 nucléotides comprenant 14 tiges-boucles.[11] Cet ARN régule l'expression de nombreuses adhésines et toxines nécessaires à ce pathogène pendant l'infection. De façon globale, l'ARNIII coordonne le passage d'un mode d'adhésion retrouvé en début d'infection à un mode de dissémination nécessaire dans une étape plus tardive de l'infection. L'ARNIII régule ses ARN messagers (ARNm) cibles par des interactions ARN-ARN qui entraînent des effets sur leur stabilité ou leur traduction par le ribosome.[12] Notamment, les duplex formés par l'ARNIII et ses cibles peuvent être clivés par l'endoribonucléase spécifique de l'ARN double brin la RNaseIII résultant dans leur dégradation[13,14,15]

Objectifs

Les objectifs principaux de ces travaux de thèse ont été de caractériser les fonctions et les mécanismes de l'action de Rho chez *S. aureus,* en identifiant les cibles ARN de Rho ainsi que d'étudier ses rôles dans la régulation de l'expression des gènes. Plus précisément, l'objectif initial fut de caractériser l'implication de Rho comme un atténuateur de la virulence. J'ai identifié les gènes affectés par la délétion de *rho*, notamment ceux impliqués dans la virulence par des analyses de transcriptomique différentielle ainsi que par l'identification de l'interactome ARN de Rho par RIP-seq. En parallèle, le but fut également d'étudier la régulation de l'expression du gène *rho*, déjà décrite dans d'autres organismes mais inconnue chez *S. aureus,* pour mieux comprendre le rôle et la place de Rho dans les réseaux de régulation de la virulence.

Résultats

I. Étude de l'expression de *rho* et de sa régulation

Afin de mieux comprendre l'importance et les rôles biologiques de Rho chez *S. aureus,* il est essentiel de déterminer comment le gène est exprimé et régulé. Ainsi, l'expression de l'ARNm de Rho a été suivie dans différentes conditions de croissance. Les résultats ont montré une diminution de l'expression en phase stationnaire de croissance, pointant vers une possible

répression du gène lorsque la densité cellulaire augmente. De plus, l'ARNm a également été suivi dans des souches délétées dans différents systèmes à deux composantes, ce qui a montré que l'expression de *rho* est dépendante du système de *quorum sensing* Agr. Comme mentionné précédemment, l'effecteur principal du système Agr est l'ARNIII. Ainsi, nous avons suivi l'expression de *rho* dans un mutant de délétion de l'ARNIII, ce qui a révélé que la répression de *rho* en phase stationnaire est dépendante de cet sARN. Des analyses *in silico* ont prédit une interaction entre les deux ARN impliquant la tige-boucle 9 de l'ARNIII (résidus 290-329) et la région codante de l'ARNm *rho*. Cette interaction ARN-ARN a été confirmée par des expériences *in vitro* de gel retard en utilisant un ARNIII radiomarqué. De plus, ce duplex formé est capable d'être clivé *in vitro* par la RNaseIII au niveau de la tige-boucle 7 de l'ARNIII. Des expériences de chromatographie d'affinité sur l'ARNIII ont également permis de mettre en évidence cette interaction *in vivo*. Pour valider le rôle de la RNaseIII et des boucles 7 et 9 de l'ARNIII dans la régulation de *rho*, l'expression du messager *rho* a été suivie dans les souches sauvage, délétées de la RNaseIII ou de ARNIII, et complémentées par l'ARNIII délété des boucles 7 et 9.

II. Les rôles de Rho dans la régulation de la virulence de *S. aureus*

A) Identification des cibles de Rho

Des analyses de transcriptomique différentielle ont été réalisées en phase exponentielle de croissance dans une souche sauvage exprimant *rho* et un mutant de délétion de *rho*. Ces données de RNA-seq ont été utilisées pour construire la plateforme de visualisation de données *S. aureus* Expression Data Browser générée par Genoscapist. De nombreux ARN antisense ainsi que des gènes de prophages ont été surexprimés dans le mutant, en accord avec le rôle de Rho dans la suppression de la transcription pervasive. De même, de nombreux gènes de virulence codant pour des toxines, des adhésines ainsi que des protéines d'évasion du système immunitaire de l'hôte, ainsi que *saeP*, un des gènes constituant le système à deux composantes SaeRS, ont été fortement enrichis dans le mutant *rho*, confirmant l'effet atténuateur de Rho décrit précédemment. Parmi les gènes régulés par Rho se trouvent l'opéron de synthèse de la riboflavine (*rib*) sous le contrôle du riboswitch FMN (Teg74) ainsi que le gène codant pour la protéine Chp pour *chemotaxis inhibitory protein*, une protéine anti-inflammatoire utilisée par la bactérie pour échapper à la réponse immunitaire innée de l'hôte. Une translecture transcriptionnelle du gène *chp* semblerait avoir lieu en absence de Rho, et pourrait être à l'origine de sa surexpression. Par ailleurs, le riboswitch FMN a déjà été associé à une

terminaison Rho-dépendante dans d'autres bactéries et il se pourrait qu'elle soit conservée chez *S. aureus*. Ainsi, les gènes *chp* et *teg74* sélectionnés pour réaliser des fusions transcriptionnelles à la GFP ont déterminé l'effet de Rho sur la terminaison de leur transcription.

Dans le but d'identifier les cibles ARN de Rho, des expériences d'immuno-précipitation de Rho (RIP-seq) ont été réalisées en utilisant une souche de *S. aureus* exprimant de façon endogène une protéine Rho portant à son extrémité C-terminale une étiquette 3xFLAG. Ceci a permis de retrouver une corrélation avec les données de transcriptomique car les ARNs de nombreux gènes surexprimés dans le mutant *rho* ont été co-élués avec la protéine. Ceci suggérerait une régulation directe de Rho sur ces candidats, entraînant potentiellement leur terminaison prématurée et ainsi la répression de leur expression.

B) Études phénotypiques du mutant *rho*

Ensuite, de manière à valider l'impact de Rho sur la virulence de *S. aureus*, différents tests phénotypiques ont été réalisés, notamment du fait que de nombreux facteurs d'évasion immunitaire et d'adhésion sont affectés par Rho (en collaboration avec l'équipe de F. Vandenesch du CIRI à Lyon). La méthode immuno-enzymatique ELISA a été appliquée à des surnageants de différentes cultures afin de doser les quantités des hémolysines HlgC et Hla (toxines dont l'expression est réprimée par Rho), ce qui a révélé une accumulation de ces toxines en absence de Rho. De plus, des essais d'adhésion au fibrinogène, un facteur de la coagulation sanguine, ainsi que des expériences de cytotoxicité sur des monocytes humains et des globules rouges ont montré que le mutant *rho* adhère plus et est plus cytotoxique que la souche sauvage. Le mutant produit également plus de coagulase et de biofilm en début de croissance que la souche sauvage. Tous ces résultats confirment ainsi l'effet répresseur de Rho sur certains mécanismes de la virulence de ce pathogène humain. Ces résultats suggèrent que Rho permettrait à la bactérie de coloniser son hôte en début d'infection, et serait inactive lorsque les enzymes nécessaires à la destruction des tissus, telles que les hémolysines, sont requises.

III. La purification d'une protéine recombinante Rho de *S. aureus*

Afin de valider ces interaction Rho-ARN et d'identifier le mécanisme de régulation des cibles par Rho, la purification de Rho de *S. aureus* est nécessaire. Différentes approches de

purification ont été employées. Une protéine recombinante de Rho possédant un 6xHIS-tag en C-ter ou N-ter a été exprimée et purifiée chez *E. coli*. Cette approche a conduit à l'obtention de faibles quantités de protéine inactive et contaminée par la protéine Rho de *E. coli*. Du fait que *rho* est un gène essentiel chez *E. coli*, et afin d'éviter la contamination par la protéine de cette bactérie, nous avons exprimé et purifié Rho possédant un 6xHIS-tag en C-ter directement chez *S. aureus*. Même si l'expression et la solubilité de la protéine furent satisfaisantes, sa purification n'a pas abouti du fait de la très faible rétention de la protéine étiquetée à la colonne de chromatographie d'affinité, ce qui est dû probablement à l'inaccessibilité stérique de l'étiquette.

Conclusion

Mes travaux portés sur Rho ont révélé l'existence de nouveaux réseaux de régulation entre les différents acteurs contrôlant la virulence de *S. aureus*. Ils ont notamment permis d'identifier *rho* comme étant une nouvelle cible de l'ARNIII. Ces travaux suscitent des questions quant à l'évolution de Rho et sa spécialisation de ses fonctions chez les Firmicutes et suggèrent que la terminaison de la transcription fait partie du répertoire de mécanismes sophistiqués que *S. aureus* a développés pour s'adapter et coloniser son hôte.

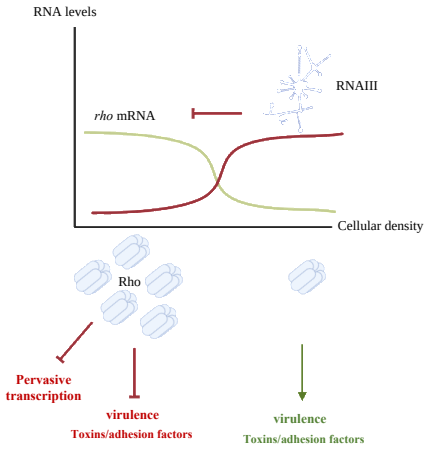

Figure 1 L'expression du transcript de *rho* dépend de la densité cellulaire grâce à l'ARNIII et contribue à la la régulation de la virulence chez *S. aureus.*

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Barrientos, L., Herrgott, L., Couzon, F., Moreau, K., Vandenesch, F., Romby, P. and Caldelari, I. (en préparation) Transcription termination factor Rho: a novel player in virulence of *Staphylococcus aureus*

Desgranges, E., **Barrientos, L**., Herrgott, L., Marzi, S., Toledo-Arana, A., Moreau, K., Vandenesch, F., Romby, P. and Caldelari, I. (2022) The 3′UTR-derived sRNA RsaG coordinates redox homeostasis and metabolism adaptation in response to glucose-6-phosphate uptake in *Staphylococcus aureus*. *Molecular Microbiology* **117**, 193–214.

Barrientos L., Mercier N., Lalaouna D., Caldelari I. (2021). Assembling the Current Pieces: The Puzzle of RNA-Mediated Regulation in *Staphylococcus aureus*. Front Microbiol 12, 706690. Revue

Communications lors de congrès

I. Communications orales

Regulating with RNA in bacteria meeting, Saint-Petersburg, Etats-Unis, Septembre 2023 - flash talk: **The termination factor Rho: a novel factor involved in stress adaptation in *Staphylococcus aureus.***

Journées de l'UPR 9002, Strasbourg, Mars 2022, présentation flash : **Transcription termination factor Rho, a novel player in *Staphylococcus aureus* stress adaptation.**

Séminaire de Microbiologie de Strasbourg (France) – mars 2022 : **RNA-binding proteins and regulation of gene expression in *Staphylococcus aureus.***

II. Posters

Regulating with RNA in bacteria meeting, Saint-Petersburg, Etats-Unis, Septembre 2023: **Barrientos**, L., Couzon, F., Moreau, K., Romby, P. et Caldelari, I. The termination factor Rho: a novel factor involved in stress adaptation in *Staphylococcus aureus.*

The New Microbiology EMBO/FEBS course, Spetses, Greece, September 2022: **Barrientos, L.**, Herrgott, L., Romby, P., et Caldelari. The Rho factor, a novel player in Staphylococcus aureus stress adaptation.

International NetRNA meeting – « RNA in gene control across kingdoms », Bischenberg (France) - mai 2022: **Barrientos, L**., Herrgott, L., Romby, P., et Caldelari, Transcription termination factor Rho, a novel player in *Staphylococcus aureus* stress adaptation.

International RiboClub Annual Meeting – « Transcriptomics and cancer biology », (visio) Septembre 2021 : **Barrientos, L.,** Herrgott, L., Romby, P., et Caldelari, I. Role of two RNA-binding proteins in the regulation of gene expression in *Staphylococcus aureus.*

Journées de l'école doctorale – avril 2021, poster: **Barrientos, L.,** et Caldelari, I. Role of two RNA-binding proteins in the regulation of gene expression in *Staphylococcus aureus.*

Chapter II

Introduction and objectives

Part 1. *Staphylococcus aureus*: a versatile human pathogen

A worldwide leading cause of nosocomial infections are attributed to the ESKAPE group of pathogens made of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter* species. The growing antimicrobial resistance associated to these pathogens place a significant burden on healthcare systems due to high mortality and morbidity rates.[1] In 2019, the six ESKAPE pathogens were responsible of 929 000 out of a total of 1,27 million deaths worldwide attributable to antimicrobial resistance and methicillin-resistant *S. aureus* (MRSA) alone counted for more than 100 000 of these deaths.[2] In high-income countries, *S. aureus* was linked to half of all fatal antimicrobial resistance cases.[2] Consequently, *S. aureus*, as a part of the ESKAPE group of pathogens, has been identified as a priority pathogen by the World Health Organization (WHO) and several global action plans to tackle antimicrobial resistance have been launched.[2]

I. *S. aureus* as a major public health concern

A) Epidemiology and diseases

Staphylococcus aureus is a Gram-positive opportunistic human pathogen with a genome consisting of a 2,8 kbp-long circular chromosome.[3] The core genome has a size of 2.3 Mbp and is composed of housekeeping genes, genes required for growth and general survival as well as many virulence genes.[4] The accessory genome contains mobile elements that include insertion sequences, plasmids, transposons, bacteriophages (i.e prophages) and pathogenicity islands that encompass genes contributing to virulence and fitness in specific environments (i.e antibiotic resistance genes such as the *SCCmec* cassette conferring resistance to β-lactam antibiotics).[4] Skin and mucous membranes of humans are a natural reservoir for this bacterium. It is stipulated that roughly 20% of general population is persistently colonized and 30% transiently.[5] Persistent colonization is found at much higher rates among patients undergoing hemodialysis, patients with type I diabetes, surgical/hospitalized patients and immunocompromised patients.[3] Notably, this colonized population faces an increased risk for consequent infections. Transmission usually requires direct contact with an infected individual and infections can be community-acquired or take place in a hospital setting. These so-called nosocomial infections are often transmitted through health care workers that have been

transiently colonized by the bacterium, the ICU environment or other patients.[3,5] Persistent carriers are at increased risk of developing nosocomial infections such as bacteremia, surgical site infections and catheter infections.[5]

Community-acquired infections can also be transmitted through contaminated food and occasionally through horizontal transfer from mothers to newborns and infants.[6,7]

S. aureus can cause a broad spectrum of infections as soon as the skin barrier or mucosa are damaged, allowing the bacterium to enter the bloodstream and reach other tissues. These infections can range from benign skin and soft tissue infections like impetigo and uncomplicated cellulitis to life-threatening conditions as bacteremia, endocarditis, osteomyelitis, sepsis and toxic shock syndrome (Fig. 1).[3] The spectrum of clinical diseases caused by *S. aureus* is singularly vast and infections are believed to remain common and serious due to growing numbers of healthcare-associated infections and community-associated skin and soft-tissue infections involving strains with particular high virulence.[8]

B) Pathophysiology

S. aureus is a highly successful pathogen due to the diverse arsenal of cell-surface proteins and secreted virulence factors that contribute to its pathogenesis. These factors allow the bacterium to evade the host immune system, adapt to different stresses encountered during infection or also respond to changes in nutrient availability. These components can act on colonization and favor adhesion or dissemination depending on the infective context.

B. 1 Colonization through cell-wall teichoic acids and microbial surface components recognizing adhesive matrix molecules (MSCRAAMS)

The first step of disease caused by *S. aureus* requires initial attachment to host tissues, mediated by teichoic acids and MSCRAAMS. MSCRAAMS are actively synthesized during exponential phase of growth and are anchored to the bacterial surface. These molecules allow the bacterium to adhere to the extracellular matrix. Among these virulence factors we find protein A (Spa), clumping factors A and B (ClfA and ClfB), fibronectin- (FnBPA and B) or fibrinogen-binding proteins. Cell-wall anchored MSCRAAMS have an N-terminal cleavable signal peptide, a C-terminal region containing a cleavable motif and a hydrophobic membrane spanning domain. **Staphylococcal protein A (Spa)** is probably the most iconic protein of this

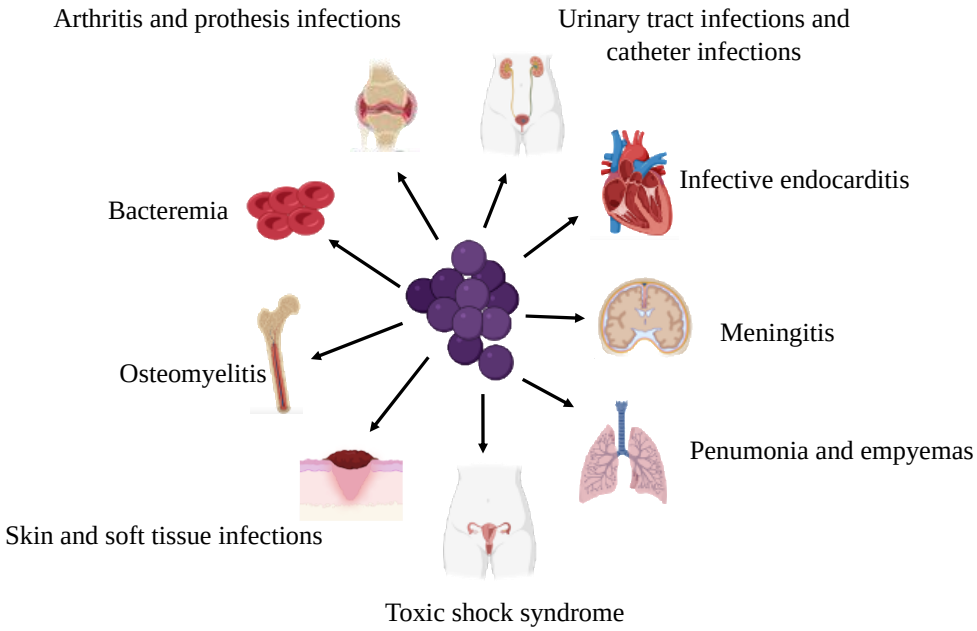

Figure 1 –Multiple infections caused by ***S. aureus*** **.**

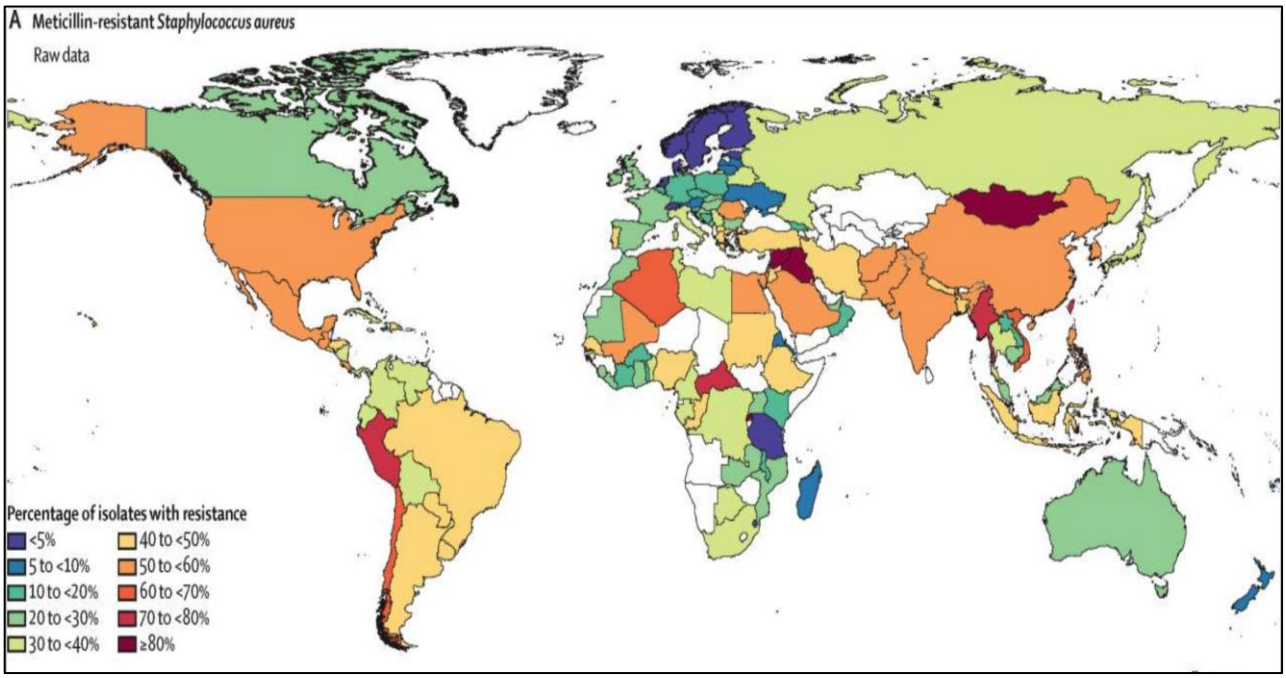

Figure 2 –Percentage of Methicillin-resistant *S. aureus* isolates by country and territory in 2019.[2]

group. This molecule recognizes the Fc of IgG to prevent opsonophagocytosis and can be found in the extracellular medium or attached to the bacterial surface to create a camouflage layer. In a similar manner, **Sbi** (Staphylococcal binder of IgG) protein also interacts with the Fc region of IgG.[9] *S. aureus* is able to attach to fibronectin, a class of extracellular matrix glycoprotein found in blood plasma and necessary for adhesion of platelets, fibroblasts and endothelial cells to accelerate wound repair, through **FnBPA and B**. These proteins can also bind fibrinogen thus contributing to adherence of *S. aureus* on plasma clots and allowing for adherence and immune evasion. Fibrinogen binding is also mediated through **ClfA and B** facilitating attachment to plasma clots and biomaterials coated with plasma such as catheters. These aggregation mechanisms are strategical to escape from immune cells in the blood. Coagulation is a key mechanism in invasive *S. aureus* infections and is used for a clinical diagnostic test of highly pathogenic strains. It is controlled by a cascade of serine proteases activated upon tissue damage. *S. aureus* secretes two proteins favoring coagulation: **coagulase (Coa)** and von **Willebrand factor binding protein (vWbp)**.[10] These proteins activate the prothrombin coagulation factor, precursor of the thrombin serine protease, leading to the polymerization of fibrin.[10] This staphylococcal coagulation mechanism not only protects bacteria from the immune system, but also promotes its dissemination to other organs.

B. 2 Evasion of the host immune system

Neutrophils, the most abundant leukocytes, play a major role in controlling and clearing *S. aureus* infection. This pathogen uses a plethora of mechanisms to avoid neutrophil action and phagocytosis. For instance, *S. aureus* can inhibit neutrophil extravasation by reducing their migration thanks to superantigen-like protein 5 (**SSL5**) and **SelX**.[11] Other factors are able to inhibit chemotaxis such as **CHIPS** (*chemotaxis inhibitory protein of Staphylococcus*) by binding and neutralizing chemo-attractants produced by neutrophils, decreasing neutrophil recruitment to the infection sites.[12]

Bacterial engulfment by neutrophils is significantly enhanced by the “marking” or opsonization of bacteria with antibodies or complement molecules (C3b). During infection, the complement system, through C3 convertase, cleaves the central complement factor C3 into C3b and this molecule is deposed on the bacterial surface which is then recognized by specific neutrophil receptors for opsonization. *S. aureus* can counteract this mechanism by synthesizing the staphylococcal complement inhibitor **SCIN**, that inhibits C3 convertase, diminishing C3b deposition. The fibrinogen binding protein **Efb** can simultaneously bind C3 and fibrinogen,

masking the C3b-covered bacterial surface with a fibrinogen coat to efficiently avoid phagocyte recognition and opsonization.[13]

S. aureus can avoid phagocytosis by macrophages through the production of capsular polysaccharides. Several clinical strains of *S. aureus* produce such capsules, with the most common capsule serotypes being 5 and 8. However, capsule production is not determinant for disease, since the main strain responsible for hospital-acquired and community infections in the USA, namely USA300, does not produce a capsule. This protection mechanism seems to be important for specific infection types such as bacteremia.[11]

Biofilm formation on medical devices is an essential component of difficult-to-treat infections. In biofilms, bacteria are surrounded with a matrix of polysaccharides such as PIA/PNAG, DNA and proteins. PIA-PNAG synthesis is regulated by the *icaADBC* operon under the control of the IcaR regulator. Biofilm structure successfully protects bacteria from phagocytosis by shielding the bacterial cells from phagocytes and prevent antibiotics diffusion.[11]

Despite the numerous mechanisms that *S. aureus* employs to avoid phagocytosis, neutrophils can eventually manage to ingest bacterial cells and entrap them in the phagosome. In this compartment, *S. aureus* must resist to attacks by reactive oxygen species (ROS) and antimicrobial peptides (AMPs). For instance, the characteristic golden color of *S. aureus* colonies is due to the production of the orange pigment **staphyloxanthin**, which protects the bacterium from oxidative killing by scavenging radicals through series of double conjugated bonds. Superoxide dismutases (SODs), catalases or lactate dehydrogenases produced by *S. aureus* allow the bacterium to maintain redox homeostasis.[11]

B. 3 Attack of the host immune system: Superantigens (SAgs) and cytotoxins

Exotoxins play a key role in causing disease since they contribute to the inactivation of the immune system responses to the infection. SAgs, such as toxic shock syndrome toxin 1 (TSST-1) are low molecular weight exotoxins with high toxicity, stability and resistance. They directly contribute to the poor prognosis of cases of severe pneumonia, infective endocarditis and menstrual toxic shock syndrome by systemically dysregulating the immune system and preventing clearance of the *S. aureus* infections, mostly resulting in fatal sepsis. These superantigenic toxins activate T-cells in a nonspecific manner and result in an exacerbated immune response including massive production and release of cytokines. There are 24 serological types of Sags, which are usually encoded on pathogenicity islands (SaPIs). *S.*

aureus is also able to cause food poisoning through the production of **Staphylococcal enterotoxins (SE)** that cause nausea and vomiting when ingested orally.

S. aureus produces a variety of cytotoxins, necessary to cause human diseases. The most studied one is **α-toxin** encoded by the *hla* gene. This cytotoxin is produced by 80% of *S. aureus* strains and is the most inflammatory molecule produced by this pathogen. Hla recognizes ADAM10 receptor on host cell membranes and forms homoheptamer structures resulting in formation of 10 nm pores. This receptor is responsible for the maturation of host pro-inflammatory signaling molecules and the recognition of Hla results in uncontrolled inflammation. Activation of ADAM10 by Hla also leads to cleavage of E-cadherin molecules, breaking adherent junctions between cells and compromising the cytoskeletons integrity leading to erythrocyte lysis. **β-toxin** (Hlb) is involved in lysis of immune cells and has also been shown to promote biofilm formation through its capacity to link extracellular DNA. ***∂*-toxin** (Hld) has been known for its haemolytic and cytolytic activity on nearly all cell types. This toxin is encoded by the gene encoding the regulatory RNA RNAIII, discussed in detail further.

Phenol soluble modulins (PSM) are three small peptides PSMα, PSMβ and Hld that form amphiphatic α-helices and result in non-specific lysis of host immune cells or red blood cells.

The bicomponent leucocidins include **Penton Valentin leucocidin (PVL)**, LukDE**, LukGH** and the complexes **HlgAB** and **HlgCB** of gamma toxin. These molecules specifically lyse leukocytes and also exhibit pro-inflammatory functions.[11]

The mechanisms that regulate the expression of all these virulence factors is discussed later.

C) Treatment of staphylococcal diseases and antibiotic resistance

Traditionally, penicillin has been the antibiotic of choice for the treatment of *S. aureus* infections. ß-lactams target the Penicillin-binding proteins (PBP) needed for cell-wall synthesis. However, due to overuses of this antibiotic, penicillin-resistant strains producing beta-lactamase, an aminoglycoside-modifying enzyme that hydrolyzes the beta-lactam ring of the drugs. were isolated as early as in 1948. Nowadays, 97% of *S. aureus* strains are resistant to penicillin. Shortly after the introduction of the beta-lactam antibiotic methicillin in the late fifties, methicillin-resistant strains emerged. MRSA strains now account for 25% of all isolates. [1] MRSA have acquired the *mecA* gene encoding PBP2a, which has a much lower affinity to

beta-lactams than the original PBP.[1] Currently, first-line treatment against MRSA infections include glycopeptide antibiotics that inhibit peptidoglycan synthesis, such as vancomycin or teicoplanin, but vancomycin intermediate/sensitive (VISA) and resistant (VRSA) are becoming more common. Resistance against vancomycin emerged after acquisition of a vancomycin resistance gene cluster *vanA* from vancomycin-resistant enterococci. This resistance is due to the production of peptidoglycan receptors with reduced affinity for glycopeptides resulting in decreased binding of vancomycin.[14] A recent study has analyzed the worldwide presence of MRSA isolates and the results are shown in Fig. 2.

II. Regulation of virulence

It has become evident that *S. aureus* is a versatile and ingenious pathogen that is able to adapt to almost any situation encountering during infection. Indeed, *S. aureus* is capable of surviving and growing in a large range of temperatures (from 4 to 44ºC), oxygen levels, pH levels (from 4.5 to 8) and salt concentrations.[15] In order to adapt to all these challenges, this pathogen has evolved a highly sophisticated and intricate network of transcriptional and post-transcriptional regulators (see below) to control the production of all the virulence factors listed above. Hence, regulation of virulence integrates host-derived and environmental signals to respond accordingly.

A) Two-component systems

Two-component systems (TCS) are the principal mechanism to sense environmental changes and adapt the physiological response. They sense an external signal that activates a membrane-bound histidine-kinase, leading to its auto-phosphorylation and subsequent phosphorylation of the response regulator. This phosphorylated response regulator is a DNA-binding protein that recognizes a specific DNA motif resulting in positive or negative alteration of gene expression. S. *aureus* encodes sixteen different TCS, of which only WalKR is essential

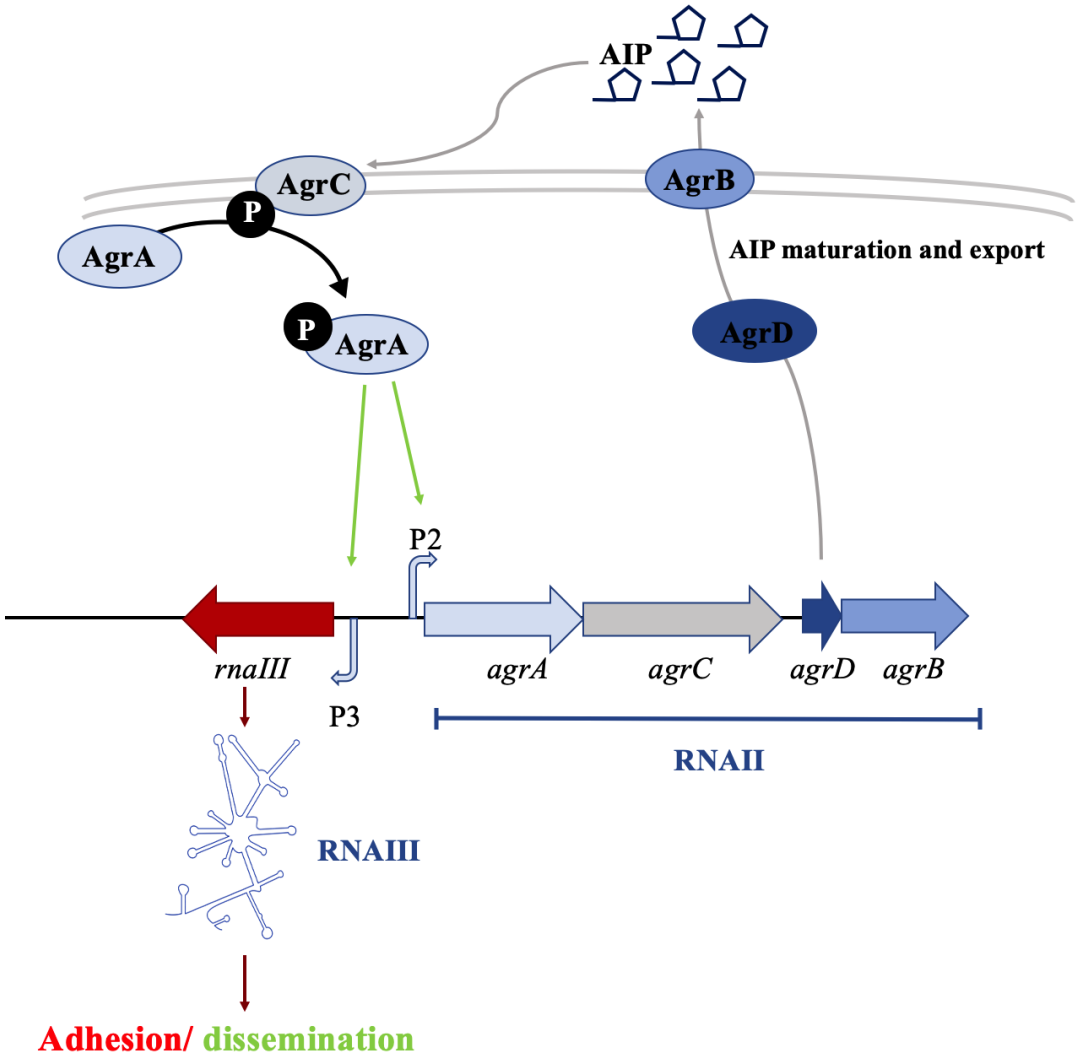

Figure 3 – Two-component quorum sensing *agr* system in *S. aureus*.
The *agr* regulon is responsible for controlling the expression of numerous virulence factors in *S. aureus*. This system is composed of two divergent operons designated P2 and P3 driving transcription of the respective RNAII and RNAIII mRNAs. The P2 operon combines a density-sensing cassette composed of the precursor peptide AgrD further matured into the auto-inducing peptide (AIP) and the transmembrane protease AgrB. The signal transduction system is composed of the histidine kinase AgrC and the response regulator AgrA. The phosphorylated and active AgrA then drives the expression of its own promoter and the divergent P3 promoter. The 514-nucleotide long transcript resulting from transcription at the P3 promoter is the main effector of the *agr* system, the regulatory RNA RNAIII. This bifunctional sRNA contains the *hld* gene encoding for the 26 amino-acid delta-hemolysin. RNAIII stimulates the expression of extracellular toxins expressed in the post-exponential phase of growth and represses the expression of surface proteins.

to growth in controlling cell wall metabolism.[15] Some TCS are directly linked to virulence, such as *agr*, *saeRS* and *arlRS*. I will mainly focus on *agr* (Fig. 3) and *saeRS* (Fig. 4) for the purpose of my thesis.

A. 1 Accessory gene regulator agr

This is the most extensively studied TCS and it acts as a major regulator of virulence. This gene cluster encodes the peptide quorum-sensing system and was first described in the mid-eighties after the finding that the inactivation of this locus by transposon insertion lead to a decrease in a significant number of exoproteins such as α, ß and ∂ haemolysins as well as TSST-1.[16] The signal sensed by this system is an 7-9 aminoacid-long autoinducing peptide (AIP) that accumulates in the extracellular environment. Once AIP concentration reaches a certain threshold (reflecting high cellular density), the system and the downstream regulatory cascades are activated. The *agr* system consists of two divergent promoters, P2 and P3, driving the expression of two transcripts named RNAII and RNAIII respectively. The RNAII transcript encodes an operon of four genes, *agrBDCA,* that build the machinery of the sensing system, composed of the density-sensing cassette (*agrD* and *agrB*) and the signal transduction system (*agrA* and *agrC*). Both components are required for the activation of the P2 promoter and transcription from the divergent P3 promoter yielding the main effector of the system, the regulatory RNA RNAIII, which regulates most *agr*-dependent targets. AgrD is the precursor of AIP and is proteolytically processed by the membrane-bound protease AgrB. AIP is secreted in the extracellular medium and is sensed by AgrC, the membrane-bound histidine kinase that autophosphorylates when the peptide reaches the critical threshold. The signal is then transferred to the response regulator AgrA, which once phosphorylated, can bind to P2 and P3 promoters and drive the transcription of the two divergent transcripts, resulting in a positive feedback loop. AgrA can also directly regulate the expression of the PSM genes, by binding to the promoters of PSM-α and ß operons and activating transcription.[17] A schematic representation of the *agr* system is depicted in Fig. 3.

Remarkably, the main effector of the *agr* system is the 514-nt long regulatory RNA molecule RNAIII, first described in 1993.[18] This regulatory RNA (sRNA) also encodes for *hld*, a 26-aminoacid long cytolytic peptide belonging to the PSM family. RNAIII regulates an

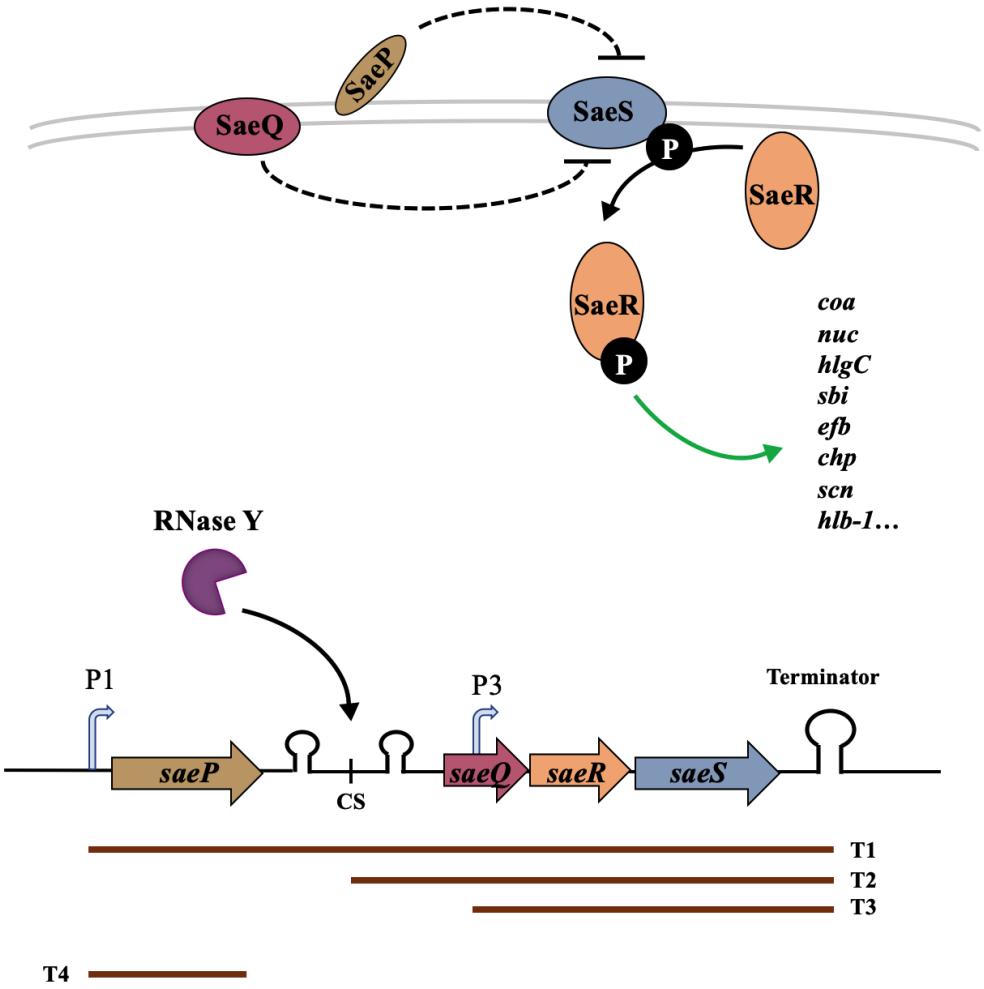

Figure 4 – Organisation of the SaeRS two-component system

The membrane-bound SaeS histidine-kinase phosphorylates its response regulator SaeR. Once phosphorylated, SaeR binds to the promoter regions of target genes and activate expression of over 20 virulence factors. SaeP and SaeQ might negatively affect the phosphorelay from SaeS to SaeR. The *sae* locus is composed of the four genes encoding the components of the system, namely *saeP*, *saeQ, saeR* and *saeS*. All four genes are transcribed from the P1 promoter, generating a long transcript comprising the four genes (T1). A second P3 promoter within the coding region of *saeQ* drives the expression of *saeR* and *saeS* generating the T3 transcript. The T1 transcript is matured by RNase Y through cleavage at its cleavage site (CS), generating the T3 transcript. The T4 transcript encoding saeP alone is generated either through processing from the T1 transcript or from premature termination of a *de novo* transcript from the P1 promoter. Thus, four different transcripts are generated and detected through transcription from the *sae* locus. Putative stem loops and intrinsic terminator are depicted.[20].

important set of well-characterized virulence factors, many of which have been described above, including Hla protein A, Coa and Sbi. The RNAIII mode of action will be discussed in detail in the next paragraph.

A. 2 SaeRS

The *sae* (*S. aureus* exoprotein expression) locus encodes the SaeRS TCS which plays a major role in controlling the expression of over 20 virulence factors including leukocidins, hemolysins, surface proteins and proteases.[19,20] The *sae* locus is composed of four genes, *saeP*, *saeQ*, *saeR* and *saeS* under the control of two promoters, P1 and P3.[19] Transcription of the histidine kinase SaeS and the response regulator SaeR is driven by the constitutive promoter P3, located in the coding region of the *saeQ* gene. The P1 promoter, located upstream the *saeP* gene, is auto-induced by the system and drives the expression of all four genes.[19] This polycistronic transcript is then processed by RNase Y downstream of *saeP*.[21] The organization of the *sae* system is schematized in Fig. 4. The roles of SaeQ and SaeP are not clear, but they are believed to assist to the return of the activated Sae system to a pre-stimulus state, by inducing SaeS phosphatase activity.[19] The activation signal of this TCS include human neutrophil peptides 1, 2 and 3 (HNP1-3) which are antimicrobial peptides produces by neutrophils, calprotectin (a calcium binding protein expressed in neutrophils suppressing *S. aureus* growth by sequestering the nutrient metal ions Zn and Mn), inhibitory concentrations of hydrogen-peroxyde and sub-inhibitory concentrations of ß-lactam antibiotics[19] The SaeRS system directly targets essential virulence factors such as *coa*, *chp*, *fnbA*, *eap*, *sbi*, *efb*, *saeP*, *hla* and *hlb* (Fig. 4).

The role of the SaeRS in *S. aureus* virulence is mainly linked to biofilm formation and host invasion. Biofilm formation can be independent of PIA/PNAG formation, through synthesis of surface or secreted proteins and extracellular DNA and the Sae regulon regulates several biofilm-promoting factors (Coa, Emp, Eap, FnBPA and B, Hla and Hlb). Additionally, by enhancing production of SCIN (staphylococcal complement inhibitor) and CHIPS (chemotaxis inhibitory protein of staphylococci) factors, the SaeRS system participates to the escape from the hosts innate immune response and disease progression.[19]

Transcriptional regulator	Description
SarA	Activates *agr* expression by binding to its P2 promoter. Post-transcriptional regulation of targets (e. g. *spa*) by binding and affecting mRNA turnover. Repression of protease production and stimulation of biofilm formation. Activation of TSST-1.[22–24]
SarR	Homolog of SarA. Negatively regulates *sarA* expression by binding to its promoters. Negatively regulates *agr* expression by binding to its P2 promoter and competes with SarA for the regulation of *agr*.[20,25]
SarT and SarU	Members of the SarA protein family. The *sarT* and *sarU* genes are adjacent and divergently transcribed. Transcription of *sarT* is repressed by *agr* and *sarA*. The agr-dependent sRNA ArtR binds to the 5'UTR of *sarT* and promotes its degradation. SarT negatively affects expression levels of RNAIII and represses *hla*. SarT represses *sarU* expression by binding to its promoter. SarU seems to positively regulate *agr* and RNAIII expression.[26–28]
SarH1/SarS	Also member of the SarA protein family, SarH1 or SarS represses *hla* and exfoliative toxins ETA and activates *spa*. SarH1 is repressed by SarA and *agr* and is activated by SarT. The *sarH1* transcript is stabilized by GdpS through interactions with its 5'UTR.[25,26,29,30]
Rot	The repressor of toxins Rot is a member of the SarA-like family and acts as a repressor of enterotoxin B, *hla*, *spl* and *ssp* proteases and lipase *geh*. Rot also represses sae transcription from the P3 promoter. Rot positively regulates spa and SarH1. Rot acts as an intermediate regulator of *agr*. Rot is repressed by SarA and by sigma factor B during stationary phase.[31–33]
MgrA	Member of the MarR/SarA protein family. Global regulator of virulence factor production, including alpha-toxin, coagulase, serine proteases and Protein A. MgrA positively regulates exoprotein production and negatively regulates surface proteins, similar to *agr*. The mgrA transcript is stabilized by RNAIII. The sRNA RsaA represses MgrA production. MgrA plays an important role in biofilm formation.[20,34–37]
SigB	The alternative sigma factor SigB responds to stresses and counterbalances the activity of the *agr* system. Expression of *sigB* (contained in a operon) is activated by the housekeeping SigA and SigB auto-regulates itself. Activity of SigB is post-transcriptionnally regulated by the binding of Rsb proteins. SigB controls expression of genes involved in virulence, biofilm formation, antibiotic resistance, among others. SigB also plays important roles in the adaptation to the lung environment durng pulmonary infections.[38–43]

Table 1– Transcriptional regulators involved in the regulation of virulence in *S. aureus*

B) Regulatory proteins

In addition, several regulatory proteins/transcription factors play a key role in regulating virulence, namely SarA, Rot, MgrA or the alternative sigma factor SigB. For details about these factors and their role please refer to Table 1.

C) Regulatory RNAs (sRNAs)

Non-coding RNAs or sRNAs have been identified and described in many bacterial species and their importance for gene regulation and physiology is now well admitted and understood. In *S. aureus* sRNAs can regulate metabolism, virulence and antibiotic resistance.[44] Their origins and mechanisms of action are varied and are reviewed in Barrientos *et al*, 2021 presented in this manuscript and compiled in Fig. 5. In summary, sRNAs can bind to their target mRNAs and affect their stability and translation, resulting in highly efficient post-transcriptional regulation and fine-tuning of gene expression. Another class of regulatory RNAs are riboswitches which are found in 5'UTR of mRNAs and regulate mRNA transcription and translation by adopting alternative structures in response to the binding of a specific ligand (metabolite, ion) (Fig. 6). In many pathogenic bacteria and for instance in *S. aureus*, sRNAs play major roles in virulence regulation, as is observed with RNAIII described below. Besides RNAIII, several other sRNAs have been linked to virulence regulation in this pathogen but I will focus solely on description of RNAIII-mediated regulation of virulence.[45,46]

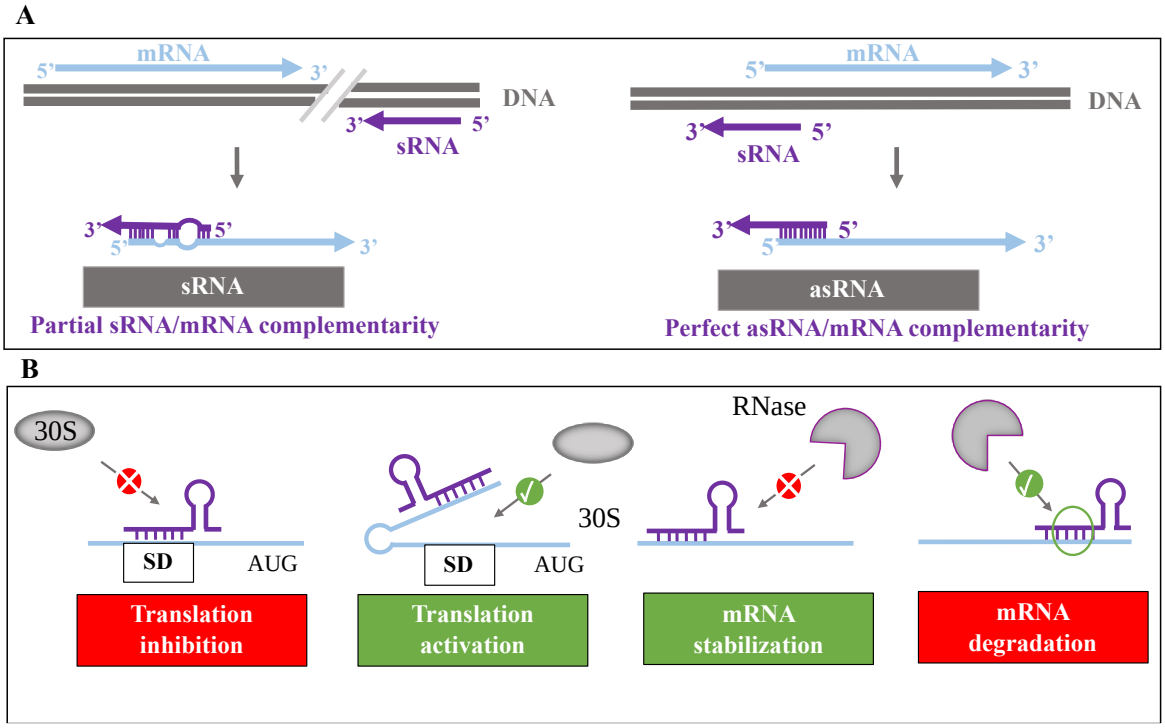

Figure 5 - Origins and mechanisms of action of sRNAs in bacteria.

A. sRNAs encoded in *trans* from their target mRNA show partial base-pair complementarity whereas asRNAs, transcribed on the opposite strand of their target mRNA show perfect base-pair complementarity. **B.** sRNA binding to their target mRNA can affect translation and/or stability. By binding to the Shine and Dalgarno (SD) region, the sRNA can either inhibit translation initiation by impeding loading of the 30S subunit of the ribosome, or it can liberate a sequestered SD sequence and activate translation of the mRNA. sRNA binding to the mRNA can also affect its stability by either protecting the mRNA from the action of an RNase (masking an RNase site) or promote degradation by creating a cleavage site for a specific RNase.

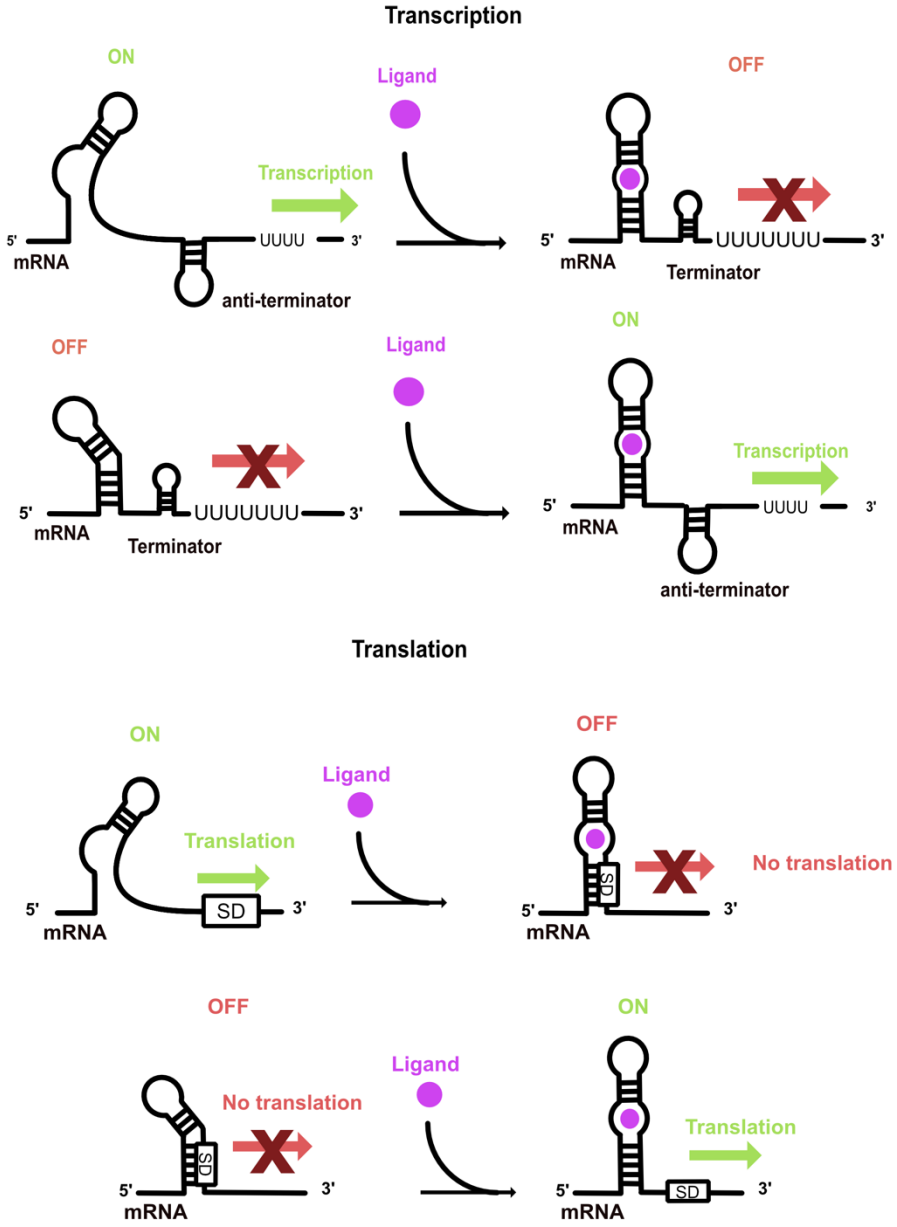

Figure 6 - Riboswitch-mediated gene regulation in bacteria.
Riboswitches are located in the 5'UTR of several mRNAs and are composed of an adapter domain acting as a receptor and binding a specific ligand and an expression platform that directs gene expression in response to binding of the ligand. Binding of the ligand leads to structural changes that impact downstream gene expression. Riboswitches can act either at the transcriptional level where binding of the ligand promotes formation or dissociation of a terminator hairpin, modulating transcription of the downstream gene. They can also act at the level of translation initiation by altering the accessibility of the SD sequence after binding of the ligand therefore modulating translation of the downstream gene.

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Assembling the Current Pieces: The Puzzle of RNA-Mediated Regulation in *Staphylococcus aureus*

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The success of the major opportunistic human *Staphylococcus aureus* relies on the production of numerous virulence factors, which allow rapid colonization and dissemination in any tissues. Indeed, regulation of its virulence is multifactorial, and based on the production of transcriptional factors, two-component systems (TCS) and small regulatory RNAs (sRNAs). Advances in high-throughput sequencing technologies have unveiled the existence of hundreds of potential RNAs with regulatory functions, but only a fraction of which have been validated *in vivo*. These discoveries have modified our thinking and understanding of bacterial physiology and virulence fitness by placing sRNAs, alongside transcriptional regulators, at the center of complex and intertwined regulatory networks that allow *S. aureus* to rapidly adapt to the environmental cues present at infection sites. In this review, we describe the recently acquired knowledge of characterized regulatory RNAs in *S. aureus* that are associated with metal starvation, nutrient availability, stress responses and virulence. These findings highlight the importance of sRNAs for the comprehension of *S. aureus* infection processes while raising questions about the interplay between these key regulators and the pathways they control.

Keywords: regulatory RNA, interconnected network, *Staphylococcus aureus*, virulence, metabolism

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INTRODUCTION

Staphylococcus aureus is a major opportunistic human pathogen capable of causing an extensive array of human infections, ranging from easy-treatable sinusitis to life-threatening endocarditis or septicemia. Its versatility in colonizing diverse human organs relies on the temporally coordinated expression of numerous virulence factors allowing the bacterium to adhere, invade and disseminate into host tissues. Regulation of virulence factors expression is conducted by two-component systems (TCS), transcriptional regulators and in particular small regulatory RNAs (sRNAs). These include *cis*-acting RNAs such as antisense RNAs or riboswitches, and *trans*-acting RNAs (Carrier et al., 2018; Jørgensen et al., 2020).

The latter generally control multiple messenger RNAs, especially by targeting their Shine–Dalgarno (SD) sequence, which results in translational repression and/or stability modulation. Indeed, many staphylococcal sRNAs contain a characteristic C-rich sequence complementary to the SD sequence of targeted mRNAs (Geissmann et al., 2009). In many bacteria, sRNA:mRNA interactions are mediated by the chaperones Hfq or ProQ. However, the role of Hfq in *S. aureus*

is still controversial and ProQ is not present (Christopoulou and Granneman, 2021). Even though staphylococcal Hfq is able to bind some sRNAs *in vivo* and *in vitro*, it does not facilitate sRNA-mRNA interactions (Bohn et al., 2007). In addition, its deletion has no effect on sRNA-mediated regulation and did not present any specific phenotype. The dispensability of Hfq may result from longer and, consequently more stable, sRNA-mRNA duplexes than the ones requiring Hfq in *Escherichia coli* (Jousselin et al., 2009).

The functions of sRNAs in gene regulation and physiological responses in bacteria are now well established. Their ability to regulate specific metabolic pathways and stress responses makes them ideal candidates to regulate virulence in pathogenic bacteria. Indeed, in *S. aureus* the bi-functional sRNA RNAIII is the main intracellular effector of the quorum sensing system and controls temporal expression of virulence genes, in addition to containing the open reading frame (ORF) for the phenol soluble modulin (PSM) hemolysin delta (Bronesky et al., 2016). Besides, RNAIII, RsaA, SprC, SprD; Teg49 and SSR42 contribute to different facets of virulence regulation in animal models of infection (Desgranges et al., 2019).

DISCOVERY OF sRNA IN *Staphylococcus aureus*

The use of predictive bioinformatic searches, microarrays and expression studies led to the discovery of the first sRNAs in *S. aureus* (Pichon and Felden, 2005; Geissmann et al., 2009; Nielsen et al., 2011). Then, the advances in high-throughput sequencing technologies opened the door to a whole new era in the small RNA field (Desgranges et al., 2020). It not only helped and accelerated the discovery of further RNAs with regulatory functions in *S. aureus* (Abu-Qatouseh et al., 2010; Beaume et al., 2010; Bohn et al., 2010; Howden et al., 2013; Carroll et al., 2016; Mäder et al., 2016), but also facilitated their characterization by promoting global analyses of transcriptional changes they induce. sRNAs are commonly encoded in intergenic regions or are originated from 3′ or 5′-UTR of mRNAs and are associated to the regulation of numerous metabolic pathways and virulence. Accessibility of these sequencing techniques accumulated huge transcriptomic data. However, the lack of a consensual and fully annotated *S. aureus* genome added to a missing unified sRNA nomenclature led to numerous redundancies and misannotated sRNAs. To overcome this matter, Sassi et al. (2015) designed the *Staphylococcus* Regulatory RNA Database (SRD) which provides a simple and non-redundant list of sRNAs identified in *S. aureus*. Sequences of transcribed sRNAs were compiled from various RNAseq analyses to yield a non-redundant catalog of ca. 500 sRNAs assigned with a single identifier. This list is drastically reduced to 50 when only *trans*-acting sRNAs are considered (Liu et al., 2018). Unfortunately, most putative 5′/3′-UTR-derived sRNAs are discarded here. Very recently, Carroll's team re-analyzed published RNAseq and ribosome profiling data scrutinizing the expression and stability or capacities to encode peptides of 303 sRNAs in different conditions, showing their diversity in behavior and functions (Sorensen et al., 2020). Altogether, these studies raise issues about the poor annotation of staphylococcal genome concerning sRNAs. Furthermore, the effort of the scientific community in sequencing genomes of many staphylococcal isolates will considerably improve it.

DIVING INTO sRNA NETWORKS

To unravel the functions of a newly identified sRNA, it is necessary to define its partners. The identification of RNA candidates as direct targets of sRNAs would provide hints of their roles and pathways, in which a specific sRNA might be involved. Several experimental techniques have been recently developed to characterize sRNA targetomes in bacteria, mostly based on the pull-down of chaperone proteins such as Hfq followed by sequencing of associated RNAs (Desgranges et al., 2020). In *S. aureus*, preference was given to a distinct approach, which relies on the co-purification of binding partners with a biotinylated/tagged sRNA. These methods called MAPS (Lalaouna et al., 2015; Mercier et al., 2021) and Hybrid-trap-seq (Rochat et al., 2018) have been used to determine the interactome of various sRNAs in *S. aureus*, generating ever more complex regulatory networks picturing many events: one sRNA involved in different pathways, several sRNA involved in the same pathway or sRNAs associated with one another (**Figure 1**). This has highlighted the complexity and intertwined nature of sRNA networks in *S. aureus*, which most certainly accounts for the versatility of this pathogen.

Overall, of the numerous sequences for potential sRNAs, only a small fraction has been experimentally confirmed and many more remain to be characterized. In this work we will review the current state of the art of the sRNA world from *S. aureus*, featuring those involved in virulence, nutrient availability, metal starvation and stress responses. We will focus predominantly on recent results deciphering functions of staphylococcal sRNAs, while others were extensively reviewed in Guillet et al. (2013), Tomasini et al. (2013), and Desgranges et al. (2019).

THE QUEST OF POWER: A LARGER ARSENAL OF sRNAs REGULATING VIRULENCE: Teg41 AND SSR42

Besides the well described RNAIII, RsaA, Teg49 and SprC/D (Desgranges et al., 2019), two sRNAs, Teg41 and SSR42, appeared recently to regulate virulence in *S. aureus*. Teg41 is a 205 nt-long sRNA that is divergently transcribed from the locus encoding alpha phenol soluble modulins (αPSM), highly potent pore-forming toxins exhibiting cytolytic activity (Zapf et al., 2019). The deletion of 24 nts in its 3′ end is sufficient to lower αPSM production at the protein level, reduce hemolytic activity and attenuate virulence in a murine abscess model. Conversely, Teg41 overexpression enhances hemolytic activity by increasing αPSM protein levels. *In silico* predictions suggest the binding of Teg41 after the start codon of αPSM4, the 4th gene of the operon and most abundant αPSM. However, this interaction remains to be confirmed as well as the activation mechanism of *psmα4*. Several

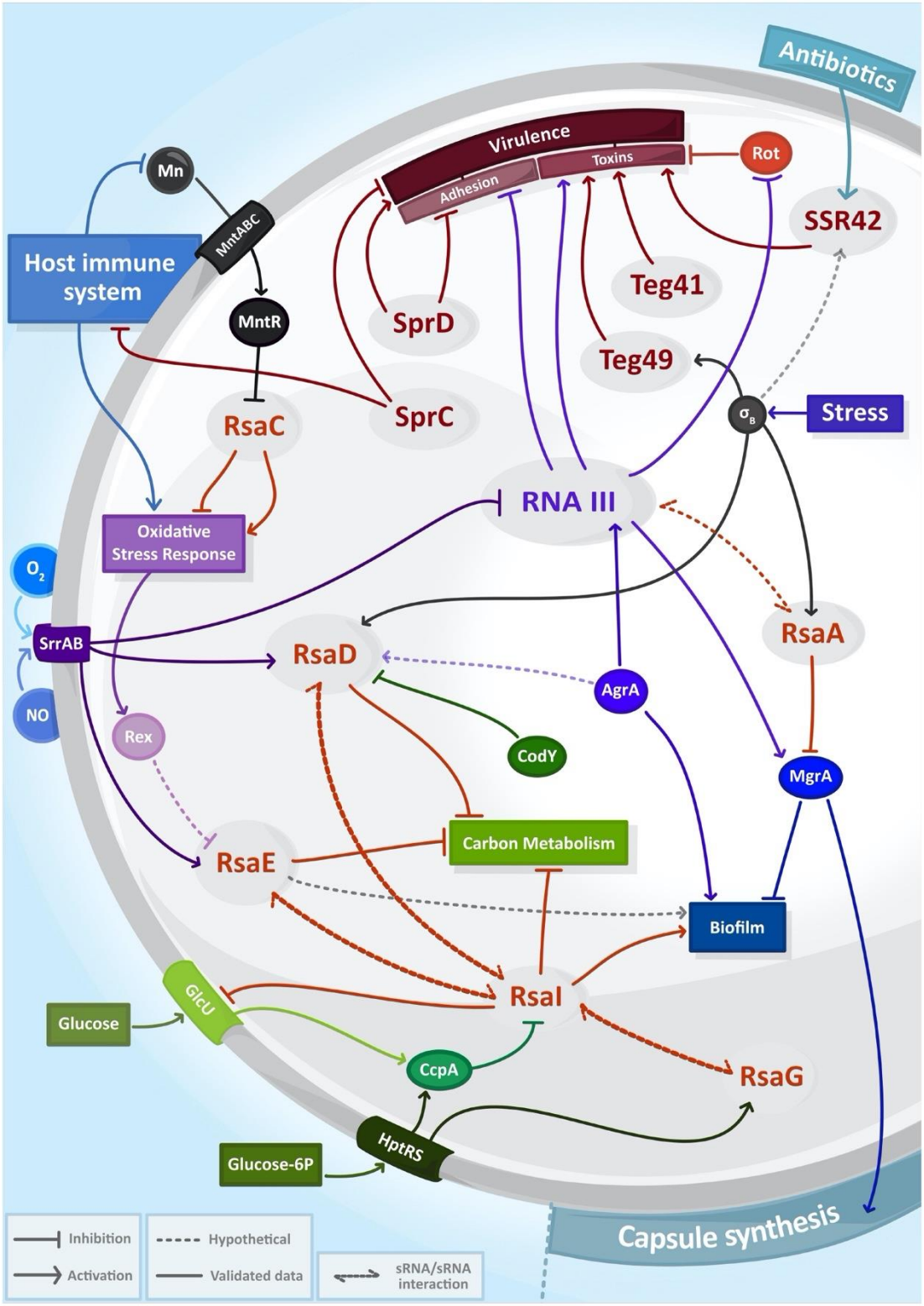

FIGURE 1 | The complexity and entanglement of regulatory RNA (sRNA) networks in *Staphylococcus aureus*. Expression of sRNAs (in gray circles) are induced by environmental signals including antibiotics, host immune system responses, exposure to reactive species (NO, O_2^-) and nutrient availability. Together with transcriptional factors (solid circle) or two-component systems, sRNAs control capsule synthesis, biofilm production, carbon metabolism, oxidative stress response or virulence and then form intricate regulatory networks.

hypotheses are raised, such as a positive regulation where Teg41 would stabilize the PSM transcript to facilitate its translation or induce conformational changes to free the ribosome binding site (RBS) together with an unknown partner. This would be the first time that an sRNA has been directly linked to the regulation of αPSM, since only protein regulators such as MgrA or AgrA are known to regulate the transcription of mRNA encoding these toxins (Jiang et al., 2018). Interestingly, the *teg41* gene is restricted to *S. aureus* and the very closely related *Staphylococcus argenteus*, and this conservation seems to correlate with the presence of the αPSM locus. This suggests that both might be genetically linked (Zapf et al., 2019).

The 1,232 nt-long SSR42 belongs to the family of small stable RNAs (SSR), a group of regulatory RNAs induced and/or stabilized during stress-related conditions such as log-phase growth, heat/cold shock or stringent response (Anderson et al., 2006; Morrison et al., 2012). SSR42 is stabilized during stationary phase where it mostly represses expression of several virulence genes through an indirect yet undescribed mechanism, probably by regulating transcriptional regulators of these virulence factors (Morrison et al., 2012). This sRNA is also required for hemolysis and for virulence in a murine model of skin infection. SSR42 is located directly upstream and in an antiparallel orientation from the gene encoding Rsp, the repressor of surface proteins, a regulator of hemolysis that positively controls the production of *hla via* the *agr* system (Li et al., 2015). Rsp activates the expression of SSR42 in presence of antibiotics as oxacillin. Consecutively, SSR42 enhances hemolysis by acting indirectly on the *hla* promoter during stationary phase (Horn et al., 2018). SSR42 therefore places itself in line with regulators such as SaeR and SarA for transcriptional activation of *hla* and RNAIII for its translation. Besides, SSR42 stabilizes the *sae* transcript encoding the major transcriptional regulator of *hla* through a yet unknown mechanism (Horn et al., 2018). This suggests that modulation of *sae* transcript impacts Hla production. SSR42 therefore participates in the complex regulation of *hla* transcription in response to antibiotics even though the molecular mechanisms remain elusive.

RsaC, THE MISSING LINK BETWEEN MANGANESE HOMEOSTASIS AND OXIDATIVE STRESS

RsaC length is highly variable across *S. aureus* isolates due to the presence of repeated sequences at its 5′ end and, consequently, ranges from 584 to 1,116 nts (Lalaouna et al., 2019). Remarkably, the characterization of RsaC provided the missing link between manganese homeostasis and oxidative stress response. The mutation of *mntABC*, coding for the major manganese ABC transporter, was previously reported as detrimental for Mn acquisition, but also for oxidative stress resistance (Handke et al., 2018). Lalaouna et al. (2019) demonstrated that RsaC derives from the 3′ untranslated region of *mntABC* after cleavage by the double-stranded ribonuclease RNase III. In manganese-limiting conditions, RsaC negatively regulates the non-essential Mn-containing superoxide dismutase A (SodA), which is involved in reactive oxygen species detoxification ($O2^-$ to H_2O_2). Concurrently, RsaC favors the SodM-dependent oxidative stress response, an alternative SOD enzyme using either Fe or Mn as cofactor. Besides helping maintain the appropriate cellular Mn^{2+} concentration, it restores the ROS detoxification pathway and counteracts Mn sequestration by host immune cells.

Noteworthy, RsaC could also interconnect and balance various metallostasis systems (Fe and Zn) (Lalaouna et al., 2019), but is apparently not involved in the MntABC-mediated increased resistance to copper (Al-Tameemi et al., 2021).

THE BLURRED LINE BETWEEN METABOLISM AND VIRULENCE: RsaD, RsaE, RsaI, AND RsaG

RsaD, a 176 nt-long sRNA, is conserved in multiple staphylococcal species (Geissmann et al., 2009). It accumulates in the late exponential phase of growth and is highly expressed in strains with an active σB factor, which is responsible of the regulation of genes involved in stress response in *S. aureus*. Nonetheless, the exact mechanism of *rsaD* regulation by this factor remains uncertain. More recently, Augagneur et al. (2020) observed that expression of *rsaD* is repressed by CodY, a global regulator activated by branched amino-acids and GTP and regulating genes involved in primary metabolism and virulence (Brinsmade, 2017). The promoter region of *rsaD* contains a putative CodY binding motif, which was detected in at least 15 staphylococcal species, indicating that the regulation of *rsaD* by CodY is probably conserved (Augagneur et al., 2020). In addition, RsaD is activated during nitric oxide (NO) stress, sensed by the TCS SrrAB (Bronesky et al., 2019) and possibly by the quorum sensing system Agr (Marroquin et al., 2019). Thus, RsaD seems to assimilate multiple signals from the environment. To determine the physiological functions of RsaD, *in silico* analyses using RNA Predator, TargetRNA2 and IntaRNA identified *alsS*, which is positively regulated by CodY and whose product is involved in carbon metabolism, as a possible target. RsaD binds the RBS of *alsSD* mRNA through its C-rich region and inhibits its translation initiation, leading to a decrease in AlsS enzymatic activity (Augagneur et al., 2020). Thus, by repressing RsaD, CodY permits AlsS synthesis. When glucose is in excess, AlsSD (acetolactate synthase/decarboxylase) generates acetoin (a neutral-pH compound) from pyruvate and therefore protects bacteria from death due to acidification of the cytoplasm by increased acetate production. Then, in these conditions, RsaD must be repressed for survival. This work revealed the *trans*-acting regulatory activity of RsaD on at least one mRNA and highlights the balancing role of this sRNA in carbon overflow and its implications in cell survival (Augagneur et al., 2020). All these mechanisms by which RsaD might be regulated, integrate, and respond to different environmental cues remain to be unveiled. Its place in the complex regulatory RNA networks of *S. aureus* awaits to be established.

RsaE is a highly conserved sRNA among the Firmicute phylum. This striking conservation emphasizes the crucial role of RsaE in metabolism adaptation. First discovered in *S. aureus*,

this 93 nt long sRNA is composed of two UCCCC motifs critical for its interaction with the RBS of its mRNA targets (Geissmann et al., 2009; Rochat et al., 2018). Its expression depends on the activation of the TCS SrrAB that responds to low oxygen concentration and NO exposure (Kinkel et al., 2013). A similar activation pattern is described in *B. subtilis* with its homolog RoxS (Durand et al., 2015). In addition, RoxS is repressed by the NAD + /NADH sensor Rex whose binding site is conserved, which suggests that Rex could fulfill a similar role in *S. aureus*.

RsaE is involved in the regulation of central metabolic pathways, in particularly by negatively regulating numerous enzymes of the TCA cycle and folate metabolism (Geissmann et al., 2009; Bohn et al., 2010; Rochat et al., 2018). Among its targets, RsaE inhibits the translation of *rocF* mRNA, which encodes an arginase responsible of converting arginine into ornithine (Rochat et al., 2018). Furthermore, the absence of RsaE stimulates growth rate in a medium containing exclusively 18 amino acids (all except glutamine and asparagine) as sole carbon sources, positioning RsaE as a major repressor of amino-acid catabolism.

Surprisingly, RsaE is processed in *S. epidermidis* and *B. subtilis* but apparently not in *S. aureus* (Rochat et al., 2018). In *S. epidermidis*, the processed form of RsaE (RsaEp) expands its targetome with the transcripts of the main biofilm repressor IcaR or of the succinyl-CoA synthetase SucCD, an enzyme involved in TCA cycle (Schoenfelder et al., 2019). Interestingly, both mRNAs only interact with RsaEp. In *B. subtilis*, RNase Y is responsible of RoxS cleavage, however, in a *S. aureus* RNase Y mutant strain, the levels of RsaE or its targets are not impacted (Marincola et al., 2012). Still, a processed RsaE could act on yet unknown mRNAs. Altogether, RsaE interferes with the TCA cycle by directly inhibiting related enzymes and by limiting the production of amino-acid alternative substrates. It has been suggested in *S. aureus* and in *B. subtilis* that RsaE balances NAD + /NADH ratio when environmental stimuli (such as O_2 concentration or NO exposure) trigger a metabolism slowdown (Durand et al., 2015).

Additionally, RsaE interacts with another sRNA named RsaI involved in sugar metabolism control (Rochat et al., 2018; Bronesky et al., 2019), that could potentially connect the regulation network of both sRNAs. It cannot be excluded that RsaE or RsaI could behave as an sRNA sponge of one another, promoting the decay or sequestration of the other partner. RsaI is a 144 nt long sRNA conserved among the *Staphylococcacea* family. The expression of RsaI is repressed by the catabolite control protein A (CcpA) in presence of glucose (Bronesky et al., 2019). When glucose has been metabolized, RsaI inhibits the translation of the main glucose uptake protein GlcU and activates enzymes acting in glucose fermentation. On the other hand, RsaI represses FN3K expression, a protein protecting the bacterium from the damages caused by high glucose concentration, positioning RsaI at the core of regulatory pathways of sugar metabolism. Interestingly, RsaI binds the 3′UTR of *icaR* mRNA and thus promotes biofilm formation by a mechanism which is still unsolved (Bronesky et al., 2019). To note, the *icaR* messenger was pulled out with RsaE *in vitro*, and sequencing suggested that it interacts with the 5′UTR of *icaR* such as in *S. epidermidis* (Rochat et al., 2018). Knowing that RsaE and RsaI form a duplex, further experiments would be necessary to decipher the intricacy of regulatory lines among all these RNAs.

In addition to RsaE, RsaD (see above) and the glucose-6-phosphate induced sRNA RsaG were enriched with RsaI in MAPS, but the relevant significance of these interactions has not been explained yet (Bronesky et al., 2019). Interestingly, it has been suggested that RsaI promotes the expression of NO detoxification or anaerobic metabolism enzymes as an indirect effect of its interaction with RsaE, RsaD and RsaG. Nevertheless, shared signals and targets between these sRNAs imply tight connections and that all these regulatory networks would rationally impact each other at different levels, connecting sugar metabolism and stress responses.

CONCLUSION

In the recent years, several tools were developed to decipher the functions of staphylococcal sRNAs. They revealed that sRNAs sense and reply to different environmental stimuli and that they mostly control mRNA translation to remodel metabolomic pathways to adapt and survive in harsh environments conditions.

The more the identified sRNAs are studied, the clearer it becomes that there is no isolated node in regulatory network or pathway, but a myriad of interconnections that we are only at the beginning to acknowledge. Exciting discoveries await for us in the years to come, as all these interrelationships will be straightened out and a clearer map of sRNA interactions will be drawn.

In the meantime, many questions about the sRNA world in *S. aureus* remain to be addressed. The significance of RNA-binding proteins in all these networks is still very uncertain, besides the established role of RNase III in sRNA maturation and target degradation. However, there could be holes in the puzzle that might be filled in by some of these proteins, which may help explaining unsolved sRNA-dependent mechanisms of action.

The study of the complex regulatory networks of *S. aureus*, in which sRNAs are at the center, is undoubtedly essential for understanding its virulence and adaptation mechanisms and will ultimately guide us in the design of treatments to fight this pathogen.

AUTHOR CONTRIBUTIONS

LB, NM, DL, and IC contributed to the manuscript writing. All authors contributed to the article and approved the submitted version.

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C.2 RNAIII

This bifunctional regulatory RNA is the main effector of the *agr* system and promotes a switch from the expression of many surface proteins needed during early stages of infection to the production of secreted toxins necessary for later stages of infection. Hence, RNAIII is considered to promote a switch from an adhesion mode to a dissemination mode during infection. It has been described that the translation of the open reading frame for the *hld* gene is delayed by one hour after RNAIII transcription, but this was abolished when deleting its 3' untranslated region (UTR), suggesting the existence of a trans-acting factor regulating *hld* translation during growth (Marenna, *et al*, unpublished). Due to the proximity of the 5' and 3' ends of the transcript, it is believed that conformational changes are required for translation of *∂*-toxin, and more precisely that both ends of RNAIII may pair and sequester the ribosomal binding site (RBS) of *hld*.[18] This already revealed the importance of the RNAIII structure for gene regulation, a main hallmark of RNA-dependent regulation.

The secondary structure of RNAIII has been determined through chemical and enzymatic probing assays which have revealed a highly structured conformation of the transcript.[47] The 514-nucleotide long RNAIII consists of 14 hairpin structures linked together by stretches of unpaired nucleotides with three long-range interactions bringing the 5' and 3' ends into close proximity.[47] RNAIII possesses several C-rich sequence motifs found in apical loops and unpaired regions that are able to bind onto the ribosome binding sites (RBS) of multiple target mRNAs. This can have a positive or negative effect on translation and/or the stability of the mRNA and multiple regions of RNAIII can simultaneously be involved in the interaction with the target transcript. For details about the mechanisms of regulation of sRNAs on their target mRNAs please refer to Fig 4. In most cases, binding of RNAIII to the RBS of its target inhibits translation initiation and leads to the double-stranded specific endoribonuclease III (RNase III, see below)-dependent degradation of the duplex. Through this mechanism, RNAIII directly represses the expression of one of the major virulence factor of *S. aureus*, namely **protein A**. Indeed, the 3' domain of RNAIII, more precisely the C-rich motif of hairpin 13 fixes the RBS of the *spa* mRNA to prevent the formation of the translation initiation complex.[48] This is then sufficient to induce degradation by RNase III.[48] Protein A is intensively produced at low density, when its mRNA is efficiently translated and protected from degradation. During high cell density, synthesis of RNAIII represses translation of ***spa*** favoring rapid degradation. In a similar manner, C-rich motifs on hairpins 7 and 13 of RNAIII can bind to the RBS of the ***coa*** mRNA encoding for staphylocoagulase and prevent translation

promoting access to RNase III-dependent degradation of the target mRNA. [49] Through the same mechanism, RNAIII represses translation initiation of the *rot* mRNA, encoding the repressor of toxins Rot, a transcription factor binding to the promoter regions of numerous exoproteins and toxins blocking their transcription.[50] In this case, the C-rich motifs of hairpins 7, 13 and 14 can bind to the SD sequence of the *rot* mRNA, preventing translation and recruiting RNase III for subsequent degradation.[50] By inhibiting Rot, RNAIII indirectly activates the expression of many exotoxins and virulence factors repressed by Rot during early growth stages. RNAIII also represses the synthesis of other major surface proteins such as Sbi, a newly-identified fibrinogen-binding protein (SA1000) and LytM, a major cell wall hydrolytic enzyme involved in peptidoglycan turnover, cell division, biofilm formation and cell death programming.[51]

Aside from the repressive functions of RNAIII, it can also directly activate expression of other targets. For instance, translation of α-toxin (*hla*) is activated by RNAIII through direct binding to the SD sequence of the *hla* mRNA and rendering the RBS accessible to the ribosome. In absence of RNAIII, the RBS is sequestered in a hairpin structure inaccessible for translation and binding of the 5' end of RNAIII results in a conformational modification that allows translation of the mRNA.[52] RNAIII was also shown to stabilize the mRNA encoding the global regulator MgrA.[35] The 5' and 3' ends of RNAIII interact with the 5'UTR of the *mgrA* mRNA and likely protects it from RNase-mediated degradation.[35] MgrA directly activates transcription of many virulence genes such as capsule genes, serine proteases or leukotoxins and downregulates the expression of surface proteins.[53] MgrA is also required for clumping and biofilm formation, essential during infection.[54] Thus, by activating the expression of MgrA, RNAIII broadens its regulon and activates virulence on a global scale. The direct RNAIII-dependent targets and their types of regulation are reported in Fig. 7.

Needless to say, through its various RNA-RNA interaction mechanisms, RNAIII is a versatile sRNA molecule that affects the expression of many virulence genes and regulates the onset of virulence in a time-dependent manner. This molecule is at the very heart of the regulatory networks of virulence in *S. aureus*, comprising many more important players, such as RNA-binding proteins.

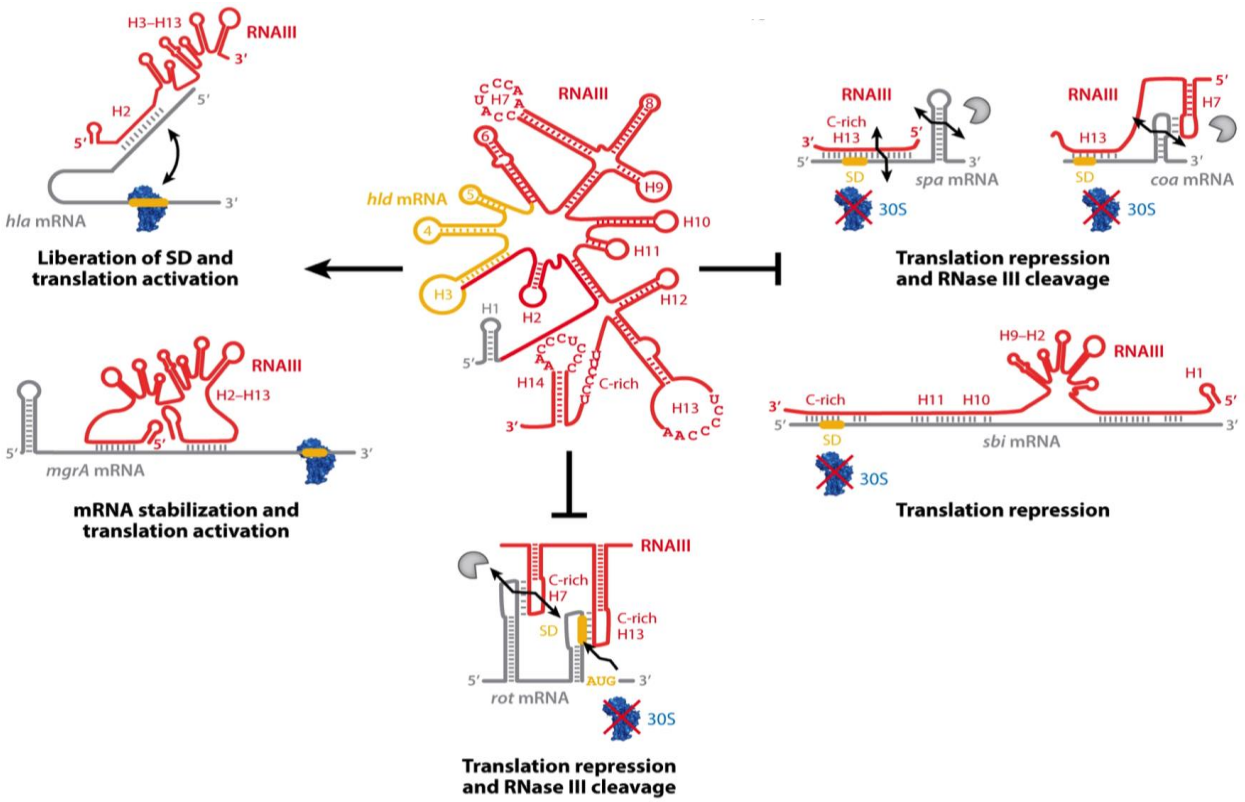

Figure 7 – RNAIII regulates its targets by direct RNA-RNA interactions and influences their stability and translation.[51]

The schematic representation of the secondary structure of RNAIII is depicted in red.[47] The hairpins are numbered from H1 to H14. The *hld* gene encoding delta-hemolysin comprised from H3 to H5 is depicted in yellow. The C-rich sequence motifs of RNAIII (notably H7, H13 and H14) bind the target mRNAs mostly at the Shine and Dalgarno (SD) sequences depicted in grey. Binding of RNAIII to *spa*, *coa*, *sbi* and *rot* mRNAs masks the SD sequence and prevents ribosomal 30S subunit from attaching, therefore inhibiting translation initiation, most often followed by rapid RNase III-mediated degradation. Conversely, RNAIII can also activate synthesis of exotoxins. By binding to the *hla* mRNA, RNAIII frees the SD from secondary structures and renders it accessible for ribosome loading. The *mgrA* mRNA is stabilized from RNase attack after RNAIII binding.[51]

Part 2. RNA-binding proteins in Bacillota (ex-Firmicutes)

RNA-binding proteins (RBPs) are ubiquitous among all living organisms and they participate in all type of cellular processes, including transcription, modification, translation and degradation of RNA. Thus, RBPs are key components of co- and post-transcriptional regulatory networks, with ribosomal proteins being the most abundant and conserved RBPs. When bound to RNAs, they form dynamic ribonucleoprotein (RNPs) complexes that are principally involved in the control of gene expression. RBPs engage with RNA molecules through distinctive domains named RNA-binding domains (RBDs) that recognize specific RNA motifs. These RBDs are usually conserved among bacteria and even eukaryotes. Such RBD include the S1 domain, the cold-shock domain (CSD) of the oligonucleotide/oligosaccharide binding (OB) superfamily, the Sm and Sm-like domains, the RNA-recognition motif (RRM), the ProQ/FinO domain, the K homology domain (KH), the double-stranded RNA-binding domain (dsRBD) or the PAZ and PIWI domains among others. (Fig. 8).[55] RBPs have been largely understudied in Gram-positive bacteria. Recent high-throughput studies have revealed that these bacteria express more RBPs than expected and they have surprisingly identified unconventional RBPs that include metabolic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or transcriptional factors as CcpA. [56] Some RBPs, although conserved in Gram-negative and positive bacteria do not exert the same functions.

I. Identification of RBPs in bacteria

In eukaryotes, RBP discovery mostly relies on RNA interactome capture (RIC) methods.[57] System-wide identification of RBPs *in vitro* involves the immobilization of RNA probes as bait incubated with cellular extracts and further mass spectrometry analyses.[58] To identify the RBP repertoire *in vivo*, ultraviolet irradiation is employed to crosslink proteins and RNA molecules in close proximity followed by the capturing of crosslinked RNPs containing poly(A)-tailed-RNAs (mRNAs) with oligo(dT) beads and final protein identification by mass spectrometry.[59] Variations of these methods have allowed the characterization of several regulatory RBPs in different organisms, as well as their RBD and the respective recognized

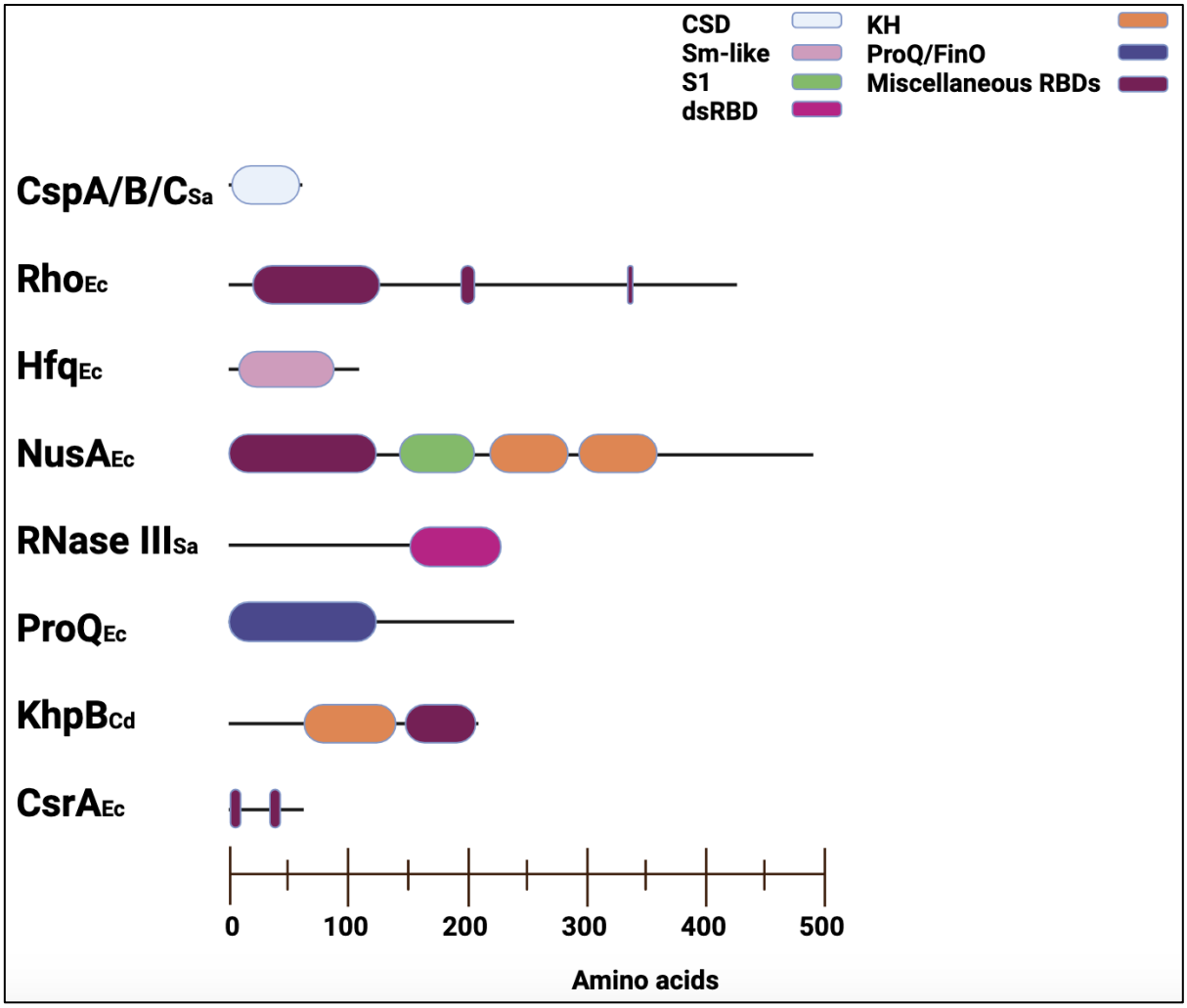

Figure 8 - Overview of RNA-binding domains (RBD) present in bacterial proteins (Adapted from Holmqvist *et al*, 2018)[55].

Schematic representation of well-characterized RNA-binding proteins in bacteria with the respective RBDs they contain. The different RBDs are characterized by colored boxes and miscellaneous RBDs refer to RBDs that do not belong to major RBD families (such as OB-fold domains for Rho or R3H domains for KhpB).

RNA motifs/sequences.[57] A similar RIC method has been applied in *E. coli*, where transient pulse-expression of poly(A) polymerase I (PAPI) allowed a broad polyadenylation of transcripts and through olido(dT) based-capture of these transcripts RNA-protein complexes could be identified, resulting in the identification of 169 putative RBPs, of which half had already been classified as RBPs.[60]

Then, more targeted methods have been developed to identify RBPs associated with a particular RNA molecule, in particular sRNAs in bacteria. They rely on a bait sRNA tagged for example with an MS2 aptamer or a streptotag and affinity chromatography to purify the complexes.[61,62] However, these methods require the altering and overexpression of the bait sRNA which could impact its native functions and interactomes. To overcome this problem, cDNA baits against specific sRNAs have been used to identify RBPs *in vivo* in *Pseudomonas aeruginosa*.[63] In short, bacterial cultures are UV-crosslinked and sRNA-protein complexes were isolated using cDNA probes designed specifically for the sRNA of interest followed by mass-spectrometry analyses to identify associated proteins.[63]

A) Global identification of RBPs

To identify RBPs on a global level in bacteria a high-throughput biochemical profiling approach named Grad-seq was applied first in *E. coli* and *Salmonella* to draw the landscape of RNA-protein interactions.[64] This method is based on the separation of RNAs and proteins in a glycerol gradient and analysis of the RNA and protein content of each gradient fraction. RNA molecules targeted by the same RBP will cluster together and can then be used to identify potential RBPs. For instance, this allowed the identification of ProQ as a global RBP and sRNA chaperone.[64] A recent modified approach named GradR is based on the same glycerol gradient separation, but a final RNase treatment is added to predict bacterial RBPs through a change in sedimentation profile when RNA partners are depleted.[65] An alternative method to Grad-seq has been recently developed mainly to overcome the burden of long ultra-centifugation times needed for glycerol gradient sedimentation. By using size exclusion chromatography (SEC), RNAs and proteins can also be separated depending on their size, similarly to the glycerol gradient, and subsequent RNAseq and mass spectrometry analyses of the fractions allow the identification of RNAs and proteins present in each fraction.[66] The power of this named SEC-seq method relies on its fast operation times, automatization potential and high resolution since it is able to differentiate similarly sized complexes.[66]

Other methods to purify RNP complexes in an unbiased manner have also been recently developed, such as the "2C" (complex capture) and PTex (phenol-toluol extraction) techniques.[67,68] 2C is a silica-based solid phase extraction that relies on the fact that silica columns are able to specifically retain nucleic acids based on charge and this interaction is strong enough to retain large RNA-protein assemblies.[68] PTex relies on the physicochemical differences of crosslinked RNPs to separate them through biphasic extractions from RNA and proteins.[67] The use of a mixture of phenol and toluol for RNA extraction alters the enrichment of biomolecule classes in the different separation phases (aqueous or interphase) and allows the specific isolation of crosslinked RNPs.[67] For instance, a first phenol:toluol extraction enriches crosslinked RNPs in the aqueous phase, which is then subjected to acidic phenol extraction that results in accumulation of crosslinked RBPs in the interphase.[67] These two methods were applied to study the global RBPome of MRSA with overlapping results.[69] Gene-ontology (GO) classification of sequenced proteins confirmed the enrichment of nucleic acid binding and RBPs.[69] Although the number of RBP candidates were rather significantly high suggesting numerous false-positives, results were validated with alternative approaches and led to the identification of new RBPs that could expand *S. aureus* regulatory networks.[69]

B) Characterization of RBPs' RNA interactome

Once an RBP has been identified, numerous methods to characterize its RNA interactome have been developed thanks to the advances in highthroughput sequencing technologies. Co-immunoprecipitation of a tagged RBP followed by RNA-seq (RIP-seq) determines the repertoire of RNAs bound to the RBP of interest and is the most common version of RBP characterization.[70] RIP-seq has been extensively used to reveal the RNA interactome of the global regulator and RNA chaperone Hfq in several Gram-negative bacteria including *E. coli*, *Salmonella*, *Pseudomonas aeruginosa*, *Neisseria meningitidis* and V*ibrio cholerae* but also the Gram-positive *Clostridioides difficile.*[71–76] This has permitted to identify novel sRNAs-mediated regulatory pathways in these organisms. RIP-seq has also been applied to other RBPs involved in sRNA-mediated regulation in other organisms such as *Legionella*, *C. jejuni*, *S. aureus* or *L. monocytogenes* to untangle sRNA-regulatory networks.[70,77–81]

A variation of this method in which the complexes are UV-crosslinked to covalently link them, and unprotected RNA is degraded (CLIP-seq) enables the identification of the precise RNA-binding sites and RBP motifs.[82] For instance, CLIP-seq of Hfq in Salmonella identified 3' Rho-independent terminators as a universal motif in Hfq-RNA interaction.[82] In

the same study, CLIP-seq was applied to CsrA, a post-transcriptional regulator in bacteria, and identified a common AUGGA motif present in apical loops of targeted RNAs.[82] CLIP-seq has also been applied for other RBPs such as ProQ and RsmA/N and in less studied organisms such as *Yersinia pestis.*[83–85]

In vitro approaches that integrate RNA sequencing have also allowed to find RNA binding sites of a given RBP by exposing the protein to libraries of RNA molecules and sequence retained/preferred transcripts.[86] This method named RNA Bind-n-Seq has been applied in eukaryotes and has proven powerful to determine structural and sequence preferences of RBPs in eukaryotes.[87] Similarly, coupling *systematic evolution of ligands by exponential enrichments* or SELEX with RNAseq for a given RBP is also a powerful method to identify preferred ligands and binding sequences.[86,88] Such a pipeline has been developed to probe helicase substrates in *E. coli*, in a method named Helicase-SELEX (H-SELEX), which was applied for the ATPase-dependent RNA:DNA helicase Rho of *E. coli*. The purified protein was exposed to a library of RNA:DNA duplexes containing sequences of the *E. coli* genome to explore the repertoire of Rho substrates.[89]

These techniques enable the successful identification of sRNA binding partners of RBPs, but lack information about the corresponding sRNA-targets. New methods to identify the sRNAs and respective targets bound to a specific RBP have been recently developed and rely on ligation of the formed duplexes. RNA interaction by ligation and sequencing (RIL-seq) is based on the ligation of sRNAs to their target RNAs, generating chimeras that are further sequenced and mapped to different genomic loci.[90] RIL-seq applied to Hfq from *E. coli* identified and significantly expanded the known sRNA-interactome of this model organism [90] and others.[91–93]A variation of this technique in which hybrids are first crosslinked (CLASH – crosslinking, ligation and sequencing of hybrids) has been used for RNaseE in enterohaemorrhagic *E. coli* (EHEC*)* and successfully captured sRNA-mRNA interactions involving specific EHEC sRNAs.[94] CLASH has also been recently applied to RNase III in *S. aureus* to unveil the sRNA-interactome of this Gram-positive pathogen (see below).[95] To bypass UV- crosslinking and *in vitro* RNA ligation, the iRIL-seq approach (intracellular RNA interaction by ligation and sequencing) applies pulse-expression of T4 RNA ligase 1 in living cells to enable *in vivo* proximity ligation of sRNAs to their interacting RNA partners.[96] This method has been used to profile the global RNA interactome of *Salmonella* and presents several advantages over other RNA interactome mapping techniques (i.e. physiologically relevant but weak interactions are maintained, simpler and more cost-efficient setup).

All these methods have allowed to explore and expand the bacterial RBP repertoire, mostly involved in sRNA-mediated regulation. Thus, the combination of these approaches to study the RNA interactome of RBPs, especially Hfq (or ProQ) have confirmed their essential/central role post-transcriptional regulatory networks mostly in several Gram-negative bacteria but was less applied to Gram-positive bacteria. Since sRNA-mediated regulation in Gram-positive bacteria is often independent of the action of RBPs, these group of proteins have been understudied in these bacteria.

II. The controversial role of Hfq in Bacillota

Recent advances in the study of RNA-RNA and protein-RNA interactions in bacteria described above have revealed the existence of extensive post-transcriptional regulatory networks centered around sRNAs including RBPs. As mentioned before, sRNA-mediated regulation involves base-pairing interactions between the sRNA and its target mRNA, often at the level of the RBS, and can result in translational repression/activation and degradation/stabilization of the mRNA. In many Gram-negative bacteria including pathogens like *E. coli*, *S. enterica* and *V. cholerae*, this post-transcriptional regulation requires the action of the chaperone protein Hfq to facilitate sRNA-mRNA interactions by independently binding to both RNA molecules.[97] This is achieved by displaying multiple surfaces for RNA binding since the distal face of the Hfq hexamer binds A-rich mRNA sequences while the proximal face recognizes A/U-rich sequences.[97] In these pathogens, Hfq is involved in many physiological processes including virulence, stress response, metal homeostasis, biofilm formation and motility.[98–102]In *E. coli*, Hfq is a global regulator modulating stability and translation of many mRNAs and inactivation of the *hfq* gene results in growth defects and sensitivity to environmental stress.[103] The role of Hfq in Gram-positive bacteria remains questionable, since *hfq* disruption in *Listeria monocytogenes*, *S. aureus* and *B. subtilis* does not produce any growth defects nor impairs sRNA-mediated regulation.[49] An exception is made for the human pathogen *Clostridioides difficile*, where Hfq has pleiotropic effects on physiology and is involved in biofilm formation, stress adaptation and sporulation.[104] A RIPseq approach applied to Hfq from *C. difficile* has allowed the identification of genome-wide associated RNAs that included sRNAs, mRNAs, 5'UTRs and antisense RNAs.[75] The sporulation-associated regulatory RNA RdC1 was found to bind Hfq and has been established as a good candidate for Hfq-dependent control of sporulation in this organism. Hfq was also found to bind to antisense RNAs, suggesting potential roles of this chaperon in antisense

regulation, especially in toxin-antitoxin systems. Further, RILseq was applied to directly identify Hfq-interacting RNA pairs in *C. difficile*.[92] This has revealed that the master regulator of sporulation, *spo0A* is subjected to post-transcriptional sRNA-mediated regulation participating in sporulation initiation. For instance, two novel sRNAs SpoY and SpoX were characterized and found to directly interact with *spo0A in vitro* and *in vivo* and modulate sporulation frequencies, reinforcing the relevance of Hfq for *C. difficile*'s physiology and sRNA regulatory networks.

In addition, although not essential in *L. monocytogenes*, Hfq contributes to pathogenicity and stress adaptation.[105] The functionality and importance of Hfq for gene regulation in one species can be assessed by its ability to complement Hfq deletions in *E. coli* or *Salmonella*. For instance, Hfq from *C. difficile* and *L. monocytogenes* are able to replace Hfq in *E. coli* but not the homologues from *S. aureus* of *B. subtilis*.[105]

In *S. aureus*, Hfq is present yet dispensable for sRNA regulation and conflicting findings have been published concerning the biological relevance of this chaperone. The global homohexameric ring structure of Hfq is conserved and the protein is able to bind RNAs such as RNAIII and *spa* mRNA even though with poor affinity *in vitro*.[103,106] However, it has been shown that Hfq is not involved in stress response and does not affect RNAIII or *spa* levels nor general production of virulence factors.[103] Thus, in *S. aureus*, sRNA-regulatory mechanisms are considered independent from Hfq.[103] This is believed to be in part due to the low GC-content of *S. aureus* genome, since Hfq homologs are absent in several low-GC Gram-positive bacteria like *Streptococcus pneumoniae* and *Streptococcus pyogenes*.[103] These differences in GC content explain the differences in binding affinities of RNAs to Hfq and therefore its requirement. Indeed, the surface of the rim domain of Hfq in Gram-negative bacteria is enriched with arginine residues, increasing the positive electrostatic charge needed for efficient RNA-binding of GC-rich sequences.[97] In addition, in Gram-positive bacteria, sRNA-RNA complementarity is usually higher (near perfect) and longer in sequence than in Gram-negative bacteria, where rather short and limited complementarity between sRNA and mRNA is predominant, which would also account for the indispensability of Hfq for sRNA-mRNA interaction in these bacteria.[97]

Besides Hfq, the Grad-seq approach applied in *S. enterica* to unveil the global landscape of RNPs identified ProQ as a global RBP involved in sRNA-mediated regulation.[107] However, ProQ is absent from *S. aureus*.

III. Examples of other RBPs in Bacillota

A) CsrA

The CsrA protein has been proposed to contribute to sRNA-mediated regulation in Gram-positive bacteria. This protein is the main component of the carbon storage regulator (Csr) system and is a global post-transcriptional regulator.[56] It is involved in many biological processes such as motility, biofilm formation and virulence.[56] CsrA binds to AG-rich sequences in the 5' UTR of mRNAs, mainly SD sequences and can lead to RNase-mediated degradation or stabilization and translation stimulation.[56] CsrA activity can be countered by sRNAs like CsrB and CsrC. This protein regulates many virulence factors in *Pseudomonadaceae*, *Salmonella* and *E. coli*.[56] CsrA homologues have been studied in *B. subtilis* and *C. difficile*, where it appears to play some role in motility and virulence in the latter.[56] In *B. subtilis*, CsrA is involved in biosynthesis of flagella by targeting the flagellin-encoding *hag* mRNA for translational repression.[56] For now, CsrA has been the only protein found to enhance sRNA-mRNA interactions in Gram-positive bacteria, however, whether it can be considered as a global chaperone and regulator of gene expression is still unclear and no homolog is found in *S. aureus*.

B) Cold-shock proteins (CSP)

This family of small DNA and RNA binding proteins is well conserved among bacteria and they contribute to adaptive responses in all kingdoms of life. As their name indicates, they are mostly expressed when temperature decreases and bind RNA molecules to prevent formation of secondary structures that would inhibit translation during low temperatures.[56] *E. coli* expresses 9 CSPs and homologues are found in many Firmicutes. *B. subtilis*, *S. aureus*, *L. monocytogenes* and *C. botulinum* contain 3 CSPs (CspA, B and C). Some are essential for viability, especially during cold exposure.[56] In *L. monocytogenes*, *C. botulinum* and *Enterococcus faecalis*, CSPs are crucial for cold-shock response, flagella biosynthesis and virulence regulation.[56] In *S. aureus*, the *cspA* mRNA levels remain unchanged by shifts in temperature[78] and CspA has been described as a global post-transcriptional regulator able to bind hundreds of transcripts *in vivo*.[108] Several CspA targets in this Gram-positive pathogen include mRNAs of genes involved in amino acid metabolism, nucleoside and carbohydrate synthesis, stress adaptation and virulence.[108] This chaperone can affect their stability and

translation and its deletion results in increased sensitivity to oxidative stress due to reduced production of the protective staphyloxanthin.[108] The production of CspB and CspC however is induced upon drops in temperature, when *S. aureus* transitions from host-related 37ºC to ambient 22/28ºC temperatures.[109] This thermoregulation is due to paralogous thermoswitches present in the 5'UTRs of *cspB* and *cspC* mRNAs and CspA is required for the temperature-dependent repression of these transcripts.[109] While RNA-binding domains were predicted in CspB and C (Fig. 8), no RNA targets were identified until now. The CSPs, although highly identical, are functionally different since a *cspA* mutant could not be restored by expression of CspB and CspC,[109] Specificity of CspA is determined by one amino acid, namely proline 58 which is conserved among CspA orthologs and suggests that this position might be fundamental for RNA target specificity.[110]

Thus, CSPs are important proteins involved in stress adaptation in many clinically significant Gram-positive pathogens.

D) KhpB in *C. difficile*

The development of the Grad-seq technique has allowed for the global identification of RNA-bound proteins first in *S. enterica* but it has been more recently adapted to the Gram-positive bacteria *C. difficile* and *Streptococcus pneumoniae.*[64,107] This has allowed the identification of a new RBP with Hfq-like functions in *C. difficile*, namely the global sRNA-binding protein KhpB [107] This protein is broadly conserved and has homologues in *S. pneumoniae* and in *Lactobacillus plantarum,* where it forms heterodimeric complexes with another RBP KhpA.[107] Inactivation of either KhpA or KhpB proteins resulted in impaired growth. KhpB was found to bind several transcripts with relevant physiological functions in *C. difficile* such as flagellar assembly, chemotaxis and cell division and was found to participate in the regulation of toxin production.[107] Even if the mechanisms of regulation by KhpB (and KhpA) remain unclear, this global and widely conserved RBP is a strong candidate for post-transcriptional regulation in Gram-positive bacteria, especially in Firmicutes, where the existence of a relevant sRNA chaperone has not been identified. However, this protein is not present in *S. aureus*.

E) CcpA in *S. aureus*

A recent study of the RNA-bound proteome in *S. aureus* revealed that most of helix-turn-helix DNA-binding and Rossman-fold domain proteins can bind RNA.[69] One of these DNA-binding proteins is the catabolite control protein A (CcpA), a major global regulator involved in carbon catabolite repression (CCR), a widespread phenomenon that modulates expression of genes and operons involved in carbon utilization in the presence of preferred carbon source.[111] During this process, the presence of a preferred carbon source induces repression of genes involved in the metabolism of less preferred carbon sources.[111] In *S. aureus*, CcpA influences transcription of several genes involved in virulence such as RNAIII, and its functions are not restricted to CCR.[111] Nevertheless, it was recently described that this factor can bind to hundreds of coding and non-coding RNAs near their intrinsic terminators and might have a role in transcription termination or stability of these transcripts.[69] CcpA is therefore stipulated to regulate expression of genes at the post-transcriptional level, as is believed for the sRNA RsaI which is downregulated by CcpA in the presence of glucose.[69,112] Binding of CcpA to terminator hairpins of RNAs could impact their stability, since strongly bound RNAs were often upregulated in a *ccpA* deletion mutant.[69] However, the extent to which binding of CcpA to RNA impacts *S. aureus* physiology as well as the binding selectivity for its targets remains unclear. The ability of DNA-binding proteins to also bind and potentially regulate RNA molecules opens the door to the discovery of new regulatory mechanisms and networks and unexpected functional RBPs.

IV. RNases

Regulation of RNA degradation is a key component of post-transcriptional gene regulation, homeostasis and adaptation of gene expression to everchanging environmental conditions. In bacteria, RNA decay is initiated through the removal of 5'end pyrophosphate, followed by endonucleolytic cleavage and exonucleolytic degradation. RNAses and composition of RNA degradation machineries greatly vary between Gram-negative and Gram-positive organisms. For instance, the major ribonuclease involved in global mRNA turnover in *E. coli*, RNase E, is absent in *B. subtilis* and *S. aureus*, and every species can have a specific set of encoded RNases.[113] Some endo- and exoribonucleases are conserved among Gram-negative and Gram-positive bacteria. These include the 3'-5' exoribonucleases PNPase, RNase R and RNase PH as well as the endoribonucleases RNase

III, RNase P and RNase Z.[113] RNA degradation can be influenced by a varied number of factors, including secondary structure, RBPs and also sRNAs.

A) RNase Y

Among the key enzymes involved in mRNA degradation in Firmicutes we find endoribonuclease **RNase Y.** This enzyme is equivalent to RNase E from *E. coli.* RNase Y is involved in degradation of bulk mRNA in *B. subtilis*, since its inactivation leads to their increase half-lives.[113] It is bound to the cytoplasmic membrane and recognizes AU-rich single-stranded regions with a preference for 5' monophosphorylated substrates.[113] RNase Y is not essential in *B. subtilis*, but its deletion affects growth, biofilm formation, aminoacid biosynthesis and other physiological traits.[113,114] In *S. aureus*, the *rny* ortholog (a.k.a. cvfaA) encoding RNase Y is not essential either, but disruption of the gene results in impaired virulence and hemolysins production. Only a small set of mRNAs and sRNAs are affected by RNase Y deletion, mostly involved in pathogenicity. [115] Indeed, RNase Y is required for the processing and stabilization of the transcript encoding the global virulence regulator SaeRS described previously and is able to activate exotoxin production independently from *agr* or *sae*.[115] RNase Y is also required for the expression of *hlgC* probably by affecting stability of the sRNA RsaA, whose half-life significantly increased in and *rny* mutant.[115] The *rib* operon, encoding for proteins involved in riboflavin synthesis and under the control of a flavine mononucleotide (FMN)-responsive riboswitch at its 5'-end was found upregulated and stabilized in the *rny* mutant.[115] Thus, RNase Y is involved in the decay of the mature *rib* operon probably in presence of riboflavin and it is speculated that the absence of this molecule induces conformational changes of the transcript that protects it from RNase Y degradation.[115]

Because of the specificity of RNase Y mostly for virulence-associated transcripts and the low global impact of its deletion, this endoribonuclease is not involved in turnover of bulk mRNA in *S. aureus* and other more relevant degradation machineries take over.

B) RNase J1/J2

RNases J1 and J2 encoded by *rnjA* and *rnjB* repectively were discovered in *B. subtilis* after screening for enzymes with RNase E-like activities, more precisely having a role in the maturation of 16S and 23S rRNAs and cleavage of the T-box of threonyl-tRNA synthetase.[114]

The bifunctional RNase J1 and RNase J2 form a complex exhibiting endonuclease and 5'-3' exonuclease activity, with RNase J1 providing most of the 5'-3' exonuclease activity and RNase J2 helping to stabilize or regulate RNase J1 in *S. aureus* and *B. subtilis*.[113] The activity is strictly dependent on 5' monophosphate (5' triphosphate ends inhibit RNase J1/J2 activity) and single stranded RNA. In *B. subtilis*, deletion of RNase J1 in absence of RNase J2 slightly increased half-lives of bulk mRNA with about thirty percent of total transcripts being affected.[114] Global RNA decay in *B. subtilis* is believed to be initiated with the endonucleolytic cleavage RNase Y generating 5' monophosphate ends targeted by RNase J1/J2 for 5' to 3' exonucleolytic cleavage and degradation from 3' to 5' by PNPase. *rnjA* mutant in *B. subtilis* is viable but shows defects in growth, sporulation, competence and cell morphology while an *rnjB* mutant grows normally.[114] In S. *aureus*, neither enzyme is essential in a growth window revolving around 37ºC and overexpression of RNase J1 can partially compensate the absence of RNase J2, probably by forming RNase J1/J1 homodimers.[114] Thus, it is believed that only RNase J1 provides enzymatic activity and RNase J2 serves mainly as a structural protein and ensures proper function of the RNase J1/J2 complex.[116] A similar global role of RNase J1 activity in mRNA turnover has also been observed in *S. aureus*, as well as a role in the maturation of 16S rRNA and RNase P.[116] A specific role of J2 acting independently of J1 is still under debate.

C) PNPase

The polynucleotide phosphorylase (PNPase) is a 3' to 5' exonuclease playing important roles in mRNA degradation in *B. subtilis* and *S. aureus*. 3' to 5' exonuclease activity is redundant in *S. aureus*, as it encodes for three nuclease orthologs, namely PNPase, RNase R and YhaM. However, PNPase plays a global role in RNA decay, with over thirty percent of total transcripts being PNPase-dependent.[114] PNPase has been linked to virulence regulation in *S. aureus* by cooperating with RNase Y to degrade virulence-associated transcripts. Indeed, the decrease in hemolysin production of a *rny* mutant was restored by disrupting the *pnpA* gene encoding PNPase.[117] It was shown that RNase Y is able to convert RNAs with a 2'-3'-cyclic phosphate group into 3'-phosphorylated RNAs, which are more resistant to PNPase activity than the original 2'-3' cyclic phosphates who are efficiently degraded by PNPase.[117] Thus, it is believed that RNase Y and PNPase competitively act to degrade some mRNAs. Indeed, targets cleaved by RNase Y are protected from PNPase degradation and in absence of RNase Y, the same RNAs are subjected to PNPase degradation.[117] Indeed, PNPase negatively alters

the expression of hemolysins and of the *saePQRS* transcript in *S. aureus*, which is believed to be degraded by PNPase in absence of maturation by RNase Y that forms protective 3' extremities.[117]

D) RNase III

The double-stranded endoribonuclease RNase III was initially discovered over forty years ago in *E. coli* and are conserved in all species of bacteria and eukaryotes.[118] RNase III-like enzymes are diverse and range from the 143 amino acid long Mini-III RNase in *B. subtilis* to the 1'922 amino acid long Dicer1 protein found in humans.[114] All these proteins are Mg^{2+}-dependent and possess a conserved catalytic RNase III domain (RIIID) characterized by a nine-residue signature motif. RNase III-cleavages are characterized by the generation of 5' phosphoryl and 3' hydroxyl ends with a two nucleotide 3' overhang in the dsRNA products.[118] In bacteria, RNase III is involved in a myriad of physiological processes including primarily the processing of rRNAs by cleaving a stem-loop inside the primary rRNAs. However, this enzyme is not essential in most bacteria studied so far with the exception of *B. subtilis*, where RNase III is required to eliminate toxins encoded by type I toxin/antitoxin systems.[113] RNase III has limited impact on mRNA expression levels in *B. subtilis* with only 11-12% of mRNAs affected.[113] In *S. pyogenes*, RNase III is an essential host factor for the prokaryotic CRISPR/Cas system to induce silencing of phage expression.[114] RNase III has been extensively studied in *S. aureus*, where its role was mainly determined through its implication in the *agr* regulatory network by degrading the duplexes formed by RNAIII and its mRNA targets leading to their repression.[18,48,50] The duplexes formed show different topologies such as imperfect base-pairings and loop-loop interactions that are efficiently recognized and cleaved by RNase III.[78]

Indeed, a deletion mutant for the *rnc* gene encoding RNase III showed decreased virulence in a murine model of peritonitis.[78] RNase III is also involved the processing of genome-wide antisense transcription and generates short RNA molecules resulting from the digestion of overlapping sense/antisense transcripts and at least 75% of all sense RNAs are concerned by this mechanism.[119] Overlapping antisense transcription can generate noncoding antisense transcripts that can include sRNAs and antisense RNAs. The latter can cover different parts of genes and this transcription can impact gene expression at different levels such as transcription by preventing RNA polymerase from transcribing the opposite strand, stability of the mRNA by blocking or promoting degradation by an RNase, transcription termination by

inducing conformational changes of the mRNA or translation by affecting the binding of the ribosome.[119] In *S. aureus*, the duplexes formed from this genome-wide overlapping transcription create double-strand substrates for RNase III which are then cleaved, generating a collection of short RNAs that mapped both strands of the genome and that can be detected through RNAseq.[119] Thus, RNase III is involved in the regulation of pervasive transcription that generates these cryptic and mostly antisense transcripts and participates in the surveillance of genome integrity. This process seems to have been conserved in Gram-positive bacteria since the same phenomenon is observed in *Listeria monocytogenes*, *B. subtilis* and *Enterococcus faecalis*.[119] In *S. aureus*, co-immunoprecipitation of RNase III as well as a catalytically inactive mutant RNase III revealed a significant number of specific sRNA and mRNA substrates of this enzyme.[78] In *S. aureus*, RNase III is required for the correct maturation of rRNA and several tRNA operons and was shown to post-transcriptionally autoregulate its own mRNA levels resulting in a negative feedback regulation of its expression and adjust intracellular levels of the protein to avoid potentially detrimental accumulation.[78] RNase III also processes the 5' UTR of the *cspA* mRNA by first cleaving within a long hairpin in this 5'UTR, encoding for the major cold-shock protein CspA to activate CspA translation by stabilizing the transcript and preventing its degradation.[78] In addition, RNase III is involved in sRNA regulation since many bona fide sRNAs were found to interact with RNase III such as RsaA, C, E, I, H and J, RNAIII and the pathogenicity-island-encoded sRNAs from the Spr family.[78] Indeed, RNase III is involved in the maturation of some of these sRNAs such as RsaA and RsaC or in their regulatory networks like for RNAIII.[78,120] Recent RNase III CLASH using this enzyme as a bait was performed in MRSA and revealed the extensive RNase III and sRNA-dependent regulon.[95] Interactions between *bona fide* sRNAs and their targets were confirmed but novel interactions were identified such as RNAIII with the *esxA* toxin-encoding mRNA, where it was found that RNAIII is needed for stabilization and correct expression of the transcript.[95] RNase III CLASH data thus revealed the extensive sRNA-dependent regulation of toxin expression in MRSA, since several sRNAs such as RNAIII or RsaE were found to interact with toxin-encoding mRNAs.[95] Interestingly, sRNA-sRNA duplexes were also identified, such as an interaction between RsaI involved in response to glucose starvation and the highly conserved RsaE that regulates several metabolic pathways.[95] RsaI along with RNase III regulate RsaE activity, thus RsaI may act as a sponge to RsaE and RNase III is hypothesized to stabilize this interaction.[95] These data also identified the long 3'UTR of the *vigR* mRNA to be involved in intermediate vancomycin resistance by positively regulating mRNAs involved in cell wall peptidoglycan synthesis leading to cell wall thickening.[121] All-in-one, RNase III is

involved in mRNA and sRNA processing, RNA turnover globally, antisense and sRNA regulation and thus, contributes to the complexity of post-transcriptional gene control networks in *S. aureus*.

In *S. aureus*, besides the role of RNase III in post-transcriptional gene regulation, no other RBP has been associated to these regulatory networks yet. However, the well-conserved transcription termination factor Rho has been recently identified as being a key player in the control of pervasive transcription and the control of virulence in *S. aureus*.[122,123] The next chapter will be dedicated to the description of this protein as well as its recently described functions in Gram-negative and Gram-positive bacteria.

Part 3. Transcription termination factor Rho

Efficient transcription termination is essential to ensure correct gene expression. This final step of transcription is necessary to prevent inappropriate expression of downstream genes that can result in the generation of antisense transcript and genetic interference It is also important for the generation of correct structures at 3'ends of sRNAs for example that can be necessary for their regulatory functions, for an efficient recycling of RNA polymerase complexes and for the prevention of collisions between replication complexes that can result in DNA damage. In bacteria, transcription termination occurs via two distinct mechanisms: intrinsic termination requiring no additional factors, and Rho-dependent termination involving the transcription termination factor Rho.

I. Transcription termination mechanisms in bacteria

RNA polymerase forms a stable but dynamic contact network with nucleic acids in the elongation complex (EC) that allows the transcription of more than 10^4 nucleotides without dissociation. The major sources of stability originate from non-specific interactions such as polar and van der Waals contacts between RNAP and the RNA-DNA hybrid in the main channel of the enzyme, H-bonds with the exiting ssRNA and long-range electrostatic and van der Waals interactions with downstream DNA.[124] These contacts are established through structural changes of RNAP following first interactions with the DNA and create an EC that is stable up to 1 M NaCl and 65ºC.[125] Thus, the challenge of transcription termination is to

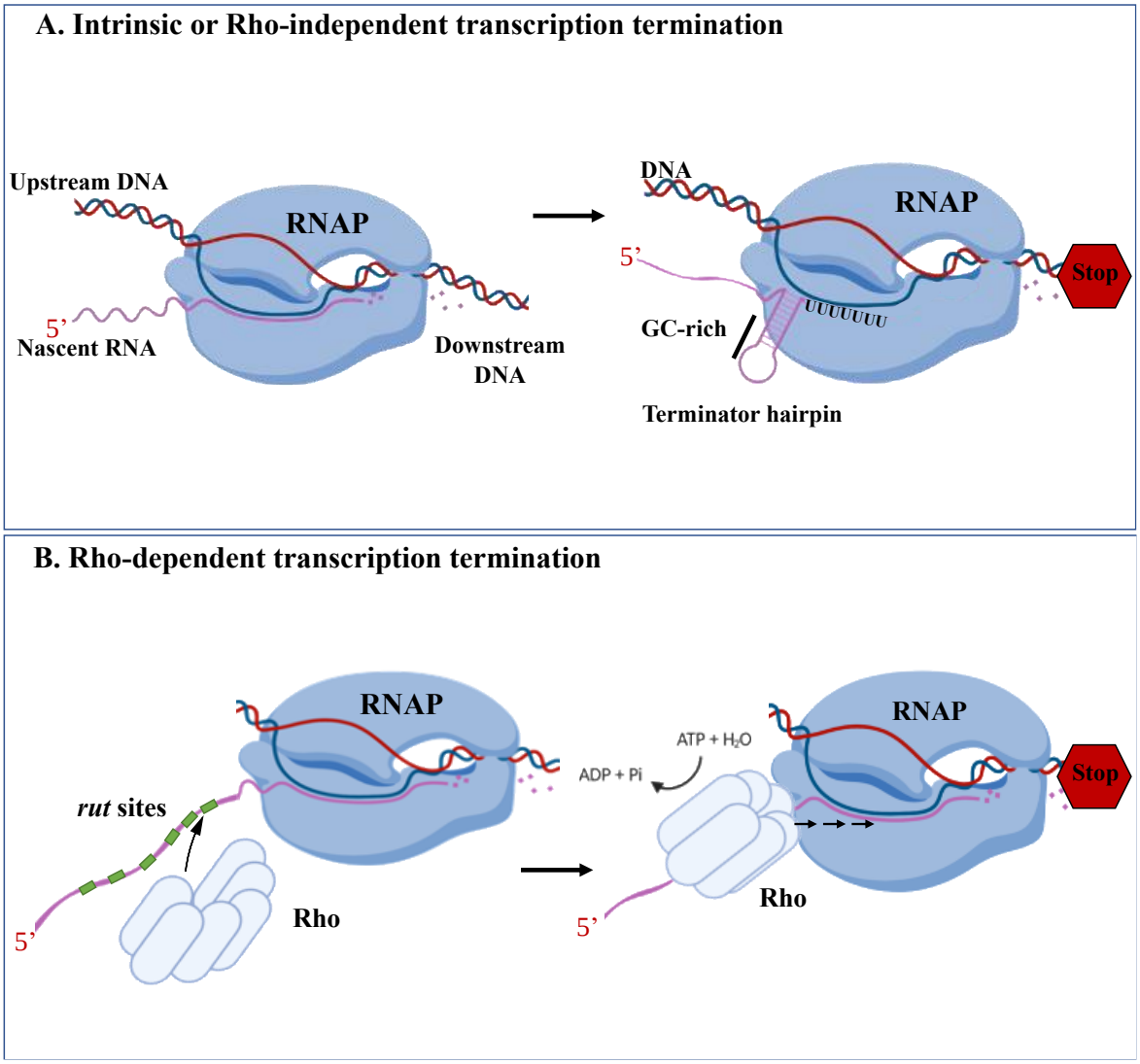

Figure 9 – Transcription termination mechanisms in bacteria.
A. Intrinsic termination depends on the interactions between the elongating RNAP and the nucleic acids from the nascent RNA. The formation of an intrinsic terminator on the nascent RNA, formed by a GC-rich stem-loop and a stretch of U residues, induces pausing of the elongation complex followed by destabilization and dissociation of the complex. **B.** Rho-dependent termination requires the action of the ATP-dependent RNA translocase Rho. Rho binds to *rut* sites (unstructured C-rich regions) on the nascent RNA. Binding to the RNA activates the ATPase activity of Rho, which then translocates along the RNA until encountering the elongation complex. Multiple models of translocation are proposed (see text). Rho then dissociates the elongation complex resulting in transcription termination.

destabilize the EC to enable dissociation of RNAP from RNA and DNA. Termination occurs when all these contacts are sufficiently destabilized such that the rate of EC pausing and eventual dissociation becomes more significant than the rate of addition of the next nucleotide to the nascent RNA.[125] A first step for efficient termination is pausing of the EC, achieved by the presence of secondary structures on the nascent RNA or by external factors.[124] An inactivated EC can compete with either elongation or dissociation, forming a termination intermediate. However, true termination requires the dissociation of the EC with the release of RNAP, DNA and the newly transcribed RNA.[125]

A) Intrinsic or Rho-independent termination

Intrinsic or Rho-independent termination refers to the dissociation of the EC without the assistance of any external factors, besides DNA and RNA at locations named intrinsic terminators. Intrinsic terminators are DNA and RNA elements at the site of termination that direct a series of steps that cause RNAP to terminate transcription in a window of 2-3 nt spanning the location of the terminator and release the enzyme from the transcript and the chromosome.[124] They are usually found at the 3' end of genes and operons and also within and upstream genes to cause a mechanisms called attenuation.[125] Intrinsic terminators can vary in length, sequence and efficiency but they exhibit canonical features. A canonical intrinsic terminator consists of a guanine/cytosine (GC)-rich hairpin immediately followed by a 7-8 nt long uracil tract on the nascent RNA. This U-rich tract causes RNAP to pause during its transcription and creates a time window for the terminator hairpin to form inside the exit channel while the U-tract is still within the DNA:RNA hybrid (Fig. 9A).[124] In absence of the U-tract, termination is not efficient so that both elements are essential. The second step in termination is hairpin nucleation, where partial terminator hairpin formation causes 3-4 bp of the RNA:DNA hybrid to melt by physically removing the hairpin from the duplex, contributing to the destabilization of the EC.[125] Finally, when the hairpin formation is complete the EC complex dissociates releasing all components. Recent cryo-EM structures revealed how the folding of the terminator hairpin in the RNA exit channel enlarges this channel and induces conformational changes in RNAP that secure the hairpin at this location, destabilize the upstream DNA:RNA interactions, stabilize the pause of the EC and increase the chances for hairpin completion and successful termination.[126]

As stated before, intrinsic terminator sequences and features are well conserved and reflect the importance of this mechanism for bacterial physiology. This conservation has

allowed the development of bioinformatical tools to predict and identify intrinsic terminators in almost all genome sequences.[125] The terminator hairpin can vary in length from 5 bp to 17 bp with an average of 8 bp and has a strong GC-bias especially in the five positions nearest the U-tract, which is important for the destabilization of the EC in the final step of termination. [125] The terminator loops range from 3 nt to 10 nt with an average of 4 nt, believed to stabilize the RNA structure.[125] Nearly half of described terminators have a perfect or near-perfect U-tract and the other half contained at least two non-U residues in what is called an imperfect U-tract with a strong bias for at least two Us immediately following the hairpin.[125] There seems to be an opposite sequence bias in the downstream DNA sequence for imperfect and perfect U-tract sequences, with a higher AT content for imperfect U-tract terminators and conversely, low AT content for perfect U-tract terminators.[125] This is believed to help compensate for the differences in U-content between both types of U-tracts and affect the melting of the DNA:RNA duplex.[125]

B) Rho-dependent termination

Rho-dependent termination requires the action of the termination factor Rho, a homohexameric ring-shaped protein that binds to the nascent RNA and translocates from 5' to 3' thanks to its ATPase and RNA-DNA helicase activity to finally dissociate the transcript from RNA polymerase (Fig. 9B). Rho was first discovered over fifty years ago during a search for factors in crude extracts from *E. coli* that would terminate *in vitro* RNA synthesis of bacteriophage lambda.[127] A few years later, the implications of Rho in operon termination and transcriptional polarity was described, since Rho was found able to terminate transcription of specific bacterial operons and the existence of Rho-specific termination sites recognized by this protein was suggested.[128] Most of the accumulated knowledge about Rho is based from numerous studies performed in *E. coli,* so unless stated otherwise, the following chapter summarizes what has been described in this Gram-negative model organism.

B. 1. Rho binds to RNA

Rho-dependent termination relies on sequence specificities on the nascent RNA that are required for binding of Rho to the transcript and activation of the translocase activity for further translocation of the RNA through Rho and pausing of the EC. Rho binds to C-rich unstructured regions of the RNA named *rut* (*R*ho *u*tilization) sites which activates the ATPase

activity that is necessary for translocation. These *rut* sites are found upstream of termination sites and are defined as regions of about 80 nts with high C content and little secondary structures. However, unlike intrinsic terminators, no consensus sequence *per se* has been defined, which makes bioinformatic predictions of Rho-dependent terminators difficult.[125,129] Rho binds the RNA in an "open" conformation, and once first contact with the RNA is made the protein adopts a "closed" ring conformation as the RNA passes through the central cavity, resulting in a translocase-competent protein (Fig. 10B).[129]

B. 2. Rho translocates along the nascent RNA

Once Rho has adopted an active form, RNA is transferred in a 5' to 3' direction through the central channel of Rho, and although Rho can be impeded by secondary RNA structures or RBP, recent evidence suggests that Rho is capable of bypassing these structural roadblocks thanks to its RNA-DNA helicase activity.[125] Several models of translocation have been proposed, that differ in the contacts made with the RNA during translocation. In a first simple tracking model, it is proposed that Rho leaves the *rut* site as it moves along the RNA to the EC and only keeps contact with the RNA though its central channel.[125,129] In a second model, named looping model, Rho is suggested to remain bound to the *rut* site during translocation, forming a loop as it reaches over to the EC.[125,129] However, the most accepted model is the tethered tracking model, in which Rho remains bound to the *rut* sites but tracks along the RNA in a zipper-like mode thanks to its helicase activity.[129] It is suggested that Rho translocates along the RNA until it encounters the exit channel of RNAP, a crucial step for inducing termination. Thus, it is suggested that there is kinetic coupling between the transcribing RNAP and the translocating Rho, such that there are competing rates between both enzymes until virtually no RNA is found between them.[129] There are remaining questions regarding a putative association of Rho with RNAP, whether Rho forms part of the elongation complex from the beginning or is recruited later, but it is admitted that Rho could make transient contacts with RNAP and induce conformational changes aiding termination to occur.[129]

B. 3. RNA release and termination

The final step for termination includes pausing of the RNAP that renders the EC susceptible to termination by Rho. Indeed, pausing RNAP or reduced elongation rates were found to increase efficiency of Rho-dependent termination.[125] However, not all halted RNAP

are subjected to Rho termination. Several additional elements contribute to the efficiency of Rho-dependent termination, such as stability of the EC, distance from the *rut* site, the sequence of the 3'end of the RNA trapped in the exit channel of RNAP or secondary structures adopted by the nascent RNA.[125]

To release the nascent RNA, the DNA-RNA helicase activity of Rho is necessary to unwind the DNA:RNA hybrid but not sufficient to dissociate the complex. As for translocation, several models are proposed to explain how the RNA is released from the EC. In the hybrid shearing model, the translocating Rho disrupts the stable DNA:RNA hybrid once the RNA is stretched tightly enough that it exerts a pulling force on the hybrid resulting in its disruption.[125,129] However, it remains uncertain if the Rho is physically capable of generating sufficient force to shear the highly stable RNA:DNA hybrids.[125] According to the hypertranslocation model, Rho exercises a pushing force that causes RNAP to move forward on the DNA without further transcription of the RNA, resulting in the collapse of the transcription bubble and RNA release.[125,129] In the so-called invasion model, Rho directly unwinds the RNA:DNA hybrid with the 3'-end of the RNA remaining in the RNAP active site.[125]

B. 4. Rho cofactors NusG and NusA

B.4.a. NusG

Rho-dependent termination at several Rho-dependent terminators *in vivo* often requires the 21 kD transcription elongation factor NusG.[130] This factor was originally discovered as a component of lambda bacteriophage-mediated antitermination and is able to accelerate transcription elongation by its anti-pausing activity.[131] In *E. coli,* NusG accelerates the elongation rate of RNAP and prevents its pausing.[132] NusG is a universally conserved transcription factor in all bacterial species and is an abundant and essential protein in *E. coli*.[130] Although initially described for its anti-pausing activity, NusG also promotes transcription termination. Depletion of NusG in *E. coli* results in reduced efficiency of transcription termination at some Rho-dependent terminators with even some terminators being fully dependent on NusG.[130] Such is the case of *galE*, the first gene of the *gal* operon in *E. coli,* whose transcription termination is totally inhibited in absence of NusG.[133] Also, NusG along with Rho participates in the silencing of genome-wide potentially harmful pervasive transcription and also participates in genome surveillance by traveling along RNAP.[134]

Bacterial NusG is composed of an N-terminal NGN domain that binds to the beta' subunit of RNAP, connected to a C-terminal KOW domain through a linker. NusG was found to directly interact with RNAP through its N-terminal domain and to Rho or the 30S ribosomal protein S10 (NusE) in a mutually exclusive way through its C-terminal domain. [135] It is proposed that NusG induces faster isomerization of the open to closed conformation of Rho resulting in stabilization of Rho-RNA interactions at suboptimal *rut* sites.[136] About one third of Rho-dependent terminators in *E. coli* are NusG-dependent and the requirement of NusG depends on the nucleotide content at these terminators.[134] NusG-dependent terminators have a lower C/G ratio whereas NusG-independent terminators exhibited a higher C/G content.[134] Thus, NusG aids Rho at low C/G sites, less likely to form *rut* sites with high affinity to Rho.

In *B. subtilis*, although not essential for viability, NusG acts a global pause-stimulating factor by enhancing pausing of RNAP at more than 1'600 sites across the genome including several operons such as *trpEDCFBA, tlrB* and *ribDEAHT*.[137–139] NusG binds to a conserved T-rich sequence on the non-template DNA within the transcription bubble upstream the pause sites and the NusG-dependent pausing is stimulated by a hairpin in the 3' end of the nascent RNA.[140] However, NusG is not believed to aid Rho in controlling gene expression, since Rho-dependent termination in this Gram-positive model is strongly restricted to C-rich RNA regions.[141]

NusG has also been recently associated to intrinsic termination. Although this mechanism does not require any external factors, NusG is able to stimulate intrinsic termination *in vitro* and *in vivo*.[142] NusG is particularly required for enhancing termination at intrinsic terminators with suboptimal hairpins containing weak base-pairs at the bottom of the hairpin stem. Indeed, NusG-dependent terminators showed terminal weak A-U/G-U base-pairs and distal interruptions in the U-tract.[142] It is proposed that NusG pausing activity provides additional time for the formation of the suboptimal hairpin.[142]

The universally conserved NusG homologs contain highly similar domains and interactions with other complexes such as RNAP or the ribosome, but they also show diverse and even opposite effects on gene expression, however no study of NusG and its role in termination in *S. aureus* is available yet.[141]

B.4.b. NusA

The 55-kDa transcription elongation factor NusA was first described in the early 1980s as both a transcription termination and antitermination factor in *E. coli*.[143] NusA was found to induce pausing of RNAP during transcription of the lambda phage, requiring the Rho factor for final transcription termination.[143] It is believed that NusA stimulates termination by assisting with hairpin folding of the nascent RNA and/or by slowing down RNAP and promoting pausing.[144] NusA versatility is explained by its ability to interact with multiple partners through various binding domains namely S1, KH1 and KH2 positively-charged motifs for RNA binding, two C-terminal acidic regions AR1 and AR2 and the N-terminal RNAP binding domain.[145] NusA is able to bind to Rho and inhibits or delays Rho-dependent thus antagonizes Rho-dependent termination *in vivo* in a genome-wide manner. NusA binds to *nut* sites that were found to overlap the Rho-dependent *rut* sites, therefore competing for recognition and reducing the efficiency of Rho loading to the mRNA.[129,146]

NusA, as NusG, is essential in *E. coli* and has also been implicated in Rho-dependent and intrinsic termination. NusA only becomes inessential in mutants with defective Rho-dependent termination.[147] Both Nus factors have similar targets and are proposed to act in concert with each other.[148]

NusA also stimulates intrinsic termination *in vitro* and *in vivo* in *B. subtilis*, where this gene is also essential. NusA proteins from *E. coli* and *B. subtilis* have similar functions and are interchangeable.[149] Deletion of *nusA* lead in an increase in antisense transcription especially at convergent loci, similar to the effects observed when deleting Rho and NusG.[144] It was also found that a large number of weak non-canonical terminators are solely dependent on NusA, including genes involved in replication and DNA metabolism.[144]

Recent studies have unveiled the transcriptome-wide effects of NusA on polymerase pausing in B. *subtilis*.[149] Transcriptome-wide NusA-stimulated pauses where limited and some of the identified sites were also NusG-dependent regions.[149] For instance, NusA was also found to stimulate pausing of the leader region of the *trpEDCFBA* operon.[149]

In *S. aureus*, NusA was found to be essential and localized to the nucleoid, as has been described for *E. coli* and *B. subtilis*.[150] However, the biological and regulatory functions of NusA in this pathogen have not been described yet, neither its implication in Rho-dependent termination.

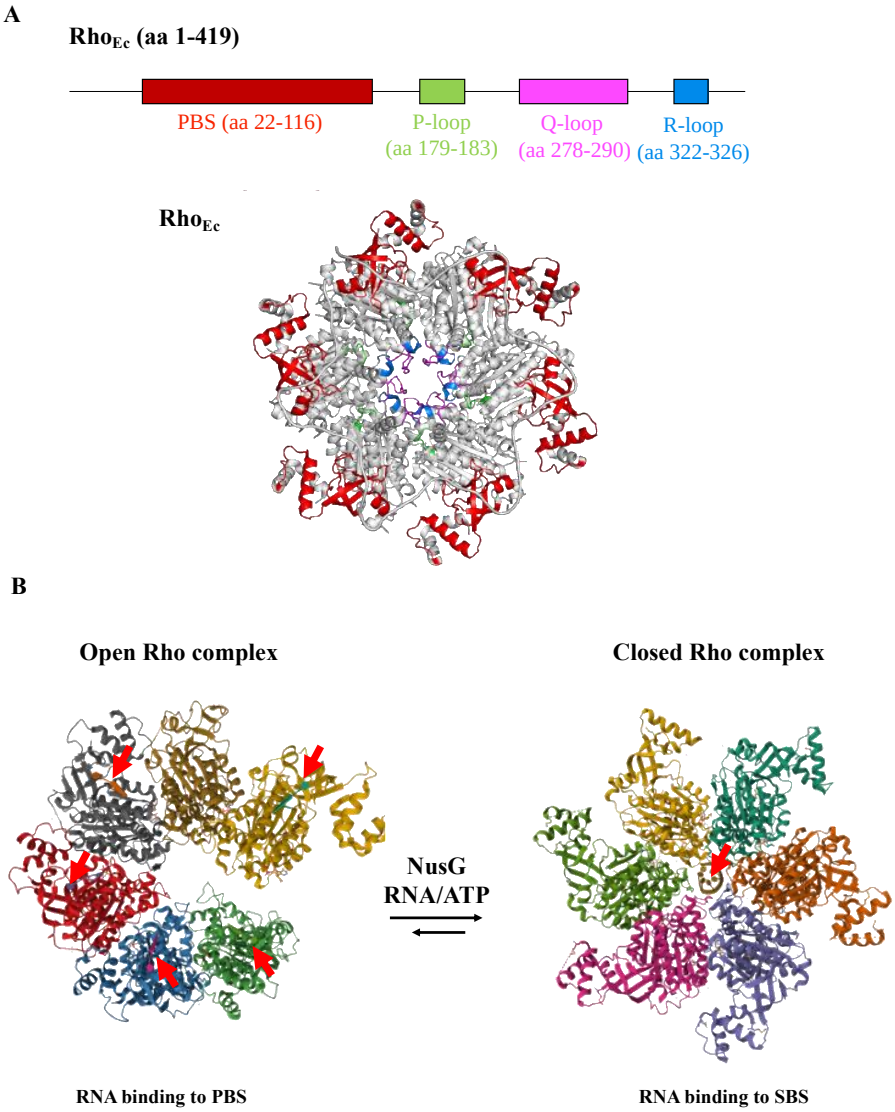

Figure 10 - Rho structure and organization in *E. coli.*

A. Domain organization of Rho in *E. coli*. The 419 amino acid-long protein is composed of the primary binding site (PBS) from aa 22 to 116 (red) that recognizes ssDNA or ssRNA and binds to *rut* sites on nascent RNAs, the P-loop motif from aa 179 to 183 (green) responsible for ATP binding and hydrolysis and the secondary binding site (SBS) composed of Q-loop (magenta) and R-loop (blue) spanning aa 278 to 290 and 322 to 326 respectively. The hexameric model of Rho in its active state is represented and the domains described above are depicted in their respective colors. (electron microscopy structure derived from Protein Data Bank ID 8E70). **B.** The Rho open and inactive complexes (X-ray crystal structure derived from PDB ID 1PVO) engage in first interactions with RNA through its PBS on the complex surface. Binding to the SBS in the central channel of the complex induces ring closure and formation of a closed and active Rho complex (X-ray crystal structure derived from PDB ID 3ICE). Presence of NusG and ATP can accelerate this isomerization to a translocation competent conformation. Bound RNA molecules are shown with red arrows. Each monomer is depicted with different colors.

II. Structure and conservation of Rho

A) Structure of Rho in *E. coli*

Rho is able to form dimers, tetramers, hexamers and even higher orders of oligomerization depending on protein and ion concentration or co-factors.[151,152] However, the homohexameric form is the principal active state in presence of ATP.[153] Rho in *E. coli* is 419 amino acids long and is composed of four functional domains pictured in Fig. 10A. The N-terminal domain contains the primary RNA-binding site (PBS) encoded by amino-acids 22 to 116. The ATP-binding and hydrolysis domains formed by Walker A and B motifs is found in the so-called P-loop in the C-terminal domain of the proteins, ranging amino acids 179 to 183. This region bears significant homology with the F1 ATPase. The secondary RNA- binding site (SBS) is formed by the Q-loop and R-loop, ranging amino acids 278 to 290 and 322 to 326 respectively.[129] The homohexameric Rho is shaped as a doughnut, with the PBS exposed on the outer ring surface, the ATPase binding site in the interface between subunits and the SBS in the inner ring of the complex (Fig. 10A).

Several crystal structures of Rho along with RNA, ATP or other protein factors have been solved over the last decades. The PBS of Rho is composed of an oligonucleotide/oligosaccharide-binding (OB) fold, that binds exclusively to two pyrimidine bases, with preference for cytosines.[154] This site ensures the very first interactions with the *rut* sites, which are rich in cytosines.[155–157] Further binding of RNA to the SBS triggers ATP-binding and hydrolysis by the P-loop domain and activation of the 5' to 3' translocation activity of Rho. Indeed, although the Q-loops exclusively interacts with RNA, the R-loop binds both RNA and ATP and is located in the interface between subunits next to the ATP binding site.[158] Even if Rho contains six ATP-binding sites, it only binds three ATP molecules.[158] The homohexamer can exist in two conformations, one inactive open-ring structure and the active closed-ring conformation requiring ATP and RNA binding (Fig. 10B).[159] Rho remains in an open-ring structure until the PBS binds to the *rut* site on the target RNA. The RNA molecule circles the outer ring of the homohexamer, passes through the central channel and is engaged in further interactions with the SBS, which finally induces ring closure and activation of the enzymatic activity (Fig. 10B).[159] The close proximity of the R-loop from the SBS to the P-loop suggests that the R-loop motif might couple RNA binding to activation of ATP binding and hydrolysis.[159]

Structure predictions of Rho in *S. aureus* show similar hexameric oligomerization as the one described in *E. coli*, with the N-terminal exposed on the surface of the protein complex. (Fig. 11A) This suggests a conservation of the structural organization of this termination factor.

B) Conservation of Rho

Rho is conserved in over 90% of bacterial genomes and nearly all bacterial phyla contain the *rho* gene, with only few species lacking this termination factor. 29 species of Cyanobacteria and 27 species of Mollicutes (Tenericutes) do not contain a *rho* gene, as well as a fraction of Firmicutes.[160] All these bacteria are believed to have lost *rho* during evolution. Some *rho*-less species include endosymbionts and parasites for which *rho* is believed to have been lost in the process of adaptation to new intracellular lifestyles.[160] However, numerous exceptions defy this hypothesis, for instance the intracellular *Rickettsia* and *Chlamydia* encode and express Rho proteins.[160]

Also, some species have duplicated and/or atypical *rho* genes containing deletions/insertions of unknown domains that are believed to alter Rho activity and target selectivity.[160] There is a seemingly correlation between genome complexity and the presence of the *rho* gene, since large G/C rich genomes are more prone to encode for a *rho* gene than genomes containing a poorer G/C ratio, suggesting that Rho might have evolved to accomplish specific functions in these bacteria. Nevertheless, low GC organisms such as *S. aureus* and *B. subtilis* encode and express functional Rho proteins.

Most Rho proteins are around 420 amino acids long but Actinobacteria and Bacteroidetes contain significantly larger homologues with the length also varying within the phyla.[160] The longest Rho sequence, with 865 amino acids, belongs to the marine extremophile member of the Firmicutes *Thermaerobacter marianensis*. As for other Firmicutes, this increased length is due to a long N-terminal domain of 490 amino acids upstream of the PBS.[160] In bacteria encoding two Rho ORFs, such as gamma-Proteobacteria *Colwelli psychrerythraea* and *Marinomonas sp*, usually one Rho ORF is incomplete, encoding for very short Rho proteins of around 300 amino acids lacking essential RNA-binding domains, and one complete *rho* gene encoding for a supposedly functional Rho protein.[160] This suggests that one copy of the *rho* gene has been inactivated, probably to avoid any deleterious effects of an overexpressed Rho factor. A few eukaryotic genomes (*Drosophila ananassae* and *Callosobruchus chinensis*) have acquired a *rho* gene through horizontal transfer, however its expression and function are not known.[160]

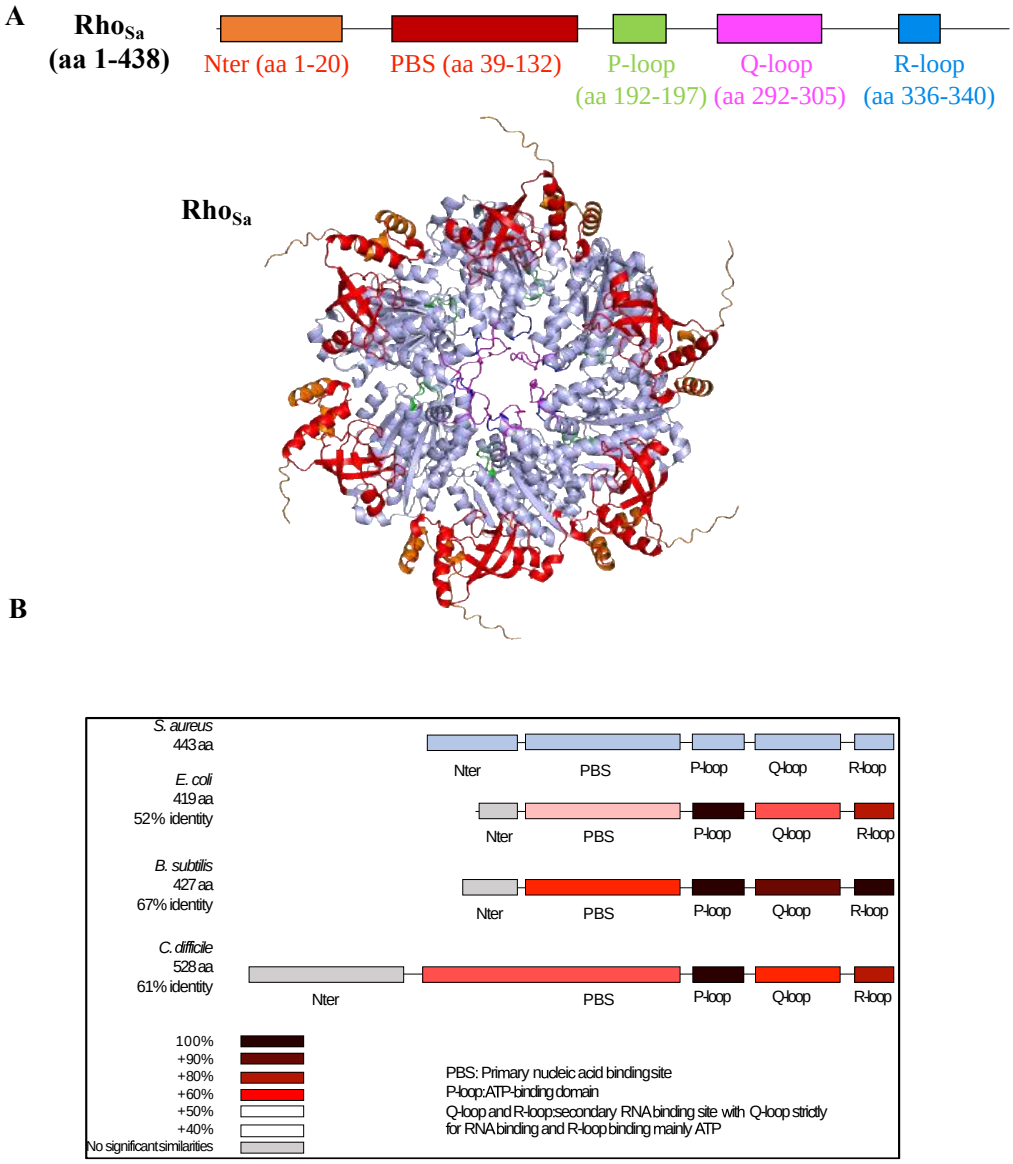

Figure 11- Conservation of Rho among bacteria

A. Predicted structure assembly of the Rho hexamer in *S. aureus* obtained with AlphaFold2. The predicted domains are represented as follows: the NTD insertion in orange, the PBS in red, the P-loop in green, the Q-loop in magenta and the R-loop in blue. **B.** Amino acid sequences of Rho from *E. coli, B. subtilis* and *C. difficile* were aligned to the protein sequence of Rho from *S. aureus* using the BLASTP tool from NCBI. The functional domains of Rho are depicted in colored boxes. ATP binding and hydrolysis domains are overall better conserved than RNA binding domains, with the P-loop being perfectly conserved among species. The N-terminal domain is not conserved at all and may confer specific functions to each bacterium and contribute to target recognition and specificity.

Overall, several regions of Rho are well conserved among the studied species, with key residues involved in oligomerization of subunits, ATP hydrolysis and RNA binding being highly conserved (Fig. 11B).[160] The C-terminal domain is much more conserved than the N-terminal one, mostly due to a large NTD insertion found in several Firmicutes. These insertions are found in 35% of the studied Rho sequences and do not correlate with the complexity of the genome. They are highly hydrophilic, positively charged and may contribute to specific behaviors and functions of Rho in their respective species. However, the length and composition of these domains are not conserved and even if it is proposed to facilitate Rho binding to G/C rich RNAs in Actinobacteria, this domain is also found in G/C poor genomes of Firmicutes and Bacteroidetes. The precise roles of these domains for most bacteria remains to be elucidated, but in *Mycobacterium tuberculosis*, the NTD is critical for RNA binding and compensates for the suboptimal motor functions of Rho in this species.[161]

The ubiquitous presence of Rho among bacterial genomes points at an ancient origin of this protein and important biological functions. The conservation of domains involved in ATPase activity indicate that the transcription termination function of Rho might be universally conserved, but variations in size of the protein and the NTD insertions might contribute to species-specific regulatory pathways in which Rho might be involved.

III. Diverse physiological roles of Rho

A) Transcription/translation coupling – attenuation

Due to the coupling of transcription and translation in prokaryotes, it seemed logical to assume that Rho-dependent termination targeted mostly non-translated mRNAs. During transcription of protein-coding genes, RNAP is immediately followed by the ribosome through the simultaneous interaction of NusG with RNAP and the ribosomal protein S10 (NusE). Actively translating ribosomes physically block the access to the *rut* sites of the mRNA, inhibiting the action of Rho on the latter. Under suboptimal translation conditions like nutrient limitation or nonsense mutations, shielding of the *rut* sites by the ribosome is lost and these sites become exposed for Rho binding, resulting in transcription termination to avoid accumulation of the transcript.[129] In the latter case, interaction of the ribosomal protein S10 to NusG is outcompeted by Rho. Because of this transcription-translation coupling and the intrinsic organization of operons, premature termination of protein synthesis of the first gene

of an operon can results in Rho-dependent transcription termination of the downstream genes of the respective operon yielding a phenomenon called translational polarity.[129] Rho-dependent terminators are therefore often found in the 5' leader regions of several operons. This is the case for the *pgaABCD* operon in *E. coli*, encoding for synthesis and export proteins of a polysaccharide adhesin involved in biofilm formation. The *rut* site found in the 5' leader region is usually sequestered in a secondary structure blocking the access of Rho and allowing for transcription and further translation.[162] However, during the shift to a planktonic environment, the global regulator protein CsrA (whose expression increases upon shift to a planktonic state) binds to the 5' UTR of the operon and unmasks the *rut* site, resulting in transcription termination and downregulation of the operon which is not needed during planktonic growth.[162]

Rho also participates in the regulation of genes encoding tryoptophanase (*tnaAB*) in *E. coli* and Mg^{2+} transporters in *Salmonella (mgtA, mgtB, corA)*.[163–165] The 5' leader regions of these operons contain a small ORF that operates as a translational sensor. If the ribosome stalls during translation of the small ORF, Rho binding is prevented due to the formation secondary structures blocking the *rut* sites, allowing transcription of the downstream ORF to take place. If the ribosome smoothly moves along the small ORF, completion of this first translational cycle creates a small time-window during which Rho can bind the 5' leader region and terminate transcription. This is known as reverse polarity. For *tnAB*, high tryptophane levels result in the blocking of the ribosome exit channel by L-Trp molecules and blocking the release of the leader peptide resulting in stalling of the ribosome that physically block access to the *rut* sites.[163] For the Mg^{2+} transporters, the ribosome stalls in the leader region when this metal ion is limiting due to its requirement for peptidyl-transferase of certain codons such as proline.[164] Ribosome stalling promotes the formation of secondary structures that sequester the *rut* sites and result in expression of the needed transporters.[165] A similar phenomenon of reverse polarity is observed for the autoregulation of the *tufB* gene in Salmonella encoding the translation elongation factor Tu. When Tu levels are insufficient, ribosomes slow down during translation of the *tufB* mRNA, promoting the formation of secondary structures masking the *rut* sites leading to effective transcription and translation of the gene. The resulting increased levels of Tu allow the ribosomes to quickly move along the transcript rendering the *rut* site structurally available for Rho binding leading to premature transcription termination and regulation of the cellular Tu amounts.[166]

Rho activity has also been associated to the flavin mononucleotide (FMN) riboswitch in *E. coli* and the Gram-positive bacterium *Corynebacterium glutamicum*.[167–169] In *E. coli*, the binding of FMN to the 5'UTR of the gene encoding for a riboflavin biosynthetic enzyme *ribB*

promotes Rho-dependent termination. It is proposed that binding of FMN to the leader region results in structural remodeling and creation of a *rut* site.[167,170] In *C. glutamicum*, although the Rho termination sites were not determined, results indicated that Rho selectively terminates transcription of the FMN riboswitch in the presence of FMN.[169]

Rho-binding sites, especially when found in 5' leading regions of genes and operons participate in regulatory mechanisms and can act as a regulatory switch. Thus, Rho is a flexible and dynamic actor in riboswitch-mediated regulation.[168,170]

B) Pervasive transcription

An important and well-conserved role of Rho among several organisms is the suppression of pervasive transcription. These non-canonical transcripts arise from transcription at cryptic promoters or from transcriptional readthrough at weak terminators and frequently result in the generation of antisense transcripts whose biological functions are still debated (Fig. 12).[171] However, at high levels, these antisense RNAs (asRNAs) can potentially interfere with sense gene expression. Thanks to the advances in high throughput and next generation sequencing technologies, especially the development of unbiased analysis independently of genome annotation, the existence of pervasive transcription has been unveiled in animals, yeast and bacteria.[171] Because mapping is often based on existing genome annotations, these transcripts were often dismissed and considered artifacts or transcriptional noise. It has been argued that contamination with genomic DNA might generate sequencing background that could be mistaken for RNA sequences or that these artifacts were due to poor microarray DNA probe specificity. However, this phenomenon has been identified through different independent methods in a wide array of bacteria including *E. coli*, *Helicobacter pylori*, *B. subtilis* and *S. aureus*.[119,172–174] More importantly, it has been found that levels of pervasive transcription are not constant, vary depending on growth conditions and are sensitive to global regulatory systems, which suggests that pervasive transcription is a regulated process and not only biological noise.[119,171] Even if tolerated by numerous bacteria, pervasive transcription needs to be suppressed and different layers of regulation exist, which go from transcription initiation, elongation and termination steps to RNA stability (Fig. 13). In Gram-negative bacteria, the histone-like nucleoid structuring protein H-NS is involved in DNA-folding and prevents RNAP from initiating transcription at AT-rich regions, where cryptic promoters are mostly found, by occluding the promoters.[175] If the RNAP evades the H-NS silencing system

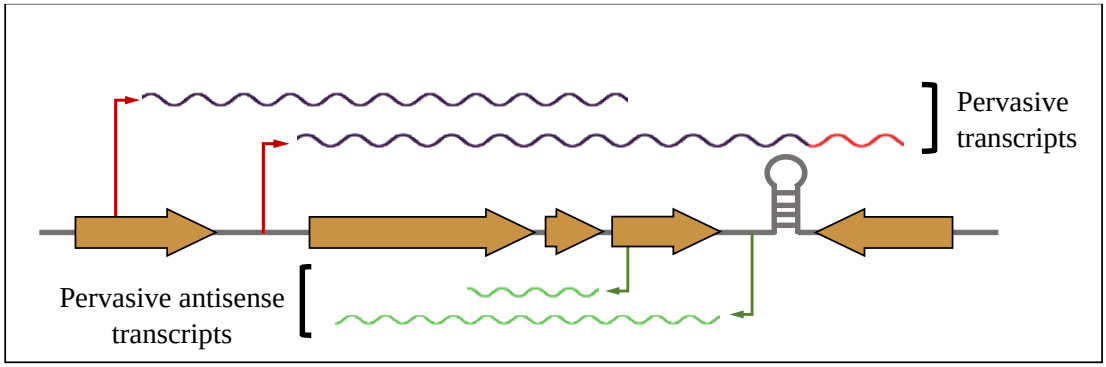

Figure 12 - Pervasive transcription in bacteria.
Pervasive transcripts can be generated following inefficient transcription termination. Read-through at these suboptimal intrinsic terminators can generate an anti-sense transcript (in red) to a downstream gene. Cryptic promoters (in red and green) can be present in inter and intragenic regions and are a major source of pervasive transcripts that can be sense (black) or antisense (green).

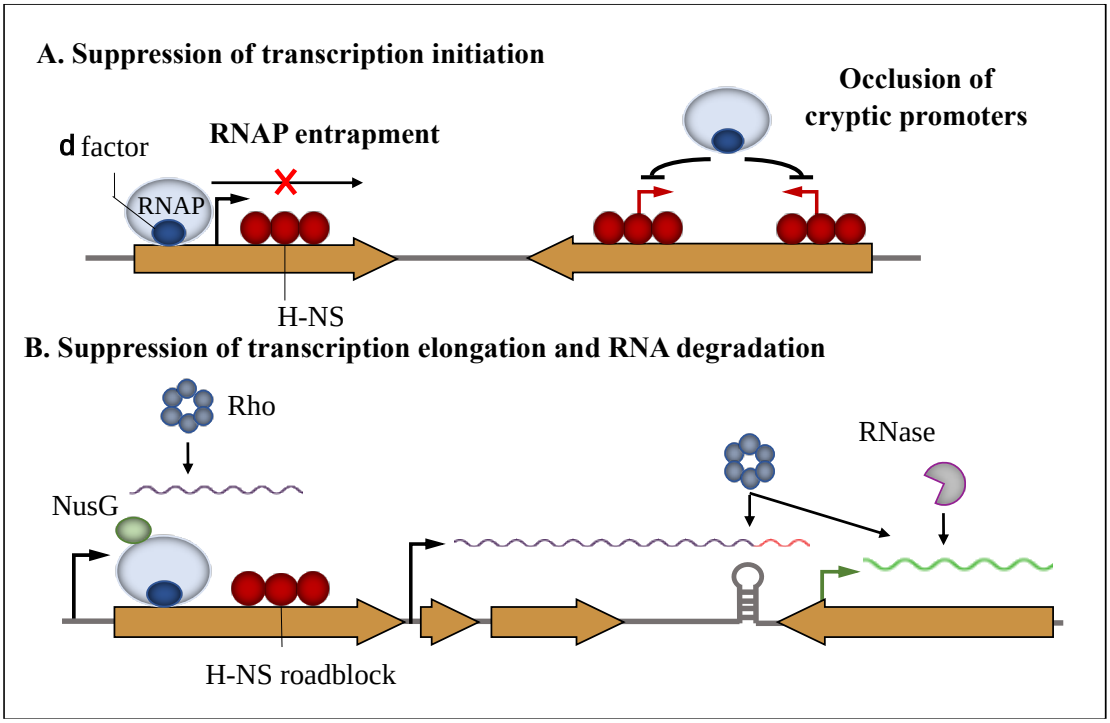

Figure 13 - Mechanisms of suppression of pervasive transcription in *Escherichia coli*.
Pervasive transcription can be regulated and suppressed at different levels. **A)** At the level of transcription initiation, H-NS protein binds to AT-rich regions of the chromosome that often harbour cryptic promoters and inhibits transcription initiation by blocking RNAP from binding to these promoters. **B)** If transcription is initiated despite early mechanisms to prevent it, H-NS binding to intragenic regions of the chromosome can slow-down RNAP and promote pausing, rendering transcripts more susceptible for Rho-mediated termination. Premature Rho termination is stimulated by the recruitment of NusG to the elongation complex. Antisense transcripts (green) and readthrough regions (red) are prone to Rho- and NusG-mediated regulation. Finally, pervasive transcripts can be degraded by RNases if not protected and translated by the ribosome.

and elongates into spurious transcripts, those are then rapidly terminated by Rho-dependent termination. H-NS can also influence Rho-dependent termination of spurious transcripts, since the presence of H-NS slows down the elongating RNAP, allowing Rho to terminate transcription at these locations.[34,176] For instance, bicyclomycin-induced inhibition of Rho results in an increase of antisense transcripts in *E. coli*.[134] This antibiotic inhibits Rho ATPase activity by binding at the center of the machinery and preventing RNA-induced conformational changes needed for the activation of ATPase activity.[177] Deletion of *rho* in *B. subtilis* and *S. aureus* resulted in a similar phenotype.[122,174] In these organisms, H-NS is absent, so deletion of spurious transcripts heavily relies on the action of RNases like RNAse III that specifically degrades duplexes formed of asRNAs with their cognate sense strand transcripts in *B. subtilis* and *S. aureus*.[78,178] RNase III-mediated degradation of pervasive and antisense transcripts is a conserved and global last-line mechanism to avoid this phenomenon.[171]

The physiological roles and relevance of spurious transcription are still a matter of debate. The numerous layers of regulation and regulatory mechanisms that exist to prevent this phenomenon suggest serious negative effects on bacterial fitness. Nevertheless, the background levels of existing pervasive transcription and the fact that the absence of Rho or RNase III, the two main players in its suppression, does not impair growth of several bacterial species points to potential functions of these spurious transcripts. It has been suggested that widespread antisense transcription could have global gene regulatory effects or that it could be a driving force for genome evolution.[171]

C) Repression of prophages and surveillance of genome integrity

Bacterial genomes contain cryptic prophages that encode for toxic genes, that when expressed are toxic and deleterious to the cell. Rho-dependent termination has evolved as a means to repress expression from these prophage genes. Bicyclomycin treatment in *E. coli* lead to the upregulation of prophage genes and death.[148] A concrete example of a prophage repressed by Rho is the *kilR* gene carried by the *rac* prophage in *E. coli*. This gene is controlled by a constitutive promoter and its expression is deadly to the bacterium. However, a Rho-dependent terminator has been described upstream the *kilR* gene, preventing its expression.[179]

In addition, Rho prevents the formation of genome-wide R-loops, a structure formed of three-strands of nucleic acids: a DNA:RNA hybrid and the non-template DNA strand. These R-loops can be formed during replication, recombination and transcription and they are believed to promote supercoiling of DNA which can harm genome stability.[129,180,181] Thus,

eliminating these R-loops is a necessity and it is proposed that Rho, thanks to its helicase activity, is capable of removing these harmful structures and preserves genome integrity.[181]

Because in bacteria the rate of DNA replication is up to 20 times faster than transcription, collisions between the replication fork and the transcription elongation complex are prone to occur. These collisions result in fork collapse and double-strand breaks (DSB), endangering genome integrity.[182] Rho is able to dissociate stalled transcription elongation complexes ahead of replication forks, thereby preventing collisions and DSB to take place. This is supported by the increase in DSB after bicyclomycin treatment.[182]

D) sRNA-mediated regulation

Rho preferentially targets relatively long, unprotected and unstructured RNA regions as stated with the examples above. It usually involves 5' untranslated regions of mRNAs. These 5'UTR are also known to be current targets for sRNA binding to the RBS and affecting mRNA translation and stability. Several studies have unveiled an existing synergy between sRNA-mediated regulation and Rho-dependent termination (Fig. 14A). The first example of this cooperation is the regulation of the *chiPQ* operon in *Salmonella*, encoding a porin transporting chitin-derived sugars (ChiP) and a small lipoprotein of unknown function (ChiQ). The sRNA ChiX binds to the 5'UTR of the *chiPQ* operon, inhibiting the ribosome from binding to the RBS of *chiP*.[183] However, it was observed that ChiX also inhibits the expression of the downstream *chiQ*. The authors of this study identified three classes of mutants that restored *chiQ* expression, which included mutations in *rho, nusG* and in a putative *rut* site within the *chiP* gene.[184] These results supported the hypothesis that Rho, along with NusG prematurely terminate transcription of the *chiP* gene resulting in inhibition of the entire operon. [184] By binding to the leader region of *chiPQ*, ChiX prevents the ribosome from binding and allows Rho to access the *rut* site.[183] This regulatory mechanism found in *E. coli* and *Salmonella*, is believed to be conserved among *Enterobacteriaceae* since the *chiP rut* site seems to be conserved.[167] The galactose operon *galETKM* in *E. coli* is regulated by the sRNA Spot 42 and Rho-dependent termination as well. Spot42 binds to the *galT-galK* boundary region and inhibits translation of *galK*.[185] The resulting translational repression of *galK* promotes Rho-dependent termination.[186]

sRNAs can also act as anti-terminators (Fig.14B). The *rpoS* gene in *E. coli* encoding for the alternative stationary phase and stress-responding sigma factor Sigma S. This gene

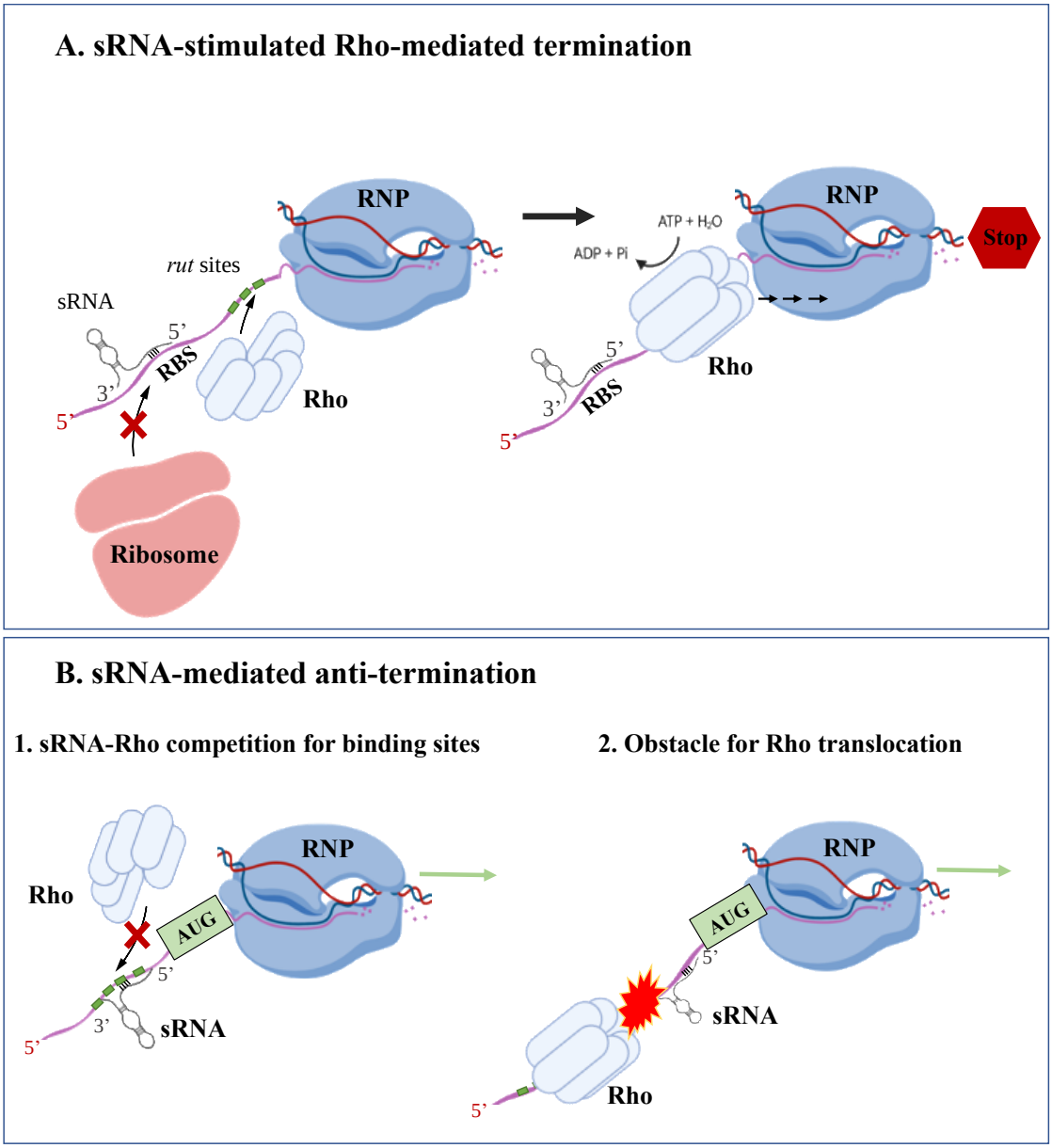

Figure 14 – Regulatory interplay between sRNAs and Rho

A. sRNAs can promote Rho-mediated termination by binding to a complementary region on the 5'UTR of the target mRNA and masking the ribosome binding site (RBS). The *rut* sites (green boxes) are no longer shielded by the ribosome and become then accessible for Rho binding and transcription termination. **B.** sRNAs can also act as transcription anti-terminators. 1. If the sRNA target sequence is in a region comprising *rut* sites, the sRNA competes with Rho for binding to the mRNA. If the sRNA binds first, the formation of an sRNA:mRNA duplex prevents Rho binding and further termination. The sRNA binding site can also be present just downstream the *rut* sites, in which case Rho can bind to the mRNA but its translocation is stopped by the sRNA:mRNA duplex, also resulting in an anti-termination mechanism. Adapted from Bossi *et al*, 2020.[167]

possesses a long 5'UTR with a RBS sequestered inside stem-loop structures resulting in poor *rpoS* translation.[187] Three different sRNAs namely DsrA, RprA and ArcZ are transcribed in response to stress and activate translation of *rpoS* by binding to its 5'UTR and liberating the occluded RBS. It has also been found that *rut* sites are present nearby the binding sites of these sRNAs onto the *rpoS* mRNA. By binding to the mRNA, these sRNAs not only free the RBS to promote translation, but they also compete with the binding of Rho, working as anti-terminators and Rho antagonists.[187] The binding of sRNAs to the mRNA is proposed either to inhibit the binding of Rho to the *rut* sites or to interfere with translocation of Rho along the mRNA.[187] Another example of sRNA-mediated anti-termination is found in the *rho* gene itself in *E. coli* and *Salmonella*, where the sRNA SraL and Rho compete for the binding to the 5'UTR.[188] The regulatory mechanisms behind the *rho* gene will be discussed in the next paragraph.

Most of the current knowledge about Rho-mediated regulatory mechanisms involving sRNAs has been accumulated from studies in Gram-negative bacteria like *E coli* and *Salmonella*. In Gram-positive bacteria little is known about the involvement of Rho in sRNA-mediated regulation. Some exceptions to this (discussed earlier) include the FMN riboswitch in *C. glutamicum* controlling the expression of the downstream riboflavin transporter gene *ribM* through Rho-dependent transcription termination and the *trpEDCFBA* operon in *B. subtilis* subjected to Rho-dependent attenuation.[167,169,189] Thus, the involvement of Rho in sRNA-dependent regulatory networks in Gram-positive bacteria needs further investigation. Promising findings are expected especially since the role of auxiliary proteins in these organisms remains unclear.

E) Regulation of the *rho* gene and Rho regulators

In *E. coli*, the *rho* gene is essential for growth and the levels of the Rho protein are maintained constant throughout growth.[190] In *E. coli* mutants with increased transcription termination activity, Rho levels decrease, which suggests that the expression of *rho* gene is autogenously regulated.[190,191] Indeed, synthesis of the Rho protein is autogenously controlled by self-mediated transcription attenuation within the leader region of the mRNA.[190] Several Rho-dependent terminators have been identified in the 5'UTR of the *rho* gene that induce in premature transcription termination.[190] Several years later, the sRNA SraL conserved in *E. coli* and *Salmonella*, was found to positively regulate the expression of the *rho* gene though base-pair interactions with the *rho* mRNA.[188] SraL directly interacts with the 5'UTR of the *rho*

mRNA near two of the predicted identified attenuators and protects the *rho* mRNA from premature termination by Rho.[188] Thus, *rho* mRNA levels decrease in absence of SraL.[188] This sRNA positively regulates *rho* mRNA levels during exponential phase when SraL is only weakly expressed, but also during stationary phase or anaerobic shock when it is activated.[188] However, no effects on the level of the Rho protein were detected. [188]The chaperone Hfq is involved in the regulation of *rho* via different mechanisms. It is associated with SraL and promotes "classical" chaperone activities for base-pair interactions. However, Hfq also regulates *rho* in a SraL-independent pathway, in which this chaperone is able to bind to the Rho protein and inhibits its ATPase, helicase and termination activities.[188] Thus, the mechanisms behind the regulation of the *rho* gene are complex. It is therefore proposed that in *E. coli* and *Salmonella,* SraL in complex with Hfq or ProQ, when expressed, binds to the 5'UTR of the *rho* mRNA near the predicted *rut* sites, and prevents either the loading of Rho to its own mRNA or its translocation, protecting the transcript from premature transcription termination. In the absence of the sRNA, the *rut* sites on the *rho* mRNA are free and exposed for Rho binding and transcription termination occurs.[188] Other factors have been described to regulate the function of Rho. The Psu protein encoded by the *psu* gene of the satellite bacteriophage P4 inhibits the enzymatic activity of Rho or the interaction of Rho with any of its cofactors (RNA, ATP, NusG or RNAP).[192] YaeO, encoded in the genome of *E. coli,* has also been found to reduce termination of several genes including the *rho* gene.[192] YaeO binds to Rho and reduces its affinity for RNA molecules by inhibiting the first contacts of RNA to Rho. [192] Recent studies have identified a third binding partner of Rho that associates transcription termination to stress response. This novel interactant is YihE, a Ser/Thr kinase activated by the Cpx pathway in *E. coli.*[193] Cpx is a stress response TCS important for maintaining cell envelope integrity in Gram-negative bacteria and thus can also contribute to virulence in several species.[193,194] Stimuli that induce this system include variations in salt concentrations, alkaline pH, carbon-monoxide or envelope stressors such as misfolded proteins, and this further drives transcription of the *yihE* gene.[193] YihE binds to Rho through electrostatic interactions involving a negatively charged NTD of YihE and a positively charged surface in the RNA binding site of Rho, and affects its interaction abilities with RNA thereby affecting global gene expression.[193] Curiously, this study showed that some stress conditions such as growth at 42ºC or pH 4.7 can induced the expression of the *rho* gene which can have negative effects on bacterial fitness, and that concomitant overexpression of YihE neutralizes the damaging effects of Rho increase and help survival.[193]

Expression of YihE, that co-localizes with Rho to the cell poles, counteracts these negative effects of Rho on cell morphology.[193] Such changes in cell morphology, for instance an elongated shape, are part of the large arsenal bacteria deploy to adapt to environmental stressors.[193] The Cpx system, through the action of YihE plays an important function in membrane homeostasis during stress.[193] It is proposed that during stress, when the expression of Rho increases and the hyperactive transcription termination damages the cell, Rho recruits YihE to neutralize these negative effects of Rho by interfering with its RNA binding abilities and allowing bacteria to survive.[193]

Overexpression of Rho was found to affect cell morphology by silencing the cell wall biosynthesis operon *waa* with the assistance of NusG.[195] The best characterized NusG paralog RfaH, counteracts the silencing of the *waa* operon by Rho by competing with NusG for binding to the RNAP leading to the activation of cell wall protein synthesis.[195]

Recent studies have found that Rho can form inactive filament structures *in vitro* and *in vivo*.[196] The stress alarmone (p)ppGpp binds to the ATP binding site of Rho and stimulates oligomerization of the protein. It has been found that ADP also promotes formation of filaments *in vitro* but the biological relevance of this phenomenon is unclear since ADP levels do not change during stress.[196] This hyperoligomerization state of Rho is reversible under stress relief resulting in the liberation of active Rho hexamers.[196] These results have shown that the Rho factor can be temporarily inactivated by forming large filaments during stress, when translation is slowed down and RNA transcripts become exposed.[196] This reversible inactivation mechanism is a cost-effective form to regulate the action of Rho during stress, avoiding the need for *de novo* synthesis of this factor when stress is relieved.[196] This stress-stimulated hibernation mechanism has already been described for other cellular machineries such as the ribosomes, RNAP and other metabolic enzymes.[196]

In *B. subtilis*, the *rho* gene is not essential, but it is also regulated autogenously. Expression of Rho in *B. subtilis* is rather low, comprising 0.004% of total soluble protein against 0.1% in *E. coli.*[197] Inhibition of Rho with bicyclomycin increased Rho expression fivefold, suggesting a negative auto-regulation of Rho on its own mRNA.[197] This autogenous regulation requires specific sequences in the leader region of the mRNA and the 5' end of the *rho* gene which suggests the presence of Rho-dependent terminators in this region.[197] Two candidate sites for potential "classical" *rut* sites, as described in Gram-negative bacteria have been identified in the leader region of the *rho* mRNA in *B. subtilis*, mostly two regions of low secondary structure and high C/U content.[197] Nevertheless, levels of Rho protein are not constitutive as observed in *E. coli*, as the amounts decrease during stationary phase compared

to exponential phase.[198] Thus, we cannot exclude that *rho* mRNA is post-transcriptionally regulated by still unknown factors in this micro-organism. It has been recently described that Rho is involved in the control of several phenomena taking place during stationary phase.[198,199] These regulatory functions of Rho in *B. subtilis* will be discussed later.

F) Rho and intrinsic termination

Recent studies have shown that the line separating intrinsic termination from Rho-dependent termination can be blurry. Efficient transcription termination is key to ensure correct gene expression. However, intrinsic terminators can be effective depending on the strength of the interactions within the hairpin. A canonical intrinsic terminator consists of a G/C-rich hairpin followed by a stretch of 7 to 9 U residues and the amount of U residues determines the strength of the terminator. Indeed, non-canonical and weaker terminators contain less U residues and hairpins with a higher energetical cost.[200] In mycobacteria, intrinsic terminators are quite inefficient and they have accordingly evolved mechanism to bypass this problem. Intrinsic termination function along with Rho-dependent termination to avoid read-through at these inefficient terminators and this phenomenon has also been identified in *E. coli*.[200] Thus, Rho stimulates transcription termination at the 3' ends of genes with suboptimal terminators in these organisms.[200] In *B. subtilis*, Rho was also found to stimulate up to 10% of intrinsic terminators *in vivo*, sometimes with the help of NusG.[201] *Rut* sites are predicted downstream these suboptimal terminators.[201] This has created a new class of "hybrid Rho-dependent termination", where Rho-dependent termination occurs after read-through at intrinsic terminators, named Rho-stimulated intrinsic terminators.[201]

Thus, transcription termination in bacteria is a complex process involving several players that can act alone or in concert with each other, to ensure correct gene expression.

IV. Rho in Bacillota

The main conserved function of Rho in Firmicutes is the control of pervasive transcription. Deletion of Rho in *B. subtilis* and *S. aureus*, in which *rho* is not essential, lead to an increase in antisense RNA production and genome-wide cryptic transcription.[122,174] Even if cells behaved well in absence of Rho, asRNA accumulation had a negative effect on sense transcripts in *S. aureus*.[122] Due to the limited impact of the absence of Rho, its physiological relevance in these bacteria has been long debated.

A) Rho in *B. subtilis*

The roles of Rho in the Gram-positive model bacterium *B. subtilis* have been recently studied and has been implicated in controlling important physiological pathways related to *B. subtilis* physiology (Fig. 15). This bacterium can adopt a motile state in which individual cells synthesize flagella or in a community-like organization in which cells form biofilm.[199] The alternative sigma factor SigD determines the motile state by driving the synthesis of the flagellar apparatus. Conversely, when adopting a biofilm behavior, flagellar genes are repressed and genes necessary for the production of the biofilm matrix are activated.[199] *B. subtilis* can also sporulate to resist stress by activating a cellular differentiation program involving the master regulator Spo0A, whose phosphorylation state is determinant for its activity. This regulator also controls biofilm formation and when its phosphorylated form (Spo0A-P) is not abundant, biofilm formation and sporulation are repressed. When the concentration of Spo0A-P increases, biofilm formation is activated and only when this form is highly abundant, cells enter the sporulation phase. The levels of Spo0A-P are thus determinant for the proportions of the *B. subtilis* population adopting either form.

It has been recently found that loss of Rho in *B. subtilis* affects cellular differentiation programs involving motility, biofilm formation and sporulation.[199] Deletion of *rho* altered the whole transcription landscape, mainly by affecting asRNA expression, although the effect on the sense strand regulation is not clear. It was found the the SigD regulon, including genes from the Spo0A and CodY regulons were significantly upregulated in the *rho* mutant.[199] Conversely, genes needed for sporulation were found over-expressed in absence of Rho both at the transcriptomic and proteomic levels.[199] Furthers analyses confirmed that inactivation of Rho impairs motility, as this process is controlled by SigD. This sigma factor constitutes a switch between sessile and motile states, since it activates motility and represses the sessile state. Activity of SigD is modulated by protein factors that can inhibit its activity. Rho has been found to control expression of sense transcripts associated with *slrR* and *slrA*, two antagonizing factors of SigD (upregulated in the *rho* mutant) and also an antisense transcript resulting from read-through at the *flhO-flhP* operon encoding for flagellar components, resulting in down-regulation of the operon (Fig. 15A).[199] Deletion of *rho* also impaired biofilm formation due to inefficient de-repression of the matrix operon and the negative effect of an asRNA of the *eps* genes encoding the main component of the biofilm matrix exopolysaccharides.[199]

Loss of Rho also led to alterations in the phosphorelay system responsible for Spo0A phosphorylation (Fig. 15A). The sensor histidine protein kinase KinB, at the basis of Spo0A is strongly upregulated in the *rho* mutant.[199] Concomitantly, the phosphotransferases Spo0F and Spo0B that transfer the phosphoryl group from sensor kinases to Spo0A are also upregulated in the absence of Rho as well as other accessory components of the phosphorelay system.[199] Taken together, Rho was found to control several components of the phosphorelay system that determines the phosphorylation state of Spo0A, leading to higher phosphorylation in absence of Rho, which has direct effects on the cellular differentiation programs adopted by *B. subtilis*. Higher levels of Spo0A-P, as are found during the transition to stationary phase, suppresses the expression of matrix components and increases *spo0A* gene expression through positive feedback loops and thus limit biofilm formation. These increased phosphorylation levels are due to readthrough at a Rho-dependent termination within the *kinB* gene, confirming that Rho prematurely terminates transcription of *kinB* to prevent its expression. Taken together these results indicated that Rho exerts specific relevant biological functions in *B. subtilis* and that its deletion leads to more sessile cells with impaired biofilm production and high sporulation efficiency.[199] Conversely, if Rho is overexpressed, cells are highly motile and poorly sporulate.[199]

These first findings lead to more in depth analysis of Rho function in *B. subtilis*, especially in the transcriptional reprogramming of stationary phase, since the phenomena described above are directly associated with this growth phase. Noteworthy, the levels of Rho were found to decrease during stationary phase with a maximal expression during exponential phase, confirming the role of Rho in this process.[198] This is in accordance with previous studies that showed an auto-regulation mechanism of *rho* expression by transcriptional attenuation.[197] Transition to stationary phase implicates significant physiological changes in metabolic pathways and the activation of stress response. In addition to Spo0A, the transcriptional regulators AbrB and CodY also sense environmental changes to adapt the metabolic response. AbrB regulates initiation of sporulation and genetic competence whereas CodY, while also being involved in these two phenomena, also regulates genes required for adaptation to nutrient starvation leading to alternative nutrient acquisition pathways during stationary phase. CodY senses the alarmone (p)ppGpp and is thus implicated in stringent response, necessary for adaptation during stationary phase.[198] All these factors were found to be regulated by Rho in a complex network necessary for transition into stationary phase. Overexpression of Rho during stationary phase lead to changes in cell morphology and decreased survival during this specific growth phase.[198] Stringent response is activated under nutrient starvation during stationary

phase through (p)ppGpp synthesis and causes a reduction of cell size. Overexpressing Rho at this stage lead to unaltered cell morphology, due to impaired production of (p)ppGpp and reduced stringent response activation.[198] However, the exact mechanisms behind the impaired production of (p)ppGpp and its downstream effects need further investigation, but it has become clear that Rho-dependent termination regulates complex physiological processes related to stationary phase in *B. subtilis*.

Given the involvement of Rho in the adaptation to stationary phase and sporulation in *B. subtilis*, it is supposed that the *rho* gene is itself subjected to fine-tuned and timely regulation. It was already described that the *rho* gene is subjected to autoregulation and that expression levels of the mRNA decrease during stationary phase. Recent studies showed that *rho* is spatiotemporally regulated to induce *rho* expression during early sporulation in each compartment of the spore.[202] Indeed, during sporulation, the cells undergo an asymmetric division into a forespore that later develops into the spore and a mother cell, that engulfs and nourishes the forespore until the mature spore is ready for release. During exponential phase, expression of *rho* is driven by the housekeeping sigma factor SigA and during sporulation its *sigA* promoter is silenced through a still unclear mechanism and *rho* expression is driven by other factors.[202] In the mother cell, expression of *rho* arises from transcriptional readthrough from the upstream promoter of transition phase-specific sigma factor SigH, and in the forespore expression of *rho* is driven by the alternative sigma factor SigF.[202] Indeed, sporulation is initiated by expression of *sigH* through Spo0A-P which then activates other alternative sigma factors such as SigF in the forespore and results in an activation cascade that ultimately leads to cell differentiation and successful sporulation. All these results prove that Rho is at the heart of specific regulatory circuits in *B. subtilis* controlling transition into stationary phase and sporulation (Fig. 15).

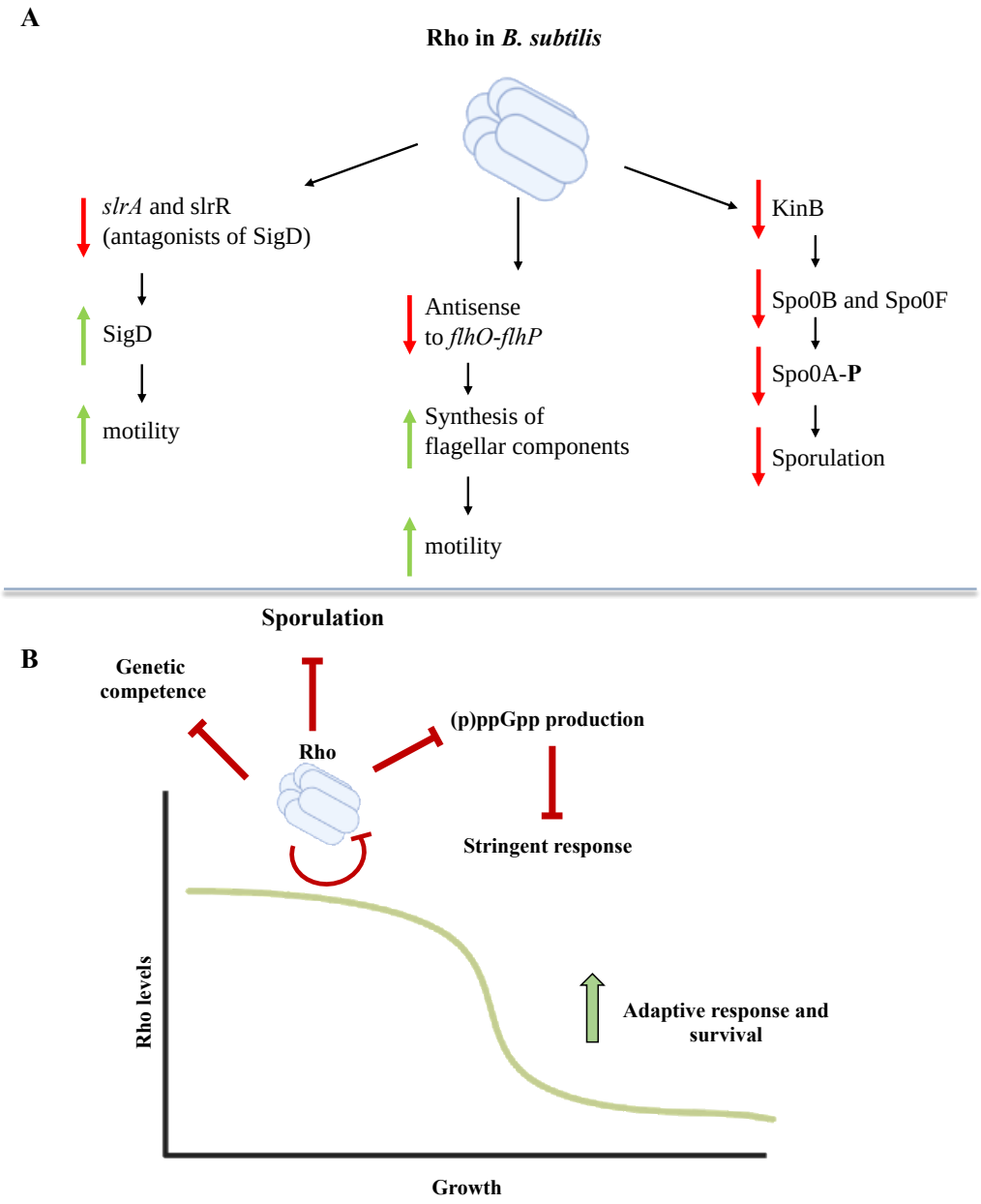

Figure 15 - Rho participates in regulatory networks controlling adaptation to stationary phase in *B. subtilis*.

A. Inactivation of Rho leads to more sessile and less motile cells. **B.** In *B. subtilis*, Rho accumulates during early phases of growth and decreases during stationary phase. High Rho levels inhibit readthrough of genes encoding global regulators needed for the activation of genetic competence, stringent response and sporulation. Thus, Rho is tightly involved in the adaptation to stationary phase events and the decision to sporulate.

B) Rho in *S. aureus*

Like in *B. subtilis*, the Rho factor is not essential for viability in *S. aureus* and its deletion also lead to increase in antisense RNA production with no growth defects.[122] The majority of transcriptional units with defined 3'-ends contain Rho-independent terminators, suggesting that Rho does not play a global role in transcription termination in this Gram-positive pathogen.[122] Genome-wide tiling array of WT and *rho* mutant strains revealed an expected strong upregulation of antisense transcription resulting from readthrough beyond mRNA ends and un-stopped transcription at cryptic promoters. Only very limited readthrough at 3'-ends of genes on the sense strand were observed.[122] This preference for the antisense strand is proposed to be due to a higher abundance of TATAAT boxes on this strand due to the high A+T composition of the staphylococcal genome compared to *B. subtilis*, resulting in more frequent initiation of spurious transcription on this strand.[122] Although a little contradictory, an increase in antisense transcription does not always affect sense transcription as reported in *E. coli*.[134] However, in *S. aureus* downregulation of genes in the absence of Rho is often due to elevated antisense transcription in this mutant.[122] It is noteworthy to mention that antisense transcription is very limited in a WT *S. aureus* strain compared to *B. subtilis* where particular asRNAs are expressed under specific conditions due to a larger repertoire of alternative sigma factors that drive their transcription.[122] Nevertheless, the negative effects of antisense transcription on the sense strand do not hinder growth, suggesting that that housekeeping genes are less impacted by antisense transcription.

A detailed comparative analysis of *S. aureus* HG001 and its isogenic *rho* deletion mutant strain revealed interesting findings linking Rho to virulence regulation.[123] Deletion of *rho* resulted in a significant increase at the proteomic levels of several virulence factors including coagulase (Coa), the extracellular matrix binding protein (Emp) or the second binding protein of immunoglobulin (Sbi), all belonging to the SaeR regulon, a key regulator of virulence in *S. aureus*. Most of the SaeR-regulated targets (29 out of 34 genes) were upregulated in the mutant in the studied conditions.[123] The impact of higher SaeRS-dependent gene expression due to *rho* deletion was studied in a murine infection model. Mice infected with the *rho* mutant exhibited decreased survival rates that the parental WT strain or a complementation strain expressing Rho from a plasmid.[123] This shows that modification in the levels of Rho can have significant pathophysiological effects and clinical relevance. It is proposed that activation of *saeP* due to a higher phosphorylation state of SaeR in the *rho* mutant

rather than the increase of expression of the systems components is responsible for higher induction of the TCS. Similar effects on the proteome and transcriptome levels of SaeR-dependent targets were observed when the parental strain was treated with the Rho-specific inhibitor bicyclomycin.[123] This finding is particularly relevant in a clinical setting, since bicyclomycin is an effective antibiotic used against Gram-positive bacteria and the use of this molecule could affect the patient's response and outcome of infections.

Thesis objectives

Regulation of virulence in *S. aureus* is an extremely complex process that involves numerous players and control mechanisms. All these regulatory networks are interconnected in order to adapt the physiological response according to the encountered situation. Especially, sRNA-mediated regulation plays a central role to fine-tune gene expression and gives this pathogen its known versatility. The role of RNA-binding proteins in this process has been questioned since sRNA-regulation does not require protein factors in this micro-organism in contrast to Gram negative bacteria such as Enterobacteriaceae. Recently, the termination factor Rho has attracted attention because it was shown to be involved in important regulatory mechanisms, including virulence. Rho has not been given much consideration in Gram-positive bacteria, as it is not essential as in Gram-negatives, but more and more evidence suggest that this ubiquitous and conserved protein has evolved to play specific functions in Bacillota. In *B. subtilis* for instance Rho has specifically been involved in the adaptation to stationary phase and sporulation and in *S. aureus* Rho seems to participate in virulence regulation. Intriguingly, these processes are associated with bacterial adaptation and stress response, and even in *E. coli*, Rho is subjected to stress-related regulation as observed with the formation of inactive Rho oligomers during stress. All these observations suggest that Rho is intrinsically related to stress response adaptation, which has allowed this factor to be involved in specific pathways in bacteria like *B. subtilis* and *S. aureus* that occupy very different niches and are exposed to different environmental cues. All-in-one, Rho rapidly became a relevant factor to be studied in *S. aureus* particularly since this pathogen is highly resilient and can grow in a wide range of conditions, implying numerous adaptation mechanisms in which Rho might be included.

With this in mind, the main objectives of my project were to better characterize the functions and mechanisms of Rho action in *S. aureus*. In particular, this included the identification of all Rho-dependent RNA targets and assessment of the role of Rho in the control of gene expression in this Gram-positive pathogen. In parallel, our goal was also to gain some insight into the regulation of the *rho* gene itself in *S. aureus*, well described in other bacteria. This was thought to help understand the role of Rho in virulence and its place in *S. aureus* regulatory networks and has been the focus of the first chapter of my thesis, that I presented as a manuscript almost ready to be submitted. In addition and in two annexes, I will present the development of the strategy to purify Rho from *S. aureus* and the attempt to identify its potential protein partners.

I. Rho, a recently discovered actor in virulence regulation in *S. aureus*

Previous studies by Nagel *et al*. showed the suppressive effect of Rho in virulence, more precisely the activation of the SaeRS regulon in absence of Rho. To understand the place that Rho has in the regulatory networks leading to the repression of the Sae system it was fundamental to study the regulation of the *rho* gene itself. We followed the expression of the *rho* mRNA in different growth conditions and strains and discovered the quorum-sensing dependent regulation of the gene. I was then able to show experimentally the existence of post-transcriptional regulatory mechanisms to control and repress the expression of *rho* by the regulatory RNA, RNAIII, when virulence activation is required. Experiments are still ongoing to fully elucidate the mechanisms behind RNAIII-dependent regulation of on *rho* expression (i.e. translational inhibition and/or degradation) as well as the transcription termination effect of Rho on *saeP*, *sbi* and *teg66* genes before submitting the paper.

To further gain insights into the functions of the Rho protein in the regulation of virulence in *S. aureus*, we aimed to identify the genes affected by a deletion of the *rho* gene, especially the virulence genes repressed in the WT strain by a differential transcriptome analysis and its RNA interactome by RIP-Seq. This aimed to determine whether the previously described effects on the Sae system were direct or indirect. Once the RNA targets of Rho had been identified, it was planned to investigate the molecular mechanism of repression of virulence genes. In addition, we also set up phenotypic assays to monitor the effect of Rho on specific virulence components in *S. aureus* such as toxin production, adhesion and internalisation by human cells. For this, a tight collaboration with François Vandenesch's team in Lyon has allowed us to present "*in cellulo*" data about the consequences of deleting the *rho* gene for the expression of virulence factors.

The surprising findings that Rho is specifically involved in virulence regulation in *S. aureus* raised further questions about its selectivity for virulence genes and the mechanisms employed to repress them.

ANNEXES: Purification of a functional Rho protein in *S. aureus* and identification of its partners

A purified and functional Rho protein from *S. aureus* (Rho_{Sa}) is required to validate possible interactions with candidate RNAs and proteins obtained after RIP experiments. This

purified protein is also required for the production of specific antibodies against Rho from *S. aureus* in order to follow its endogenous expression, especially because of the lack of cross-reactivity with antibodies against Rho from *B. subtilis* (Rho_{Bs}). This purified protein is also necessary to elucidate the mechanism of action of Rho in *S. aureus,* especially its transcription termination, ATPase or helicase activities. Previous attempts to purify a functional Rho complex have been attempted by expressing N-terminal His-tagged Rho protein in *E. coli*. However, several problems were encountered that will be discussed in this chapter. To overcome problems related to heterologous expression of Rho_{Sa} in *E. coli,* we plan to set up a purification protocol for a His-tagged Rho protein directly from *S. aureus.*

Purification of Rho from *S. aureus* is an essential step to understand the selectivity of Rho_{Sa} for the virulence genes described in the first chapter as it might help to identify domains involved in the recruitment of mRNA targets. This will provide key elements to understand the evolutionary divergence of Rho in Bacillota and how this protein has specialized in the regulation of particular pathways.

Another aspect that I will detail in this annex is an attempt to purify protein complexes associated with Rho by co-immunoprecipitation and to determine regulatory pathways in which Rho might be involved. I speculated that Rho might be associated to RNases to remove spurious and antisense RNAs as well as to repress expression of Rho-dependent targets.

All-in-one, the aims of this thesis project were to elucidate the specific regulatory functions of Rho in *S. aureus,* including the regulatory mechanisms of the *rho* gene itself. This project has set up the foundations to understand the particularities of this conserved factor in *S. aureus* and *B. subtilis* in the context of a French research consortium (ANR CoNoCo). These collaborations have been essential to build a genome browser tool to visualize our RNAseq data, to investigate protein partners with Rho_{Sa} and also for protein purification. The existence of such a consortium focused on Rho in these two key members of Bacillota highlights the relevance of investigating the mechanisms in which Rho participates in these bacteria, so different from Gram-negative.

Chapter III

Manuscript

Quorum-sensing dependent regulation of Rho connects transcription termination to pathogenicity in *Staphylococcus aureus*

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Keywords: transcription termination, Rho, *Staphylococcus aureus*, virulence

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ABSTRACT

In Gram-positive bacteria, such as the opportunistic pathogen *Staphylococcus aureus*, sRNA-mediated regulation has not been majorly linked to the action of RNA-Binding-Proteins, known to be essential for these regulatory networks in Enterobacteriaceae. However, *S. aureus* transcription termination Rho factor has been described as an attenuator of virulence, making this universal protein a new player in *S. aureus* pathophysiology. The protein is also required for the repression of “pervasive" transcription, a mechanism that results from transcription at cryptic promoters or from transcriptional readthrough. In our study, we have shown that the *rho* transcript is specifically repressed by the quorum sensing dependent RNAIII at stationary phase of growth allowing exotoxins to be activated at this stage. Rho-dependent RNA targets have been identified using RIP-seq and differential transcriptomic analyses, and several of them are involved in adhesion, dissemination, and host-response of the pathogen. Our data provide a molecular explanation for the functions of this well-conserved termination factor that have evolved in this human pathogen.

INTRODUCTION

Any RNA molecule, to exist and function in the cell must be first transcribed, elongated and properly terminated. Thus, transcription termination is an essential step in gene regulation. In bacteria, it happens through two mechanisms long considered independent from one another: intrinsic termination and factor-dependent termination. Intrinsic termination requires the formation of a terminator hairpin at the 3' end of the nascent RNA followed by a stretch of U residues, and this secondary structure alone promotes the dissociation of the transcription elongation complex. Conversely, Rho-dependent termination involves the transcription termination factor Rho, an ATP-dependent RNA-DNA helicase, that binds specific features on the nascent RNA named *rut* sites - composed of non-structured cytidine-rich regions - and releases the RNA polymerase from the DNA-RNA hybrid.[1] The Rho factor has been extensively studied in *Escherichia coli* since this protein is essential and mediates the widespread termination of about half of *E. coli* transcription events. In this Gram-negative model, the transcription elongation factor NusG has been found to stimulate Rho-dependent termination *in vitro* by directly interacting with the termination factor and accelerating RNA release at termination sites and stalled elongation complexes.[2–4] About one third of Rho-dependent terminators are NusG-dependent in *E. coli*. It is believed that the interaction of NusG with Rho induces a rapid conformational change to the active form of Rho to help the stabilization of the Rho-RNA interaction at suboptimal *rut* sites.[5] NusG is universally conserved among bacteria and its absence has global effects on gene expression and bacterial physiology, like motility in *Bacillus subtilis*.[6] NusG together with NusA are also known to enhance intrinsic termination at weak terminators by inducing transcriptional pausing. [7,6] More recently, it has been shown that it exist an interplay between intrinsic termination and Rho to enhance termination at 3' ends of genes with inefficient intrinsic terminators in *E. coli*, *Mycobacterium tuberculosis*, and in *B. subtilis* to prevent readthrough at these locations.[8,9]

The significance of Rho beyond simple transcription termination is also well established now. This factor has been proven to be important for regulation of gene expression through transcriptional polarity, riboswitch-mediated attenuation and sRNA-mediated regulation.[10] For instance, Rho regulates transcription of several genes such as the *tna* operon in *E. coli* or the *mgtA* riboswitch in *Salmonella*.[11] By terminating transcription in the leader region of these operons, Rho prevents transcription of the downstream genes in a mechanism known as attenuation. Some sRNAs such as ChiX from Salmonella can influence Rho-

dependent termination by affecting the accessibility of the *rut* sites on target mRNAs.[12] This sRNA binds to the ribosome binding site of the first gene of the *chiPQ* operon, thereby inhibiting access to the ribosome and exposing the transcript to Rho-dependent termination.[12] In addition, Rho is also a guardian of genome integrity since it has been described to limit the expression of exogenous DNA that could be deleterious to the cell, and recycles stalled RNA polymerase to facilitate DNA repair and other DNA-dependent processes essential for cell survival.[13,14] Most notably, Rho is involved in the suppression of pervasive transcription, a mechanism that frequently produces antisense (asRNAs) and can be detrimental by interfering with gene expression, genome stability and replication.[6] The spurious transcription of asRNAs can lead to the formation of asRNA-mRNA duplexes which are then targeted by the double-stranded endonuclease RNase III for degradation.[15,16]

Even though Rho is well conserved among bacteria, with over 90% of genomes containing a Rho homologue, the essential nature of this factor is not.[17] Most notably, Rho is not essential for viability in the Gram-positive models *B. subtilis* and the opportunistic human pathogen *Staphylococcus aureus,* but it still accomplishes relevant biological functions.[17,18] In *B. subtilis*, Rho regulates motility, sporulation, and cell fate determination.[19] Furthermore, Rho is necessary for the adaption of *B. subtilis* to stationary phase, a phase characterized by physiological remodeling allowing the bacterium to adapt to limited resources.[20] In *S. aureus,* the mechanism of action of Rho is not well studied. Although NusG and NusA are present, their significance and roles in transcription termination have not been investigated yet. Rho is required to decrease antisense transcription in accordance to its conserved role in suppressing pervasive transcription.[21] More strikingly, it has recently been shown that in an *S. aureus rho* deletion mutant the expression of the SaeRS regulon is induced.[22] The Sae two component system (TCS) responds to several stimuli, especially induced by the host defenses, and controls the expression of multiple virulence genes encoding toxins, adhesins and immune evasion proteins.[23] This system is composed of four genes: the sensor histidine kinase SaeS, the response regulator SaeR and two auxiliary proteins, SaeP and SaeQ, necessary for the activation of the phosphatase activity of SaeS.[23] Hence, Rho acts as a suppressor of general virulence because the *rho* mutant appeared more virulent than its cognate WT strain in a murine model of bacteremia.[22] This activation of the Sae regulon was shown to be directly dependent on the transcription termination activity of Rho, since its inhibition by bicyclomycin, an antibiotic that specifically blocks ATPase activity of Rho, had the same virulence-inducing effect.[22]

The physiological significance of Rho in all these different bacterial species supposes the existence of tight regulatory mechanisms to ensure the correct expression of this protein. For instance, in *E. coli rho* is autogenously regulated by transcriptional attenuation of the *rho* mRNA leading to premature termination.[24,25,26] Conversely, the sRNA SraL binds to the 5'UTR of the *rho* mRNA and counteracts premature transcription termination by competing with Rho for the *rut* sites.[27] Another layer of regulation has been recently described, where Rho can be transiently inactivated upon stress by undergoing oligomerization.[28] This sequestration mechanism is reversible and allows modulation of Rho activity during stress conditions, where the latter could be detrimental.[28] Similarly, in the Gram-negative *Acinetobacter baumanii*, where *rho* is essential, Rho levels are maintained constant but the activity of Rho is modulated by external factors to decrease Rho-dependent termination and promotes a switch between virulent and avirulent states.[29] A similar auto-regulation mechanism has been described in *B. subtilis*, where Rho levels are kept low by the cell.[30] In addition, the levels of the *rho* mRNA and protein decrease upon entry intro stationary phase, suggesting the existence of regulatory pathways controlling the expression of the gene.[20] In a similar manner, the level of *rho* mRNA in *S. aureus* appeared to be at its lowest during stationary phase, suggesting that the *rho* gene is subjected to specific regulation once bacterial population increases, when important physiological remodeling takes place and notably when virulence is activated in this human pathogen.[21]

Regulation of virulence factors in *S. aureus* comprises complex regulatory networks involving numerous players including TCS, transcription factors, and regulatory RNAs (sRNAs). The accessory gene regulator (Agr) or quorum-sensing system responds to cellular density and is a global regulator and activator of virulence.[31] The system senses an auto-inducing peptide (AIP) and only when its extracellular concentration reaches a certain threshold (reflecting high density), the system is activated. The *agr* locus is composed of two divergent operons under the control of the promoters P2 and P3 initiating the transcription of transcripts named RNAII and RNAIII, respectively. The RNAII mRNA encompasses the four genes that encode the TCS machinery, namely *agrBDCA*, AgrC being the sensor and AgrA, the response regulator.[31] The main effector of the system is RNAIII, which is a 514 nt-long bifunctional sRNA that also encodes for ∂-hemolysin (*hld*).[32] RNAIII post-transcriptionally regulates many mRNA targets usually by direct RNA-RNA interactions that have an effect on stability and/or translation of the target mRNAs.[33,34,35] For instance, it activates α-hemolysin (Hla) synthesis by favoring translation of its mRNA and represses the translation of other

mRNAs such as *rot*, encoding the transcriptional repressor of exotoxins, or *spa*, the mRNA encoding the immunoglobin binding protein A.[36,37] All-in-one, RNAIII coordinates the switch from the adhesion mode of early infection to a disseminative state necessary during later stage of infection.

As mentioned before, in *S. aureus* the levels of *rho* mRNA decrease during stationary phase, when the expression of most exotoxins is activated. Concomitantly, Rho has been described as a suppressor of this phenomenon by inhibiting the expression of the Sae regulon, while bacteria proliferate. Taken together, these observations suggest the existence of fine-tuning mechanisms of the levels of Rho to ensure proper expression of virulence genes. In this work, we show that the *rho* gene is subjected to post-transcriptional regulation during stationary phase, mainly by RNAIII to allow its repression. We have also identified RNA targets of Rho that point to the direct involvement of this protein in the repression of the *saePQRS* locus and virulence genes, confirmed by phenotypic assays studying specific virulence-associated mechanisms.

Our findings place Rho in the main regulatory network of virulence in *S. aureus* and add transcription termination to the vast repertoire of sophisticated mechanisms this human pathogen has evolved to adapt and colonize its host.

RESULTS

Expression of *rho* mRNA is quorum-sensing dependent

In *S. aureus*, Rho is most probably involved in the regulation of virulence pathways, therefore we first analysed the steady state levels of *rho* mRNA during cell growth. The expression of the *rho* mRNA was followed at different time points during growth of a HG001 WT strain (Fig. 1A). Culture samples were taken at early exponential (2 hours of growth), mid exponential (4 hours) and early stationary phases (6 hours) for RNA extraction and visualization on *Northern blot*. We observed an accumulation of the *rho* mRNA during early exponential phase followed by a significant decline during stationary phase (Fig. 1A). This suggests that *rho* is not constitutively expressed and that its expression is repressed during stationary phase, when cellular density is at its highest. Under these conditions, bacteria must face several stress-related signals.

To sense environmental factors and respond accordingly, *S. aureus* genome encodes 16 two component systems, that connect environmental changes to physiological responses. Among the 16 TCS, only one (*walRK*) is essential.[38] Since the expression of *rho* is growth-

dependent, we investigated the levels of the mRNA in several HG001 mutant strains lacking each TCS (Fig. S1). Among the TCS, we found that only the *agr* mutant strain alleviates the repression of *rho* at late stationary phase of growth (Fig. S1, Fig. 1A). This result is consistent with the fact that the quorum sensing *agr* system accumulates at high density. Since the regulatory bi-functional RNAIII is one of the main intracellular effectors of the *agr* system, we analysed the yields of *rho* mRNA in a deletion mutant strain for *rnaIII* in HG001, as well as a complemented strain where RNAIII was expressed from a plasmid. We found that in the Δ*rnaIII* mutant strain, expression of *rho* becomes constitutive during active growth. The phase dependent repression of *rho* was restored in the complemented strain expressing high levels of RNAIII (Fig. 1B). These data suggested that the growth-phase dependent inhibition of *rho* is RNAIII-dependent, similarly to the HG001 WT strain (Fig. 1B). Note that RNAIII transcribed from the plasmid is slightly longer than the chromosomal copy most probably due to the weakness of its terminator leading to the usage of the terminator sequence encoded on the plasmid. However, this longer RNAIII is still able to regulate key virulence factors as the activation of *hla* was restored in the complemented strain (Fig. S2). The RNAIII-dependent repression of *rho* during stationary phase was also confirmed in *S. aureus* RN6390 WT and *⊿rnaIII* strains, strain in which RNAIII expression is naturally stronger than in the HG001 background (Fig. S3).

All in all, our results show that the quorum sensing system Agr, via its main effector RNAIII, represses *rho* during stationary phase.

RNAIII binds to *rho* mRNA at multiple sites

RNAIII is a long highly structured sRNA composed of 14 hairpins and contains the coding region for the *∂*-hemolysin gene (*hld*).[39] To assess the existence of a *rho*-RNAIII interaction *in vivo,* we first applied the MAPS technique (MS2 affinity purification coupled with RNA sequencing) which was previously used to determine the RNA targetome of several staphylococcal sRNAs.[40,41,42] Briefly, RNAIII was tagged with an MS2 tag at its 5’ end and expressed in the Δ*rnaIII* strain from a plasmid under the control of its own P3 promoter. This tagged version of RNAIII exhibited similar steady-state levels as compared to the WT RNAIII, and as expected its yield accumulates during stationary phase (Fig. S4). After purification by affinity chromatography, RNAs from each fraction were extracted and analyzed by *Northern blot* using specific probes against RNAIII and *rho* mRNA. The data showed that MS2-RNAIII was strongly retained by the MS2-MBP fusion protein and that *rho* was significantly enriched

in the elution fractions of MS2-RNAIII (Fig. 2) suggesting that *rho* mRNA is one of the mRNA targets of RNAIII.

In silico predictions of base-pairing interactions between RNAIII and *rho* were then performed using IntaRNA.[43–46] The CU-rich unpaired region of hairpin 9 of RNAIII was predicted to interact with two distant regions of the coding sequence of the *rho* mRNA. The two regions included respectively nucleotides 146 to 182 (Fig. 3A) and nucleotides 1267 to 1324 near the 3'-end of *rho* mRNA (Fig. S5A). To verify that RNAIII and *rho* can form a stable complex, we performed gel retardation assays using *in vitro* transcribed *rho* mRNA and the full length radioactively 5'-end-labeled RNAIII. Two different fragments of *rho* mRNA were used respectively, the 5'-end containing nucleotides 1 to 468 and the 3'-end of the mRNA encompassing nucleotides 965 to 1417. A stable interaction with the 5'-part of *rho* could be visualized *in vitro* (Fig. 3B and Fig. S5B) and relative quantification suggested that the k_d is around 150 nM. To validate the base pairing of *rho* with the hairpin loop 9 of RNAIII, gel retardation assay was done with a 5' end-labeled RNAIII mutant lacking hairpin 9 (RNAIII △9). Unexpectedly, the deletion of hairpin 9 had only a minor effect on the binding to *rho* (Fig. 3C) that evoked the existence of an alternative binding site on RNAIII.

Previously, RNase III has been shown to specifically cleave various RNAIII-mRNA complexes. We thus tested its ability to cleave the RNAIII-*rho* complex *in vitro* (Fig. 4A). The hybrid formed *in vitro* was incubated with increasing concentrations of purified *S. aureus* RNase III. In parallel, controls were done with RNAIII alone incubated with RNase III (lane 2) or with *rho* in the absence of RNase III (lane 3). The cleavage positions were assigned using 5' end radiolabeled RNAIII subjected to RNase T1 cleavage and alkaline hydrolysis. A strong RNase III cleavage was detected in the hairpin loop 7 of RNAIII at the C residues (Fig. 4A). This cleavage is specific since the replacement of Mg^{2+} by Ca^{2+} ions, which inhibits the enzyme activity, causes disappearance of the cleavage signal (lane 8 in Fig. 4A). This result shows that the RNAIII-*rho* duplex formed *in vitro* is cleaved by RNase III and that hairpin 7 is part of the *rho* binding site.

In order to evaluate the contribution of hairpins 7 and 9 in the formation of a stable complex with *rho* mRNA, we have synthesized RNAIII mutants lacking either hairpin 9 (RNAIII △9), hairpin 7 (RNAIII △7) or both hairpins (RNAIII △7△9). We then performed gel retardation assays and RNase III cleavage assays. For the gel retardation assays, the 5'-end radiolabeled *rho* mRNA was incubated in presence of increasing concentrations of each RNAIII variant. The absence of either hairpin alone had only a minor effect on the binding affinity of *rho* to the RNAIII mutants as compared to the WT RNAIII (Fig. 3C). However,

deletion of both hairpins in RNAIII strongly impairs the interaction with *rho* mRNA, but still a weak binding was detectable, suggesting the existence of additional binding sites on RNAIII that may compensate binding to *rho* in absence of the deleted hairpins (Fig. 3C). Nevertheless, both hairpins are required for stable interactions with the *rho* mRNA. To assess the effect of these mutations on the cleavage by RNase III, we incubated each RNAIII mutant with or without *rho* and subjected the formed complexes to RNase III cleavage. For this experiment, reverse-transcription using a 5'end-radiolabeled primer complementary to RNAIII was performed to detect the RNase III cleavages (Fig. 4B). The deletion of hairpin 9 had no visible effect on the cleavage observed in hairpin 7 of RNAIII bound to *rho* (Fig. 4B). This result suggests that hairpins 7 and 9 do not share the same binding site on *rho*.

All these data point to a direct interaction of RNAIII with the *rho* mRNA. We propose that the hairpin loop 9 of RNAIII would interact with the region encompassing nucleotides 146 to 182 of *rho*. In addition, *rho* mRNA contains several G-rich repeats in its 5' UTR and in the coding sequence, which are complementary to the redundant C-rich motifs present in the hairpin loops 7, 13 and 14 (Fig. 3A). Taken together, our data strongly suggested that RNAIII interacts at multiple sites of *rho* mRNA and may affect its stability and/or translation efficiency/translation ability.

Identification of Rho-dependent pathways

Previous tiling-array studies have shown that *rho* deletion lead to an overall increase in antisense transcription as well as increased levels of transcripts belonging to the SaeRS regulon at the transcriptional and proteomic levels.[22] To have a genome-wide view of Rho function, we constructed a deletion mutant of *rho* in HG001 by homologous recombination and performed RNAseq on total RNA extracts from cultures of HG001 WT and the isogenic HG001⊿*rho* mutant grown in BHI medium at 37ºC until early exponential phase, when *rho* is mostly expressed. The experiments were done in duplicates with high reproducibility. The data were analyzed using the Galaxy platform and Genoscapist interactive platform was employed for an interactive exploration and visualization of the RNAseq data.[47,48] We have considered a gene to be regulated by Rho when there is at least a two-fold expression change between both strains with adjusted p-values (padj) below a 0.05 threshold. This has allowed us to identify different scenarios and gene classes in which Rho is involved: 1) pervasive and antisense transcripts, mostly associated to prophage genes, 2) indirect transcriptional differential regulation and 3) direct differential regulation due to readthrough at putative Rho-dependent sites sometimes associated with 4) Rho-independent terminators.

Antisense RNAs repressed by Rho negatively impact gene expression

Several genes were downregulated upon deletion of *rho* with the criteria defined above (Table S1). In particular, genes involved in anaerobic metabolism were significantly affected (Fig. 5A). These include genes encoding fermentation enzymes such as lactate dehydrogenase *ldh1* (FC= 0.164) or alcohol dehydrogenase *adh* (FC=0.124). The operon *srrAB* encoding the two-component system SrrAB (staphylococcal respiratory response) was also downregulated in absence of Rho (FC= 0.449 and 0.424 for *srrA* and *srrB*). Genes involved in nitrate respiration (*narG*, *narH*, *narI* and *narJ*), nitrate transport (*nirT*) and nitrate reduction (*nirD*) were also significantly downregulated in the *⊿rho* mutant (FC of 0.011, 0.01, 0.072, 0.013, 0.014 and 0.013 respectively). The *nreABC* operon encoding a two-component system 1involved in the regulation of nitrate/nitrite reduction in *S. aureus* was also downregulated (FC= 0.085, 0.102 and 0.127 respectively). Concomitantly, we observed an elevated production of antisense transcripts for most of these genes in the *⊿rho* mutant. An example is depicted for the *narGHJI* and *nreABC* operons (Fig. S6A). Another example is the overexpression of an antisense RNA to the genes *mbtS*, BSR_RS14940, and BSR_RS14945 in the Δ*rho* (Fig. S6B). Interestingly, the yields of these mRNAs are significantly decreased in this strain (Fig. S6B). MbtS is a membrane bound transcriptional factor which regulates the cysteine protease staphopain A and serine proteases SplA-F, all involved in the response to host defenses.[49] Downregulation of *mbtS* in the Δ*rho* mutant strain might induce indirect regulatory effects accounted to Rho.

Rho represses mRNAs involved in virulence

The differential RNA-seq revealed significant changes in expression consisting primarily in the upregulation of virulence factors, antisense transcripts and prophage genes (Table S1). Virulence factors are an over-represented class of upregulated genes in Δ*rho* strain. Most differentially expressed genes in the *⊿rho* mutant are depicted in the volcano plot (Fig. 5A). A large proportion of genes belongs to the *sae* regulon including the *saeP* and *saeQRS* transcripts, with 25 out of 39 SaeRS-dependent genes being significantly upregulated in the *⊿rho* mutant (Fig. 5B). Among the four genes of the *saePQRS* operon, *saeP* appears to be the most upregulated gene (FC=6.466) (Fig. 5B, S7).

Among the most highly expressed genes from the *sae* regulon, we found *chp* encoding the chemotaxis inhibitory protein, an anti-inflammatory agent that impairs the recruitment of neutrophils and monocytes to infection sites[50], *sbi* encoding the second binding protein of

immunoglobulin involved in immune evasion[51] and *hlb-1* encoding the non-pore forming hemolysin beta[52]. All three genes are upregulated in the Δ*rho* strain. For some genes such as *sbi*, the upregulation due to Rho deletion might seem mostly transcriptional (Fig. S8). For the *chp* gene, we observed a clear readthrough in the mutant strain (Fig. S9), which generated a long transcript antisense to the downstream genes encoding a putative phage amidase (BSR30_RS10675) and *sak* encoding staphylokinase (BSR30_RS10680). Surprisingly, this antisense RNA has no significant effect on the cognate sense RNA (Fig. S9). Noteworthy, a Rho-independent terminator structure is predicted at the 3'-end of the *chp* gene and at the same position on the opposite strand at the 3'-end of BSR30_RS10675, generating "mirror" terminator hairpins on both strands. A readthrough transcript from the BSR30_RS10675 gene was generated on the opposite strand in the Δ*rho* mutant and terminated at the Rho-independent terminator of the *scn* gene (Fig. S9). This readthrough transcript generates an antisense RNA to the *chp* gene and comprises the sRNA SprD encoded at this locus known to modulate *sbi* expression (Fig. S9).[53,54] Therefore, this complex profile suggested that Rho contributed to transcriptional arrest at this location to prevent pervasive transcription. A similar readthrough event might take place in the absence of Rho at the 3'end of the *hlb-1* gene (Fig. S9). In addition, we also detected upregulated mRNAs encoding virulence factors whose expression is independent of SaeRS, such as the staphopain A (SspP), and its inhibitor SspC, the siderophore receptor SirA (Table S1).

The riboflavin synthesis operon, comprising the genes *ribD*, *ribE ribAB* and *ribH* under the control of the transcriptional FMN riboswitch was upregulated in the Δ*rho* mutant (Fig. 5A, Table S1). Deletion of *rho* causes higher transcription levels of Teg74 and the downstream *rib* genes, resulting from putative transcriptional readthrough at Teg74 (Fig. S10). We looked for variations in the expression of other known riboswitches in *S. aureus* and detected slight downregulation of the PyrR-, Lysin- and GlmS-riboswitches in the Δ*rho* mutant strain (Table S1). Only slight upregulation of antisense transcripts to the riboswitch-dependent genes in the Δ*rho* mutant was observed.

In summary, differential RNA-seq allowed us to get a global overview of the effect of Δ*rho* deletion on the transcriptomic landscape and identify several Rho-dependent pathways essentially linked to metabolism and virulence traits.

The RNA targetome of Rho studied by RIP-Seq

Besides the RNA-seq approach, we unveiled the RNA interactome of Rho by performing RIP-seq (RNA immunoprecipitation followed by RNAseq). In this experiment,

Rho was co-immunoprecipitated along with its RNA partners which were identified by deep sequencing. For RIP-seq, we constructed a strain expressing a chromosomally encoded tagged version of the Rho protein by using homologous recombination. A triple FLAG-tag was added to the C-terminal extremity of the protein. We have verified that the mRNA levels of the recombinant Rho-3xFLAG protein showed similar steady-state levels as the WT *rho* mRNA (Fig. S11A) and that the Rho-3xFLAG protein was well expressed (Fig. S11B). The immunoprecipitation of the Rho-3xFLAG was executed from cultures grown until early exponential phase using anti-FLAG antibodies and a 3xFLAG peptide was used to elute the recombinant protein-RNAs complexes. A control Co-IP was performed using the untagged Rho protein from a WT strain. The Rho-3xFLAG protein was successfully enriched in the elution of the modified strains (Fig. S12). The co-immunoprecipitated RNAs were extracted for sequencing. The enrichment of putative Rho targets was measured by comparing the number of reads obtained from the Rho-3xFLAG purification and from the untagged Rho. We have considered as putative Rho targets, the RNAs that were enriched at least two-fold with padj < 0.05. The RIP-Seq data revealed that 326 RNAs were enriched with Rho using these criteria (Table S1). Many RNAs were considered as antisense RNAs or overlapping genes which is correlated with the major function of Rho as a suppressor of pervasive transcription. To identify putative direct targets of Rho, we crossed our RNAseq and RIPseq datasets to obtain a list of significantly enriched RNAs upon RIP with at least a two-fold transcriptomic change expression in the Δ*rho* mutant (Table S1). This yielded 121 RNAs that co-precipitated with Rho and whose expression was significantly affected by *rho* deletion. Among this reduced list of RNA candidates, eight of them were known to be regulated by the *sae* operon. They include *saeP* itself, *hlb-1*, *sbi*, *scn*, and *chp* for which a significant readthrough had been identified in the Δ*rho* mutant strain (Fig. 5B). Other interesting candidates such as *ribH* from the riboflavin synthesis operon, which is under the control of FMN riboswitch, has been enriched in the RIP-seq (FC=2.2) although with a padj value above 0.05.

Transcripts for most of the downregulated genes in the *rho* were not enriched upon RIP, with only six of their corresponding transcripts co-precipitating with Rho (Table S1). Among these we found the *narT* mRNA encoding a nitrate transporter, and the mRNAs *cydB*, *ktrA* and BSR30_RS05090 localized in the same operon.

Therefore, the RIP-Seq data is well correlated with our differential transcriptomics with a selectivity for RNAs repressed by Rho. The vast majority (115/121) of enriched RNAs were indeed highly upregulated in the *rho* mutant, suggesting that our RIP is enriched in targets

repressed by Rho. Besides its role in pervasive transcription, the data also suggested a direct role of Rho in the regulation of virulence gene expression.

Rho-dependent termination of targets leads to gene repression

To validate potential Rho effects (i.e., *chp*, the FMN riboswitch *teg74*, *saeP*, *sbi*) occurring in the mutant Δ*rho* strain, we constructed a GFP reporter, in which we have introduced a sequence of each Rho target mRNA (Fig 6A). These genomic regions were cloned upstream the *gfp* sequence (Fig. 6A). All plasmids were transformed into the strains HG001 pMKX, (wild-type strain containing an empty pMKX plasmid), HG001 ⊿*rho* pMKX (*rho* deletion mutant containing an empty pMKX plasmid) and HG001 ⊿*rho* pMKX::*rho* (*rho* deletion mutant strain complemented with the pMKX::*rho* plasmid expressing *rho* under the control of a xylose-inducible promoter).[21] As previously published we confirmed that the *rho* transcript is expressed in the complemented strain as well as in the WT strain without promoter induction (Fig. S13).[21] The strains were grown in BHI medium until exponential phase and cells were lysed to measure GFP expression. If Rho terminates transcription at the predicted locations of the constructs, elongation through the *gfp* gene should be affected. Conversely, Rho transcriptional readthrough should lead to increased elongation through the *gfp* gene resulting in higher expression of the GFP protein.

To analyze the effect of Rho on the *teg74* riboswitch, we have cloned the sequence of the riboswitch including the Rho-independent terminator in the reporter fusion construct. The data showed a significant increase of the GFP synthesis in the Δ*rho* mutant strain. Conversely, the GFP synthesis was reduced in the WT strain and decreased slightly more in the complemented strain (Fig. 6B). For the *chp* operon, we used a fragment comprising the 3'-end of the gene and the intergenic region downstream containing a Rho-independent terminator hairpin. We chose this region based on the data visualization in which transcriptional readthrough is observed (Fig. S9). The data revealed a tendency for an increased GFP synthesis in the Δ*rho* mutant strain in comparison to the WT strain or complemented strain (Fig. 6B). These data agree with the existence of Rho-dependent termination of transcription of specific mRNAs*.

Rho expression impacts *S. aureus* virulence

To assess the impact of Rho on the physiology of *S. aureus*, various phenotypic tests were performed to compare the behaviors of the WT HG001 strain, the isogenic Δ*rho* mutant strain and the complemented strain. Based on the functions of several Rho-dependent targets

identified above, different phenotypic assays were chosen in relation with the identified Rho targets to define its impact.

The amounts of alpha (Hla) and gamma (Hlg) hemolysins were first measured by ELISA tests. A 2,8-fold increase in Hla and a 16-fold increase in HlgC was observed in the Δ*rho* mutant strain compared to the WT strain or the mutant strain overexpressing Rho (Fig. 7A). These protein levels correlated with an increase in the cytotoxic activity of the Δ*rho* mutant strain on specific monocytes expressing gamma hemolysin receptors C5aR1 (3-fold increase) and CxCR2 (4-fold increase) (Fig. 7B) and on rabbit red blood cells sensitive to alpha hemolysin (4,6-fold increase) (Fig. 7C). We also tested the fibrinogen adhesion phenotype *in vitro* and observed an increased ability of the Δ*rho* mutant strain to adhere to fibrinogen compared to the WT strain (Fig. 7D). Finally, we have tested the ability of the same strains to coagulate rabbit plasma and observed a higher coagulase titer in the Δ*rho* mutant strain compared to the WT strain (Fig. S14). Noticeably, we observed a restoration of most of the phenotypes with the HG001Δ*rho*::pMKX-*rho* complemented strain albeit with a reduced effect as compared to the WT strain. One cannot exclude that the expression of Rho from the pMKX plasmid led to a less active protein in the tested conditions.

All-in-one, these data are in favor of an attenuation role of Rho in virulence.

* While I am finishing my thesis manuscript, transcriptional GFP fusion constructs to assess the role of Rho at the *saeP, and sbi* locations are available and investigations are currently ongoing in the laboratory. First, for the *saeP* construct, we used a fragment including the 3' end of the *saeP* gene, the intergenic region containing a Rho-independent terminator and part of the downstream coding sequence of *saeQ*. Secondly, the *sbi* gene seems to be affected on a transcriptional level in the *rho* mutant with no apparent readthrough events occurring, however this mRNA was enriched upon RIP. To assess the direct involvement of Rho on this gene, we also constructed a GFP fusion plasmid in which the 3' end of the coding sequence of *sbi* and a downstream intergenic region including a Rho-independent terminator were used. In addition, we used the Teg66 riboswitch as a negative control to assess the specificity of Rho for Teg74. Indeed, *S. aureus* encodes for a second FMN-dependent but translational riboswitch controlling expression of the *ribU* gene encoding an uptake transporter for riboflavin. These genes do not seem affected upon *rho* deletion; thus, for the Teg66 GFP fusion construct, the complete riboswitch sequence including the beginning of the *ribU* coding sequence was cloned into the vector and experiments are currently ongoing.

DISCUSSION

In this study, we have investigated and characterized the regulation and the roles of the transcription termination factor Rho in *S. aureus*. This highly conserved protein is found in over 90% of bacterial genomes and accomplishes diverse biological functions including regulation of specific operons, participation in sRNA-dependent mechanisms and prevention of spurious transcription. The later explains why Rho is essential for viability in *E. coli* since the expression of prophage genes can be deleterious to the cell.[55] In this Gram-negative model, Rho plays a broad role since it terminates transcription of up to 50% of all genes and often cooperates with other factors such as NusA and NusG.[17] It can also associate under specific circumstances with other proteins such as RNases, as Rho can be part of the RNaseE-based degradosome in *E. coli* and other bacteria, demonstrating that Rho is a versatile and global regulator.[56]

In *S. aureus* however, besides its role in keeping pervasive transcription under control, the functions of Rho have been narrowed down to virulence regulation and more precisely to its attenuation. Indeed, in this Gram-positive pathogen, the deletion or inactivation of Rho resulted in increased expression of the SaeRS dependent-targets and its entire regulon, resulting in enhanced virulence as tested in a murine model of infection.[22] This system controls the expression of over 20 virulence factors including hemolysins, surface proteins and immune evasion factors and the absence of Rho has an impact on their expression. Since virulence is a tightly controlled and time-dependent feature, the specificity of Rho for virulence regulation suggests that this protein must also be regulated accordingly.

Rho as a novel target of RNAIII

We have shown that the expression of the *rho* gene is dependent on the *agr* quorum-sensing system. The *rho* mRNA levels decrease over time in a HG001 wild-type strain in contrast to a deletion mutant for the *agr* system, where the *rho* mRNA levels appear constitutive over time. The main effector of this system is the 514-nt long sRNA RNAIII, whose regulatory functions are now well established.[33–37] In a mutant for this sRNA, the time-dependent regulation of the *rho* mRNA is abolished, suggesting an RNAIII-mediated repression of *rho* during stationary phase. Indeed, we have shown the formation of a stable *rho*-RNAIII complex which can be cleaved by RNase III *in vitro*. However, some uncertainties remain regarding the topology of the interacting RNAs leading to the repression of *rho*. While the early coding sequence of *rho* (nts 46-88) is predicted to interact with a region comprising hairpin loop 9 of RNAIII, the

deletion of this hairpin in RNAIII only partially affected the binding to *rho*. Using RNase III cleavage as a proxy for the formation of a *rho*-RNAIII duplex, we unexpectedly found a single cleavage occurring in the loop of hairpin 7 of RNAIII. Again, deletion of the hairpin loop 7 had only a minor effect on the binding affinity *in vitro*. However, the concomitant deletion of hairpins 7 and 9 of RNAIII showed a synergic effect as the binding to *rho* mRNA was strongly affected but not completely abolished. Our data suggested that multiple and compensatory binding sites might exist between RNAIII and *rho* mRNA. Interestingly enough, the 5' UTR and the coding sequence of *rho* mRNA contain redundant GGGA motifs that are potentially complementary to the UCCC motifs found in hairpin loops 7, 13 and 14 of RNAIII. Moreover, this GGGA motif has been previously shown to be part of the binding sites of other mRNA targets of RNAIII including *coa* and *rot* mRNAs, and specific RNase III cleavages have been observed in the hairpin loop 7 at the C-rich motif.[35,37,57] As for now, the importance of each hairpin of RNAIII for a successful interaction with *rho* demands to be clarified. We nevertheless hypothesize that hairpin 9 of RNAIII binds to the coding region forming at least 10 consecutive base pairing interactions which are further stabilized by the binding of hairpin 7 to the $_{314}$GGGAU$_{318}$ motif (Fig.S15). Whether this interaction leads to a long interhelical helix appropriate for RNase III cleavage remains to be demonstrated.[57] We do not exclude that the hairpin loops 13 and 14 of RNAIII contribute to the overall stability of the complex through the binding of the UGGG motifs present in the 5'UTR of *rho* including its SD sequence in a way similar to *rot* mRNA.[37]

Post-transcriptional impact of RNAIII on *rho* mRNA

The interaction of *rho* mRNA with RNAIII could as well affect its stability than its translation. The *in vivo* relevance of RNase III cleavage on the expression of the *rho* mRNA is still under investigation. Preliminary data revealed that a stronger accumulation of *rho* during exponential and stationary phase in a deletion mutant of RNase III have been observed and the stability of the *rho* mRNA weakly varied in the Δ*rnaIII* mutant strain but intriguingly not in the Δ*rnc* strain (not shown). While the *rho* gene is under the control of the housekeeping sigma factor SigA[21], there is also clear regulation of the *rho* mRNA levels relying on the quorum sensing-dependent RNAIII.

Specific antibodies against Rho from *S. aureus* are not available and no cross-reactivity with available antibodies against Rho from *B. subtilis* was observed (data not shown). We have followed the expression of the recombinant Rho-3xFLAG protein used for RIP-seq and we have observed a constitutive expression of the protein over time (Fig S11). Yet we cannot

exclude that insertion of tags at the C-terminal or N-terminal regions affects the degradation/stability of the protein. To investigate endogenous Rho levels, preliminary proteomic data of total protein content of WT and *rnaIII* mutant strains during exponential and stationary phase point to a light decrease in Rho protein during stationary phase in the WT strain and to a higher accumulation in the mutant (data not shown). We will investigate *in vitro* the role of RNAIII on *rho* translation by toeprinting experiments and translation assays.

It has been shown in *Enterobacteriaceae* that binding of non-coding RNA to the ribosome binding site blocks the ribosome from accessing to the mRNA, which in turn favors the recruitment of Rho. Indeed, examples of sRNA-mediated premature transcription termination by Rho, sometimes in conjunction with NusG have already been described (ChiX, Spot42 examples)[58]. It is tempting to propose that binding of RNAIII to *rho* might favor the recruitment of Rho to promote a negative feedback regulation. We cannot exclude that during stationary phase, some *rho* transcripts are targeted by RNAIII to inhibit translation initiation or elongation, facilitating the binding of Rho to its own mRNA and terminate its transcription, as it has been described for *E. coli* and *B. subtilis*.[26,27,30] The *rho* mRNA was not enriched upon RIP; however, the experiment was performed during exponential phase when RNAIII is not present yet. Thus, if this hypothesis is true, Rho-dependent termination of the *rho* mRNA might require RNAIII and therefore only occurring during stationary phase to limit translation of Rho.

Common roles of Rho in phenotypic switches in different bacterial species

Interestingly the growth phase dependent repression of Rho is conserved in *B. subtilis*, and is required for cell adaptation, although the mechanism of regulation is not known.[20,59] In *Acinetobacter baumanii* which can adopt a virulent and an avirulent phenotypes, it has been recently shown that Rho is necessary for premature termination of transcriptional regulators and their repression in the virulent status.[29] Conversely, when switching to an avirulent phenotype, Rho-dependent termination is reduced to allow correct expression of these regulators. Interestingly, levels of Rho remained unchanged in both phenotypes, suggesting the existence of mechanisms that inactivate the protein or reduce its binding to the mRNA targets.[29] As demonstrated in *E. coli*, Rho can be transiently and reversibly inactivated through oligomerization and aggregation.[28] Also, in *Clostridium botulinum*, Rho can adopt free or aggregated conformations due to the presence of a prion-like domain in the NTD which can impact activity of the protein and act as a regulatory switch of Rho functions.[60] Further analyses will be needed to investigate if a similar phenomenon exists in *S. aureus*, especially since Rho also contains an NTD insertion that could mediate a similar regulatory switch independently

of RNAIII. This might be highly relevant especially if the levels of the Rho protein do not significantly vary between exponential and stationary phase, suggesting that other mechanisms might exist to ensure the inactivation of Rho during stationary phase to allow virulence factors in particular to be properly expressed at this stage.

Downregulation of antisense transcription by Rho

Deletion of *rho* increased the expression of antisense transcripts, whose functions are still controversial, consequently the global effect of this increased transcription is difficult to evaluate. Even if antisense transcription is expected to modulate expression levels of the sense transcripts and could represent another layer of gene regulation, we did not always observe a correlation between overexpression of an antisense and downregulation of the corresponding sense transcript. For instance, the antisense produced in the Δ*rho* mutant strain after readthrough at the *chp* gene, did not have any effect on the levels of the sense transcript (Fig S9 and Table S1). Intriguingly, we often detected enhanced antisense transcription in poorly transcribed regions, which renders the identification of any effect on the sense transcript more difficult. Hence, we cannot exclude that the effects of antisense transcription are masked by the low basal levels of sense transcription in the concerned regions. The coprecipitation of many antisense transcripts with Rho suggests that this factor is needed to suppress their expression through transcription termination and is in accordance with the already described roles of Rho in suppressing pervasive transcription.

Inhibition of anaerobic gene expression by Rho

Several genes involved in anaerobic metabolism were negatively affected by the deletion of *rho*, suggesting that these genes are positively regulated by this factor. Of note, we observed that *rho* transcription does not seem to rely on the SsrAB TCS known to sense low O_2 level (Fig. S1). We frequently observed the generation of antisense transcripts to these genes, pointing to possible sense/antisense RNA regulation occurring. This hypothesis is supported by the absence of any of the concerned mRNAs co-precipitating with Rho but the enrichment of several antisense transcripts to the affected genes upon RIP. Thus, Rho could inhibit the synthesis of these antisense transcripts to ensure the proper expression of anaerobic genes. However, we cannot exclude that the effect on these genes is indirect through other affected upstream-lying pathways. Upon RIP experiment, the nitrate transporter encoding mRNA *narT* was enriched suggesting a direct effect of Rho on this gene. Similarly, the mRNAs of the genes encoding *cydB*, *ktrA* and BSR30_RS05090 also involved in metabolism adaptation

co-precipitated with Rho. Interestingly, these genes are found in an operonic organization, suggesting that Rho acts on this genomic location. These data could indicate that for a reduced number of genes (mostly involved in metabolism/anaerobic respiration), Rho might be required for proper transcription termination and activates expression of these respective genes.

Anaerobic gene expression allows *S. aureus* to adapt to changes in oxygen concentration and is particularly relevant in an infective context, since oxygen concentration varies within the human host and these variations can affect bacterial physiology and virulence factor production.[61]Thus, potential regulation of anaerobic gene expression by Rho might have consequences for *S. aureus* adaptation to different host tissues and pathogenesis. Therefore, expression of Rho in low oxygen environments such as abscesses might be necessary for bacterial adaptation and it would be highly interesting to study the expression of Rho in oxygen-dependent conditions to determine its impact on bacterial physiology.

Nevertheless, we believe that our RIP experiment mostly reveals targets degraded by Rho and that genes activated by this factor are not direct targets of the protein.

Direct and indirect repression of the SaeRS regulon by Rho

Our RNA-seq analysis together with the RIP-seq revealed that the absence of Rho lead to an increased production of virulence factors, confirmed by the phenotypic assays (Fig. 7). We mainly focused on virulence associated genes and only considered up-regulated targets in both datasets. Since Rho is a repressor of virulence in *S. aureus*, our rationale is that Rho induces transcription termination of these targets, which somehow impairs their expression. Since almost the entire *sae* regulon is upregulated in the absence of Rho, it was stipulated that the effect of Rho would probably be indirect on the synthesis of virulence factors.[22] Our data indicates that Rho binds to the *saeP* mRNA and to none of the other *sae* components. The *saePQRS* operon contains two promoters: P1 inducing the expression of *saeP* and P3 the expression of *saeQ*, *saeR* and *saeS*. The *saeP* transcript can be produced by two mechanisms involving a specific RNase Y cleavage leading to *saeP* and *saeQRS* mRNAs and a termination event from a Rho-independent terminator yielding *saeP*.[62] Our data suggested that in the Δ*rho* mutant, a transcriptional readthrough event occurred at *saeP* allowing further transcription into the *saeQRS* genes (Fig S7). Thus, *saeP* can be transcribed separately from *saeQRS* and it was already hypothesized that premature transcription termination could lead to the individual *saeP* mRNA.[62] The fact that only this mRNA co-precipitated with Rho supports this idea and suggests that Rho might terminate transcription of *saeP*. Even if the effect of SaeP on the SaeRS system remains unclear, it could be possible that premature termination and/or

degradation of *saeP* alone would have a major impact on the functioning of this system. This would explain seemingly indirect effects observed for some *sae* regulated genes such as *sbi* for which we see a major transcriptional effect with no visible readthrough event (Fig. S8). However, several of these targets also co-precipitated with Rho, including the *sbi* mRNA, pointing to a simultaneous direct effect of Rho (Fig. 5B).

The genomic region depicted in Figure S9 has retained our attention, not only because a readthrough effect is observed on both strands, downstream the *chp* and BSR30_RS10675 genes as well as on the *hlb_1* gene, but also because many of the depicted genes are upregulated in the RIP-seq data (Fig. 5 and Table S1). We believe that Rho is involved in transcription termination of several loci in this region, for instance at the *chp*, BSR30_RS10675 and *scn* genes, containing Rho-independent terminators and also on the *hlb_1* gene containing no predicted intrinsic terminator hairpin. We used a transcriptional GFP reporter plasmid to identify readthrough events due to the absence of Rho *in vivo*. We selected the *chp* gene that we cloned upstream the *gfp*-encoding sequence and measured GFP expression in presence or absence of Rho. Although preliminary, our results support the direct involvement of Rho in the regulation of the *chp* gene and point to Rho-dependent transcription termination.

Therefore, we propose that Rho might be involved in transcription termination of the *saeP* which could promote degradation of the transcript. Although only *saeP* seems to be a direct target of Rho, destabilization/degradation of *saeP* alone might be sufficient to diminish the expression and function of the whole TCS. Thus, overexpression of SaeRS targets in the Δ*rho* mutant, especially of those that were not enriched upon RIP, might be indirect through repression of *saeP*. However, since several SaeRS targets also co-precipitated with Rho, we deduce that Rho can also directly act on these transcripts and terminate their transcription (i.e. *chp*), adding another layer of direct repression of these genes by Rho.

Effect of Rho on FMN riboswitch regulation

In bacteria, homeostasis of riboflavin or vitamin B2 is regulated by the widely distributed class of FMN riboswitches. The FMN riboswitches regulate the biosynthesis and uptake pathways of riboflavin, which is then converted into flavine mononucleotide (FMN) and flavine adenine dinucleotide (FAD), two essential co-factors for bacterial growth as they are critical for many redox reactions of cellular metabolism.[63] FMN constitutes the primary ligand regulating FMN riboswitches by binding to cognate aptamer when concentrations are high and inducing conformational changes that inhibit downstream gene expression.[64]

Conversely, low cellular FMN levels allow expression of downstream genes resulting in *de novo* riboflavin synthesis or import.[64]

S. aureus encodes for two highly related but different FMN-responsive riboswitches, Teg74 and Teg66 that regulate the riboflavin biosynthetic operon (*ribDEBAH*) at the transcriptional level or the uptake transporter (*ribU*) at the translational level respectively.

The FMN-responsive transcriptional riboswitch *teg74* gene along with the downstream *rib* operon were upregulated in the Δ*rho* strain and some transcripts enriched upon RIP, although with a non-significant padj value, which may be explained by the relatively low expression of the operon in non-depleted riboflavin conditions such as ours (rich growth medium and early exponential phase). We cloned the *teg74* gene upstream the *gfp*-encoding sequence and measured GFP expression in presence or absence of Rho, where we observed a significant increase of GFP signal in the Δ*rho.* These observations strongly suggest that Rho-dependent termination of *teg74* determines downstream expression of the *rib* operon and links Rho-dependent termination to riboswitch-mediated regulation in *S. aureus*. Although both riboswitches share 96% identity[65] Teg66 and *ribU* were not affected upon *rho* deletion nor enriched upon RIP, suggesting the specific action of Rho on the transcriptional FMN riboswitch. Intriguingly, the association of Rho to this particular riboswitch has been conserved in different bacteria such as *E. coli* [66], *C. botulinum*[67] and *B. subtilis* (personal communication).

Riboflavin synthesis and uptake are essential for bacterial growth and even required for virulence in *E. coli*.[68] Although disrupting riboflavin biosynthesis in *S. aureus* does not reduce virulence, simultaneously disrupting biosynthesis and uptake pathways using FMN riboswitch inhibitors like roseoflavin or ribocil-C efficiently impairs MRSA growth and virulence in a murine septicemia model.[65] Thus, specifically targeting FMN riboswitches in *S. aureus* might be a promising mechanism to treat drug-resistant bacteria. All in all, Rho-dependent regulation of the transcriptional FMN riboswitch in *S. aureus* might contribute to modulation of virulence and pathogenesis.

Potential role of PNPase in Rho-dependent transcripts degradation

It has been recently described that Rho can stimulate intrinsic termination in *B. subtilis* by inhibiting the formation of secondary structures that could compete with the terminator hairpin, making Rho-dependent and intrinsic termination not mutually exclusive.[69] In addition, recent studies have demonstrated that 3’ ends generated from Rho-dependent termination are easily subjected to 3’ to 5’ degradation by PNPase until encountering a stable hairpin that inhibits RNase activity.[70] This renders the identification of Rho-dependent termination sites by

RNA-seq difficult since the discrimination between a real intrinsic terminator and the formation of a PNPase-inhibiting hairpin is more difficult and, in both cases, a sharp decrease in transcription signal is observed.[70] Nevertheless, PNPase seems to be a strong candidate for degradation of Rho-targets in various bacteria and has been associated with virulence repression and stress response in several organisms.[71] More precisely, in *Salmonella enterica* PNPase suppresses plasmid virulence genes[72] and in the sheep pathogen *Dichelobacter nodosus* it is also involved in virulence repression.[73] In the Gram-positive pathogen *Streptococcus pyogenes*, PNPase is involved in degradation of virulence transcripts during exponential phase[74] and in *Listeria*, PNPase favors biofilm formation.[75] In *S. aureus*, PNPase negatively alters the expression of hemolysins and of the *saePQRS* operon.[76] The *saePQRS* transcript is believed to be degraded by PNPase in absence of maturation by RNase Y that forms protective 3' extremities.[76] Thus, it is tempting to propose that Rho-dependent termination of *saeP* might induce degradation by PNPase affecting expression of the entire operon. It could be therefore possible that during exponential phase in *S. aureus*, virulence genes, including the ones with intrinsic terminators, are subjected to Rho-mediated termination, generating PNPase-sensitive 3'ends leading to transcript degradation. During stationary phase, when Rho is believed to be absent because of RNAIII-mediated repression of the *rho* mRNA, intrinsic termination could become dominant, yielding transcripts with a more stable 3'end protected from degradation. For instance, this could explain the visible sole transcriptional effect we observe in the *rho* mutant for some targets that were nevertheless enriched after RIP-seq such as *sbi*. We can imagine that Rho-dependent termination of the *sbi* gene occurs downstream of the predicted intrinsic terminator and induces PNPase-mediated degradation until the inhibitory terminator hairpin (or another secondary structure) is encountered. In absence of Rho (*rho* mutant or stationary phase), intrinsic termination occurs and PNPase does not degrade the transcript, resulting in mRNA stabilization and thus higher transcript levels. This would explain why we observe significant differences in transcript levels and no visible readthrough, even though our data point to a direct effect of Rho on the *sbi* transcript. However, we have to take into account the complex regulatory mechanisms behind *sbi* expression which also involve the action of regulatory RNAs such as RNAIII and SprD which inhibit *sbi* expression.[53,54] For instance, SprD negatively affects translation of *sbi* and curiously in our *rho* mutant dataset, SprD is found in a region in which readthrough occurs in the absence of Rho. Thus, we believe that in this mutant, SprD is contained in a long readthrough transcript and might not be able to regulate its targets *in trans*. Thus expression of *sbi* increases both at a transcriptomic and proteomic level (Fig. 5).[22] Interestingly, Hence, a Δ*pnp* deletion as well as

a double *pnp, rho* deletion would be needed to discriminate between direct and indirect effects of Rho on its targets and also to investigate a potential interplay between both proteins. Indeed, in *B. subtilis*, the effect of Rho on its target stability only became clear in a *pnp* mutant (S. Durand, personal communication).

CONCLUSION

It is known that in *S. aureus* as well as in *B. subtilis* and *E. coli*, the role of Rho in the control of genome surveillance through the regulation of antisense transcription has been conserved. These pervasive transcripts are often associated to horizontally acquired genetic material or mobile elements. Through such evolutionary mechanisms, *B. subtilis* and *S. aureus* have acquired accessory genes necessary for sporulation or virulence, which would explain why in these bacteria Rho seems to have specialized in the regulation of these very specific pathways.

Our findings have unveiled the *rho* mRNA as a novel target of RNAIIII, the main sRNA implicated in the regulation of virulence in *S. aureus*. This temporary modulation of *rho* expression adds another level to the already complex networks controlling the activation of exotoxins during stationary phase. It has become clear that this ubiquitous termination factor has evolved to be specifically involved in the adaptation of Bacillota to the physiological remodeling taking place in the transition to stationary phase. The RNAIII-dependent repression of *rho* at high cell density allows for Rho-dependent targets to be fully de-repressed. Moreover, our RNA-seq and RIP-seq data highlight the existence of specific targets for potential Rho-mediated transcription termination, especially virulence factors down-regulated during exponential phase. Overall, our results give a first glimpse of the roles and functions of Rho in *S. aureus*. Although several questions remain to be addressed, such as the exact RNAIII-dependent mechanism of repression of *rho* and mainly the selectivity of Rho for its targets and how they are repressed, we have placed Rho as another key component of the main regulatory networks of virulence of *S. aureus* (Fig. 8).

MATERIALS AND METHODS

Strains and plasmids

All strains and plasmids used and constructed in this study are described in Tables S2 and S3. The primers designed for cloning and mutagenesis are provided in Table S4. *E. coli* strain IM08B was used as an intermediate strain for plasmid amplification before electroporation in *S. aureus*. Plasmids were extracted from chemically transformed *E. coli* pellets with the NucleoSpin Plasmid kit according to the manufacturer's protocol (Macherey-Nagel). Electrocompetent *S. aureus* strains were then transformed with the purified plasmids by electroporation (Bio-Rad Gene Pulser).

The HG001 *rho* and HG001 *rnaIII* deletion mutants as well as the HG001 *rho-3xflag* strain were constructed by homologous recombination using plasmid pMAD in *S. aureus.* HG001Primers used are listed in Table S4.

The pCN51-P3::*rnaIII* and P3::MS2-RNAIII plasmids were constructed by ligating PCR-amplified fragments (see Table S4 for primers) containing the 514 nt-long sequence of *rnaIII* previously digested with SphI/BamHI or the sequence of *rnaIII* fused to an MS2 tag at the 5'end digested with PstI/BamHI into a pCN51-P3 plasmid digested with the same enzymes respectively. The pCN51-P3::*rnaIII⊿H7*, pCN51-P3::*rnaIII⊿H9* and pCN51-P3::*rnaIII⊿H7⊿H9* plasmids containing deletion mutants of hairpins 7, 9 or both, of RNAIII were constructed by site directed mutagenesis using plasmid pCN51-P3::*rnaIII* as a template. A PCR using Phusion High Fidelity DNA polymerase (Thermo Scientific) was performed using the primers listed in Table S1 to introduce the deletions. One denaturation step (30 s at 90ºC) was followed by eighteen cycles of amplification of the fragment (30 s at 95ºC, 1 min 55ºC, 6 min at 68ºC) and ultimately an elongation step (5 min at 72ºC) was performed. PCR products were then treated with 10U/µL DpnI to eliminate the template pCN51-P3::*rnaIII* plasmid. Reactions were then transformed into *E. coli* TOP10 and positive clones were selected and sequenced. These plasmids were used as templates for PCR amplification to introduce the T7 promoter for templates in *in vitro* transcription assay.

Plasmids pCN57::GFP-*chp,* pCN57::GFP-*teg74,* pCN57::GFP-*saeP* and pCN57::GFP-*teg66* were constructed by ligating PCR-amplified fragments of the *chp, teg74, saeP and teg66* genes (see Table S4 for primers) previously digested with EcoRI/BamHI into a pCN57::GFP plasmid digested with the same enzymes.

Growth conditions

E. coli strains were grown in Lysogeny-Broth (LB, Roth) medium supplemented with ampicillin (100 μg/mL). *S. aureus* strains were grown in Brain-Heart infusion (BHI, BD) media containing 10 μg/mL of erythromycin or chloramphenicol when needed. To measure the half-life of the RNA of interest, cultures were grown in BHI (BD) with the needed antibiotics until late exponential phase. Rifampicin was added to a final concentration of 300 μg/mL to stop transcription initiation and samples were taken at different time points.

pMAD mutagenesis

Strains HG001 *⊿rho*, HG001 *⊿rnaIII* and HG001 *rho-3xflag* were constructed by allelic recombination using the shuttle vector pMAD as previously described.[77] To generate the mutants, we amplified fragments of the flanking upstream and downstream regions of the targeted genes by PCR using chromosomal DNA of *S. aureus* HG001 as template. The final PCR products were obtained by flanking PCRs or double digestion/ligation steps (Table S4 for primers) and cloned into pMAD shuttle plasmid with BamHI/BglII or BamHI/EcoRI restriction enzymes. Plasmids were amplified into RN4220 after electroporation and then transferred in HG001 for homologous recombination steps as previously described.[77]

Northern blot

Isolation of total RNA of *S. aureus* cultures was performed accordingly to the FastRNA pro blue kit (MP Biomedicals) using the FastPrep machine (MP Biomedicals) for mechanical lysis of bacterial cells. Electrophoresis of 15 μg of total RNA was run in 1% agarose-TBE 1X gel containing 25 mM guanidium thiocyanate (Sigma). After migration at 150 V, RNAs were transferred on Hybond+ nitrocellulose membrane (Cytiva) by capillarity with saline-sodium citrate 2X buffer (SSC 2X) and then fixed by UV-crosslinking (Stratalinker 1800 Stratagene). RNA detection was performed by hybridization with specific digoxigenin (DIG)-labeled RNA probes complementary to each targeted RNA as previously described.[42] (see S4 for primers used for DIG-probe production).

Western blot

Total proteins were extracted from *S. aureus* cultures grown for 2, 4 or 6 h in BHI. After harvesting by centrifugation, bacterial pellets were washed once wit 1X PBS, then resuspended in 1X PBS and finally lysed using the Fastprep (MP Biomedicals). Quantification of proteins

was performed with Bradford reagent following the manufacturer protocol (BioRad) and using BSA as standard. 10 µg of total protein extract were migrated on 12 % polyacrylamide-SDS gel and transferred onto a PVDF membrane using the Trans-blot Turbo Transfer system (BioRad). Membranes were incubated with anti-FLAG mouse antibodies (Sigma) at the final dilution of 1:2500 followed by the incubation with a goat anti-mouse peroxidase (HRP) antibody (BioRad) at the final dilution of 1:5000. Membranes were revealed using the ChemiDoc (BioRad) and were then stained by Coomassie blue as loading controls of samples.

MAPS

Cultures of HG001 pCN51::MS2 or *⊿rnaIII* pCN51::MS2-RNAIII strains were grown in BHI medium for 6 h and then centrifuged. Pellets were then harvested, lysed and purified by affinity as previously described.[78] RNA was isolated, purified from the collected fractions with PCI (Carl Roth, pH 4,5-5), precipitated with EtOH and used for Northern blot.[78]

Transcriptomics

Cultures of HG001 and HG001*⊿rho* strains were grown in BHI medium until OD_{600}=0.5 and harvested by centrifugation. Total RNA was extracted as described above and treated with DNAse I (Sigma) prior to RNA-seq. RNAs were treated to deplete abundant rRNAs and the cDNA libraries were performed using the NEBNext Ultra II directional RNA kit. The libraries were sequenced using NextSeq2000 P2. Each RNAseq reaction was performed in duplicates. The reads were then processed to remove adapter sequences and poor-quality reads using Trimmomatric[79], then converted to FASTQ format using FASTQ Groomer[80], and aligned to the HG001 genome[81] using Bowtie2[82]. Finally, the number of reads mapping to each annotated feature was determined with HTSeq.[83] To perform the differential expression analysis, we used DESeq2.[84]All processing steps were performed on the Galaxy platform.[47]

RIPseq

Cultures of HG001 WT and HG001 *rho-3xflag* strains were grown in BHI medium until early exponential phase (OD_{600}=0,5) and harvested by centrifugation. Pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitors) and transferred into flacon tubes containing Lysing Matrix B silica beads (MP Biomedicals) to proceed to lysis of the cells using the Fastprep (MP Biomedicals). After centrifugation to remove cell debris, the supernatant was incubated for 2 h at 4ºC on a rotator with pre-equilibrated ANTI-FLAG M2 affinity Gel (Sigma) as described by the manufacturer

protocol. The suspension was centrifuged, the supernatant discarded, and the beads were washed twice with cold 1X TBS. The antibody-bound fusion protein Rho-3xFLAG was then eluted by competition with 100 µg/mL of triple FLAG peptide (Sigma) in 1X TBS. A sample of each fraction was used for Western blot to assess the enrichment of the tagged protein after elution. RNA was purified from the elution fraction with PCI precipitation (Carl Roth, pH 4.5-5) and treated with DNase I (Sigma) prior to RNA-seq analysis. RNAs were treated to deplete abundant rRNAs and the cDNA were performed sequenced using the NEB Next Ultra II directional RNA kit. The libraries were sequenced using HiSeq 1000 Single read 50 bases. Each RNAseq was performed in duplicates. Treatment of RNAseq data was performed as described above. To identify co-precipitating asRNAs, the RNAseq analysis was performed as described above but in a reverse orientation.

Preparation of RNAs for *in vitro* experiments

Transcription of the different RNAs used (RNAIII, RNAIII△H7, RNAIII△H9, RNAIII △H7△H9, *rho5'* and *rho3'*) was achieved using PCR products containing the sequence of the RNA downstream the T7 promoter, introduced with the primer (Table S4). These PCR products were used as templates for *in vitro* transcription using T7 RNA polymerase. In the case of RNAIII transcription, reactions were supplemented with 5 mM of ApG RNAs to avoid the 5'-end dephosphorylation step. RNAs were treated with DNase I and purified from an 8% polyacrylamide-8 M urea gel and eluted with 0,5 M ammonium acetate pH 6.1, 1 mM EDTA, and 0.1% SDS and precipitated in cold absolute ethanol. Precipitated RNAs where then labeled with T4 polynucleotide kinase (Fermentas) and [γ^{32}P] ATP and ultimately purified from a 8% polyacrylamide-8M urea gel and eluted as described above.

EMSA

The 5'-end radiolabeled RNAs (10'000 cps/sample, <1pM), and cold RNAs were denatured separately by incubating at 90ºC for 1 min in 100 mM Tric-HCl pH 7.5, 300 mM KCl, 200 mM NH_4Cl, then cooled down for 1 min on ice and renatured at 20ºC for 10 min after addition of 10 mM $MgCl_2$. Complexes were formed at 37ºC for 15 min and 1 volume of glycerol blue was added. Samples were then loaded on a native 6% polyacrylamide gel containing 10 mM $MgCl_2$ and migrated at 300 V and 4ºC in 1X TB buffer with 10 mM $MgCl_2$.

RNase III cleavage

The 5'-end radiolabeled RNAIII (10'000 cps/sample, <1 pM) and cold *rho* mRNA were denatured separately at 90ºC for 1 min, cooled down on ice for 1 min and renatured in 1X TMK buffer (Tris HCl 100 mM pH7, $MgCl_2$ 100 mM, KCl 1M) at 20ºC for 10 min. Cold RNAs are added to the labeled-RNA mix and RNA-RNA complexes are formed at 37ºC for 15 min. RNAs are then incubated with increasing concentrations of purified *S. aureus* RNase III (12.5 nM, 17 nM, 25 nM and 50 nM) in presence of Mg^{2+} or Ca^{2+} and 1 µg of yeast total tRNA at 37ºC for 15 min. RNAs are purified with PCI precipitation (Carl Roth, pH 4.5-5) and loaded on a 12 % polyacrylamide-8 M urea gel.

For the alkaline ladder, radiolabeled RNA is incubated with Alkaline hydrolysis buffer 1X (Ambion) at 90ºC for 10 min before addition of 1 volume of loading buffer (Ambion).
For the T1 ladder, radiolabeled RNA is denatured at 90ºC for 1 min in sequencing buffer 1X (Ambion). After renaturation at 20ºC for 1 min, 1 U of RNase T1 (Sigma) is added and incubated at 37ºC for 5 min before addition of one volume of loading buffer (Ambion).

For RNase III cleavage experiments with RNAIII mutants, unlabeled *rho* and RNAIII variants to final concentrations of 400 nM and 200 nM were used to form duplexes and RNase III cleavage was performed as described above. Cleavage signals on RNAIII were detected by primer extension. Precipitated RNAs were then hybridized with a 5' end-labeled primer complementary to the RNAIII 3' end (Table S4) and reverse transcription with AMV reverse transcriptase was performed at 37ºC for 30 min. Sanger sequencing reactions were performed using DNA template for each RNAIII and Vent (exo-) DNA polymerase (NEB) (25 cycles, 1 min denaturation at 95ºC, 1 min annealing at 52ºC and 1 min elongation at 72ºC).

GFP fusions

S. aureus strains containing the pCN57 plasmids derivatives were grown in BHI medium supplemented with 10 µg/mL erythromycin and chloramphenicol until exponential phase. Total proteins were extracted and quantified as described above. For each sample, 200 µL of dilutions of 1 mg/mL, 0.5 mg/mL and 0.25 mg/mL of total proteins were loaded on a black uCLEAR 96 wells-plate (GreinerBioOne) and GFP signal was measured with the platereader Spectramax Id3.

HlgC and Hla quantification by an ELISA

Bacterial strains were cultured in CCY broth supplemented with chloramphenicol at 10 µg/mL at 37°C overnight with shaking to stimulate toxins production.[85] Samples were centrifuged at

10’000 g for 10 min and supernatants were collected. HlgC and Hla quantification was performed by a sandwich ELISA using custom-made antibodies. 96-well Nunc MaxiSorp plate (Thermo Scientific) were coated with anti-HlgC (R&D Biotech) or anti-Hla (MedImmune) HlgC monoclonal or Hla polyclonal antibodies at 10 µg/mL and 2 µg/mL respectively, and incubated overnight at 20°C. After 5 consecutive washes with phosphate-buffered saline (PBS)–0.05% Tween (PBS-T), wells were saturated for 1 h 30 min at 20°C with a blocking solution containing PBS-T, low-fat milk (5 g/L), and bovine serum albumin (BSA) (1 g/L). Standard dilutions from 15 to 1’000 ng/mL of recombinant HlgC or from 0,3125 to 20 ng/mL of recombinant Hla or the culture supernatant were denatured for 1 h at 95°C, loaded in duplicate, and incubated for 2 h at 37°C. For HlgC quantification, after washing, polyclonal rabbit $F(ab)'_2$ biotinylated antibody (1.55 µg/mL, R&D Biotech) was added to each wells, and the plate was incubated for 1 h 30 at 37°C, washed, and ExtrAvidin-peroxidase antibody (Sigma) targeting the biotin molecule and conjugated with horseradish peroxidase (HRP) was added. The plate was incubated for 1 h at 20°C. For Hla quantification, the plate was washed, monoclonal anti-Hla antibody (2 µg/mL) was added, and the plate was incubated for 1 h 15 at 37°C. After washing, polyclonal goat $F(ab)'_2$ ant-human Fcg-HRP conjugated with horseradish peroxidase (HRP) antibody (Jackson Immuno-Research) was added and the plate was incubated for 1 h 15 min at 37°C. For both, a final wash was performed then 75 µL of the substrate tetramethylbenzidine (KPL SureBlue; SeraCare) were added. The reaction was stopped with sulfuric acid at 1 N. The plates were read at 450 nm in a Bio-Rad model 680 microplate reader.

Red blood cells lysis

Bacterial strains were cultured in CCY broth supplemented with chloramphenicol at 10 µg/mL at 37°C overnight with shaking, centrifuged at 10’000 g for 10 min and supernatant were collected. Activity of alpha-hemolysin was confirmed by cell permeability assays using rabbit erythrocytes. In 96-well round bottom plate, bacteria culture supernatants were diluted in PBS (1/2 to 1/2048) and mixed with 50 µl of washed rabbit erythrocytes solution (10^8 cells/ml; Atlantis). After 60 min of incubation at 37°C, the plate was centrifuged and the OD_{415nm} of the supernatants measured. The cut off (corresponding to lysis of 25% of red blood cells) was determined using the following formula: (OD lysis control – OD neg control)/2, where the lysis control is the total lysis of red blood cells with pure water and the negative control is the mix of red blood cells with PBS. For each condition, the HLA activity value corresponds to the inverse of the exact dilution causing the lysis of 25% of the red blood cells.

Cytotoxicity assay

Bacterial strains were cultured in CCY broth supplemented with chloramphenicol at 10 µg/mL at 37°C overnight with shaking, centrifuged at 10'000 g for 10 min and supernatant were collected. U937 Human monocytes expressing C5a or CXCR2 receptor[86] were routinely cultured in Roswell Park Memorial Institute (RPMI ; Thermofisher) 1640 Medium supplemented with 10% foetal bovine serum at 37°C with 5% CO_2. U937 cells were routinely cultured in DMEM growth medium supplemented with 10% foetal bovine serum at 37°C with 5% CO_2. Cells were diluted at 1.10^6 cell/ml and Iodure Propidium (IP) was added for a final concentration at 25 µg/mL. A 96-well plate was seeded with 90 µl of this solution and 10 µl of culture supernatant diluted at ½ in DMEM medium were added. IP incorporation into cells was measured using TECAN plate reader during 24 h.

Adherence assay

Adherence of bacterial cells to immobilized fibrinogen was performed as described by Hartford *et al*,1997.[87] Briefly, 96-well plate was coated with 5 µg/ml of fibrinogen (SIGMA) in PBS and incubated overnight at 4 °C with shaking. Bacterial strains were cultured in BHI broth at 37°C overnight. The plate was incubated with bovine serum albumin solution (1% in PBS) for 1 h at 37 °C. 1 ml of bacterial cell adjusted at 10^8 CFU/ml were labelled with 2 µl of BacLight RedoxSensor Green Vitality for 15 min at 37°C with gentle shaking. 100 µl bacterial cell suspension was added per well in duplicate and the plate was incubated for 1 h at 37°C, in the dark. After incubation, the plate was washed three times with PBS and the adherence of bacterial cells was quantified at 485 nm using TECAN plate reader.

Coagulase quantification

Bacterial strains were cultured 5 h at 37°C with agitation in Brain Heart Infusion (BHI; Difco) supplemented with chloramphenicol at 10 µg/mL and then centrifuged. Coagulase activity was assayed by adding 0.5 ml of two-fold serial dilutions of culture supernatant in PBS to 0.5 ml of rabbit plasma (Biorad) diluted 1 in 3 in distilled water. The titer was the reciprocal of the highest dilution of the supernatant that showed evidence of clotting after incubation for 18 h at 37°C.

Statistical Analysis

The statistical analyses were performed using GraphPad Prism 10 software. Data from 3 strains were compared using multiple comparisons of the means through a Brown-Forsythe and Welch ANOVA tests. The significance threshold was set at 0.05 for all tests.

FUNDING

This work was supported by the Centre National de la Recherche Scientifique (CNRS), by the French National Research Agency ANR (ANR-18-CE12-0025-04 CoNoCo to [P.R.]). This work of the Interdisciplinary Thematic Institute IMCBio, as part of the ITI 2021-2028 program of the University of Strasbourg, CNRS and Inserm, was supported by IdEx Unistra (ANR-10-IDEX-0002) and by SFRI-STRAT'US (ANR 20-SFRI-0012), and EUR IMCBio (IMCBio ANR-17-EURE-0023) under the framework of the French Investments for the Future Program. LB were supported by the "Fondation pour la Recherche Médicale" (ECO202006011534 and FDT202304016346).

Figures and legends

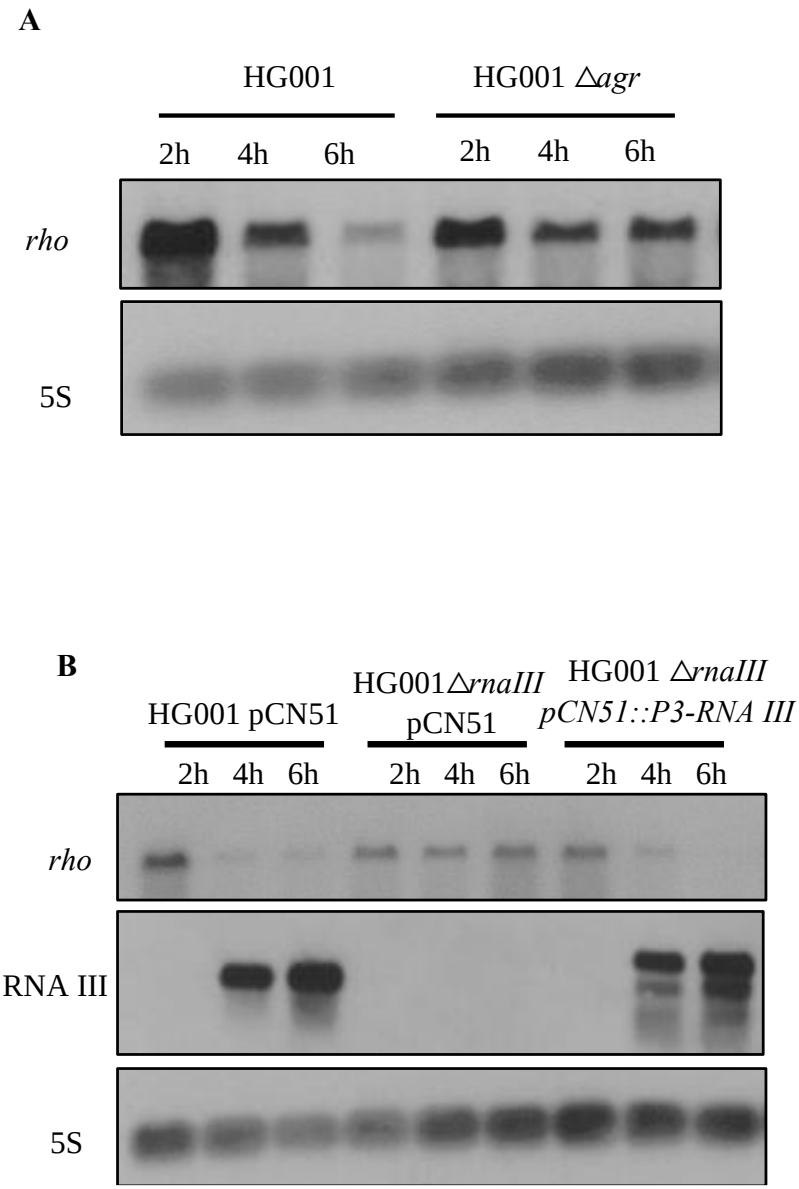


Figure 1. *rho* mRNA is regulated by the quorum sensing two component system Agr
A. *Northern blot* analysis of *rho* in HG001 WT and HG00△*agr* strains. Total RNA was extracted at 2h, 4h and 6h of growth in BHI medium at 37ºC and loaded on a 1% agarose gel. DIG-labeled RNA probes against *rho* and the loading control 5S rRNA (5S) were used. **B**. *Northern blot* analysis of *rho* and RNAIII in HG001 pCN51, △*rnaIII* pCN51 *and* △*rnaIII* pCN51::RNAIII. Total RNA was extracted at 2, 4 and 6 hr of growth in BHI medium supplemented with 10 ug/uL erythromycin at 37ºC and loaded on a 1% agarose gel. DIG-labeled RNA probes against *rho* and the loading control 5S rRNA (5S) were used.

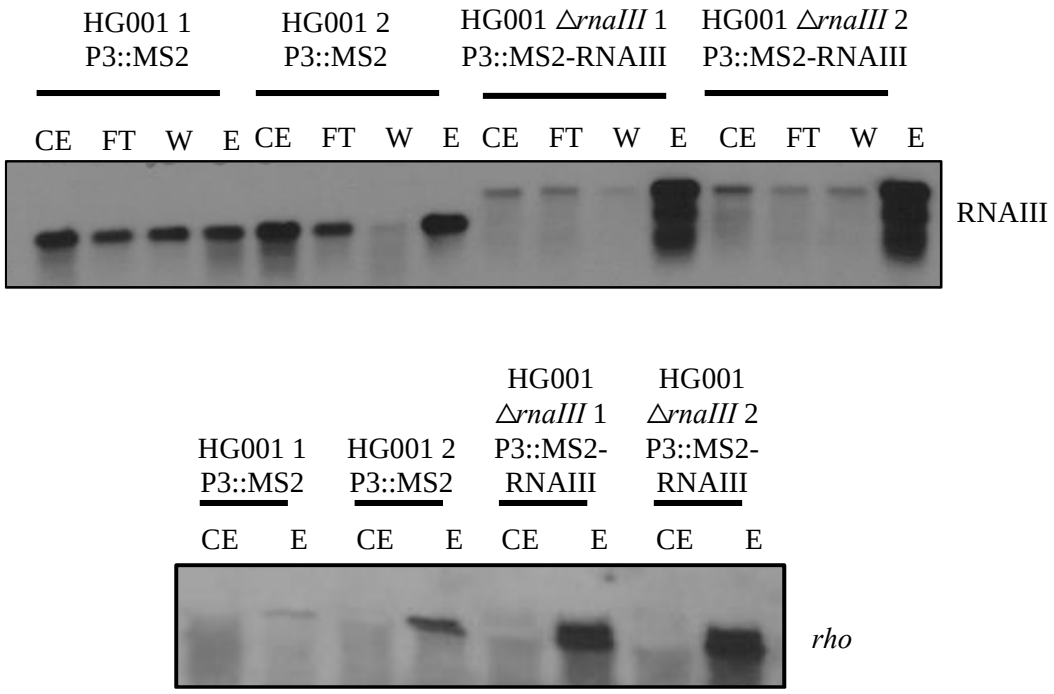


Figure 2. RNAIII binds to *rho* mRNA *in vivo*

MS2-affinity purification of MS2-RNAIII was performed in a △*rnaIII* background using HG001 strain expressing the MS2-tag alone as a control. HG001 pCN51::P3 MS2 and △*rnaIII* pCN51::P3 MS2-RNAIII strains were grown in BHI medium supplemented with 10 ug/µL erythromycin for 6 hr. Cells were harvested, lysed and the supernatant was subjected to affinity purification. RNAs from each fraction (CE: crude extract, FT: flowthrough, W: wash, E: elution) were extracted and loaded on a 1% agarose gel to perform *Northern blot.* Specific DIG-labeled RNA probes against RNAIII and *rho* were used.

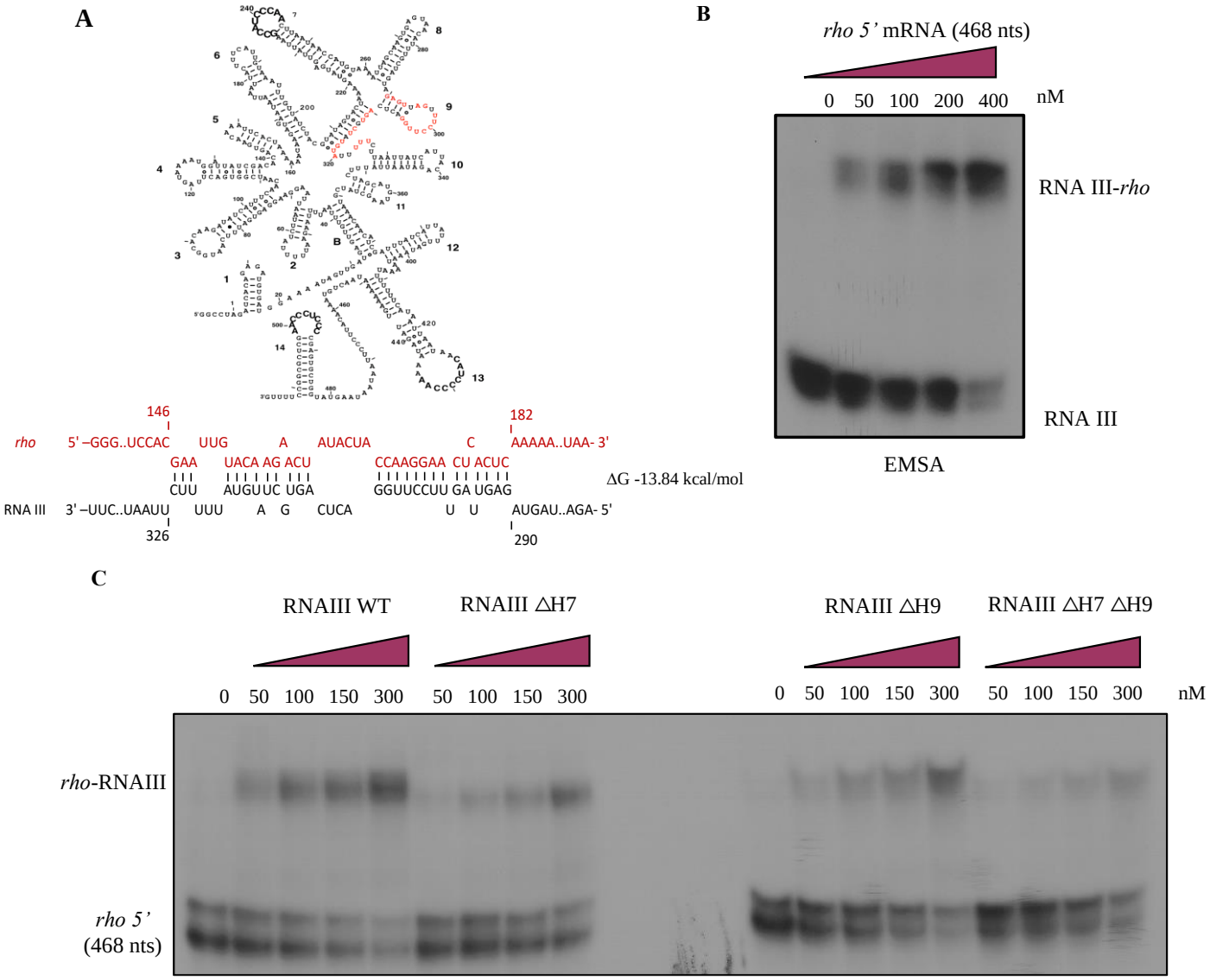
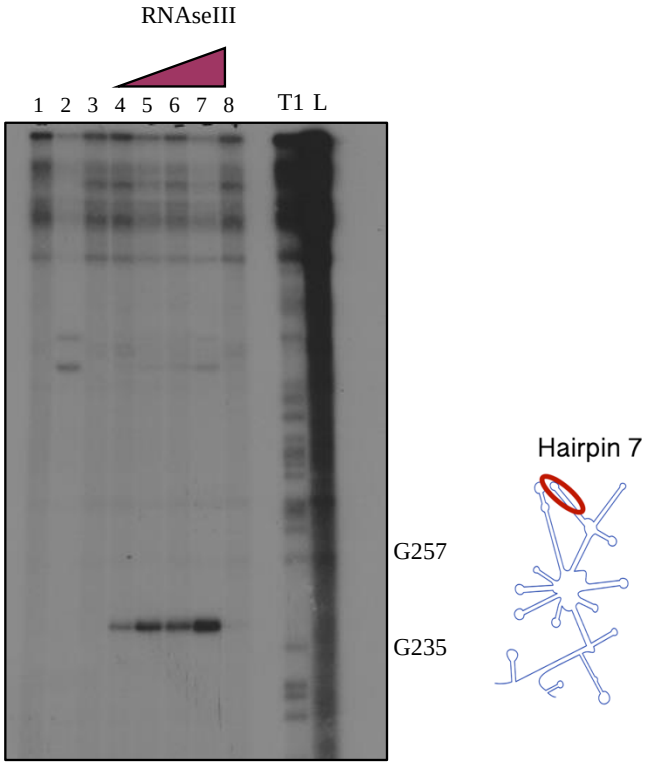


Figure 3. RNAIII binds to *rho* mRNA *in vitro*.

A. Secondary structure model of RNAIII determined by Benito *et al*, 2000. The predicted regions of RNAIII complementarity to *rho* are shown in red. Below is the predicted interaction performed with IntaRNA. **B.** Electrophoretic mobility shift assay (EMSA) shows the formation of the complex between 5'-end radiolabelled RNAIII and a 498 nt-long fragment of the *rho* mRNA comprising the region of *rho* predicted to interact with RNAIII. The 5'-end radiolabeled RNAIII was incubated with increasing concentrations of cold *rho* mRNA (50 to 400 nM). The primers used for *in vitro* transcription of the *rho* mRNA fragment and RNAIII are described in Table S3. **C**. Electrophoretic mobility shift assay (EMSA) shows the formation of complexes between 5'-end radiolabelled 498 nt-long fragment of the *rho* mRNA comprising the region predicted to interact with RNAIII and the different versions of RNAIII deleted of either or both hairpins. The 5'-end radiolabeled *rho* was incubated with increasing concentrations of cold RNAIII (50 to 300 nM).

A


RNAseIII
1 2 3 4 5 6 7 8
T1 L
G257
G235
Hairpin 7

B

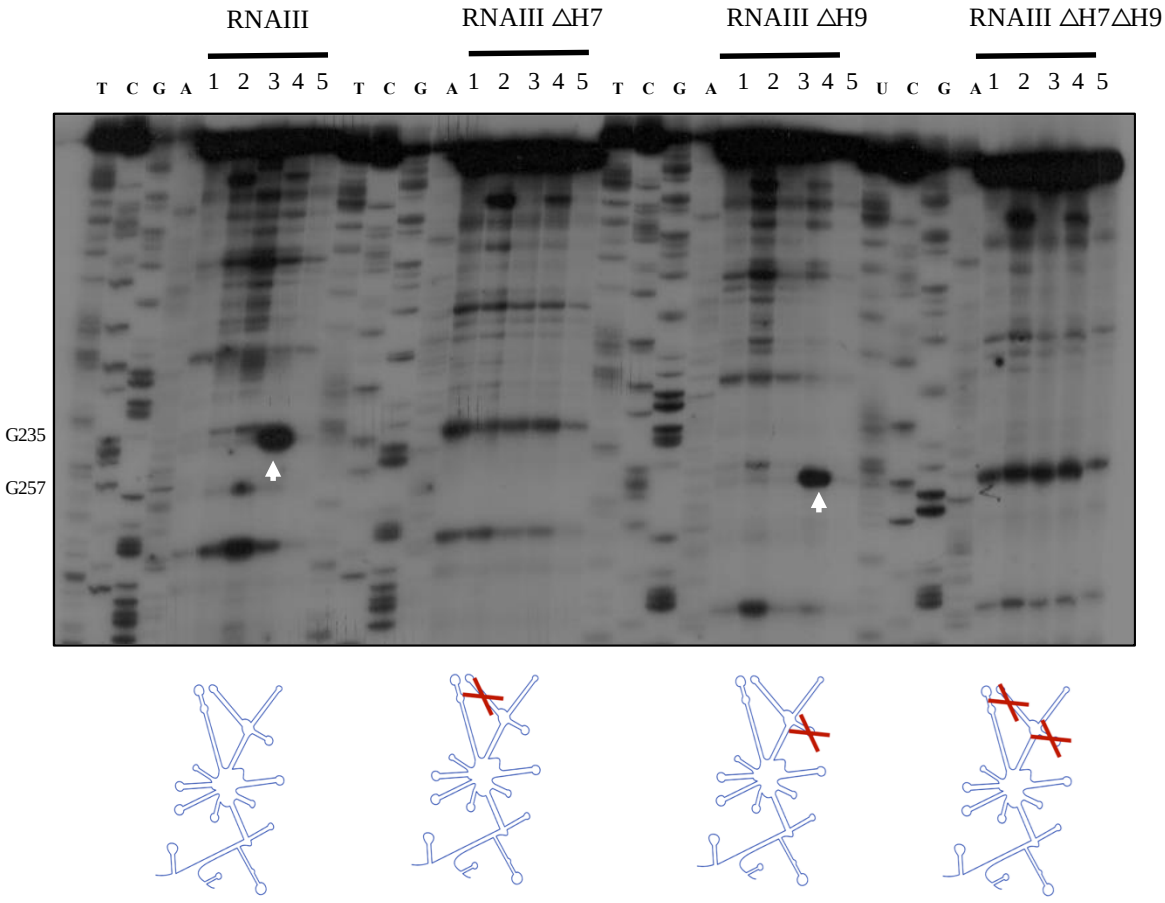
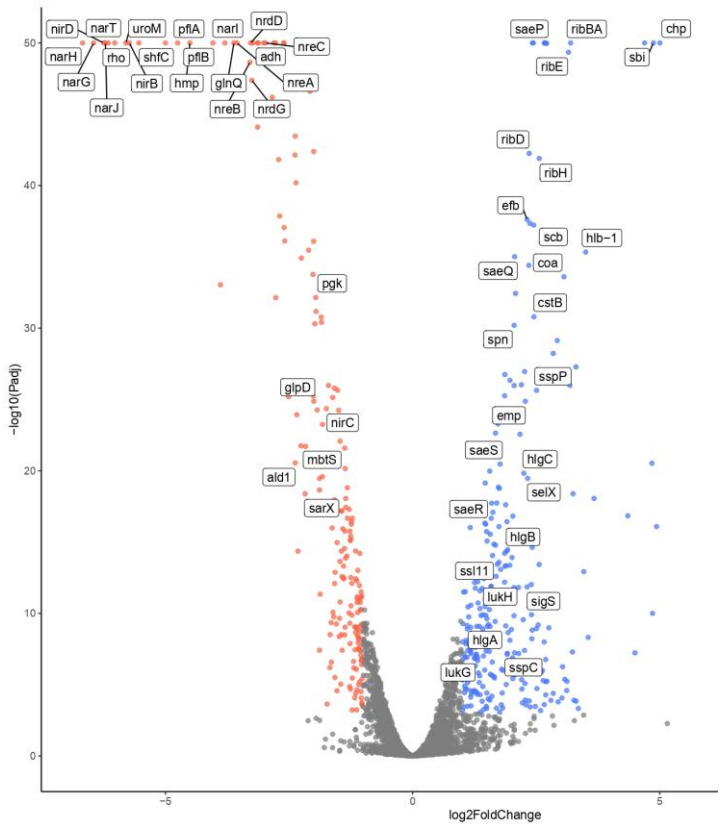

RNAIII
RNAIII ΔH7
RNAIII ΔH9
RNAIII ΔH7ΔH9
T C G A 1 2 3 4 5
T C G A 1 2 3 4 5
T C G A 1 2 3 4 5
U C G A 1 2 3 4 5
G235
G257

Figure 4. RNAIII binding to *rho* mRNA leads to its degradation by RNase III
A. The RNAIII-*rho* duplex is cleaved by RNase III *in vitro.* 5'-end radiolabeled RNAIII is incubated with the *rho* fragment described previously with or without increasing concentrations of purified *S. aureus* RNase III.
Lane 1: incubation control of RNAIII in absence of RNase III; Lane 2: incubation control of RNAIII in presence of 50 nM RNase III; Lane 3: incubation control of RNAIII with 400 nM *rho* in absence of RNase III; Lanes 4-7: cleavage of the RNAIII-*rho* complex in presence of increasing concentrations (12,5 nM, 17 nM, 25 nM and 50 nM) of purified RNase III. Reactions were performed in the presence of Mg^{2+} required for RNase III activity. Lane 8: reaction control using Ca^{2+} instead of Mg^{2+} to specifically block RNase III activity. T1, L: RNase T1 and alkaline ladders respectively, under denaturing conditions. **B**. H9 of RNAIII is not necessary for cleavage on H7. 200 nM of *in vitro* transcribed RNAIII, RNAIII△H7, RNAIII△H9 and RNAIII△H7△H9 were incubated with *rho* with or without 50 nM of RNase III in presence of Mg^{2+} or Ca^{2+}. Lane 1: incubation control of RNAIII in absence of RNase III; Lane 2: incubation control of RNAIII in presence of 50 nM RNase III; Lane 3: incubation control of RNAIII with 400 nM *rho* in absence of RNase III; 4: incubation of RNAIII with 400 nM *rho* in presence of 200 nM RNase III; Lane 5: reaction control using Ca^{2+} instead of Mg^{2+} to specifically block RNase III activity; Lanes T, C, G, A are sequencing ladders. White arrows depict the RNAse III specific cleavage site in hairpin 7.

A

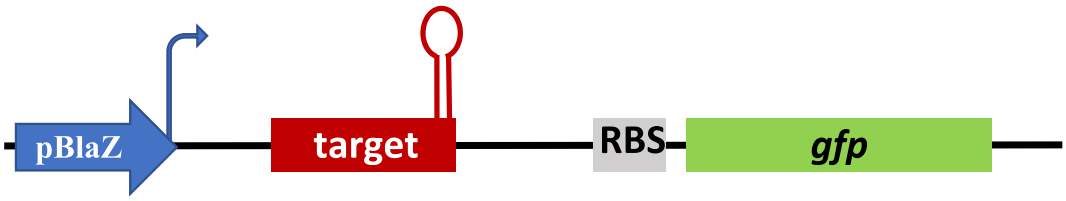


B

ID	*sae* regulon genes	Transcriptomics	RIP
BSR30_RS00935	*coa*	4.505	0.711
BSR30_RS01720	*selX*	2.328	3.741
BSR30_RS01910	*ssl7*	1.418	1.078
BSR30_RS01920	*ssl9*	1.157	1.317
BSR30_RS01945	*ssl11*	3.356	0.59
BSR30_RS01970	*lpl9*	1.213	0.792
BSR30_RS03490	*saeS*	3.414	1.075
BSR30_RS03495	*saeR*	2.755	1.132
BSR30_RS03500	*saeQ*	4.181	1.093
BSR30_RS03505	*saeP*	6.466	2.702
BSR30_RS04000	*emp*	4.86	1.819
BSR30_RS04010	*nuc1*	1.066	3.636
BSR30_RS05450	*ecb*	2.839	0.675
BSR30_RS05470	*efb*	4.98	0.737
BSR30_RS05475	*scb*	5.465	0.713
BSR30_RS05505	*hla*	2.169	0.827
BSR30_RS09370	*splD*	1.667	2.002
BSR30_RS09375	*splC*	1.716	1.936
BSR30_RS09385	*splA*	1.408	3.073
BSR30_RS09380	*splB*	1.594	1.917
BSR30_RS09450	*lukD*	1.002	1.056
BSR30_RS09455	*lukE*	1.098	5.181
BSR30_RS10635	*map2*	5.281	1.016
BSR30_RS10640	*eaplmap*	7.598	1.749
BSR30_RS10645	*hlb-1*	11.333	5.439
BSR30_RS10665	*scn*	5.371	2.875
BSR30_RS10670	*chp*	32.076	2.326
BSR30_RS10695	*sak*	1.155	1.722
BSR30_RS11035	*lukG*	2.379	3.22
BSR30_RS11035	*lukH*	3.634	3.158
BSR30_RS13280	*sbi*	29.265	2.656
BSR30_RS13295	*hlgA*	2.435	0.978
BSR30_RS13300	*hlgC*	4.76	1.194
BSR30_RS13305	*hlgB*	3.735	1.114
BSR30_RS13770	*fnbB*	1.604	1.049
BSR30_RS13775	*fnbA*	2.000	1.255
BSR30_RS09395	SAOUHSC_01944	1.877	5.173
BSR30_RS01950	SAOUHSC_00400	1.721	0.784
BSR30_RS00885	SAOUHSC_00182	6.413	3.347

Figure 5. Rho regulates the expression of numerous virulence genes.
A. Volcano plot of differentially expressed genes between HG001 WT and HG00*⊿rho* strains. RNAseq analysis was performed on purified total RNAs from duplicates of each strain grown in BHI medium at 37ºC until exponential phase. Each point represents a gene. In squared are some of the significantly up- and downregulated genes studied in this work. Depicted in blue are strongly upregulated genes in the *⊿rho* mutant and in red downregulated genes. In grey genes not significantly affected. B. Differential expression of genes from the SaeRS regulon after RNAseq and the enrichment of their respective mRNAs upon RIPseq of Rho-3xFLAG protein. An endogenously 3xFLAG-tagged Rho protein was immunoprecipitated from cultures grown in BHI medium at 37ºC until exponential phase, and co-precipitating RNAs were isolated and sequenced. In blue are genes presenting FC>2 with padj<0.05 in the RNAseq data and in yellow the genes with FC>2 with padj<0.05 in the RIPseq data.

A



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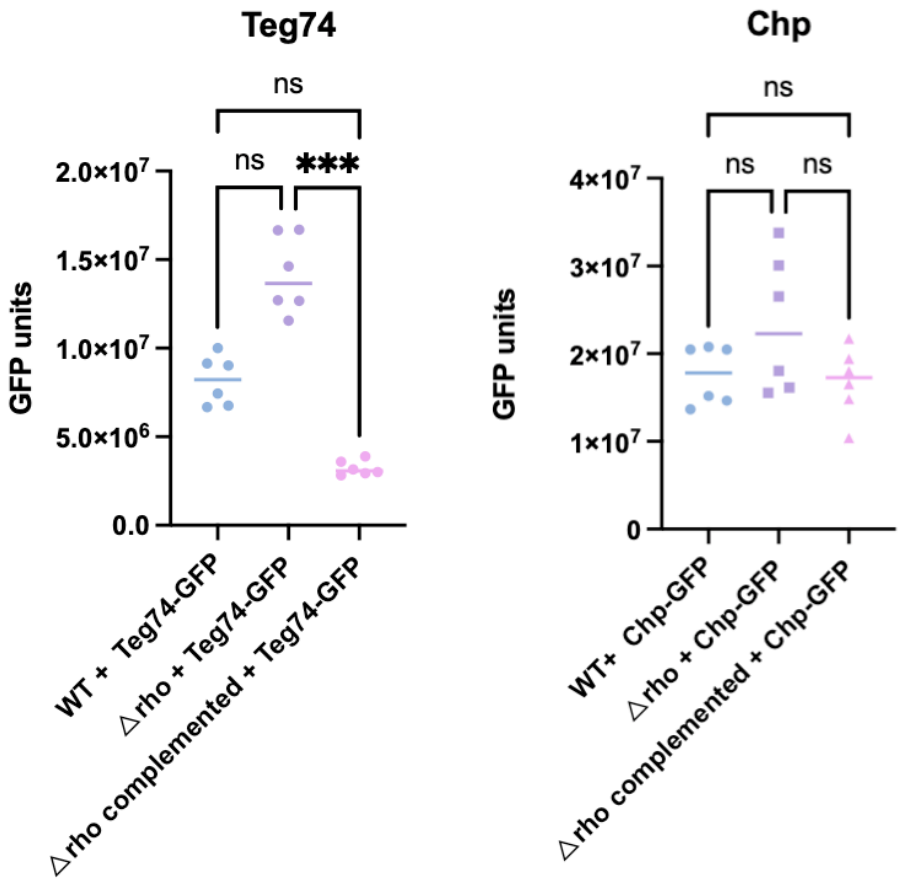


Figure 6. Rho terminates transcription of *teg74* and potentially *chp*.
A. Schematic representation of pCN57::*gfp* reporter fusion plasmids. Target sequences of the *chp* and *teg74* genes comprising their own Rho-independent terminator hairpin are cloned upstream the *gfp* sequence. **B.** GFP levels of cellular extracts from strains HG001 WT pMKX, HG001⊿*rho* pMKX and HG001⊿*rho* pMKX::*rho* transformed with plasmids illustrated in A containing the sequences of *chp* and *teg74*. Cells were grown until exponential phase and lysed extracts were used for GFP measurement (n=6). Statistical analysis was performed with Kruskal-Wallis test. *** = p-value<0.001, ns = not significant.

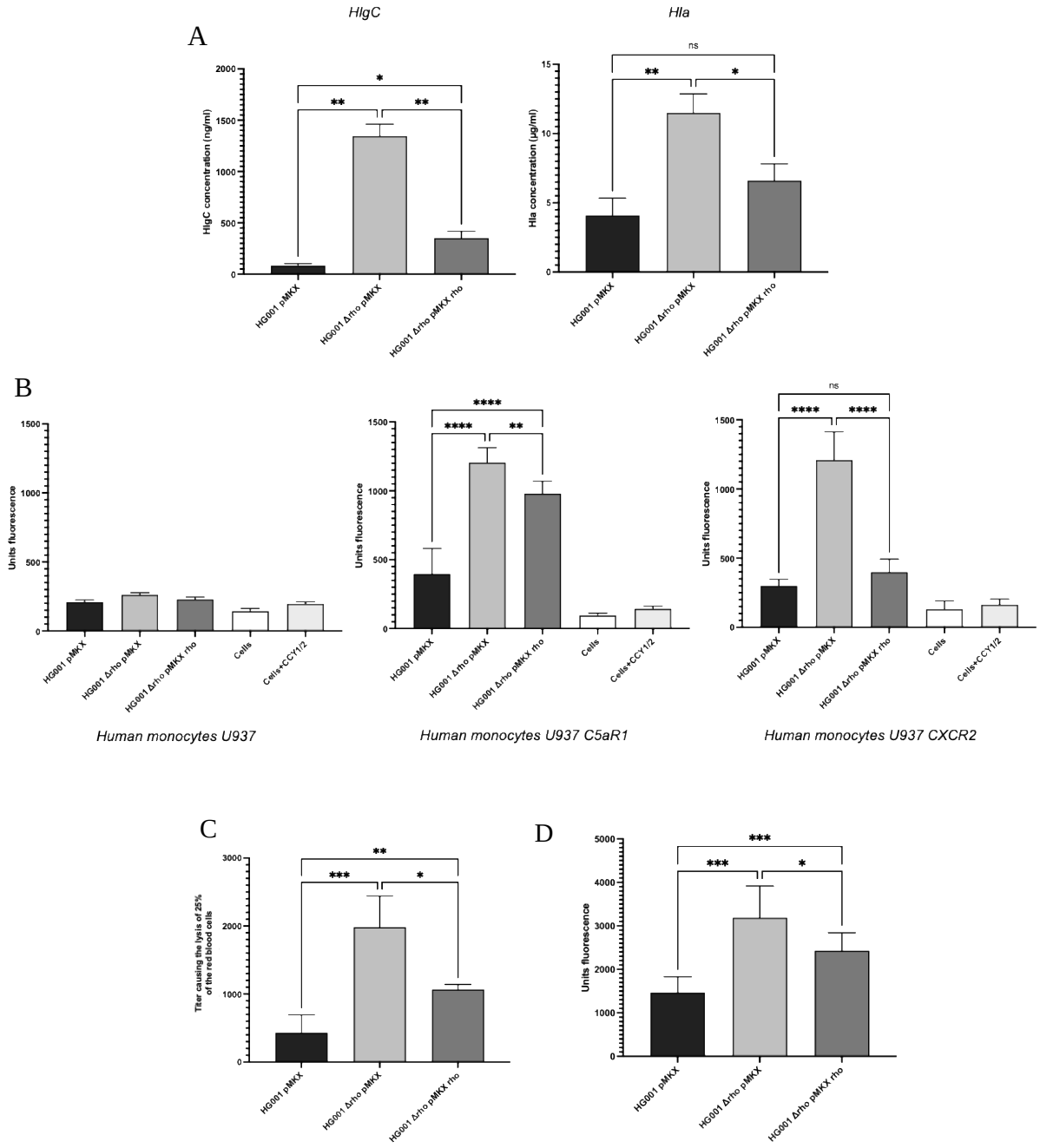


Figure 7. Impact of Rho on *S. aureus* cytotoxicity

A. Quantification of HlgC and Hla proteins (in nanograms per milliliter) from supernatants of HG001 WT (HG001-pMKX), mutant deleted for *rho* gene (HG001D*rho* pMKX) and complemented strain (HG001D*rho* pMKX::*rho*) cultures grown during 18 h in CCY medium (n=3). **B.** Bacterial supernatant cytotoxicity was quantified by measuring propidium iodide incorporation into U937 monocytes, C5aR1-transfected U937 monocytes and CXCR2-transfected U937 monocytes (n=9). **C.** Graphic representation of the Hla hemolytic activity of the supernatants from cultures grown during 18 h in CCY medium on rabbit red blood cells (n=3); the Hla activity value corresponds to the inverse of the exact dilution causing the lysis of 25% of the red blood cells. **D.** Isogenic strains of *S. aureus* examined for their binding capacity to fibrinogen coated on 96-well plates, at concentration of 5 µg/ml. The adherence of bacterial cells was quantified at 485 nm using TECAN plate reader. Mann-Whitney tests performed: * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001, **** = p-value < 0.0001.

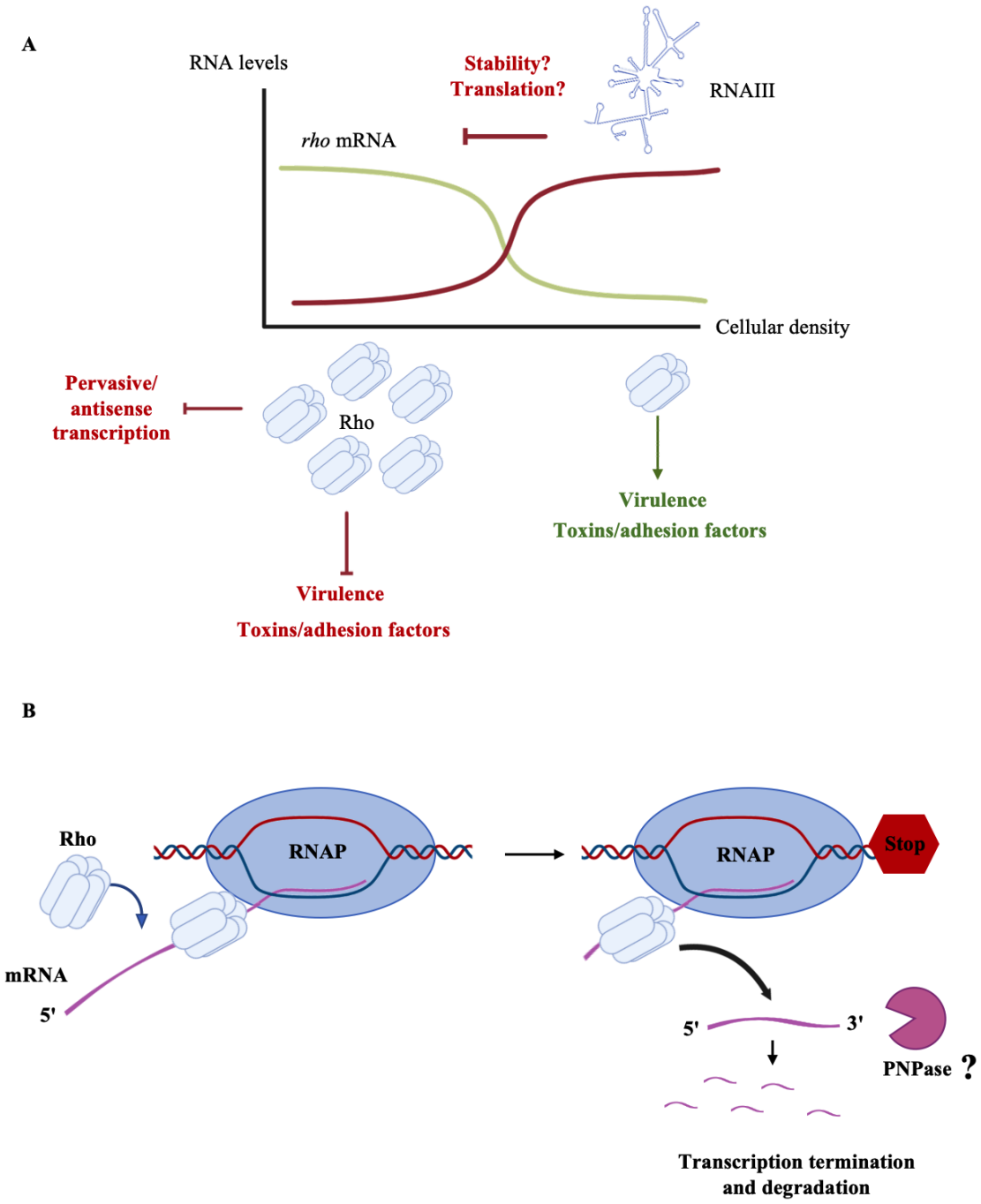


Figure 8. Proposed model of regulation of *rho* during growth (A) and regulation of its target mRNAs during exponential phase (B).

Supplementary data

Table S1: Table of total RNAseq and RIPseq data
Table S2: Strains used in this study
Table S3: Plasmids used in this study
Table S4: Primers used in this study
Figures S1 to S15

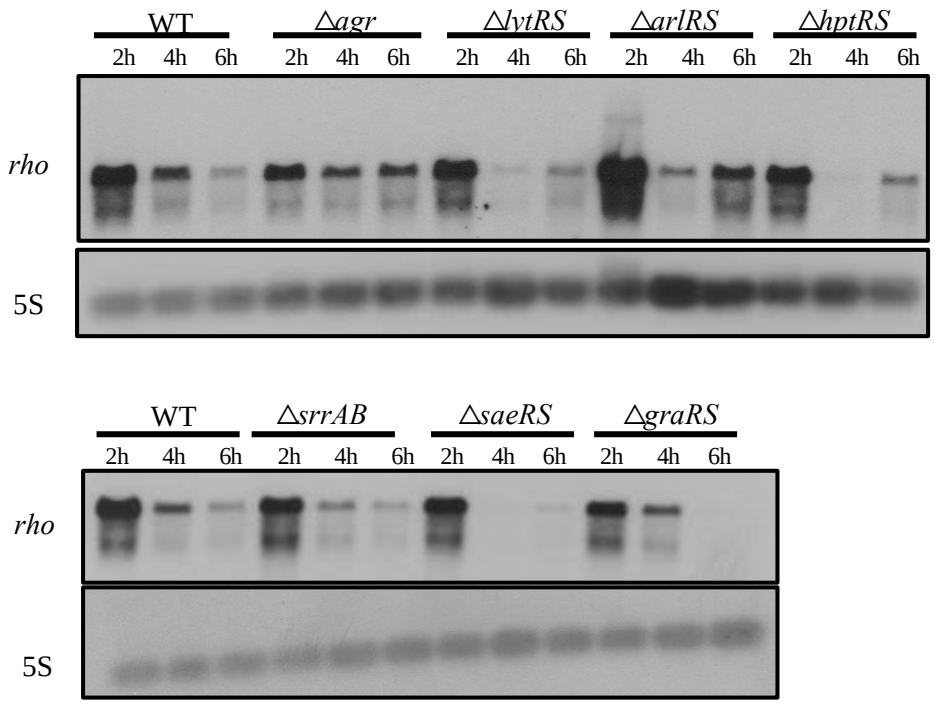


Figure S1. Expression of *rho* in various TCS mutants. A. *Northern blot* analysis of *rho* in HG001 WT and mutant strains for different two component systems. Total RNA was extracted from samples taken at 2 h, 4 h and 6 h of growth in BHI medium at 37ºC and loaded on a 1% agarose gel. DIG-labelled RNA probes against *rho* and the loading control 5S rRNA (5S) were used.

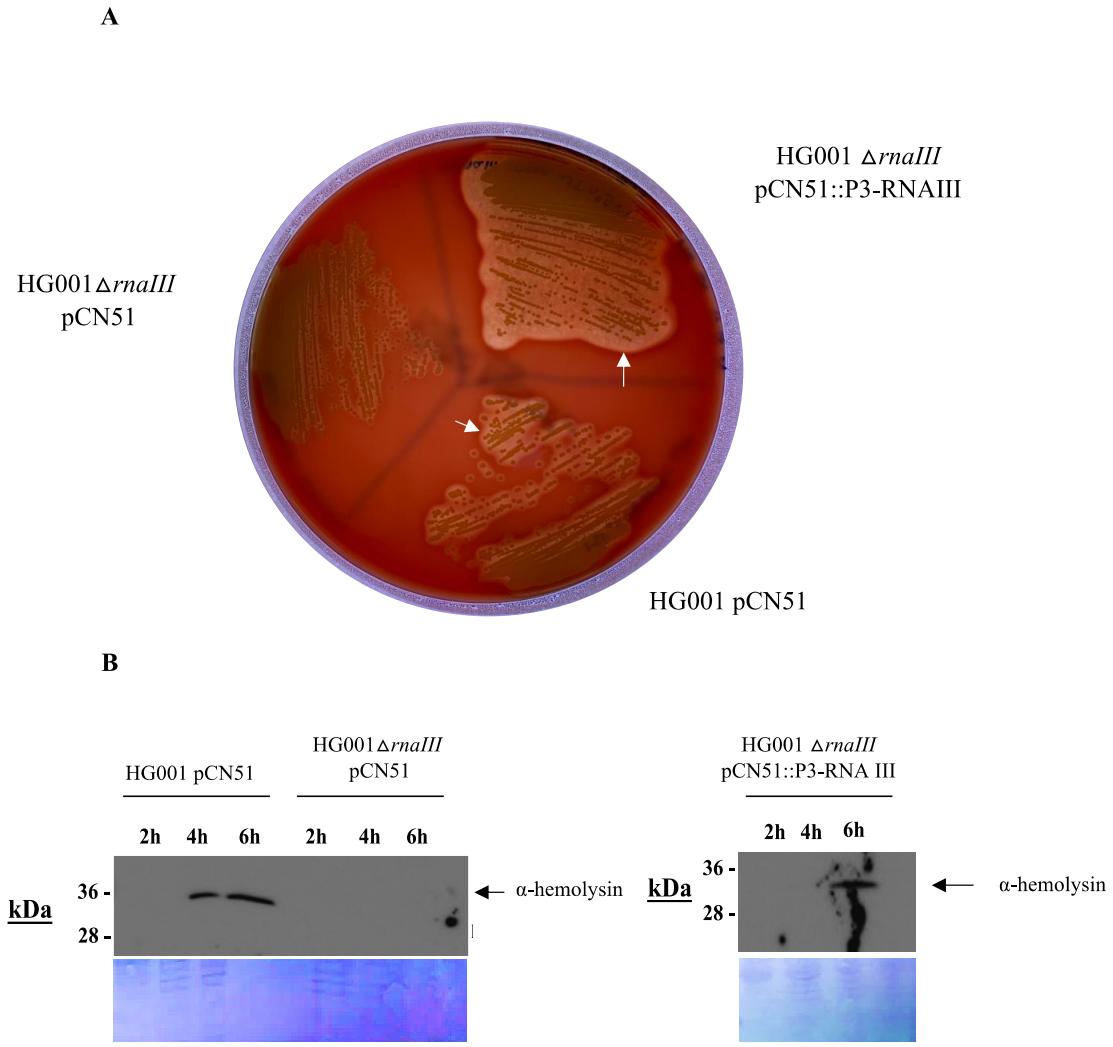


Figure S2. Plasmid complementation of RNAIII restores hemolytic activity of *ΔrnaIII* mutant.

A. HG001 pCN51, HG001*ΔrnaIII* pCN51 and HG001Δ*rnaIII* pCN51::P3-RNAIII strains grown on blood-agar medium. Hemolytic halos (white arrow) were observed for HG001 pCN51 and the complemented HG001 Δ*rnaIII* pCN51::P3-RNAIII strains but not for HG001Δ*rnaIII* pCN51. **B**. *Western blot* analysis of *in vivo* expression of α-hemolysin in HG001 pCN51, HG001*ΔrnaIII* pCN51 and HG001Δ*rnaIII* pCN51::P3-RNAIII strains. Strains were grown in BHI medium with 10 mg/uL erythromycin and protein samples were taken at different time points (2 h, 4 h and 6 h) and separated on 12% SDS-PAGE gel. α-hemolysin hemolysin was detected using a primary monoclonal *@hla* antibody. Membranes were stained with Coomassie for loading controls.

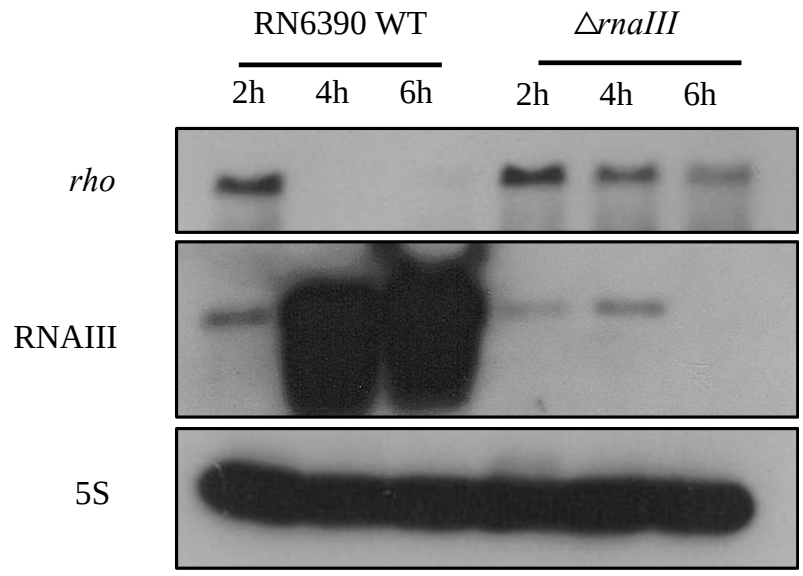


Figure S3. Expression of *rho* is RNAIII-dependent in *S. aureus* RN6390.
Northern blot analysis of *rho* in RN6390 WT and △*rnaIII*. Total RNA was extracted at 2, 4 and 6 h of growth in BHI medium at 37ºC and loaded on a 1% agarose gel. DIG-labelled RNA probes against *rho*, RNAIII and the loading control 5S rRNA (5S) were used.

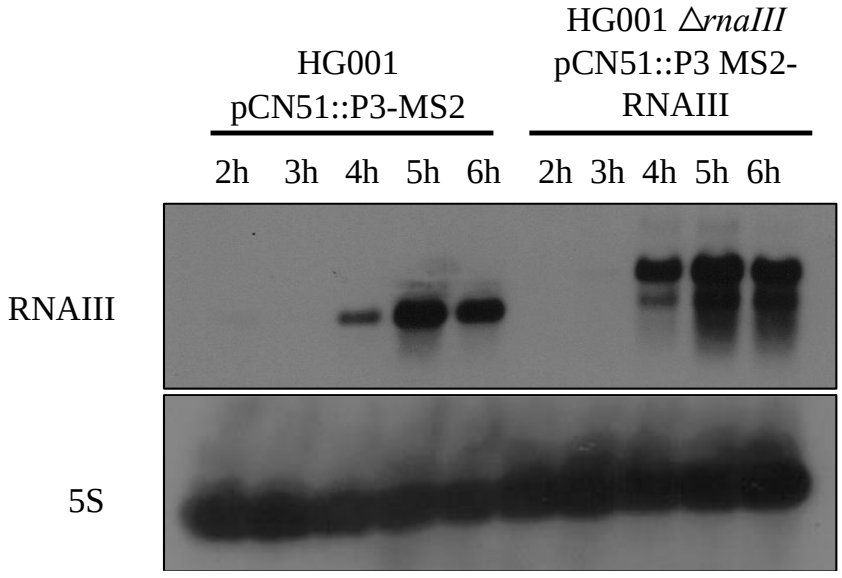
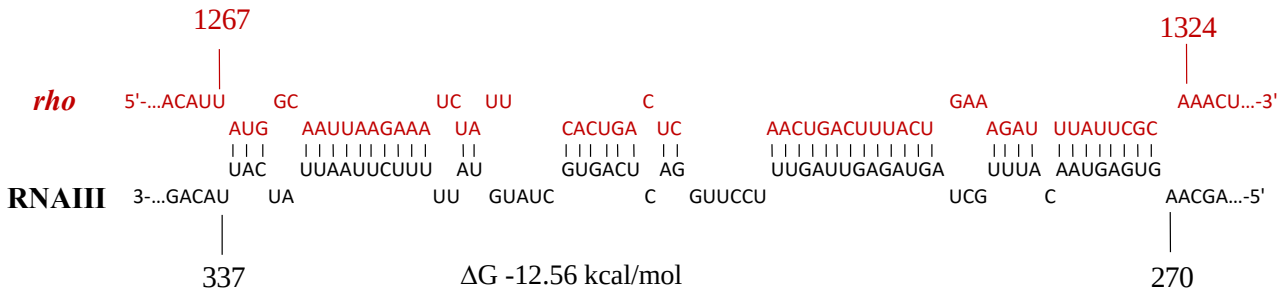


Figure S4. Expression of RNAIII in strains used for MAPS.
Northern blot analysis of RNAIII in HG001 pCN51::P3-MS2 expressing the MS2 tag alone and HG001 *⊿rnaIII* pCN51::P3-MS2-RNAIII expressing MS2-RNAIII. Cultures were grown in BHI medium supplemented with 10 mg/uL erythromycin at 37ºC and samples were taken at different times. Total RNA was extracted and loaded on a 1% agarose gel to perform *Northern blot* to assess the expression of the MS2-tagged RNAIII. DIG-labelled RNA probes against RNAIII and the loading control 5S rRNA (5S) were used.

A



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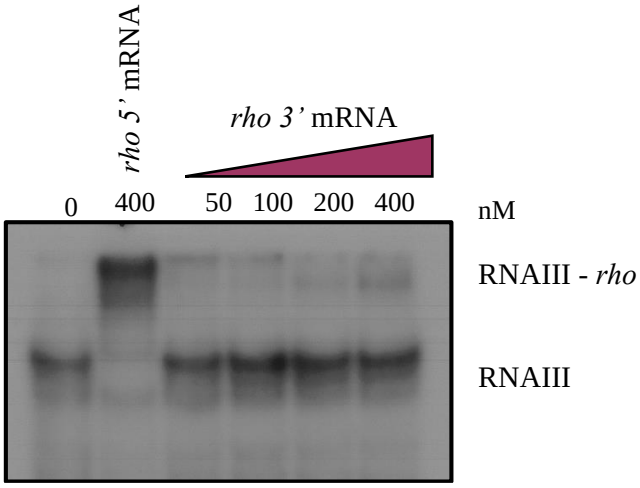

Figure S5. The 3' region of the *rho* mRNA does not interact with RNAIII *in vitro*.
A. Predicted interaction of the 3' region of the coding sequence of the *rho* mRNA with RNAIII using IntaRNA. B. Electrophoretic mobility shift assay (EMSA) shows the absence of a complex between the 5'-end radiolabeled RNAIII and the 3' region predicted to interact (453 nts). The 5'-end radiolabelled RNAIII was incubated with increasing concentrations of cold *rho 3*' (50 to 400 nM) and 400 nM of cold *rho 5'* as a control. The primers used for *in vitro* transcription of the *rho* mRNA fragment are described in Table S4.

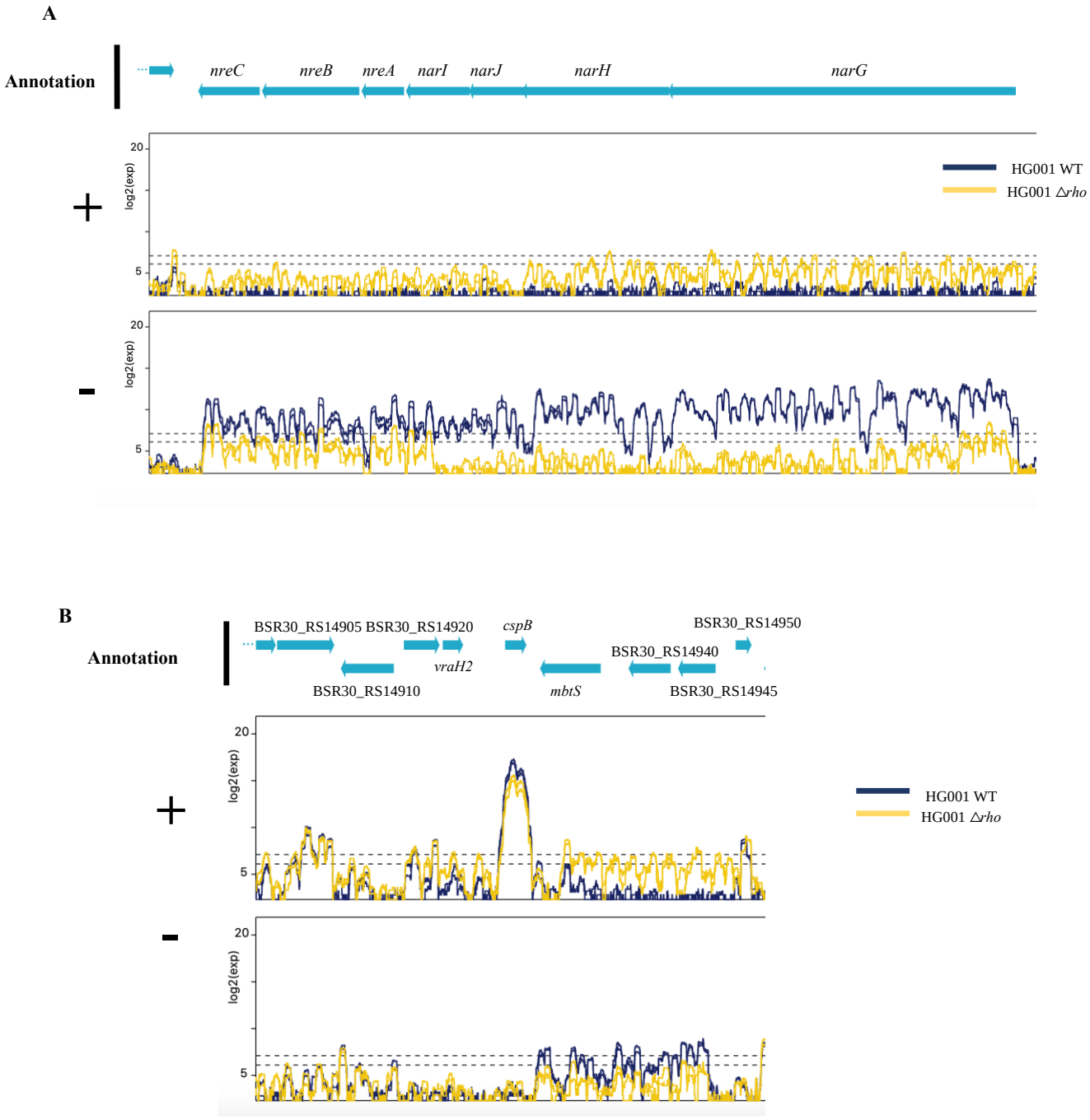

Figure S6. Antisense RNAs generated upon *rho* deletion impact opposite strand gene expression.

Expression profiles of HG001 WT and HG001△*rho* strains determined by RNAseq during exponential phase of growth and visualised by Genoscapist browser. Both strands (+ and -) are shown. The annotation of the genomic region is detailed at the top. Genes of unknown function (hypothetical genes and prophages) are shown with their reference number (BSR_RSXXXXX). Profile of each strain is represented with a different color, blue for WT and yellow for the △*rho* mutant. Decreased expression of the depicted regions are observed in absence of *rho* with concomitant increase of an antisense RNA to the same region.

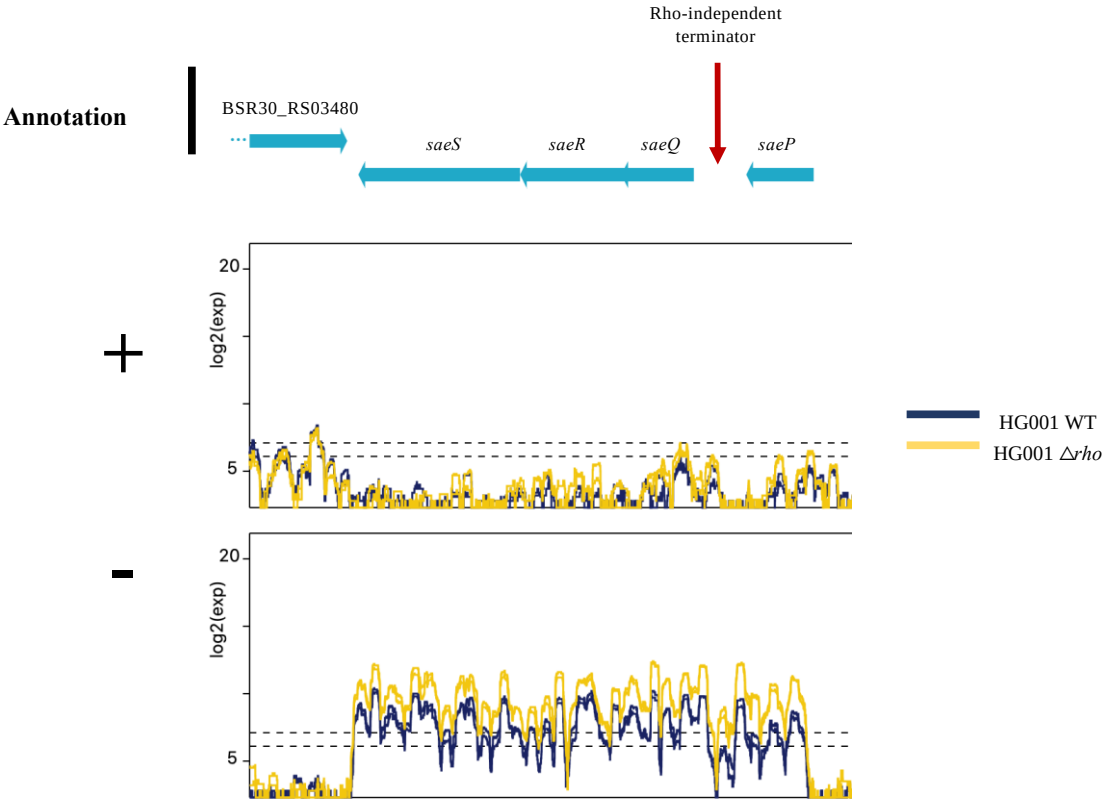

Figure S7. The *saePQRS* operon is upregulated upon *rho* deletion.
Expression profiles of HG001 WT and HG001△*rho* strains at the *saePQRS* locus determined by RNAseq during exponential phase of growth and visualised by Genoscapist browser. Both strands (+ and -) are shown. The annotation of the genomic region is detailed at the top. Genes of unknown function (hypothetical genes and prophages) are shown with their reference number (BSR_RSXXXXX). Profile of each strain is represented with a different color, blue for WT and yellow for the △*rho* mutant. Rho-independent terminators are pointed with a red arrow.

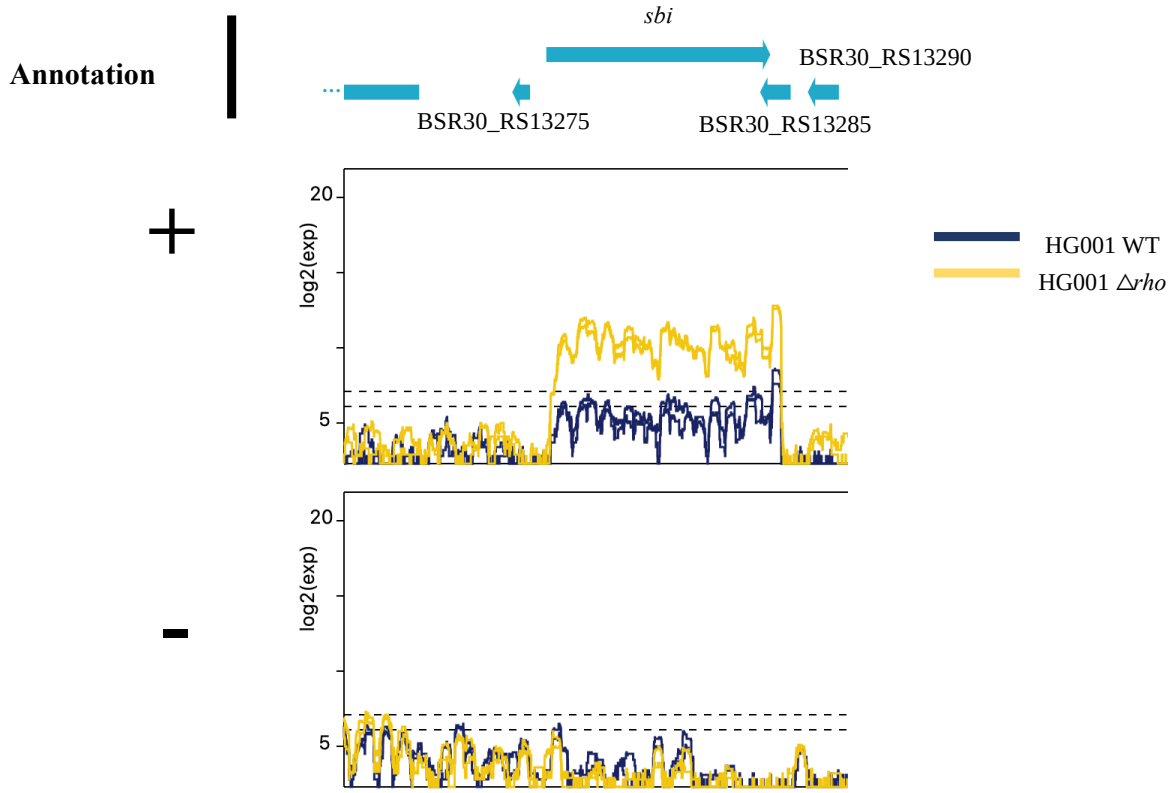

Figure S8. Increased expression of the *sbi* mRNA in the △*rho* mutant.
Expression profiles of HG001 WT and HG001△*rho* strains at the *sbi* locus determined by RNAseq during exponential phase of growth and visualised by Genoscapist browser. Both strands (+ and -) are shown. The annotation of the genomic region is detailed at the top. Genes of unknown function (hypothetical genes and prophages) are shown with their reference number (BSR_RSXXXXX). Profile of each strain is represented with a different color, blue for WT and yellow for the *rho* mutant.

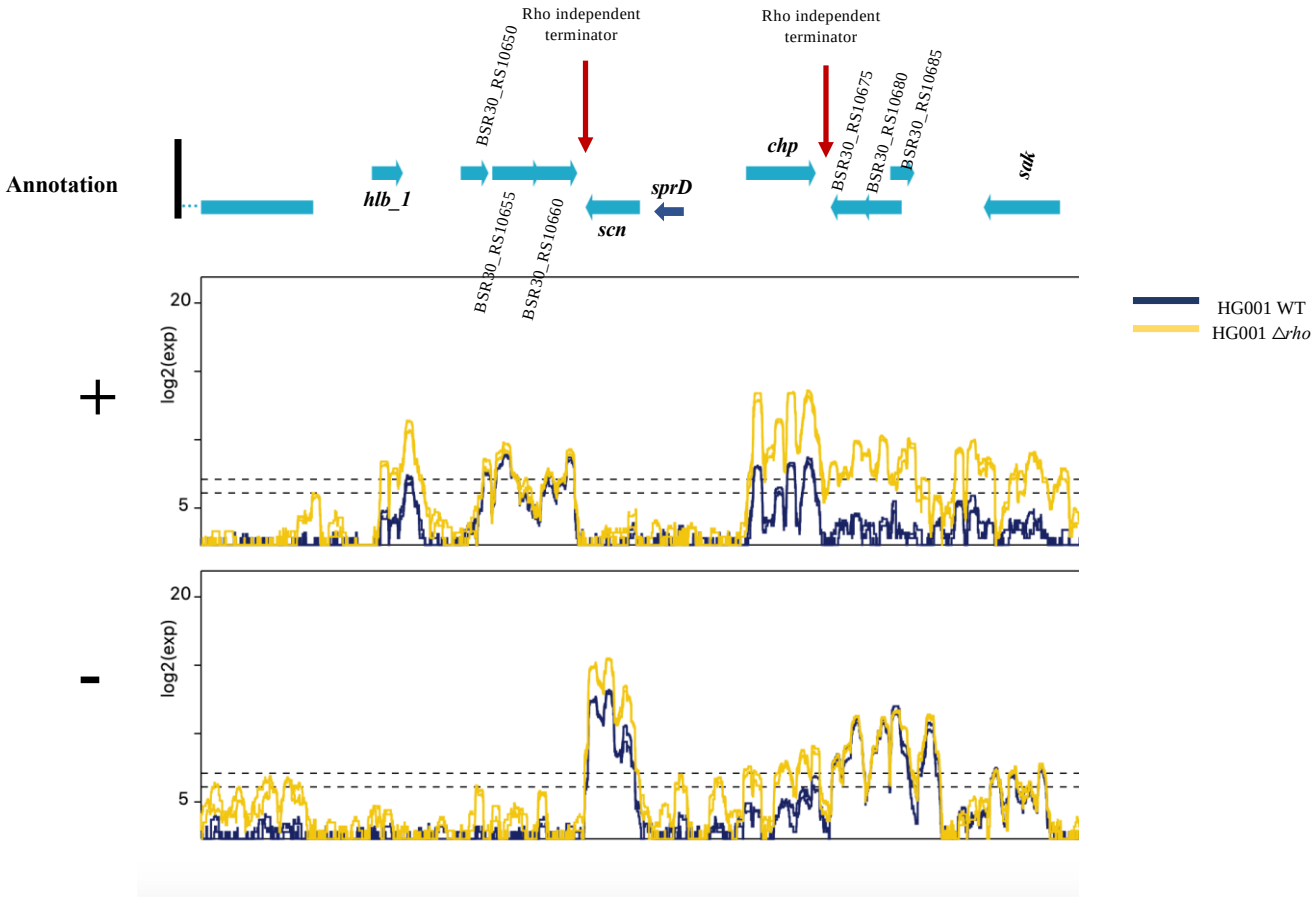

Figure S9. Direct Rho-mediated control virulence genes.

Expression profiles of HG001 WT and HG001△*rho* strains at the *sbi* locus determined by RNAseq during exponential phase of growth and visualised by Genoscapist browser. Both strands (+ and -) are shown. The annotation of the genomic region is detailed at the top. Genes of unknown function (hypothetical genes and prophages) are shown with their reference number (BSR_RSXXXXX). Profile of each strain is represented with a different color, blue for WT and yellow for the *rho* mutant. Rho-independent terminators are pointed with red arrows.

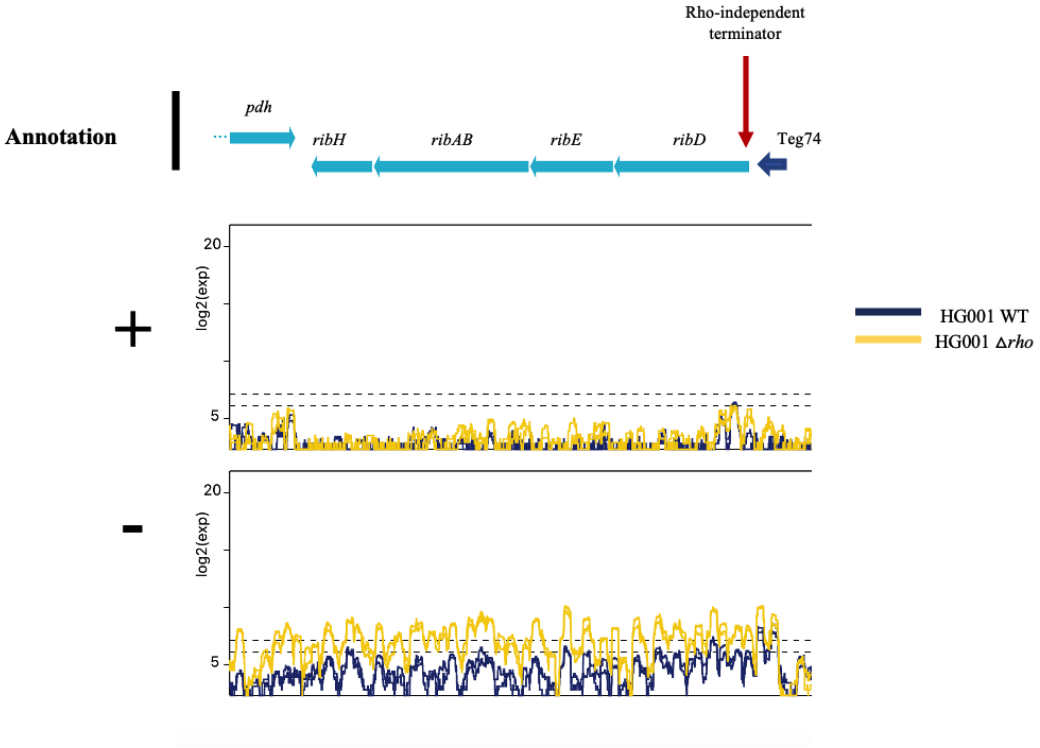

Figure S10. Rho-mediated control of the FMN riboswitch.

Expression profiles of HG001 WT and HG001△*rho* strains determined by RNAseq during exponential phase of growth and visualised by Genoscapist browser. Both strands (+ and -) are shown. The annotation of the genomic region is detailed at the top. Genes of unknown function (hypothetical genes and prophages) are shown with their reference number (BSR_RSXXXXX). Profile of each strain is represented with a different color, blue for WT and yellow for the △*rho* mutant. Rho-independent terminators are pointed with a red arrow.

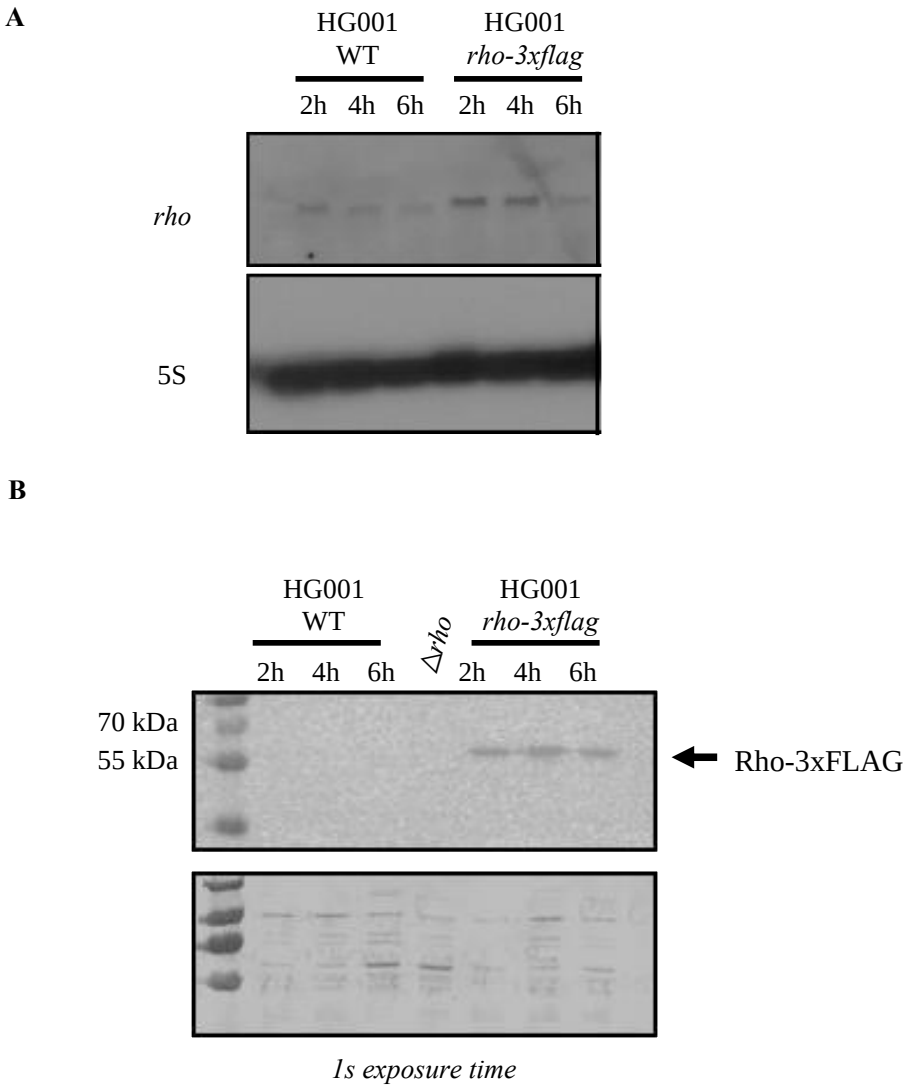

Figure S11. Expression of Rho3x-FLAG.

A. *Northern blot* analysis of *rho* in in HG001 WT, HG001 *rho-3xflag*. Total RNA was extracted at 2, 4 and 6 h of growth in BHI medium at 37ºC and loaded on a 1% agarose gel. DIG-labeled RNA probes against *rho* and the loading control 5S rRNA (5S) were used. **B**. HG001 WT and HG001 *rho-3xflag* strains were grown in BHI medium at 37º C and samples were taken at different times. Cells were harvested, lysed and the supernatant was used for measuring total protein concentration. 10 mg of total proteins loaded on a 12% polyacrylamide-SDS gel to perform *Western blot*. Membranes were incubated with anti-FLAG mouse antibody followed by the incubation with a goat anti-mouse peroxidase (HRP) antibody. A protein extract from HG001 Δ*rho* was also loaded as a control.

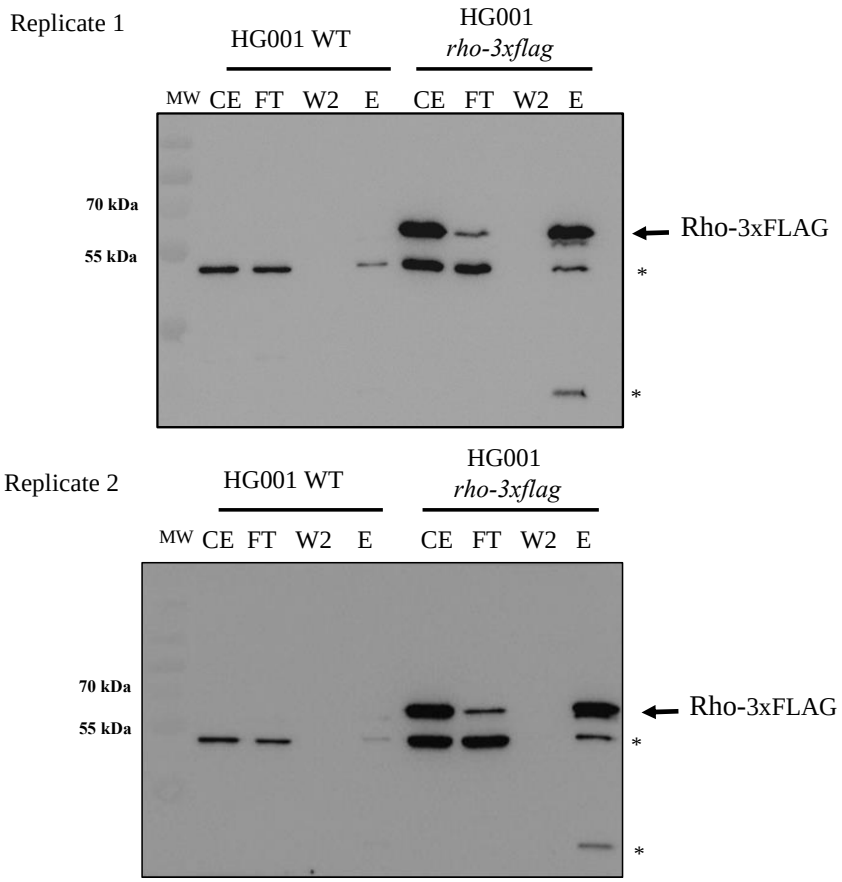

Figure S12. Immunoprecipitation of Rho 3x-FLAG.
HG001 WT and HG001 *rho-3xflag* strains were grown in duplicate in BHI medium at 37ºC until exponential phase. Cells were harvested, lysed and supernatant was used for immunoprecipitation using ANTI-FLAG M2 affinity gel. Samples from each fraction (CE: crude extract, F: flowthrough, W2: wash 2 and E: elution) were collected and loaded on a 12% polyacrylamide-SDS gel to perform *Western blot*. Membranes were incubated with anti-FLAG mouse antibodies followed by the incubation with a goat anti-mouse peroxidase (HRP) antibody. The recombinant Rho-3xFLAG protein was successfully recovered after elution. RNAs from this fraction were isolated and sequenced. *=non-specific signal

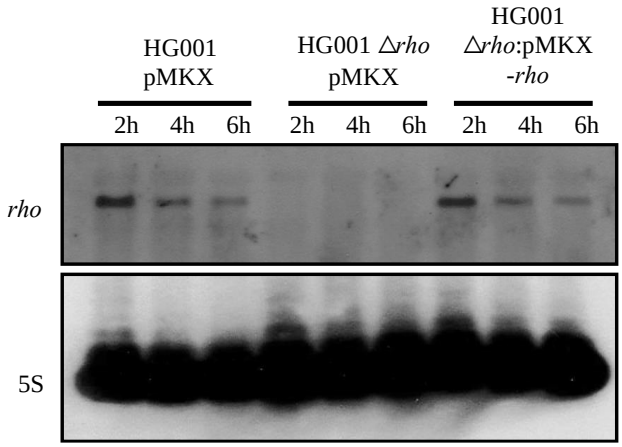

Figure S13. Leaky expression of *rho* from complementation plasmid results in the same RNA levels as in the WT strain during growth.

Northern blot analysis of *rho* in in HG001 pMKX, HG001 ⊿*rho* pMKX and HG001 ⊿*rho* pMKX-*rho* strains. Total RNA was extracted at 2, 4 and 6 h of growth in BHI medium containing 10 mg/uL chloramphenicol at 37ºC without xylose induction and loaded on a 1% agarose gel. DIG-labelled RNA probes against *rho* and the loading control 5S rRNA (5S) were used. Expression of *rho* follows the same pattern of regulation in WT and in the complemented strain without xylose induction.

Strains	Culture 1	Culture 2	Culture 3	Culture 4
HG001 pMKX	32	16	16	16
HG001 Drho pMKX	128	64	128	64
HG001 Drho pMKX rho	32	32	32	32

Figure S14: Quantification of coagulase activity

The coagulase tube test was performed with four supernatants from cultures of HG001 pMKX, HG001⊿*rho* pMKX and HG001⊿*rho* pMKX-*rho* strains grown during 5 h in BHI (n=4). These supernatants were diluted in serial two-fold dilution and mixed with rabbit plasma. The titer is the reciprocal of the highest dilution of the supernatant that show evidence of clotting after incubation for 18 h at 37°C.

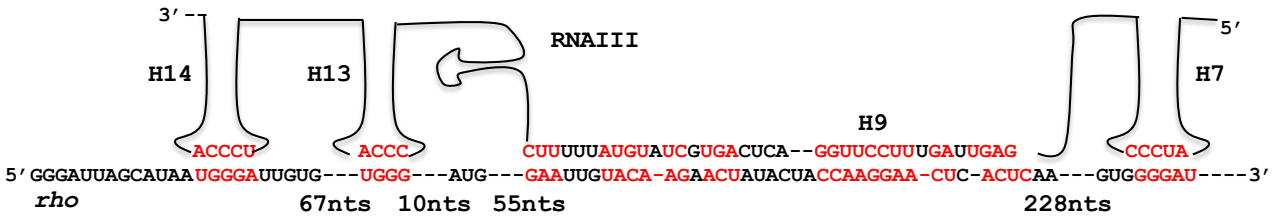

Figure S15. Proposed hypothetical interaction model of RNAIII and *rho* mRNA.
Multiple interaction sites might mediate RNAIII-*rho* base-pairing. They might include hairpins 9 and 7 of RNAIII and accessory interactions with hairpins 13 and 14. These binding sites might include the 5'UTR and the coding sequence of *rho*.

Supplementary information

Strains	Characteristics	Reference
Escherichia coli		
TOP10	F- *mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG* P3: KanR AmpR (am) TetR (am)	Invitrogen
IM08B	SA08BΩPN25-*hsdS* (CC8-1) (SAUSA300_0406) of NRS384 integrated between the *essQ* and *cspB* genes.Cloning intermediate for *S. aureus.*	88
Staphylococcus aureus		
HG001	Derivative of 8325-4, *rsb*U restored RN1, *agr* positive	89
RN4220	Restriction mutant of 8325-4 that accepts foreign DNA	90
HG001 △agr	Deletion of *agr* in HG001	38
HG001 △lytRS	Deletion of *lytRS* in HG001	38
HG001 △hptRS	Deletion of *hptRS* in HG001	38
HG001 △arlRS	Deletion of *arlRS* in HG001	38
HG001 △srrAB	Deletion of *srrAB* in HG001	38
HG001 △saeRS	Deletion of *saeRS* in HG001	38
HG001 △graRS	Deletion of *graRS* in HG001	38
HG001 △rnc	Deletion/replacement △rnc::cat in	33
HG001 △rnaIII	Deletion of rnaIII in HG001	This work
HG001 △rho	Deletion of rho in HG001	This work
HG001 *rho-3xflag*	Chromosomal insertion of 3xFLAG at the 3'end of the *rho* gene	This work
HG001 pMKX	Wild-type HG001 strain containing empty pMKX vector	This work

HG001 △*rho* pMKX	Deletion of *rho* in HG001 containing empty pMKX vector	22
HG001 △*rho* pMKX::*rho*	Deletion of *rho* in HG001 containing complementation plasmid pMKX::rho	22
RN6390	Derivative of NCTC8325-4, agr positive	91
LUG950	Deletion/replacement of rnaIII in RN6390 (nts 1570 to 1015 in the *agr* sequence)	92

Table S2 –Table of strains used in this study

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Name	Characteristics	Reference
pCN51 P3	pCN51 with P3 promoter	42
pCN51::P3-*rnaIII*	pCN51 P3 plasmid expressing RNAIII	This work
pCN51::P3-*rnaIII⊿H7*	pCN51 P3 plasmid expressing RNAIII△H7	This work
pCN51::P3-*rnaIII⊿H9*	pCN51 P3 plasmid expressing RNAIII△H9	This work
pCN51::P3-*rnaIII⊿H7⊿H9*	pCN51 P3 plasmid expressing RNAIII△H7△H9	This work
pCN51::P3-MS2	pCN51 expressing MS2 tag under P3 promoter	42
pCN51::P3-MS2-RNAIII	pCN51 expressing MS2-RNAIII under P3 promoter	This work
pMKX	Derivative of pMK4 vector, xylose-inducible promoter of the Staphylococcus xylosus xylA gene	22
pMKX::*rho*	Rho complementation plasmid.	22
pMAD	Thermosensitive origin of replication, constitutively expressed bgaB gene	77
pMAD::*rho-3xflag*	pMAD containing flanking sequences of the 3'end region of the rho gene wit 3xFLAG sequence.	This work
pMAD::*rnaIII*	pMAD containing flanking sequences of rnaIII to delete rnaIII	This work
pCN57 GFP	*E. coli-S.aureus* shuttle vector to express genes under the control of the PblaZ promoter and for transcriptional fusions with gfp reporter gene. Low copy number. AmpR-ErmR	93
pCN57 GFP::*chp*	pCN57 plasmid expressing the 5'end and downstream intergenic region of the chp gene.	This work

pCN57 GFP::*teg74*	pCN57 plasmid expressing teg74	This work

Table S3 –Table of plasmids used in this study.

	Plasmid contructions
	Construction of pCN51::*rnaIII*
P3-SphI	TTTGCATGCATACGTGGCAAACT
Rev RNA III-BamHI	CGCGGATCCAAGGCCGCGAGCTTGGGAG
	Construction of pCN51::*rnaIII⊿H7* QC
QC-dH7 RNAIII rev	CTTCGTATAGTACTAAAAAAAATTAGCAAGTGAGTAAC
QC-dH7 RNAIII for	GTTACTCACTTGCTAATTTTTTTTAGTACTATACGAAG
	Construction of pCN51::*rnaIII⊿H9* QC
QC-dH9 RNAIII rev	GAGTAACATTTGCTAGTAAGTGCTATGTATTTTTCT
QC-dH9 RNAIII for	AGAAAAATACATAGCACTTACTAGCAAATGTTACTC
	Construction of pCN51::MS2-RNAIII
Rev RNA III BamHI	CGCGGATCCAAGGCCGCGAGCTTGGGAG
For MS2-RNA III (PstI)	CGCCTGCAGCGTACACCATCAGGGTACGTTTTTCAGACACCATCAGGGTCTGTTTAGATCACAGAGATGTGATGG
	Construction of pMAD::*rho-3xflag*
E-rho 3xFLAG 3' For (BamHI)	CGCGGATCCGAACAGGTAACATGGAG
F-rho 3xFLAG 3' Rev (NcoI)	CGCCCATGG**TTA**TTTATCATCATCATCTTTATAATCTTTATCATCATCATCTTTATAATCTTTATCATCATCATCTTTATAATCAATTATAGGTCGACCCG
G-rho 3xFLAG 3' For (NcoI)	CGCCCATGGTAAACATTATATAGGGGCTTG
H-rho 3xFLAG 3' Rev (BglII)	CGCAGATCTCGTCCAGTGTAGAATG
	Construction of pMAD::rnaIII
UP for RNA III pMAD (BamHI)	CGCGGATCCGGATTCGATGGTAACACAG

UP rev RNA III pMAD	GTAATGAAGAAGGGATGAGTTAATC
DOWN for RNA III pMAD	GATTAACTCATCCCTTCTTCATTACAGTTATATTAAAACATGCTAAAAGC
DOWN rev RNA III pMAD (EcoRI)	CGCGAATTCGTGCACCATGTGCATGTC
	Construction of pMAD::rho
Rho pMAD For (BamHI)	CGCGGATCCGTTCTTAGAAGTGAAATCTATAG
rho deletion rev	ATAGTTTACACCCATTTCATTA
rho deletion for	TAATGAAATGGGTGTAAACTATTAAACATTATATAGGGGCTTG
H-rho 3xFLAG 3' Rev (BglII)	CGCAGATCTCGTCCAGTGTAGAATG
	Construction of pCN57 GFP::*chp*
For EcoRI chp GFP fusion	CGCGAATTCCTAATGAATATGCATACTAATAG
Rev BamHI chp GFP fusion	CGCGGATCCCCAGTGCCAGCAGGTTATAC
	Construction of pCN57 GFP::*teg74*
Rev BamHI Teg74 GFPfusion	CGCGGATCCAAATTGACTCAAATGATCAC
For EcoRI Teg74 GFP fusion	CGCGAATTCCAGAAAACATATAGTATCATTT
	Construction of pCN57 GFP::sbi
Rev BamHI sbi GFP fusion	CGCGGATCCGCGAAGAAGCATTTAATTG
For EcoRI sbi GFP fusion	GCGGAATTCCAGTACTAGGTAGTGGTTCT
	Construction of pCN57 GFP::teg66

Rev BamHI teg66 GFP fusion	CGCGGATCCATCTAAAGTTAAGTATGGTGGC
For EcoRI teg66 GFP fusion	GCGGAATTCGTATCACTCTCCAATTACGTAAC
	Construction of pCN57 GFP::saeP
For EcoRI saeP GFP fusion	CGCGAATTCCGCAATGGTTGACTACGAT
Rev BamHI saeP GFP fusion	CGCGGATCCCCCACACGAATGATAAATGTAAC
	In vitro trancription
	RNAIII
For RNA III	AAGGCCGCGAGCTTGGG
Rev T7 RNA III	*TAATACGACTCACTATAGGG*AGATCACAGAGATGTGATGGAAAATAG
	Rho 5'
For T7 Rho EMSA	*TAATACGACTCACTATAGGG*ATTAGCATAATGGGATTGTGC
Rev Rho short EMSA	CCATAATATTTTTCGTTATCTTTAGGTTTTC
	Rho 3'
For T7 Rho 3' EMSA	TAATACGACTCACTATAGGGCCAAGTGGTCGTACATTATCAG
	DIG-probes
	Rho
rho-dig (Rev)	ATGCCTGAAAGAGAACGTAC
rho-dig-T7 (For)	*TAATACGACTCACTATAGGG*AAAACGACGAATTTGGCTAG
	RNAIII
RNAIII rev	GAAGGAGTGATTTCAATGGC
RNAIII fw T7	*TAATACGACTCACTATAGGG*GGACTAAGTGTTAAAG
	5S
5S-dig (Rev)	GTAAGTTATTTTGTCTGGTGGCTATAGC
5S-dig-T7 (For)	*TAATACGACTCACTATAGGG*GATTTGTCATTTGCCTGGC

	Verification primers
pMAD1 (For)	GGAAGCGAGAAGAATCATAATG
pMAD2 (Rev)	CTAGCTAATGTTACGTTAC
	Primer extension RNAIII
RNAIII-PE	ATACTTATTATTAAGGGAATGTTTTACAGTTA

Table S4 – Table of primers used in this study. Underlined are the restriction sites, in italic the sequence for the T7 promoter and in red the *3xflag* sequence.

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Chapter IV

General conclusion and perspectives

Selectivity of Rho targets in *S. aureus*

Our data have confirmed the role of Rho in the repression of pervasive antisense RNAs transcription as well as the SaeRS regulon in *S. aureus*. We observed numerous *sae*-dependent but also *sae*-independent virulence genes positively affected by the deletion of Rho. More interestingly, our RIP-seq data suggests that Rho binds to several of these mRNA targets and that their repression is most likely due to a combination of direct transcription termination by Rho and also indirect effects through dysregulation of transcriptional regulators. In contrast to *E. coli* and other Gram-negative bacteria in which Rho-dependent termination affects more than 30% of genes, Rho-dependent termination in *S. aureus* seems restricted to a specific set of transcripts. This could in part explain why Rho is not essential in *S. aureus* in the tested conditions. Yet preliminary experiments indicated that the growth of the *rho* deletion mutant is impaired in RPMI, a medium mimicking human serum.

Numerous questions arise concerning this apparent selectivity of Rho for virulence associated genes in this pathogen. In Gram-negative bacteria for instance, the binding sites of Rho on the nascent RNA are well described and are composed of C-rich unstructured regions named *rut* sites.[158] Such *rut* sites have not been described in *S. aureus* nor Gram-positive bacteria. If these *rut* sites should maintain the same requirements in *S. aureus*, on one hand the low GC content of the *S. aureus* genome would explain why Rho-dependent termination is less frequent in this bacterium and why this factor does not have a global effect on gene expression. However, it would not quite explain the conserved effect on terminating pervasive transcription nor the specificity for virulence genes, unless their sequences or structures have specifically evolved to match Rho-dependent termination sites. Thus, we propose that the composition of *rut* sites in *S. aureus* might differ from Gram-negative bacteria at the levels of sequence and/or structure. The poor conservation of RNA-binding domains of Rho in *S. aureus* compared to the *E. coli* Rho (Fig. 11, Chapter II) also suggests different binding mechanisms. The Rho protein in *S. aureus* contains a long arginine-rich N-terminal insertion[160] that is predicted to extend outside the ring-shape structure of the hexamer (Fig. 11). Such insertions are found in several other Rho proteins of other bacterial species and they are believed to confer specific behaviors depending on preferred ecosystems, metabolisms or genome structures.[160] For instance, the N-terminal insertion of Rho in the high GC *Micrococcus luteus* facilitates termination of structured RNA transcripts near their respective promoters and in *Mycobacterium tuberculosis* the efficient termination activity of Rho despite poor "motor"

function is attributed to this N-terminal insertion.[160,161] Hence, we cannot exclude that this N-terminal domain of Rho in *S. aureus* is involved in the specific recognition of its RNA targets. Although the exact functions of this N-terminal insertion are not fully understood, this region is essential for the proper expression and stability of the protein in *S. aureus*. The modification of the N-terminal end of the endogenous protein by introducing a FLAG-tag strongly affected its expression and transferring the tag to the C-terminal end was sufficient to enhance protein expression. It would be deeply interesting to investigate the complementarity of Rho between Gram-positive and Gram-negative bacteria.

Thus, to understand the role of this N-terminal region in substrate recognition and understand how this protein might have evolved to specialize to regulate virulence factors in this *S. aureus*, it seems necessary to identify particular sequences or structures that might be predominant in Rho-dependent targets. Recent Helicase-SELEX (H-SELEX, Systematic Evolution of Ligands by Exponential enrichment) has been developed and applied to Rho from *E. coli* to discover functional substrates of the protein and to screen and generate a genome-wide map of putative *rut* sites.[89] This proof-of-concept study successfully identified previously described, as well as new Rho binding sites. A classical SELEX technique allows to isolate nucleic acids that specifically bind to a target molecule from a large library of sequences. The target molecule is usually immobilized on a surface, incubated with a library of nucleic acid sequences and nonbinding sequences are eliminated whereas bound molecules are eluted and amplified to be ultimately converted into a new pool of sequences used in a further round of selection.[203] Several rounds of selection and amplification are applied to enrich the final pool with sequences binding with high affinity to the target molecule. H-SELEX enables the identification of functional helicase substrates by combining the classical SELEX approach with a selection based on helicase activity. In this approach, a library of RNA:DNA duplexes containing 5'-tailed variable sequences is generated and preferred sequences are isolated based on the strand separation ability of helicases.[89] These duplexes are trapped on streptavidin beads, so that nucleic acid sequences interacting with the helicase of interest will be unwound and released in the supernatant, which will then be isolated, amplified and used in further rounds of selection. In this prototype study, a library of RNA:DNA duplexes harboring 5'-tailed sequences from the *E. coli* genome was used, so that each RNA:DNA duplex contained a different 5'end overhanging RNA extremity. After 10 rounds of selection hundreds of *rut* peaks (H-SELEX enriched peaks) were identified that mapped to both strands of the genome and were especially found in prophages and CRISPR loci.[89] *Rut* peaks were found at expected locations for already described Rho-dependent terminators, with 81% of experimentally

validated candidates being detected, such as the 5'UTR of the *rho* gene or the *trp* operon.[89] The peaks that were not identified mapped to 5'UTRs corresponding to attenuator signals controlled by riboswitches which require specific folding, thus in the applied experimental conditions they might not have folded adequately to properly interact with Rho and might therefore not have been detected.[89] Interestingly, the inclusion of Rho cofactors such as NusG was found to influence the nature of enriched sequences, providing a powerful tool to assess the effect of other cellular factors on Rho selectivity and activity (stimulation or inhibition). For instance, addition of NusG to the experimental setup increased the number of detected *rut* peaks by 20%, consistent with the stimulation of Rho by NusG at weak rut sites.[89] Rut peaks enriched specifically in presence of NusG were usually shorter and smaller than NusG-independent peaks, which explains the requirement for Rho stimulation to terminate these transcripts.[89] Considering the preference of virulence-associated transcripts and the lack of data regarding preferred Rho substrates in *S. aureus*, it would be of tremendous interest to apply this H-SELEX method to Rho_{Sa} to identify possible consensus sequences in this pathogen that might explain its preference for these genes. The genome-wide mapping of Rho-dependent sites might also reveal if the involvement of Rho in 5'UTR regions of genes is conserved in *S. aureus*. However, to apply this method to $Rho_{Sa,}$ non-negligible amounts of purified protein are required, which constitutes a current limitation to our study. Another concern might be the absence of real strong consensus sequence that would explain the preference of Rho for the corresponding sequences, as has been observed in *E. coli*.[89] Indeed, Rho-dependent sites are generally U-rich and G-poor, contain higher YC dinucleotides and are less prone to form secondary structures but no clear consensus motif has been associated to *rut* sites.[89] In addition, termination sites that require the action of NusG might deviate from these rules, rendering the characterization of a consensus Rho site even more complicated.[89] H-SELEX has been applied to Rho from *B. subtilis* and similar observations were made regarding the lack of consensus motifs recognized by Rho_{Bs} (M. Boudvillain, personal communication). Hence, even if sufficient amounts of Rho_{Sa} were purified to perform H-SELEX experiments, we might as well not be able to characterize specific sequences to which our protein binds. Nevertheless, combining this approach to the already available data that we have accumulated about the selectivity for virulence factors of Rho in *S. aureus*, is a crucial step to understanding and characterizing the function of the enzyme in this clinically relevant bacterium.

Evolution of Rho in Bacillota

The importance of Rho in the control of pervasive transcription has been universally conserved, suggesting that Rho-dependent termination is an efficient mechanism to limit potentially harmful antisense transcription. These pervasive antisense transcripts (that might escape Rho-dependent control) can form base-pair interactions with their cognate sense strand and these duplexes have previously been shown to be degraded by RNase III.[77,78] Strikingly, the long antisense transcripts generated in absence of Rho do not seem to be degraded, which questions the role of RNase III in preventing the accumulation of these molecules, as if Rho could be required for RNase III-dependent cleavage. However, no interaction between these two proteins has been detected yet (data not shown). The observed effects on the readthrough transcripts might just be a question of a balance between relative amounts of antisense and sense RNAs. In our conditions by comparing transcriptomes of Δ*rho* and Δ*rnc*, RNase III does not seem to influence Rho-dependent antisense RNA levels, suggesting that both enzymes might act independently from one another to regulate pervasive transcription (data not shown).

Due to the post-transcriptional regulation of *rho* during stationary phase, we expect a decrease in Rho-dependent termination at this stage and an increase of pervasive antisense transcription at this stage. Since deletion of Rho and consequent accumulation of pervasive transcripts do not impair *S. aureus* viability, we might speculate that allowing pervasive transcription to increase during stationary phase might confer bacteria supplementary adaptability to circumvent different stresses encountered. Thus, pervasive transcription usually considered as harmful might in the contrary provide evolutionary advantages and the acquisition of new functions during this challenging growth phase or when stress must be endured. In this manner, pervasive transcription might be a motor for evolution and adaptation to new environments. This being said, in *S. aureus* Rho could become non-essential in specific environments during which the bacteria might rely on pervasive transcription to survive.

The seemingly specialized role of Rho in virulence in *S. aureus* raises questions about the roles of Rho in other pathogenic staphylococci such as *S. epidermidis*, *S. simiae* or *S. argenteus*. Is Rho also involved in virulence repression in these species? Is this virulence-centered function of Rho conserved? Could *rho* contribute to the switch from commensalism to pathogenic lifestyles? More interestingly, what about non-pathogenic staphylococci that do not encode *agr* such as *S. warnerii*? How is *rho* expressed and regulated and what are its main functions in such bacteria (restricted to pervasive transcription)? Is *rho* essential in these

species? Naturally, it is tempting to speculate that in *S. aureus*, Rho might be a key component in adjustment to different niches. Indeed, in *B. subtilis* the involvement of Rho in the regulation of sporulation and the tight spatiotemporal regulation of *rho* expression during this process points to a crucial role of Rho for bacterial adaptation, which might be conserved in *S. aureus*.[202]

Rho might affect biofilm formation

Bacterial biofilm consists of an assembly of bacterial cells contains within a polymeric matrix that provides a protective physical barrier against environmental conditions and particularly against the host immune response.[204] *S. aureus* is able to produce two types of biofilms classified as *ica*-dependent or *ica*-independent. *Ica*-independent biofilm is often composed of extracellular DNA (eDNA) and involves the action of surface proteins such as fibronectin binding proteins A and B FnbA/B[205], surface and adhesion protein SasG[206], staphylococcal protein A[207], clumping factor B ClfB[208] and also eDNA[209]. Ica-dependent biofilm is composed of polysaccharide intercellular adhesion (PIA) (also known as poly-N-acetylglucosamine; PNAG), whose synthesis is encoded by the *icaADBC* operon, in turn regulated by the *icaR* repressor.[204]

eDNA acts as an electrostatic polymer and maintains cells together due to its negative charge and its main source is cell lysis.[209] *S. aureus* is capable of programmed cell death and lysis, controlled by the *lrg* and *cid* operons which have opposing effects on murein hydrolase activity and antibiotic tolerance.[210,211] The products encoded by the *cidA* and *lrgA* genes encode two transmembrane proteins that function as a holin and anti-holin respectively.[209] *cidA* promotes cell lysis and eDNA release allowing biofilm formation.[212] CidA oligomerizes and forms pores in the cytoplasmic membrane, activating murine protein hydrolase, cell lysis and consequent eDNA release and biofilm development.[204] The anti-holin encoded by *lrgAB* operon counteracts the activity of CidA therefore inhibiting all the processes mentioned above.[204]

Our RNAseq data shows the upregulation of *cidA* and *cidB* (log2FC= 1.866 and log2FC= 1 respectively) and the downregulation of *lrgA* and *lrgB* (log2FC= -1.883 and log2FC= -3.137 respectively) in the *rho* mutant. This is in accordance with preliminary results obtained that showed an increased biofilm production in the *rho* mutant when performing crystal violet staining (results not shown). These findings are not surprising considering that biofilm development is a virulence mechanism deployed to avoid bacterial killing and enhance

antibiotic resistance. However, the effect of Rho on these targets is most probably indirect, since none of them were enriched upon RIPseq. Different pathways activate *cidAB* and *lrgAB* expression, they include the LytRS two component regulatory system and the CidR regulator.[213] These regulators are slightly affected in the *rho* mutant, with almost two-fold decrease in expression for *cidR* (log2FC= -0.901) and for *lytS* (log2FC= -0.789). An impact on the LytRS system might explain the effect observed on the *lrgAB* operon but the increase in *cidA* is probably accounted to other regulatory pathways. Since none of these regulators were enriched upon RIP, the effects of Rho are most probably indirect affecting upstream regulatory molecules. Moreover, the upregulation of several adhesion factors in absence of Rho most probably contributes to the observed phenotype, such as Emp which is needed for late infection process and is negatively regulated by Rho.

The *icaADBC* operon expression was not affected by *rho* deletion, suggesting that the increased biofilm formation is solely due to *ica*-independent factors.

Rho might regulate an antisense to the type VII secretion system (T7SS)

The type VII secretion system of *S. aureus* allows the export of extracellular proteins across the membrane, in particular exotoxins, and has been associated with virulence in this pathogen.[214] It is encoded by the highly conserved but variable *ess* locus.[214] T7SS usually consists of four integral membrane proteins composing the core machinery (EsaA, EssA, EssB and EssC), two cytosolic proteins (EsaB and EsaG), five secreted substrates (EsxA, EsxB, EsxC, EsxD and EsaD) and EsaE necessary to target the substrates to the secretion machinery.[215] Our RNA-seq data has shown the production of a long antisense transcript to the entire *ess* operon in the *rho* mutant. This affected the levels of *esxA* and *esaG1* whose expression decreased more than two-fold in the mutant (Table S1). The other T7SS genes were not affected and this is probably due to the very low basal levels of expression of the *ess* locus in the WT strain in our growth conditions. Furthermore, several of these antisense transcripts to the T7SS genes were enriched upon RIP, suggesting direct involvement/action of Rho in the regulation of the entire genomic locus.

T7SS is key for *S. aureus* virulence since strains lacking the entire system or specific elements are less able to form abscesses in mouse infection models and exhibit impaired persistence, although the underlying mechanisms are not known to date.[216] A recent role of the toxin-antitoxin pair EsaD and EsaG in intraspecies competition has been demonstrated.[214,215] EsaD is a nuclease substrate that is coproduced with EsaG, whose role is to protect the producer

strain from nuclease activity prior to secretion.[214] Although some *S. aureus* strains do not encode EsaD, they all encode at least one copy of EsaG.[214] These orphan EsaG homologs protect their cognate strain from nuclease attack by strains secreting the EsaD toxin.[214] This intraspecies competition is highly relevant since *S. aureus* is a frequent colonizer of nasal cavities and is also able to colonize and persist in the lungs of cystic fibrosis patients, which are all highly competitive environments.[214] Thus, the potential regulation of an antisense to the *ess* locus by Rho could impact the abilities of *S. aureus* to colonize and persist in these different niches. Thus, the action of Rho, especially to avoid expression of antisense transcription of these genes, might be fundamental to ensure proper expression of the system and might confer adaptability/competition advantages to the strains properly expressing this termination factor. Thus, we might consider comparing the expression of *rho* in different niches occupied by *S. aureus*. Regulation of the *rho* gene and thus, controlling repression of virulence genes and specific antisense transcripts by Rho might be a regulatory switch to ensure bacterial adaptation and colonization and might be crucial for initial steps of infection.

The outcomes of my thesis project have raised more questions and opened the door to further research about the role of Rho in *S. aureus* and other staphylococci. These exciting discoveries show how a universally conserved transcription factor has evolved to regulate specific pathways in bacteria that have to face highly variable and sometimes hostile environments. More research is needed to understand how and why Rho-dependent termination has been selected for these processes and whether modulation of pervasive transcription is linked to this high adaptability.

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Chapter V

Annexes

ANNEX I. Identification of protein partners of Rho in *S. aureus*

Numerous proteins, including components of RNAP, have been identified as binding partners of Rho in *E. coli* and they mostly act as modulators of Rho activity. As mentioned before, NusG binds Rho near the interface between both subunits and stimulates Rho-dependent termination.[1,2] Other Rho partners have been identified and antagonize with its activity, such as YaeO that binds near the PBS and competes for RNA binding[3], the phage-derived Rho inhibitor Psu that binds onto the SBS and inhibits RNA binding and translocation[4], the chaperone Hfq that prevents ATPase activity of Rho [5,6] or NusA that has contradictory roles and can stimulate and inhibit Rho termination.[6] In *B. subtilis*, Rho is also believed to interact with NusG to stimulate its termination activity as described for *E. coli*.[7,8]

Rho has also been found to cooperate with RNases to conjointly regulate protein-coding genes. Rho and RNaseE/G from the Gram-positive bacterium *Corynebacterium glutamicum* cooperate to modulate the expression of the FMN riboswitch controling the expression of the downstream *ribM* gene.[9] In *B. subtilis*, Rho-dependent termination of the *slrA* mRNA is required for PNPase-mediated decay, again pointing to an existing synergy between Rho and RNase action.[10] However, a physical interaction in the mentioned organisms of Rho with these RNases has not been examined yet. Interestingly, Rho has been identified as a component of the RNaseE-based degradosome in some Gram-negative bacteria such as *Rhodobacter capsulatus, Caulobacter crescentus* and in *E. coli* under specific conditions.[11] This suggests that Rho could cooperate with RNases to degrade specific RNA targets. This is not surprising considering that both Rho and RNases are involved in the suppression of pervasive transcription. Thus, it is possible that Rho-dependent termination of pervasive transcripts could be followed by RNase-mediated degradation.

Thus, to gain more insight into the specificities and mechanisms of Rho-mediated gene regulation in *S. aureus*, we aimed to identify the RNA and protein-binding partners of Rho. For this matter, an *S. aureus* strain expressing a chromosomal 3xFLAG-tagged Rho protein has been constructed, as described previously in the Chapter 3, which described the identification and analysis of the RNA molecules that co-precipitated with Rho. Thus, here we will focus on the identification of the staphylococcal protein partners of Rho *in vivo*.

Experimental procedures

Bacterial growth and lysis

Duplicates of 100 mL cultures of HG001 WT and HG001 *rho-3xflag* strains were grown until exponential phase (OD=2) when *rho* transcript is mostly expressed and centrifuged at 3'700 rpm at 4ºC for 15 minutes. Two lysis buffers differing in the type of detergent were used for each culture. 50 mL of each culture was resuspended and lysed with 1 mL of Triton X-100 1% lysis buffer (Triton X-100 1%, NaCl 50 mM, Tris 50 mM pH=8, $MgCl_2$ 2 mM and protease inhibitors) or with 1 mL of Igepal 0,1% buffer (Igepal 0,1%, NaCl 50 mM, Tris 50 mM pH=8, $MgCl_2$ 2 mM and protease inhibitors). Resuspended cells were transferred into 2 mL tubes containing silica beads and mechanically lysed with the FastPrep (MP Biomedicals) with 2 cycles of 40 s at 4 m/s. Tubes were then centrifuged for 20 min at 20'000 g at 4ºC, the supernatant was transferred into a new tube and total proteins were quantified by Bradford technique (Biorad). The washing buffers used were Wash Igepal 0,1% (Igepal 0,1%, NaCl 50 mM, Tris 50 mM pH=8, $MgCl_2$ 2 mM and protease inhibitors) and Wash Triton X-100 0,1% (Triton X-100 0,1%, NaCl 50 mM, Tris 50 mM pH=8, $MgCl_2$ 2 mM and protease inhibitors).

Co-immunoprecipitation

Co-IP of Rho-3xFLAG with bound proteins was performed using the uMACS technology and kit using antiflag beads from Miltenyi. Protein isolation was performed according to the manufacturer's protocol. Briefly, 200 µL of bacterial lysate was incubated with 50 µL µMAC Anti Flag-tag MicroBeads for 1 h at 4ºC. The labeled cell lysate was applied to a MACS column placed in the magnetic field of the uMACS separator previously conditioned with washing buffer. The column is then rinsed four times with the respective washing buffer and proteins were eluted with 35 µL of hot 95ºC-pre-heated Elution buffer. Elution fractions were then subjected to nanoLC-MS/MS by the Strasbourg-Esplanade Proteomics Facility.

Bacterial Two-hybrid (BACT2H)

To assess primary protein-protein interactions *in vivo*, the *Bordetella pertussis* adenyl cyclase-based bacterial two hybrid system (BACT) was applied. In short, the T18 and T25 fragments of the adenyl cyclase protein were fused to the N- or C-terminus of the full-length Rho and candidate proteins. DNA fragments used for cloning were amplified using *S. aureus*

HG001 chromosome as DNA template. Plasmids containing both adenyl cyclase domains fused to the proteins of interest were transformed into *E. coli*. Bacteria containing the appropriate pairs of plasmids were then tested for beta-galactosidase activity on X-gal containing plates.

Results

Co-IP

Two different lysis buffers containing different detergents (Igepal or Triton X-100) were used in order to aid bacterial lysis while maintaining the integrity of the Rho-associated complexes. Both non-ionic detergents are relatively mild, non-denaturing and are used to solubilize membrane proteins and isolating cytoplasmic proteins. However, Triton X-100 is considered slightly stronger and more hydrophilic than Igepal. Since we did not possess any information about the behavior of Rho-dependent protein complexes in *S. aureus*, we decided to compare the use of both detergents. Samples of the crude extract and elution fractions of each Co-IP were loaded onto a 10% SDS-PAGE gel to perform an anti-FLAG *Western blot* to detect the Rho-3xFLAG protein. The tagged Rho-3xFLAG protein was detected only in the HG001 *rho-3xflag* strain as expected and was successfully enriched after IP (Fig.1)

Mass spectrometry analysis of the Co-IP elutions identified a high number of proteins pulled down for both strains. The most significantly enriched proteins in both lysis conditions are shown in Table 1. Only slight differences in the Rho/WT ratios of eluted proteins were observed between the two lysis conditions. Rho was successfully enriched in the Rho-3xFLAG with a logFC of 4.18 (Fig. 1). However, Rho was still identified in the IP of the WT strain, suggesting non-specific interactions of this protein with the uMACS column. Interestingly, we observed numerous transcription factors enriched in the Rho-3xFLAG IP (Table 1), especially proteins involved in virulence regulation. Among the most significantly pulled-down proteins we found the global activators of virulence SarA (and also SarS/SarR), the major repressor of biofilm MgrA and the repressor of toxins Rot, described previously.

		Rho/WT		Rho/WT Triton		Rho/WT Igepal	
Accession	**Description**	**LogFC**	**adjp**	**LogFC**	**adjp**	**LogFC**	**adjp**
HG001_00749	HG001_00749| conserved hypothetical protein	6.55	7.43E-27	8.05	6.43E-19	5.67	9.49E-14
HG001_02667	HG001_02667|gene_marR|Transcriptional regulator MarR	6.02	9.28E-10	5.27	1.62E-03	6.57	1.24E-07
HG001_01028	HG001_01028|gene_mraZ|Transcriptional repressor MraZ	5.76	2.19E-08	5.96	3.83E-05	5.39	8.33E-04
HG001_00207	HG001_00207|gene_gntR|putative HTH-type transcriptional regulator GntR	5.26	3.08E-06	4.31	3.41E-02	5.90	4.59E-05
HG001_01684	HG001_01684|gene_rot|HTH-type transcriptional regulator rot	5.26	2.91E-06	4.62	1.54E-02	5.75	1.18E-04
HG001_00352	HG001_00352|gene_NONE|Type I restriction modification DNA specificity domain protein	4.84	2.44E-18	5.25	7.40E-11	4.44	3.34E-09
HG001_00029	HG001_00029|gene_NONE|hypothetical protein	4.79	8.70E-05	5.09	3.34E-03	4.22	4.02E-02
HG001_01608	HG001_01608|gene_mutM|Formamidopyrimidine-DNA glycosylase	4.68	8.48E-16	3.54	2.51E-06	7.37	1.18E-12
HG001_01415	HG001_01415|gene_NONE|hypothetical protein	4.52	9.87E-08	3.80	6.15E-04	5.75	1.18E-04
HG001_01664	HG001_01664|gene_NONE|hypothetical protein	4.51	1.30E-15	4.67	1.07E-08	4.31	1.44E-10
HG001_01210	HG001_01210|gene_NONE|1,4-dihydroxy-2-naphthoyl-CoA hydrolase	4.45	6.68E-09	5.27	1.62E-03	4.44	2.77E-09
HG001_01422	HG001_01422|gene_xerD_3|Tyrosine recombinase XerD	4.27	1.10E-08	6.57	1.93E-07	3.07	2.94E-03
HG001_02490	HG001_02490|gene_NONE|hypothetical protein	4.19	2.52E-10	6.89	6.73E-09	3.04	4.48E-04
HG001_02148	HG001_02148|gene_rho|Rho	4.18	1.61E-114	3.88	8.31E-85	4.45	4.71E-169
HG001_01063	HG001_01063|gene_priA|Primosomal protein N'	4.10	1.01E-17	3.82	2.55E-08	4.31	2.84E-11
HG001_01735	HG001_01735|gene_NONE|EcoKI restriction-modification system protein HsdS	4.06	4.35E-19	4.67	2.15E-11	3.58	1.25E-09
HG001_00637	HG001_00637|gene_glcR|HTH-type transcriptional repressor GlcR	3.83	1.26E-06	5.08	3.92E-03	3.65	4.98E-05
HG001_01972	HG001_01972|gene_NONE|65 kDa membrane protein precursor	3.78	2.40E-12	3.50	2.42E-19	4.13	1.55E-12
HG001_00834	HG001_00834|gene_oppD_1|Oligopeptide transport ATP-binding protein OppD	3.66	2.62E-04	4.87	7.23E-03	3.16	2.04E-02
HG001_00625	HG001_00625|gene_mgrA|HTH-type transcriptional regulator MgrA	3.64	1.85E-58	4.24	5.24E-38	3.20	1.37E-39
HG001_00556	HG001_00556|gene_sarA|Transcriptional regulator SarA	3.59	2.10E-44	3.97	3.02E-36	3.22	3.51E-32
HG001_01103	HG001_01103|gene_xerC_1|Tyrosine recombinase XerC	3.54	1.87E-05	5.08	3.48E-03	3.21	1.29E-03
HG001_01214	HG001_01214|gene_parC|DNA topoisomerase 4 subunit A	3.49	1.10E-77	3.16	6.19E-31	3.75	2.77E-46
HG001_01917	HG001_01917|gene_NONE|hypothetical protein	3.44	2.36E-10	3.78	6.40E-06	3.17	1.34E-05
HG001_02527	HG001_02527|gene_srmB|ATP-dependent RNA helicase SrmB	3.39	1.40E-46	3.36	1.12E-22	3.37	6.65E-28
HG001_01780	HG001_01780|gene_NONE|Putative transcription factor, Xre family	3.23	3.05E-47	3.20	6.56E-32	3.19	1.55E-23
HG001_00006	HG001_00006|gene_gyrA|DNA gyrase subunit A	3.20	8.40E-50	3.26	1.53E-25	3.07	1.27E-61
HG001_00674	HG001_00674|gene_yclQ|putative ABC transporter solute-binding protein YclQ precursor	3.12	4.56E-07	6.49	1.31E-06	2.21	3.58E-03
HG001_01481	HG001_01481|gene_nfo|putative endonuclease 4	3.07	2.44E-15	2.41	6.90E-06	3.61	8.59E-18
HG001_01111	HG001_01111|gene_uppS|Isoprenyl transferase	3.00	5.05E-18	2.78	4.45E-08	3.15	4.18E-12
HG001_01407	HG001_01407|gene_lexA_2|LexA repressor	2.99	4.13E-08	2.99	4.74E-05	2.85	3.09E-04
HG001_00730	HG001_00730|gene_ssp|Extracellular matrix protein-binding protein emp precursor	2.88	6.63E-29	2.65	6.79E-42	3.07	8.22E-52
HG001_02348	HG001_02348|gene_NONE|DNA-binding transcriptional activator FucR	2.85	1.43E-08	2.63	3.03E-04	3.00	1.03E-05
HG001_01625	HG001_01625|gene_NONE|Cobalt-dependent inorganic pyrophosphatase	2.81	1.89E-15	2.78	3.95E-07	2.84	1.45E-11
HG001_01905	HG001_01905|gene_immR_1|HTH-type transcriptional regulator ImmR	2.81	4.87E-10	2.11	1.66E-03	3.45	1.37E-08
HG001_02330	HG001_02330|gene_sarR|HTH-type transcriptional regulator SarR	2.80	1.08E-26	2.49	2.78E-12	3.04	1.28E-17
HG001_01609	HG001_01609|gene_polA_2|DNA polymerase I	2.71	1.19E-39	2.44	2.19E-24	2.94	1.54E-35
HG001_00956	HG001_00956|gene_NONE|hypothetical protein	2.71	2.92E-17	2.73	1.53E-10	2.57	2.12E-09
HG001_01621	HG001_01621|gene_accD|Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	2.68	1.34E-17	2.63	4.32E-10	2.66	8.06E-11
HG001_01775	HG001_01775|gene_cbF1|3'-5' exoribonuclease CbF1	2.60	3.50E-05	3.84	2.89E-06	2.25	8.56E-04
HG001_01938	HG001_01938|gene_ligA|DNA ligase	2.52	3.19E-11	2.30	1.05E-04	2.68	2.97E-09
HG001_01604	HG001_01604|gene_dnaB|Replication initiation and membrane attachment protein	2.51	7.16E-04	2.21	2.99E-02	2.76	1.40E-02
HG001_00327	HG001_00327|gene_NONE|hypothetical protein	2.50	6.41E-16	2.22	1.32E-07	2.74	9.51E-19
HG001_00697	HG001_00697|gene_uvrA|UvrABC system protein A	2.46	2.63E-78	2.25	4.50E-18	2.61	4.79E-82
HG001_00813	HG001_00813|gene_addB|ATP-dependent helicase/deoxyribonuclease subunit B	2.45	6.15E-05	2.06	2.38E-02	2.76	7.29E-04
HG001_01333	HG001_01333|gene_hup|DNA-binding protein HU	2.44	6.92E-43	2.40	3.65E-76	2.42	3.48E-20
HG001_01911	HG001_01911|gene_queE_2|7-carboxy-7-deazaguanine synthase	2.44	6.71E-04	2.60	1.75E-02	2.24	3.64E-02
HG001_00145	HG001_00145|gene_hsdR|Type-1 restriction enzyme R protein	2.44	1.50E-20	2.32	2.48E-09	2.50	1.17E-12
HG001_02126	HG001_02126|gene_fabZ|3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ	2.41	2.88E-11	2.28	6.77E-06	2.46	7.00E-07
HG001_01445	HG001_01445|gene_argR_1|Arginine repressor	2.41	1.22E-08	2.17	1.20E-03	2.58	3.78E-07
HG001_00351	HG001_00351|gene_hsdM|Type I restriction enzyme EcoKI M protein	2.40	1.36E-18	2.34	5.51E-15	2.37	3.27E-13
HG001_01736	HG001_01736|gene_NONE|putative type I restriction enzymeP M protein	2.37	4.76E-18	2.32	2.17E-15	2.33	5.78E-13
HG001_00061	HG001_00061|gene_sarS|HTH-type transcriptional regulator SarS	2.34	2.63E-04	2.44	9.33E-03	2.18	2.05E-02

HG001_01213	HG001_01213|gene_parE|DNA topoisomerase 4 subunit B	2.29	6.97E-33	2.15	5.55E-15	2.37	3.32E-43
HG001_00410	HG001_00410|gene_rsmA|Ribosomal RNA small subunit methyltransferase A	2.25	7.96E-07	2.67	7.00E-06	1.71	7.70E-03
HG001_00814	HG001_00814|gene_addA|ATP-dependent helicase/nuclease subunit A	2.25	4.01E-08	2.63	8.41E-07	1.72	1.30E-03
HG001_02749	HG001_02749|gene_noc|Nucleoid occlusion protein	2.14	2.34E-09	2.32	3.69E-05	2.01	1.69E-05
HG001_00028	HG001_00028|gene_rlmH|Ribosomal RNA large subunit methyltransferase H	2.11	1.28E-04	2.30	2.59E-03	1.77	4.02E-02
HG001_01408	HG001_01408|gene_dnaQ|DNA polymerase III subunit epsilon	2.00	3.64E-05	1.74	7.57E-04	2.29	3.46E-04
HG001_01080	HG001_01080|gene_plsX|Phosphate acyltransferase	1.96	4.90E-09	1.75	1.68E-04	2.10	5.08E-09
HG001_01149	HG001_01149|gene_NONE|Glycerol-3-phosphate responsive antiterminator	1.86	1.46E-04	1.89	1.48E-02	1.84	4.37E-03
HG001_01128	HG001_01128|gene_ftsK|DNA translocase FtsK	1.83	6.15E-06	0.92	4.05E-02	2.41	2.11E-17
HG001_01509	HG001_01509|gene_lepA|Elongation factor 4	1.82	5.47E-13	1.40	4.90E-05	2.18	2.24E-13
HG001_00413	HG001_00413|gene_purR|Pur operon repressor	1.80	3.01E-11	1.91	1.00E-10	1.57	8.28E-07
HG001_02117	HG001_02117|gene_yidC|Membrane protein insertase YidC precursor	1.80	8.70E-06	1.78	1.97E-03	1.74	2.92E-04
HG001_01939	HG001_01939|gene_pcrA|ATP-dependent DNA helicase PcrA	1.78	8.71E-18	1.48	1.80E-06	2.03	7.87E-14
HG001_00621	HG001_00621|gene_NONE|hypothetical protein	1.77	2.45E-08	1.62	1.06E-05	1.87	1.66E-08
HG001_02456	HG001_02456|gene_sbi|Immunoglobulin-binding protein sbi precursor	1.72	3.21E-07	1.43	1.22E-10	2.10	1.13E-53
HG001_01564	HG001_01564|gene_tgt|Queuine tRNA-ribosyltransferase	1.71	1.10E-05	1.20	4.93E-02	2.13	4.06E-05
HG001_02351	HG001_02351|gene_RpiR|Putative transcription factor, RpiR family	1.70	3.62E-06	1.46	3.51E-03	1.88	2.63E-04
HG001_00579	HG001_00579|gene_tagX|Putative glycosyltransferase TagX	1.68	2.26E-03	1.28	3.77E-02	2.26	1.15E-03
HG001_02345	HG001_02345|gene_lytR_2|Transcriptional regulator LytR	1.65	2.88E-03	1.47	1.21E-02	1.68	4.02E-02
HG001_02062	HG001_02062|gene_agrA|Accessory gene regulator protein A	1.64	2.00E-14	1.74	1.55E-09	1.47	4.13E-08
HG001_01485	HG001_01485|gene_sigA|RNA polymerase sigma factor SigA	1.63	1.95E-06	1.29	5.52E-03	1.93	5.43E-05
HG001_01620	HG001_01620|gene_accA|Acetyl-coenzyme A carboxylase carboxyl transferase subunit alph	1.62	8.02E-05	1.43	7.57E-04	1.75	1.60E-03
HG001_00455	HG001_00455|gene_NONE|DNA repair protein	1.57	1.40E-07	1.14	1.72E-04	2.00	6.85E-07
HG001_01418	HG001_01418|gene_rluB|Ribosomal large subunit pseudouridine synthase B	1.54	4.19E-08	1.20	1.94E-03	1.83	7.64E-07
HG001_01440	HG001_01440|gene_pdhC_2|Dihydrolipoyllysine-residue acetyltransferase component of py	1.52	8.81E-07	1.55	2.19E-05	1.42	3.31E-04
HG001_01311	HG001_01311|gene_ponA|Penicillin-binding protein 1A/1B	1.51	8.38E-05	1.14	3.30E-09	2.06	9.18E-13
HG001_00310	HG001_00310|gene_ssb_1|Single-stranded DNA-binding protein ssb	1.50	3.81E-04	0.91	2.53E-03	2.38	1.05E-17
HG001_01765	HG001_01765|gene_hemY|Protoporphyrinogen oxidase	1.47	1.44E-07	1.01	1.05E-03	1.85	8.32E-07
HG001_02091	HG001_02091|gene_rpsA_2|30S ribosomal protein S1	1.45	6.31E-16	1.34	9.26E-09	1.51	3.43E-09
HG001_00656	HG001_00656|gene_kipA_1|KipI antagonist	1.43	3.00E-08	1.46	3.20E-04	1.34	3.49E-05
HG001_01094	HG001_01094|gene_rbgA|Ribosome biogenesis GTPase A	1.40	4.34E-05	1.13	3.79E-02	1.60	4.11E-04
HG001_00005	HG001_00005|gene_gyrB|DNA gyrase subunit B	1.38	1.50E-11	1.21	4.74E-05	1.51	2.70E-12
HG001_00829	HG001_00829|gene_fabH|3-oxoacyl-[acyl-carrier-protein] synthase 3	1.35	3.40E-08	1.06	1.95E-03	1.58	5.31E-08
HG001_02298	HG001_02298|gene_femX|Lipid II:glycine glycyltransferase	1.34	2.05E-08	0.85	1.33E-03	1.77	9.03E-11
HG001_01235	HG001_01235|gene_femB|Aminoacyltransferase FemB	1.24	9.98E-05	1.29	8.75E-03	1.20	7.73E-04
HG001_01955	HG001_01955|gene_alkH|Aldehyde dehydrogenase	1.22	2.47E-04	1.28	1.30E-07	1.02	8.33E-04
HG001_01234	HG001_01234|gene_femA_2|Aminoacyltransferase FemA	1.21	1.22E-05	1.12	9.08E-04	1.26	9.59E-05
HG001_01120	HG001_01120|gene_infB|Translation initiation factor IF-2	1.21	1.02E-06	0.99	3.30E-09	1.43	5.78E-06
HG001_01249	HG001_01249|gene_cvfB|Conserved virulence factor B	1.13	8.58E-06	1.00	9.67E-03	1.21	2.16E-04
HG001_01101	HG001_01101|gene_topA|DNA topoisomerase 1	1.13	1.37E-10	1.06	7.89E-06	1.12	5.05E-06
HG001_00201	HG001_00201|gene_epsJ|putative glycosyltransferase EpsJ	1.09	3.84E-08	1.09	7.55E-05	1.01	1.64E-04
HG001_02043	HG001_02043|gene_NONE|putative leukocidin-like protein 1 precursor	1.08	1.75E-04	1.51	2.00E-05	0.71	2.82E-02
HG001_00858	HG001_00858|gene_fabI|Enoyl-[acyl-carrier-protein] reductase [NADPH] FabI	1.07	1.43E-03	1.07	1.57E-03	0.97	1.79E-03
HG001_01031	HG001_01031|gene_pbpB|Penicillin-binding protein 2B	1.02	1.16E-05	0.90	8.55E-03	1.07	1.22E-03
HG001_01441	HG001_01441|gene_bfmBAB|2-oxoisovalerate dehydrogenase subunit beta	1.02	1.90E-08	0.81	2.88E-03	1.16	2.15E-06
HG001_02241	HG001_02241|gene_adhR|HTH-type transcriptional regulator AdhR	5.36	1.72E-06	3.92	7.21E-02	6.16	6.27E-06
HG001_01447	HG001_01447|gene_xseB|Exodeoxyribonuclease 7 small subunit	2.04	3.42E-04	1.64	6.90E-02	2.36	2.94E-03
HG001_01095	HG001_01095|gene_rnhB|Ribonuclease HII	1.52	8.15E-03	0.42	7.18E-01	2.39	9.89E-04

Table 1. Proteins co-precipitating with Rho in Triton and Igepal lysins conditions.
Regulatory factors are depicted in orange and in green ribonucleases. LogFC values above 1 are represented in yellow.

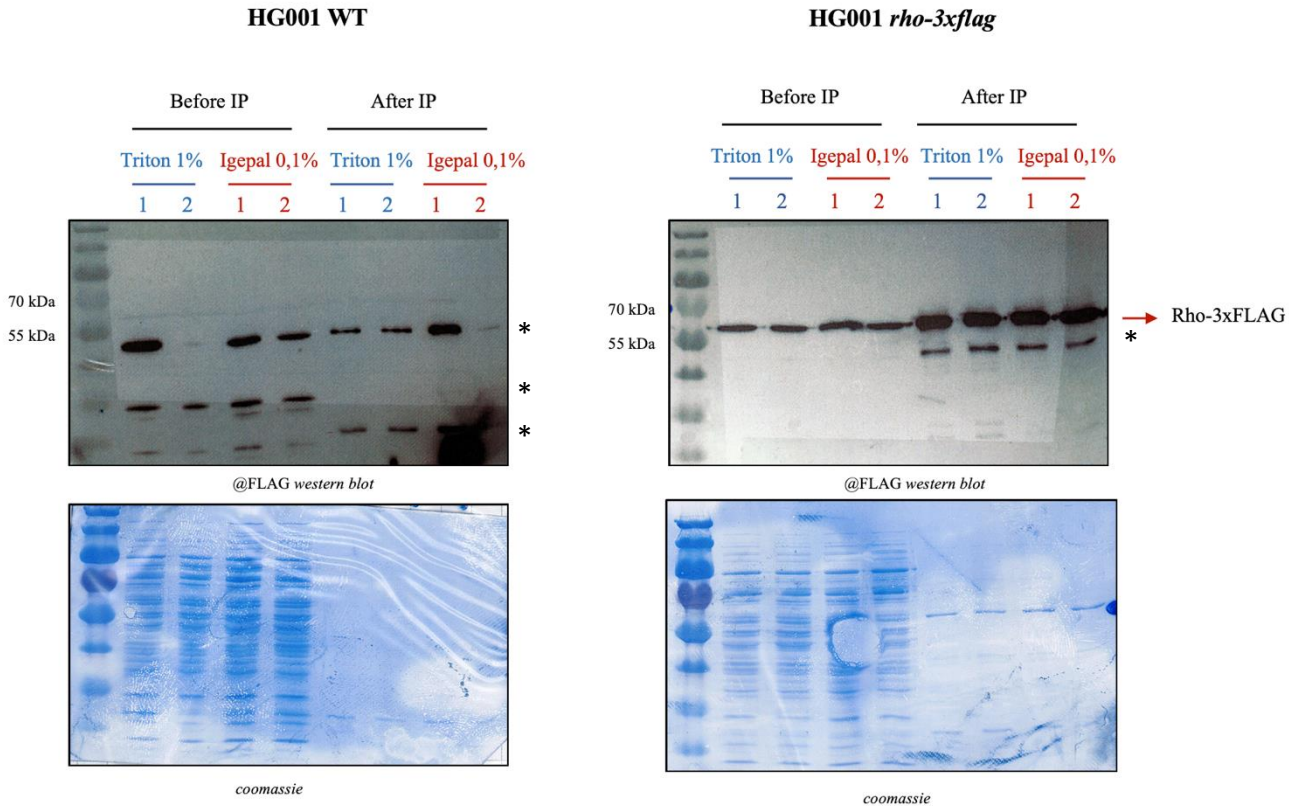

Figure 1. Rho-3xFLAG is enriched after IP in HG001 *rho-3xflag* strain.
Protein duplicate samples of the crude extract (before IP) and elution (after IP) fractions of the performed anti-FLAG immunoprecipitations in the HG001 WT (left) and *rho-3xflag* strains (right) were loaded onto a 10% SDS-PAGE gel and *western blot* with anti-FLAG antibodies was performed to detect the tagged protein. Two different lysis buffers were used (Triton-X100 1% and Igepal 0,1 %). *=non-specific signal.

In other bacteria, Rho has been found to cooperate with RNases and even be a component of the degradosome. Thus, we looked for potential co-precipitating RNases in our Co-IP dataset. We found some RNases to be enriched in the Rho-3xFLAG samples: ATP-dependent helicase and deoxyribonuclease Add A and B with a logFC of 2.25 and 2.45, the 3' to 5' exoribonuclease Cbf1 with a logFC of 2.6, ribonuclease HII with a logFC of 2.39 (but only with one detergent), exodeoxyribonuclease 7 XseB with a logFC of 2.36 (but only with one detergent). However, no major RNases described to be involved in virulence regulation were enriched, especially RNase III which is essential to restrain pervasive transcription.

BACTH (Performed by Vladimir Bidnenko, Micalis, Jouy-en Josas)

To assess a potential direct interaction between Rho and the pulled-down proteins, a bacterial two hybrid system was adapted. As described previously, the catalytic domains of the adenyl cyclase protein of *B. pertussis* were separately fused to the N- or C-terminal domains of Rho and the selected target proteins. Both proteins are co-expressed in *E. coli* and bacteria are grown on plates containing X-gal. If both recombinant proteins physically interact, the fused domains of adenyl cyclase are brought into close proximity and are able to reconstitute a functional protein, restoring cyclic AMP (cAMP) production, needed for downstream activation of the reporter gene. Thus, if two proteins interact, beta-galactosidase activity will be activated and detected.

Rot and the most differentially expressed protein corresponded to protein 00749 (aka MgsR) of unknown function encoded by the HG001_00749 gene were selected to perform BACTH. The T25 and T18 were cloned at the N-ter and C-ter of Rho, MgsR and Rot proteins and all plasmid combinations were transformed into *E. coli*, including control empty pUT18C and pKT25 plasmids. We observed high intensity signals for a Rot-Rot interaction. Indeed, Rot acts as a homodimer to bind and repress promoter expression.[12] An important signal was also observed for a Rho-Rho interaction, in accordance to its homohexameric structure (Fig. 2). No signal was detected for a Rho-Rot interaction (Fig. 2). Regarding the MgsR protein, a stronger signal was observed for a MgsR - MgsR interaction, suggesting the multimerization of this protein. The interaction of Rho with itself was confirmed again. However, no interaction between Rho and MgsR was detected either (Fig. 2).

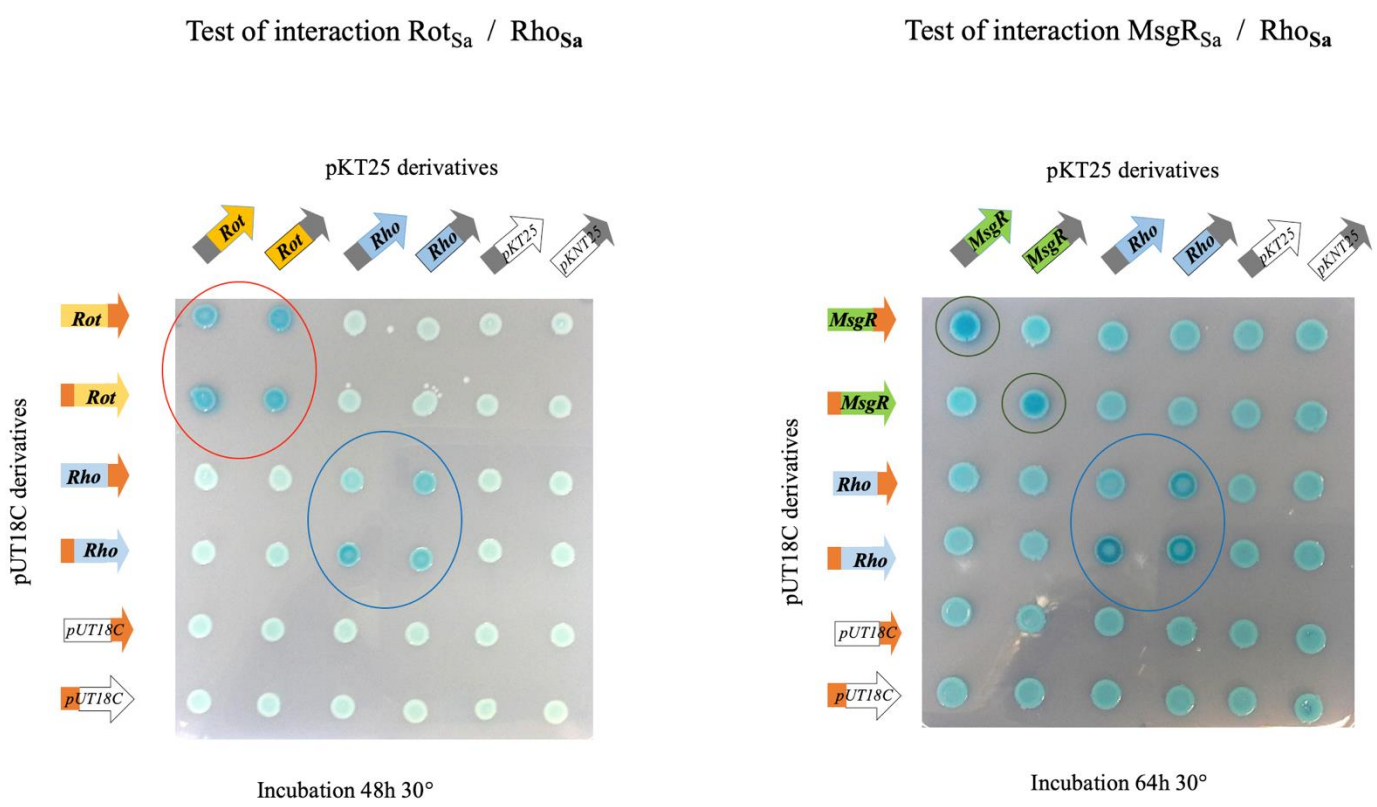

Figure 2. Bacterial two-hybrid assay to identify direct interactions of Rho with Rot and MgsR. *E. coli* strains co-expressing pUT18C and pKT25 derivative plasmids containing domains T18 and T25 of *B. pertussis* adenyl cyclase fused to the N-terminal or C-terminal domains of the studied proteins are grown on X-gal containing plates. After incubation at 30ºC beta-galactosidase activity is detected. The considered interacting proteins that show higher intensity of beta-galactosidase activity are circled.

Discussion

Co-immunoprecipitation of Rho-3xFLAG protein was performed in *S. aureus* to identify protein binding partners of Rho *in vivo.* Elutions from the Co-IPs were subjected to nanoLC-MS/MS and the spectra were analyzed to identify the eluted proteins. The tagged Rho factor was successfully enriched after IP using anti-Flag magnetic beads (Miltenyi) in the corresponding strains. However, native Rho was also found to engage in non-specific interactions with the column as it was also detected in the elution of the WT strain in a ten times less abundant amount than in the tagged strain. Our first observation was the significant amount of detected proteins in every elution. We expected a limited amount of co-precipitating protein in the Rho-3xFLAG strain. However, numerous proteins co-precipitated with Rho in both lysis conditions with a FC>2 and pdj<0.05 (Table 1). Several regulatory proteins were identified with high FC values, which could lead to hypothesize that Rho might cooperate with or be recruited by transcription factors to silence their target genes. In order to verify the existence of physical interactions of Rho with the pulled-down proteins, a bacterial two hybrid assay was performed by Vladimir Bidnenko in Elena Bidnenko's team. We selected the most enriched protein in our CoIP 00749 (aka MgsR), annotated as a conserved protein of unknown function and Rot, due to the function of the latter in the repression of virulence pathways. No interaction between Rho with any of these proteins could be detected with this assay. The functionality of this assay could be validated since interactions of Rot, Rho and MgsR with themselves were confirmed. However, we cannot exclude that other staphylococcal factors or RNAs are needed for the interaction between the proteins and that they might be missing in the *E. coli* model.

We speculated that the bacterial lysis method might not be adequate to purify stable Rho-dependent complexes in *S aureus*. Indeed, we used mechanical lysis with the FastPrep to break cellular membranes, however this could have been too brutal, breaking up protein complexes and leading protein identification. In order to counteract this problem, we could break cellular envelopes using an enzymatic lysis with lysostaphin instead of mechanical lysis. The direct interactions of Rho with potential interacting RNases was not assessed by two hybrid assay and the biological significance of these potential interactions has not been investigated. However, investigating a possible interaction of Rho with ribonuclease HII (RNase HII) could be of interest, since in *E. coli* this RNase has been proven to be important for R-loop or

DNA:RNA hybrid removal.[13] Indeed, *E. coli* encodes for two RNase H enzymes (RNase HI and RNase HII) that remove DNA:RNA hybrids to avoid unresolved R-loops generated during DNA-replication and transcription that can lead to potential irreparable chromosomal lesions.[13] RNase HII is involved in the degradation of DNA:RNA hybrids resulting from the misincorporation of ribonucleotides into the DNA during replication.[13] Rho-dependent termination is known to be required to avoid formation of R-loops to maintain chromosomal integrity. *S. aureus* encodes for three RNase H enzymes (RNase HI, HII and HIII) and cooperative functions of Rho with these enzymes could exist in order to avoid the formation of these structures and permanent chromosomal damage and the existence of interactions between both proteins should be further investigated.

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ANNEX II. Purification of Rho from *S. aureus*

Results and discussion

The purification of a functional Rho protein of *S. aureus* is crucial to validate protein-RNA interactions with the potential RNA targets previously identified through RIPseq. A purified protein is also needed to verify the mechanisms of action of Rho on these potential candidates, and more precisely to confirm the transcription termination activity on them. The production of specific antibodies against Rho_{Sa} also requires a purified protein, and the availability of such antibodies is essential to follow the *in vivo* native expression of Rho in different strains and conditions, to fully understand the regulation of Rho as well as its functions in *S. aureus*.

We previously attempted to purify a recombinant Rho_{Sa} containing a His-tag followed by a TEV cleavage site at the N-terminal end. The gene was cloned in the pET28a- by our collaborator, Marc Boudvillain (CBM, Orléans). Recombinant plasmid was transformed in the the BL21-CodonPlus(DE3) *E. coli* strain. Cultures were grown at 16°C in LB and induced by IPTG 0,5 M at OD_{600}=0,2 O/N. The N-terminal tagged protein was expressed very poorly and impacted growth of *E. coli*. However, small amounts of this recombinant His-Rho_{Sa} protein were purified from *E. coli* through purification on a NiNTA column but heavily precipitated after a dialysis step performed to concentrate the protein. To increase the solubility, salt concentration in the elution buffers was increased as the pH to 9, which somewhat improved the precipitation issue, but protein concentration remained relatively low. The resulting elution fraction was subjected to mass spectrometry to verify the nature of the eluted protein(s), which revealed a 10% contamination with the Rho protein from *E. coli (*Rho_{Ec}) (Fig. 1A). Also, the N-terminal His-tag was not able to be removed through TEV cleavage, suggesting that the tag might be inaccessible due to structural rearrangements. To test the activity of the purified protein, an ATPase activity test was performed in which the protein fraction was incubated with a polyC RNA molecule (to mimic molecules containing *rut* sites) and a mix of cold ATP/ γP^{32}-ATP. The reactions were stopped at different time points and the ratio of free P^{32} and ATP was compared. In the presence of an active Rho protein able to hydrolyze ATP, higher free P^{32} levels are expected. However, very little ATPase activity was observed for the recombinant purified $Rho_{Sa,}$ especially when compared to the Rho_{Ec} ATPase activity (data not shown). After 90 minutes P^{32}-ATP was still significantly present in the reaction mixture for Rho_{Sa} (Fig. 1B)

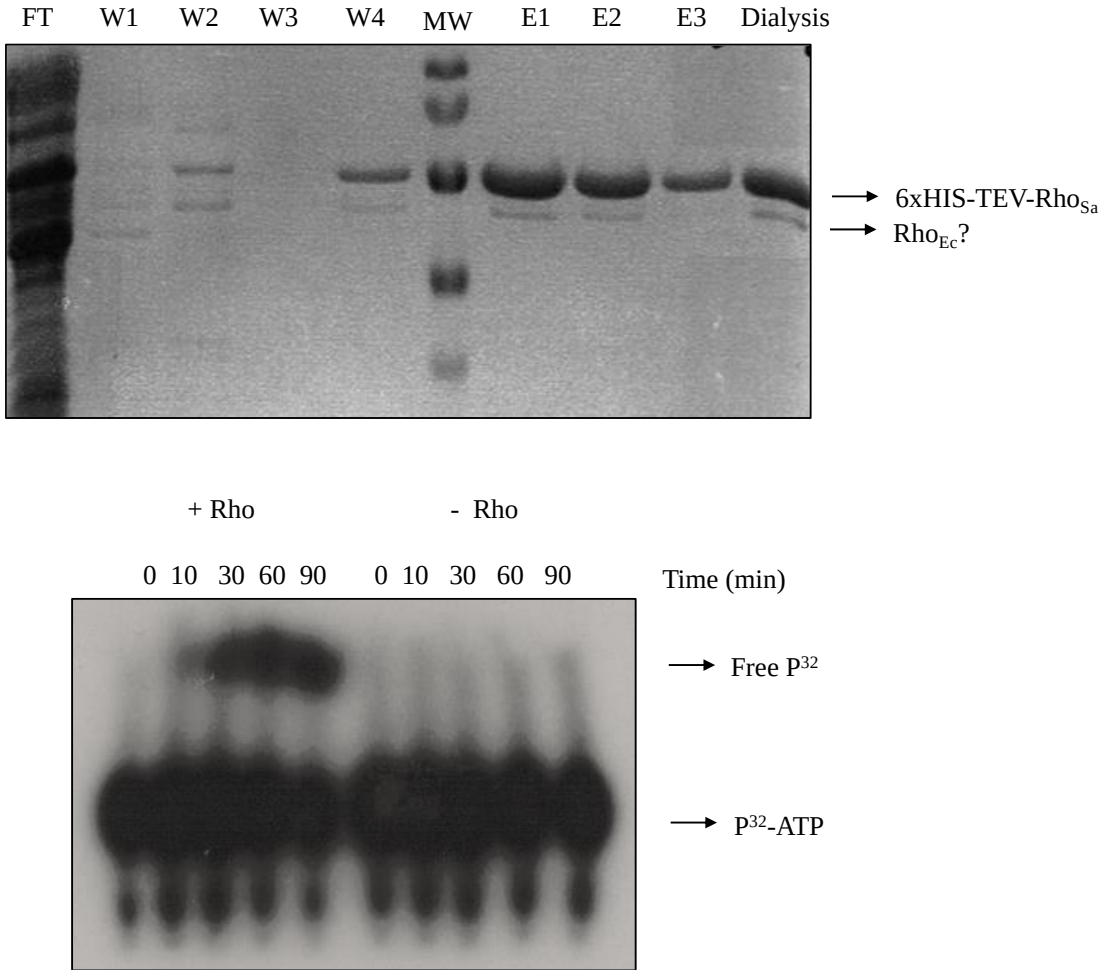

Figure 1 - Purified 6xHIS-TEV-Rho$_{Sa}$ from *E. coli* has weak ATPase activity *in vitro*.

A. Different fractions of 6xHIS-TEV-Rho$_{Sa}$ purification from *E. coli* strain were loaded on a 10% SDS-PAGE gel followed by coomassie staining. The recombinant Rho protein was purified using a NiNTA matrix and was enriched after elution with 250 mM imidazole. The eluted protein was concentrated using a dialysis cassette. FT=flowthrough, W=wash, E=elution, MW=molecular weight. **B.** ATPase activity test of the previously purified 6xHIS-TEV-Rho$_{Sa}$ protein at a concentration of 18 uM per reaction. A control ATPase test without protein was performed.

whereas with Rho_{Ec} no more P^{32}-ATP could be detected after only 10 minutes (M. Boudvillain, personal communication).

Thus, we cannot exclude that the observed ATPase activity is actually accounted to the contaminant Rho_{Ec} protein. Whereas the activity of Rho relies on its hexameric conformation, the eluted protein was subjected to dynamic light scattering (DLS) to assess the oligomeric assembly. It was found that the protein formed mainly dimers instead of hexamers, which could also explain the low ATPase activity observed (Figure 1B). Also, we cannot exclude that Rho_{Sa} binding sites on RNA might differ from the classical *rut* sites recognized by Rho_{Ec}. Thus, the polyC fragment used in the reaction to mimic Rho-specific RNA targets might not be adequate for our recombinant protein. Since RNA-binding is crucial for the protein ATPase activity, a low affinity for the RNA fragment used might result in low ATPase activity, as observed. All these experiments were conducted in collaboration with Lucas Herrgott, an engineer in the team.

To address these encountered problems, we designed a new purification strategy using a C-terminal tagged Rho protein directly expressed and purified from *S. aureus*. that will avoid the Rho_{Ec} contamination problem. To overexpress Rho-6xHis in *S. aureus*, we will use the pRAB11 plasmid that allows the overexpression of the desired protein under the control of an anhydrotetracycline (ATc)-inducible promoter.[1] The Tet regulation system, involving the tetracycline repressor TetR is frequently used for inducible gene expression in bacteria. In short, TetR is a homodimeric transcriptional repressor that binds to *tetO* sites on DNA to repress transcription. Upon interaction with the inducer, namely tetracycline or a tetracycline-derivative such as ATc, TetR detaches from the *tetO* sites allowing for gene expression to be initiated.[2] The gene encoding for TetR is present on the pRAB11 plasmid and two *tetO* sequences control the expression of the cloned gene.[1] Thus, we cloned the coding sequence of the *rho* gene containing an RBS consensus sequence to favor protein production and TEV cleavage site followed by a *6xhis* tag-encoding sequence at the 3'end. Additionally, a SUMO (Small Ubiquitine-like Modifier) tag has been introduced to the construct to increase the solubility of the protein and perhaps to add a linker and increase the tag accessibility. The resulting plasmid will be introduced into HG001 △*rho* and HG001 ⊿*rnaIII* strains. Because of the translational negative regulation of the *rho* mRNA by RNAIII, we believe that overexpressing the recombinant Rho-6xHis protein in the strain lacking RNAIII might result in higher protein levels since any post-transcriptional regulation might be avoided.

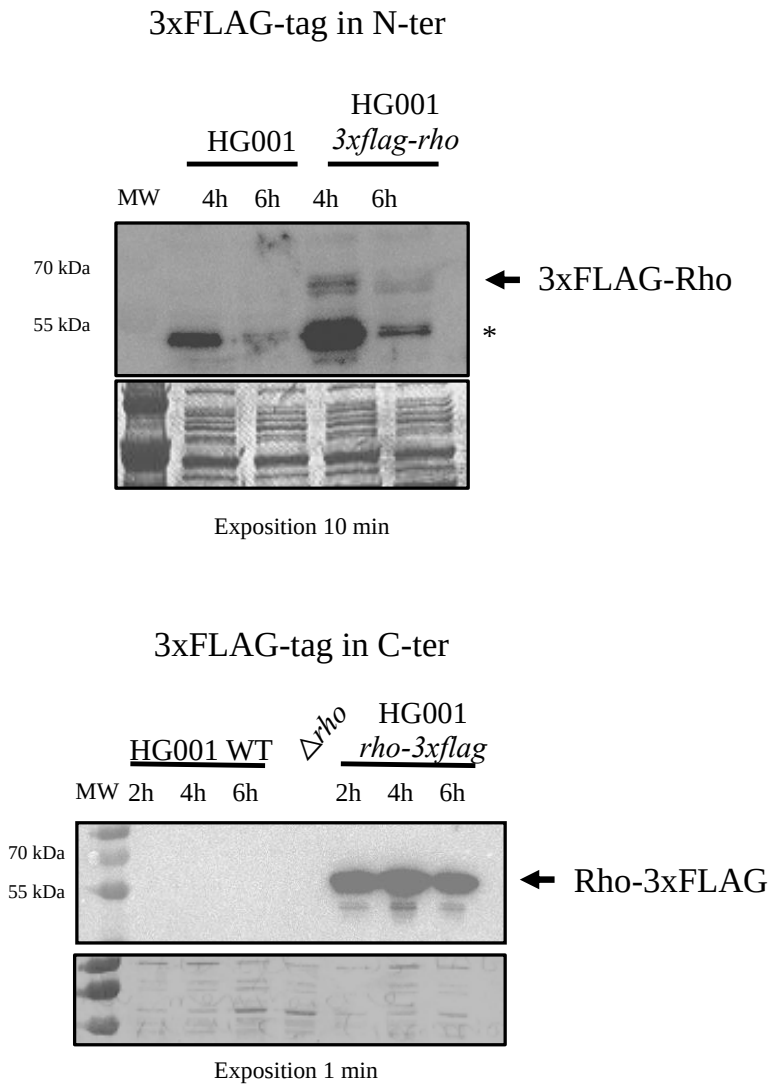

Figure 2 - Expression of Rho_{Sa} containing a 3x-FLAG at either at the C-terminal or N-terminal extremities.

HG001 WT, HG001 *3xflag-rho* (N-terminal) and HG001 *rho-3xflag* (C-terminal*)* strains were grown in BHI medium at 37º C and samples were taken at different times. Cells were harvested, lysed and the supernatant was used for measuring total protein concentration. 10 mg of total proteins loaded on a 12% polyacrylamide-SDS gel to perform Western blot. Membranes were incubated with anti-FLAG mouse antibodies followed by incubation with a goat anti-mouse peroxidase (HRP) antibody. A protein extract from HG001 Δ*rho* was also leaded as a control (lower panel). Coomassie staining of the membranes are shown below. Exposition times for each western blot are specified. *=non-specific signal.

The construct is available and once it has been introduced in the respective *S. aureus* strains, its expression will be followed during growth and its solubility will be assessed prior purification assays. We expect this C-terminal tagged protein to be well-expressed in *S. aureus* since previous C-terminal modifications were not found to affect expression of Rho in this bacterium. For previous RIPseq experiments, we first introduced a 3xFLAG-tag at the N-terminal of Rho in *S. aureus* by introducing a *3xflag* coding-sequence at the 5'end of the *rho* gene on the chromosome through homologous recombination (See Chapter III). We then followed the expression of the 3xFLAG-Rho protein in *S. aureus* and observed very low expression, suggesting that the protein was unstable, probably due to the modification of its N-terminal region (Fig. 2). The fusion of the FLAG-tag at the C-terminal end instead significantly improved the stability (Fig. 2).

The N-terminal end of Rho from *S. aureus* contains a long, positively charged insertion that is suggested to confer specific binding properties to this termination factor.[3] In structural prediction models of Rho hexameric assembly in *S. aureus*, the N-terminal region is shown to extend outwards, creating tentacle-like structures that could be involved in RNA recognition and binding (Figure 11, Chapter II). This could be even more important since no specific *rut* sites have been identified for Gram-positive bacteria. Thus, the N-terminal insertion could play crucial roles for substrate binding in *S. aureus*. Since RNA-binding is critical for Rho activity and oligomerization, it is possible that modifying the N-terminal region in *S. aureus* could lead to destabilization and degradation of the protein.

Hence, experiments are ongoing to purify a functional Rho_{Sa} protein from *S. aureus* to perform gel shift and transcription termination assays on Rho RNA targets.

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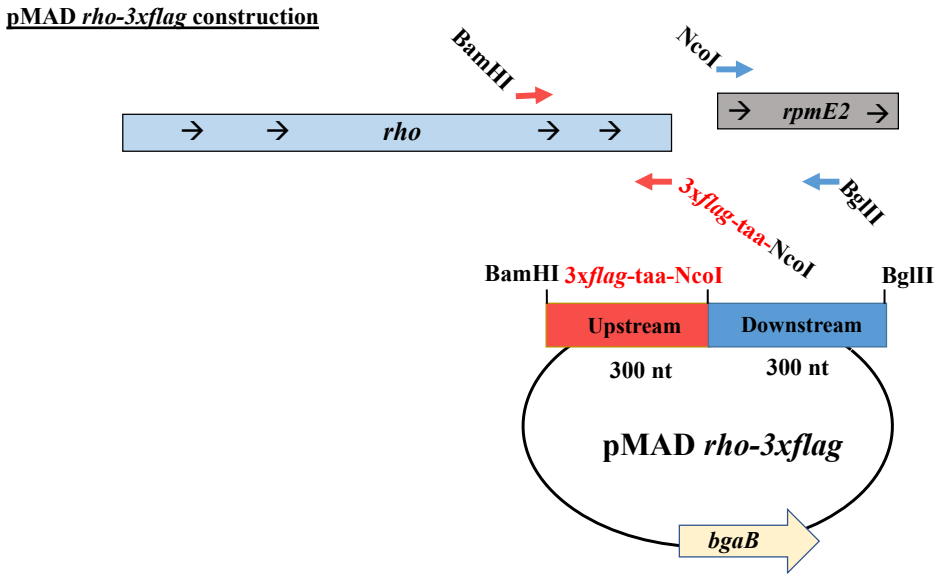
ANNEX III. Cloning strategies

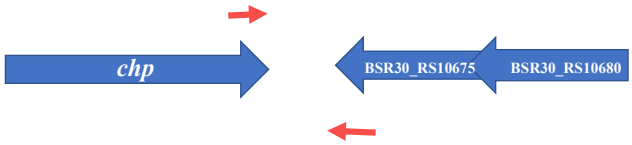


1. Cloning strategy for the chromosomal insertion of the *3xflag* sequence at the 3'end of the *rho* gene.

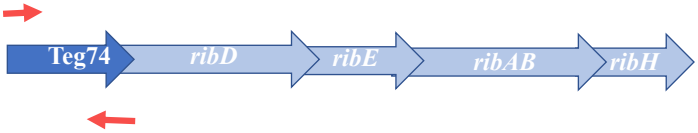
pCN57 *teg74* and pCN57 *chp* construction

A) pCN57::GFP-target construction scheme

B) pCN57::GFP-chp construction

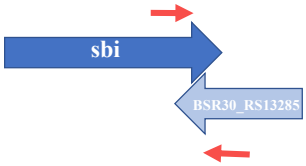


C) pCN57::GFP-Teg74 construction



D) pCN57::GFP-Teg66 construction

E) pCN57::GFP-sbi construction



2. Cloning strategies for the GFP transcriptional fusion plasmids.

ANNEX IV. Contributions to Desgranges *et al*., 2022

The 3'UTR-derived sRNA RsaG coordinates redox homeostasis and metabolism adaptation in response to glucose-6-phosphate uptake in *Staphylococcus aureus*.

Desgranges E., **Barrientos L.,** Herrgott L., Marzi S, Toledo-Arana A., Moreau K., Vandenesch F., Romby P. and Caldelari I., Mol. Microbiol., 2022 Jan;117(1):193-214. doi: 10.1111/mmi.14845.

L'ARN régulateur RsaG de *Staphylococcus aureus* est dérivé de la région 3' non traduite du gène *uhpT,* très conservé parmi les bactéries et codant pour un transporteur transmembranaire du glucose-6-phosphate (G6P) produit en réponse à la présence extracellulaire de cette molécule. Le transcrit *uhpT*-RsaG est soumis à une dégradation de 5' en 3' par l'exoribonucléase J1/J2 dont l'action est probablement inhibée par la formation d'une structure en tige-boucle à l'extrémité 5' de RsaG, permettant la stabilisation et l'accumulation de ce petit ARN en présence de G6P. RsaG et *uhpT* sont également produits suite à l'internalisation de la bactérie par des macrophages et des myoblastes ou en présence de cellules pulmonaires humaines sécrétant du mucus. En appliquant pour RsaG la technique du MAPS (*MS2 affinity purification coupled with RNAseq*), une purification d'affinité MS2 couplée au séquençage haut débit des ARNs, de nombreux ARNs ont été identifiés comme des cibles potentielles de RsaG. Parmi ces cibles de nombreux ARNm codant pour les facteurs de transcription Rex, CcpA et SarA ont été identifiés ainsi que RsaI, un autre ARN régulateur bien décrit chez *S. aureus*. Les données de cette étude suggèrent que RsaG contribue au contrôle et au maintien de l'homéostasie redox et permet d'adapter le métabolisme bactérien aux changements des conditions environnementales. Il a été montré que RsaG, à travers différents mécanismes, est capable de stabiliser, dégrader et aussi de réprimer la traduction de ses ARNm cibles. Des études comparatives ont également mis en évidence la conservation de RsaG uniquement chez des espèces proches. Néanmoins, la région 3' non-traduite de l'ARNm *uhpT* du pathogène de singe *S. simiae* contient également un petit ARN de séquence très différente et qui ne répond pas au G6P, dont la fonction reste à découvrir. Ainsi, ces résultats suggèrent que les régions 3' non traduites des ARNm codant pour le transporteur UhpT auraient pu évoluer rapidement pour permettre l'adaptation de *S. aureus* aux différentes niches rencontrées, notamment celles de l'hôte.

Contributions à l'étude de Desgranges *et al*, 2022.

Concernant ma participation à cette étude, j'ai contribué à compléter la validation des données générées par le MAPS de RsaG en vérifiant l'interaction *in vitro* de RsaG avec certaines cibles fortement enrichies suite à la chromatographie d'affinité par des expériences de retard sur gel. J'ai pu confirmer l'interaction de RsaG avec les ARNm *mscL*, *ndh*, *arcR*, *arcC2* et *slyA*. Même si l'importance biologique de ces interactions n'a pas été explorée pour toutes les cibles, la validation *in vitro* des interactions entre les ARN a été essentielle afin de confirmer la robustesse de la méthode du MAPS. Ainsi, mes expériences de retard sur gel ont pu renforcer la validité de l'étude.

J'ai également reproduit et confirmé l'effet de RsaG sur la stabilité de l'ARNm *rex*, codant pour le régulateur central du métabolisme anaérobie Rex, en effectuant des expériences de rifampicine dans différentes souches de *S. aureus* (souche sauvage, souche mutante de RsaG et souche complémentée) et en suivant la stabilité de cet ARNm par *Northern blot*. Cette expérience a été nécessaire pour comprendre et valider l'effet *in vivo* de RsaG sur cet ARNm. Ainsi, j'ai pu confirmer la stabilisation de *rex* en présence de RsaG.

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RESEARCH ARTICLE

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The 3′UTR-derived sRNA RsaG coordinates redox homeostasis and metabolism adaptation in response to glucose-6-phosphate uptake in *Staphylococcus aureus*

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Abstract

Staphylococcus aureus RsaG is a 3′-untranslated region (3′UTR) derived sRNA from the conserved *uhpT* gene encoding a glucose-6-phosphate (G6P) transporter expressed in response to extracellular G6P. The transcript *uhpT-RsaG* undergoes degradation from 5′- to 3′-end by the action of the exoribonucleases J1/J2, which are blocked by a stable hairpin structure at the 5′-end of RsaG, leading to its accumulation. RsaG together with *uhpT* is induced when bacteria are internalized into host cells or in the presence of mucus-secreting cells. Using MS2-affinity purification coupled with RNA sequencing, several RNAs were identified as targets including mRNAs encoding the transcriptional factors Rex, CcpA, SarA, and the sRNA RsaI. Our data suggested that RsaG contributes to the control of redox homeostasis and adjusts metabolism to changing environmental conditions. RsaG uses different molecular mechanisms to stabilize, degrade, or repress the translation of its mRNA targets. Although RsaG is conserved only in closely related species, the *uhpT* 3′UTR of the ape pathogen *S. simiae* harbors an sRNA, whose sequence is highly different, and which does not respond to G6P levels. Our results hypothesized that the 3′UTRs from *UhpT* transporter encoding mRNAs could have rapidly evolved to enable adaptation to host niches.

KEYWORDS

3′UTR-derived sRNA, redox homeostasis, *Staphylococcus aureus*

1 | INTRODUCTION

Staphylococcus aureus is usually described as an extracellular opportunistic pathogen, infecting a wide range of organs and tissues. However, it also invades and replicates in various phagocytic or nonphagocytic host cells (Hamza & Li, 2014). To be successful as a pathogen, this bacterium needs to adapt to the hostile environment of the host and must acquire imposed nutriments for its survival. Consequently, the staphylococcal genome encodes several transporters for metabolites (sugars, metals, amino acids, etc.), which are

often primordial for virulence. For instance, a mutant strain deprived of the four glucose transporters (*glcA*, *glcB*, *glcC*, and *glcU*) is avirulent in a murine skin infection model (Vitko et al., 2016).

Metabolic and virulence genes are tightly controlled by transcriptional factors (TFs), two-component systems (TCS), metabolite-sensing proteins, and regulatory RNAs to adapt to various environmental situations (Richardson, 2019; Villanueva et al., 2018). *S. aureus* possesses a plethora of TFs, such as CcpA, CodY, and Rex, responding to diverse stimuli (for review, see Richardson, 2019). In response to glucose, the catabolite control protein CcpA activates glycolytic genes, represses the expression of genes, whose products act in the TCA cycle, gluconeogenesis, and amino acid catabolism (Seidl et al., 2009). It mediates catabolic repression allowing bacteria to use the preferred carbon source. Besides, CodY, sensing both branched amino acids and GTP, represses genes involved in the amino acid synthesis and secreted virulence factors (Richardson, 2019). Furthermore, Rex is considered as the central regulator of anaerobic metabolism and inhibits transcription of genes-encoding fermentative enzymes such as lactate dehydrogenase Ldh1 or alanine dehydrogenase Ald1 under aerobic conditions (Pagels et al., 2010). At high NADH/NAD level, when Rex is inhibited, survival of *S. aureus* depends on a metabolic switch to promote fermentation in response to NO or to face the aerobic low-redox potential of host cytosol (Christmas et al., 2019; Crooke et al., 2013). Although immune radicals damage the terminal oxidase of the electron transport chain, leading to the disruption of redox homeostasis, *S. aureus* produces predominantly L- or D-lactate as defenses. The three lactate dehydrogenases Ldh1, Ldh2, or Ddh generate, respectively, L- or D-lactate from glucose fermentation and support NAD^+ recycling. Therefore, CcpA activates transcription of the key enzyme Ldh1 only if *rex* is repressed (Crooke et al., 2013). In contrast, when bacteria are internalized and glucose unavailable, Ldh1 is not active, and the amino acids are preferred as nutrients reflecting the derepression of the CodY and CcpA regulons (Michalik et al., 2017). Alongside TFs, the staphylococcal core genome encodes 16 two-component systems (TCS), which sense and respond to diverse stimuli and various metabolites or small compounds (Villanueva et al., 2018). In glucose-limiting conditions, when *S. aureus* penetrates host cells, the bacteria could utilize cytosolic glucose-6-phosphate (G6P) instead of glucose, whose uptake relies on the hexose phosphate antiporter UhpT. Expression of *uhpT* is induced by the TCS HptRS, which senses extracellular G6P (see below and Garzoni et al., 2007; Park et al., 2015; Yang et al., 2016). The response regulator HptR binds to a consensus sequence called HptR box, localized between −67 and −96 before the beginning of the transcriptional start site of *uhpT* (Yang et al., 2016). The disruption of *hptRS* operon impairs survival into various host cells, suggesting that sensing and uptake of G6P are critical for *S. aureus* virulence (Park et al., 2015).

Regulatory RNAs (sRNA) cooperate with TFs and TCSs to quickly adjust the bacterial physiology to the surrounding conditions and especially to the availability of carbon sources (Bobrovskyy & Vanderpool, 2013; Desgranges et al., 2019; Wagner & Romby, 2015). They often act at the posttranscriptional level and hybridize to their mRNA targets through imperfect complementarities to regulate positively or negatively their stability or translation. The vast majority of sRNAs derives from intergenic regions (IGR) and possesses their own promoter, otherwise, they are processed from 5′- or 3′-untranslated regions (UTRs) of mRNAs (Desgranges et al., 2019; Miyakoshi et al., 2015). Their transcription is often induced by sensing external stimuli via TCS or TF (Brosse & Guillier, 2018). The targetomes of different staphylococcal sRNAs were identified and reflected the diversity of pathways in which they intervene (e.g., Augagneur et al., 2020; Bronesky et al., 2019; Lalaouna, Baude, et al., 2019; Rochat et al., 2018; Tomasini et al., 2017). Several of them are part of large regulatory networks that connect major regulatory proteins of virulence gene expression (CodY, CcpA, SigB, AgrA, and SrrAB) in response to wide arrays of metabolic and environmental signals (Desgranges et al., 2019). For instance, the sRNA RsaI is repressed by CcpA in the presence of glucose. When glucose decreases, RsaI downregulates glucose uptake and activates fermentation, energy production, and NO detoxification. RsaI binds to other sRNAs such as RsaD, RsaE, and RsaG (Bronesky et al., 2019). SrrA, which senses nitric oxide, activates RsaE to repress the synthesis of many TCA enzymes and to reduce NADH production. In turn, low activity of the TCA cycle has a positive effect on the *agr* system to adjust virulence factor production under stress conditions. Conversely, the presence of NAD+ enhances the binding of Rex to an RsaE promoter region to repress its expression (for a review see Marincola et al., 2019).

Here we show that the 3′UTR-derived sRNA, RsaG, conserved in *S. aureus* and in very close relative species, accumulates when G6P is available extracellularly. This sRNA is derived from the degradation of *uhpT* mRNA encoding the hexose phosphate antiporter UhpT. Using the combination of approaches, we propose that RsaG contributes to the regulation of redox homeostasis, and metabolism adaptation when G6P is metabolized. The consequences of RsaG functions on *S. aureus* pathogenesis will also be discussed.

2 | RESULTS

2.1 | RsaG expression is induced by G6P upon the *uhpT* promoter

RsaG was first identified as an intergenic region that is conserved only in *S. aureus* (Geissmann et al., 2009). RsaG is located downstream the *uhpT* gene and was shown to accumulate in the late exponential phase of growth in a rich medium (Geissmann et al., 2009) or in the presence of G6P (Bronesky et al., 2019). Because no obvious predicted promoter sequence was detected for *rsaG* and because *uhpT* is induced in response to extracellular G6P (Bronesky et al., 2019), we have revisited the mechanism leading to the accumulation of RsaG.

We first tested RsaG expression by Northern blot analysis in MHB (Muller-Hinton Broth) with or without G6P in the wild-type (HG001) strain, the mutant strains carrying either deletion of the *hptRS* system (HG001 Δ*hptRS*) or a deletion of the *uhpT* promoter

containing the *hptR* box recognized by the transcriptional regulator HptR (HG001 Δ*PuhpT*) (Figure 1a). In MHB, in which starch is added as a carbon source, RsaG is constitutively and weakly expressed. In contrast, when the medium is supplemented with G6P, we observed a large increase of RsaG transcription, which is almost completely abolished in the HG001 Δ*hptRS* or HG001Δ*PuhpT* mutant strains (Figure 1a). Furthermore, we also detected bands with higher molecular weights, showing the presence of a longer transcript containing RsaG and corresponding to the size of *uhpT* (1782 nucleotides). We then constructed various transcriptional fusions carrying *gfp* in place of RsaG and determined the effect of successive deletions within *uhpT* and its promoter on GFP synthesis (Figure 1b). All the constructs were expressed from a plasmid that was transformed into HG001. The data were analyzed by Western blot using an anti-GFP antibody (Figure 1c). GFP bacteria were detected only with *uhpT* full length containing its promoter and the *hptR* box.

Taken together, these data evoked that RsaG is mainly transcribed together with *uhpT* under the control of the same promoter responding to G6P via HptRS and that RsaG accumulated most probably after a rapid degradation of *uhpT* mRNA.

2.2 | The *uhpT* mRNA and RsaG are enhanced under conditions mimicking infection

G6P is the activated form of glucose in numerous cellular metabolic pathways and hexose-phosphate sugars are predominant in the cytosolic environment (Chico-Calero et al., 2002). Upon internalization into host cells, *S. aureus* senses cytosolic G6P and may consume it as a carbon source. We first compared the growth rate of HG001, HG001Δ*rsaG*, and HG001Δ*hptRS* strains in the glucose-depleted LB medium or in the presence of 0.5% G6P. Without G6P, the three strains grew equally. In contrast, the wild-type HG001 strain and the Δ*rsaG* deletion mutant grew faster in the presence of G6P than the HG001Δ*hptRS* strain under identical conditions (Figure 2a). These data indicated that RsaG is not required for G6P catabolism

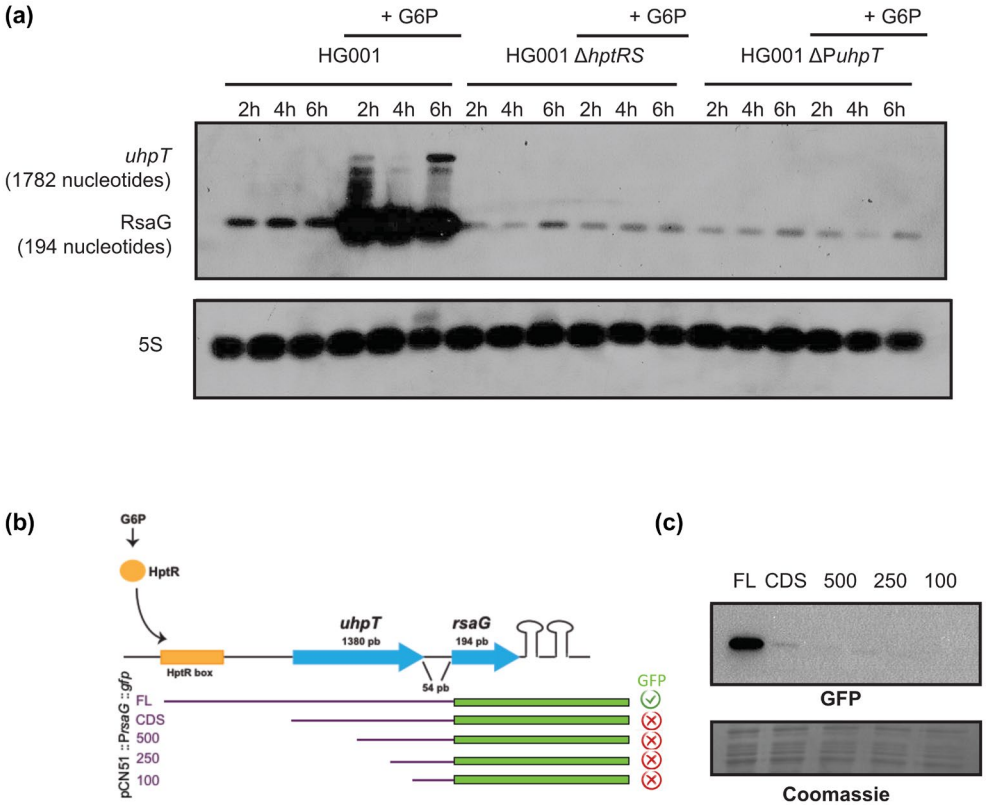


FIGURE 1 RsaG is expressed under *uhpT* promoter activation by HptRS upon sensing external glucose-6-phosphate (G6P). (a) Northern blot analysis of RsaG in HG001 wild-type strain, HG001Δ*hptRS* and HG001Δ*PuhpT*. Total RNA was extracted at 2, 4, and 6 hr of growth in an MHB medium with or without the addition of 0.5% G6P. 5S rRNA (5S) was used as a loading control. However, for this control, we used aliquots of the same RNA preparations but the migration of the samples was performed in parallel to the experiments on a separate agarose gel because RsaG and 5S rRNA have very similar sizes. (b) Genomic context of *uhpT-RsaG* locus and mapping of the different DNA fragments transcriptionally fused to *gfp* in pCN51 plasmid. Only the full-length construct carrying the HptR box provided a positive fluorescence signal. The various sizes of the DNA fragments are given: FL is for full-length, CDS is for the coding sequence of *uhpT*, and 500, 250, and 100 are the number of nucleotides just upstream rsaG. (c) Western blot experiment detecting GFP synthesis in the different transcriptional fusion constructs (FL, CDS, 500, 250, 100) expressed in HG001 wild-type strain. Total proteins were separated by SDS-PAGE (10%) and were revealed using a GFP-specific antibody. We used Coomassie staining as a loading control. However, we used aliquots of the same protein preparations, but the migration of the samples was performed in parallel to the experiments on a separate SDS-PAGE gel

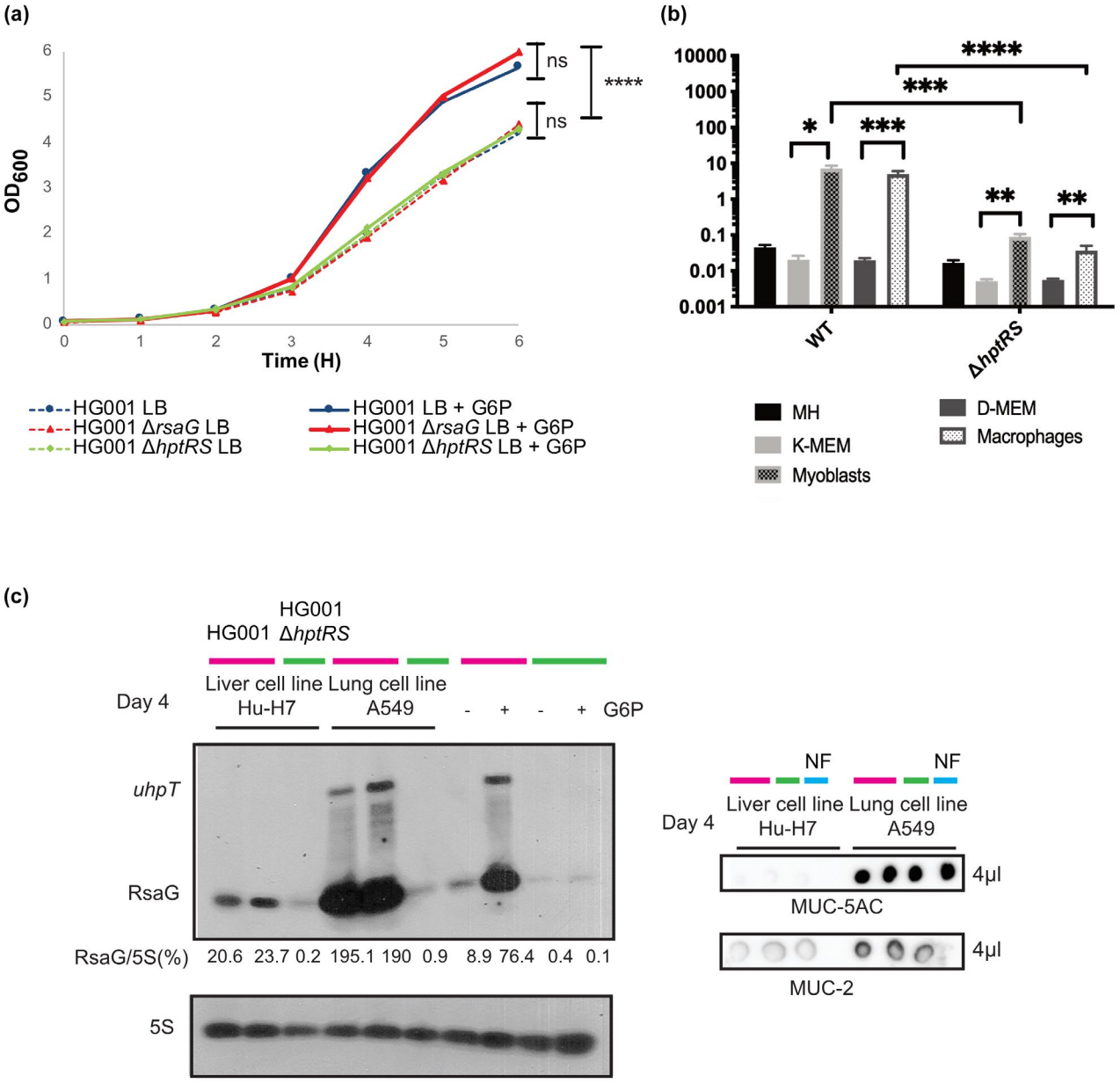


FIGURE 2 The *uhpT* mRNA and RsaG are enhanced under conditions mimicking the infection. (a) Growth curve of HG001 wild-type strain, HG001Δ*rsaG* and HG001Δ*hptRS* strains in an LB medium with or without the addition of 0.5% glucose-6-phosphate (G6P). ns is for nonsignificant, statistical analysis with ANOVA (****p <.0005). (b) Levels of RsaG in HG001 and HG001Δ*hptRS* strains as determined with qRT-PCR. Samples were taken after 1 hr of *S. aureus* internalization into RAW 264.7 macrophages or CTi400 myoblasts. As a control, the yield of RsaG was also quantified for HG001 wild-type or HG001Δ*hptRS* cultured for 1 hr in a K-MEM or D-MEM medium. Data were normalized to *gyrB* and represented the mean of at least three independent experiments, statistical analysis with *t* test (*p <.05). (c) Left panel, Northern blot analysis of RsaG in HG001 (pink bars) and HG001Δ*hptRS* (green bars). Total RNA was extracted after 1 hr incubation with liver cell line Hu-H7 and lung cell line A549 at Day 4 of culture. As a control, RNA was extracted from HG001 and HG001Δ*hptRS* incubated 1 hr in an MHB medium supplemented or not with 0.5% glucose-6-phosphate (±G6P). Right panel, Mucin 2 (MUC-2) and Mucin-5AC (MUC-5AC) proteins were quantified by dot blot assay. One microliter of supernatant from the Hu-H7 or A549 cells incubated with HG001 (pink bars) and HG001Δ*hptRS* (green bars) were spotted on a nitrocellulose membrane. MUC-2 and MUC-5AC were specifically detected with anti-MUC-2 or anti-MUC-5AC antibodies. NF, noninfected Hu-H7 or A549 cells

in contrast to the TCS HptRS. We next performed internalization assays with HG001 and HG001Δ*hptRS* strains in (nonphagocytic) myoblasts and (phagocytic) macrophages cell lines cultured in a K-MEM or a D-MEM medium, respectively. Quantification of RsaG levels in intracellular bacteria was monitored by qRT-PCR. The data showed that RsaG is induced 100-fold under these conditions and that the activation is dependent on HptRS, which allows the expression of the *uhpT-RsaG* cotranscript (Figure 2b and Figure S1a).

Because *S. aureus* is primarily an extracellular pathogen, we postulated that bacteria might face G6P in contact with host

cells producing mucus either in the intestine or lungs. Four days-cultivated A549 airway epithelial cells, which produce high levels of mucin as measured with two different antibodies MUC-5AC and MUC-2 (Figure 2c, right panel), were incubated with the HG001 and HG001Δ*hptRS* strains. Bacteria were gently recovered, and total RNA was extracted. Northern blot experiments were performed with a probe-specific RsaG. The data showed that the expression of RsaG and of a higher band corresponding to *uhpT*-RsaG transcript was highly induced when HG001 was mixed with A549 cells in contrast to HG001 incubated with liver HU-H7 cells that do not synthesize mucin (Figure 2c, left panel). This effect is strictly dependent on the TCS HptRS because no significant signal was observed for RsaG in the mutant strain HG001Δ*hptRS*. We also compared the accumulation of RsaG when staphylococci were incubated in the presence of colon cell lines HT-29 or HT-29MTX cultivated for 4 days and for 14 days to allow differentiation and the production of mucus (Figure S1b, right panel) (Behrens et al., 2001; von Kleist et al., 1975). HT-29 cells are heterogeneous cells as they contain <5% of mucus-secreting cells. HT29-MTX cells have been obtained from HT-29 cultures after treatment with methotrexate to give more homogeneous and stable mucus-secreting cells. Strong induction of RsaG was observed when HG001 strains were incubated with either HT-29 or HT-29 MTX cells cultivated for 14 days compared with HG001 strains incubated with either HT-29 or HT-29 MTX cells cultivated for 4 days, respectively (Figure S1b). This effect is linked to G6P entry because RsaG accumulation was no more observed when the mutant strain HG001Δ*hptRS* was incubated with HT-29 and HT-29 MTX cells (Figure S1b).

In summary, we defined two different environmental conditions in which *uhpT* mRNA and consequently RsaG are induced. Because RsaG is not essential for growth in the presence of G6P, we proposed that RsaG is not required for G6P uptake and catabolism but might contribute to adapting the cells in new environments during the infection, that is, the presence of mucus and/or internalization into host cells.

2.3 | RsaG is derived from the maturation of the 3′UTR of *uhpT*

Because only primary transcripts have triphosphates at the 5′-end, we used the 5′-phosphate-dependent exonuclease Terminator™ (Tex) to discriminate the phosphorylation status of the 5′-end of RsaG. Total RNAs extracted from HG001 grown in BHI for 4 hr (when RsaG is present) were treated with or without Tex and run on an agarose gel. Surprisingly, RsaG level was only slightly reduced in the presence of Tex (Figure 3a, left panel). This result might be due to the fact that the activity of Tex is altered by the presence of a helical structure present at the 5′-end, as found for 5S rRNA, which is also resistant to Tex treatment (Figure 3a, right panel). A longer exposition of the autoradiography showed that the *uhpT-rsaG* transcript and shorter fragments were digested by Tex (Figure S2a). However, a faint band can still be observed at the top of the gel in treated samples, which could correspond to the 5′-triphosphate *uhpT-RsaG* transcript, which is not fully degraded. As positive controls, ethidium bromide staining revealed that the bulk 16S and 23S rRNAs (carrying a monophosphate at their 5′-ends) were fully degraded by Tex (Figure 3b, left panel). In contrast, Northern blot analysis showed that the *bona fide* sRNA RsaI (containing a 5′-triphosphate end) was resistant to Tex (Figure 3b, right panel). These data suggested that the degradation process of the long *uhpT* transcript involves a 5′ exoribonuclease, which would be partially blocked by the 5′ stem-loop structure of RsaG.

In the following experiments, we have tested the involvement of the major ribonucleases from *S. aureus*, namely RNase P, RNase III, RNases J1/J2, and RNase Y (Figure 3c and Figure S2b–d). An in vitro transcribed *uhpT-rsaG* RNA was used in the assay with the RNA component of RNase P, which is sufficient to induce cleavage (Guerrier-Takada & Altman, 1984). The data showed that the cotranscript was not cleaved in contrast to the premature tRNA, which was used as a positive control (Figure S2b). For the other enzymes, Northern blot experiments were performed on total RNAs prepared from various mutant strains carrying deletion at specific genes encoding RNase III (HG001Δ*rnc*), RNase Y (HG001Δ*rny*), and RNases J1 or J2 (RN4220Δ*j1*, Sa624Δ*j1*, or Sa624Δ*j2*) and their parent wild-type strains (HG001, RN4220 or Sa624). The strains were grown in MHB containing G6P, and RsaG was revealed using a specific probe. Although RNase Y and RNase III did not significantly alter the maturation of *uhpT*-RsaG transcript (Figure S2c), a significant decrease of RsaG level was observed in the single deletion mutants of either RNase J1 (Δj1) or RNase J2 (Δ*j2*), and concomitantly an accumulation of *uhpT*-RsaG was visualized (Figure 3c and Figure S2d). Because the growth of the double-mutant strain (Δ*j1*Δ*j2*) was strongly impaired, we did not manage to obtain enough bacteria to extract a sufficient amount of RNAs for further analysis (Linder et al., 2014). In the mutant Δ*j1* and Δ*j2* strains, the size of RsaG appears to be slightly longer than in the parental WT strain, suggesting that the J1/J2 heterodimer is required for RsaG complete maturation (Figure 3c and Figure S2b).

Taken together the data strongly suggested that RsaG is a product of the rapid degradation of the full-length *uhpT* mRNA and involves the 5′-3′ exoribonuclease activities of RNases J1/J2 (Figure 3d), which are both able to degrade mono- or triphosphate 5′-end of transcripts (Linder et al., 2014). The 5′ hairpin structure of RsaG (Figure 4a) most probably blocks the progression of the exoribonucleases.

2.4 | Defining the RsaG targetome

In order to identify RsaG functions, the MAPS approach ("MS2 affinity purification coupled to RNA sequencing") was applied to identify the RsaG targetome (Lalaouna et al., 2018). Briefly, the MS2 tagged version of RsaG was expressed from a plasmid under the control of the P3 promoter in the Δ*rsaG* mutant strain. To mimic the inducible conditions of RsaG, the growth was performed for 5 hr in a BHI

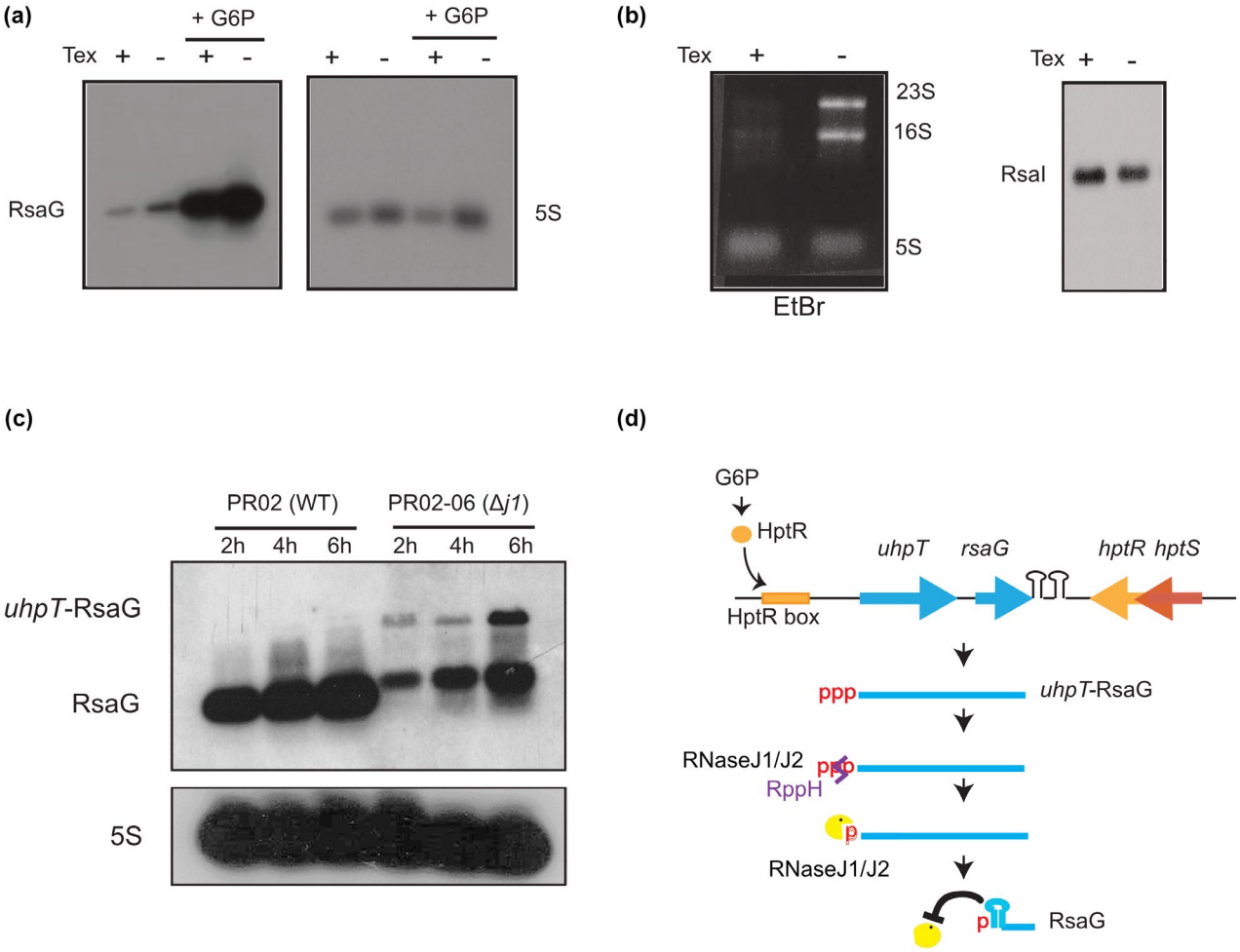


FIGURE 3 RsaG is maturated from *uhpT* by 5′-3′exoribonucleases J1 and J2. A, B. Determination of the 5′ end status of various RNAs. Ten micrograms of total RNA extracted after 4 hr of growth in an MHB medium (±glucose-6-phosphate, G6P) were treated with the Terminator™ 5′-phosphate-dependent exonuclease (±Tex). RsaG, 5S (a), and RsaI (b) were revealed by Northern blot analysis using specific probes, whereas 5S, 16S, and 23 rRNAs were visualized by ethidium bromide staining (EtBr) of the agarose gel (b). (c) Northern blot analysis of RsaG in PR02 wild-type strain and PR02-06 (Δ*j1*). Total RNA was extracted at 2, 4, and 6 hr of growth in an MHB medium supplemented with 10 mg/L uracils and 0.5% glucose-6-phosphate. 5S rRNA (5S) was used as a loading control (see Figure 1a). (d) Genomic context of *uhpT-RsaG* locus and the sequential steps involved in the maturation leading to the accumulation of RsaG. The degradation of *uhpT* might involve the RppH enzyme to remove the pyrophosphate although RNases J1/J2 can also degrade 5′ triphosphate RNA. The 5′ hairpin of RsaG blocked the progression of the two exoribonucleases leading to its accumulation

medium and then G6P 0.5% was added for 1 hr before harvesting. As controls, we showed that the MS2-RsaG is specifically retained by the column (Figure S3a) and is as stable as the chromosomal copy (Figure S3b). After elution and extraction, RNAs were sequenced, and the data were analyzed using the tools of Galaxy platform (https://usegalaxy.org/) (Afgan et al., 2016). The sequencing reads were mapped, counted per feature, and normalized using the HG001 genome as previously described (Tomasini et al., 2017). The enrichment of putative RsaG targets was derived by the comparison of the number of reads obtained from the MS2-RsaG purification and the MS2 alone as a control. In the latter case, the MS2 tag alone was expressed in the wild-type HG001 strain. We have considered as RsaG targets, the RNAs that were enriched at least twofold and were reproducibly and significantly detected in two independent experiments (Table S1).

Among the most-enriched RNAs, we identified several mRNAs encoding transcriptional regulatory proteins (SarA, SlyA, SarX, SarV, Rex, TcaR, CcpA, and RpiR). In addition, the PTS operon (phosphoenolpyruvate [PEP]-dependent phosphotransferase system) encoded the genes *ptsH* and *ptsI*, allowing carbohydrates transport and phosphorylation before entry into glycolysis. G6P promotes the phosphorylation and activation of the histidine-containing phosphocarrier protein HPr, encoded by *ptsH*, which is the activator of CcpA. Hence, HPr connects glycolytic activity with carbon catabolite repression (Deutscher et al., 1995). Other metabolic operons were also enriched including the *arcABDCR* operon, which is involved in arginine catabolism when *S. aureus* grows in anaerobic conditions (Makhlin et al., 2007), the *Thi* operon required for thiamine biosynthesis, an indispensable cofactor of enzymes involved in amino acid and carbohydrate metabolism, and various dehydrogenases (Ald2,

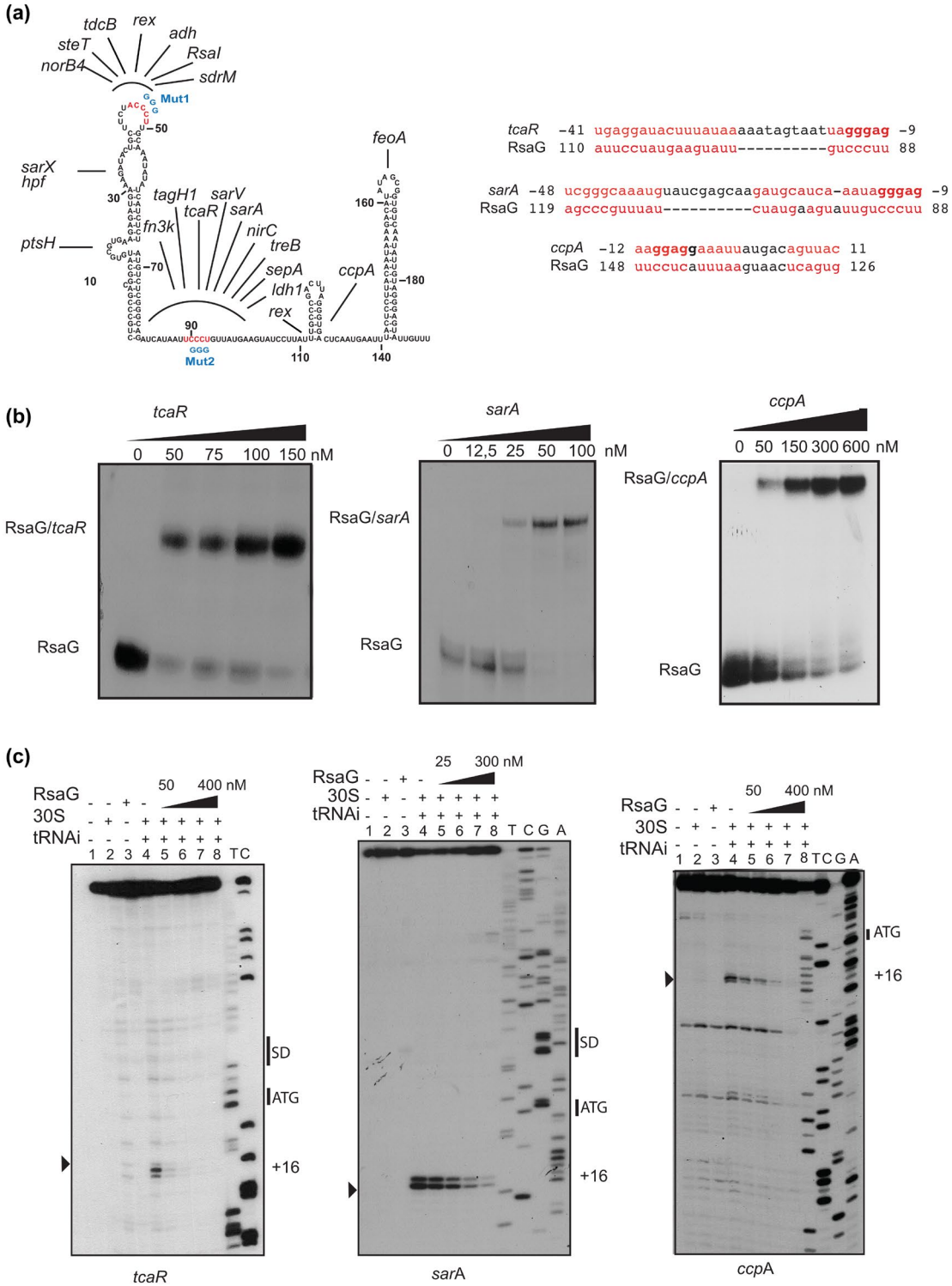


FIGURE 4 RsaG binds to *tcaR*, *sarA*, and *ccpA* mRNAs and inhibits their translation. (a) Secondary structure model of RsaG. The red cytosines have been substituted by guanines (in blue) in the RsaG mutants (mut1 or mut2). The predicted regions of RsaG complementary to its RNA targets are depicted. On the right side, three examples of basepairing interactions are given for *tcaR*, *sarA*, and *ccpA*. The Shine and Dalgarno sequences are in bold characters. (b) Electrophoretic mobility shift assays (EMSA) show the formation of the complex between RsaG and *tcaR*, *sarA*, and *ccpA* mRNAs. The 5′-end-labeled RsaG was incubated with increasing concentrations of cold mRNA (given in nM). (c) Toe-print assays showing RsaG effect on the formation of the ribosomal initiation complex of *tcaR*, *sarA*, and *ccpA* mRNAs. Lane 1: incubation control of mRNA alone; Lane 2: incubation control of mRNA with 30S subunits; Lane 3: incubation control of mRNA with RsaG; Lane 4: formation of the ribosomal initiation complex containing mRNA, 30S, and the initiator $tRNA^{fMet}$ (tRNAi); Lanes 5–8: formation of the initiation complex in the presence of increasing concentrations of RsaG: 50 (Lane 5), 100 (Lane 6), 200 (Lane 7), and 400 nM (Lane 8) for *tcaR* and *ccpA* and 25 (Lane 5), 100 (Lane 6), 200 (Lane 7), and 300 nM (Lane 8) for *sarA*. Lanes T, A, C, and G: sequencing ladders. The Shine and Dalgarno (*SD*) sequence, the start site of translation (ATG), and the toe-printing signals (+16) are indicated

Ndh, Hom). Besides, we have pulled out several polycistronic transcripts encoding transporters including the mechanosensitive ion channel MscL, the ABC transporters for K^+ and Mn^{2+}, KdpBAF and MntABC, respectively (Ando et al., 2003; Xue et al., 2011), the Pmt operon that exports the PSM toxins (phenol soluble modulins) causing host cell lysis (Chatterjee et al., 2013), and the multidrug efflux pumps SrdM-SepA. Finally, although RsaI is weakly expressed in the presence of G6P, it is among the best candidates enriched with RsaG (Bronesky et al., 2019). We also considered the two poorly enriched *ldh1* and *fn3K* mRNAs because *ldh1* encoding lactate dehydrogenase is repressed by Rex and activated by CcpA, whereas *fn3k* translation is repressed by RsaI (Table S1) (Bronesky et al., 2019).

We then verified that RsaG is able to form stable complexes with 39 copurified RNAs. Gel retardation assays were performed with a 5′-end-labeled RsaG incubated with increasing amounts of the potential target RNAs. The data showed that the majority of mRNA candidates (28 out of 39) formed stable basepairings with RsaG with binding affinities ranging from 50 to 600 nM (Table S1, Figure 4a and Figure S4). The binding strength was not correlated with the enrichment factors since the poorly enriched mRNAs *ldh1* and *fn3k* form also stable complexes with RsaG (Table S1). Eleven RNAs were not able to form stable complexes with RsaG in vitro, and among them, *glcU_2* and the hypothetical proteins HG001_01242, HG001_02210, and HG001_02520 were shown to be direct targets of the sRNA RsaI (Bronesky et al., 2019). These mRNAs were most probably pulled down with RsaI as their binding sites are different from RsaG (Bronesky et al., 2019). Surprisingly, among the first 20 most-enriched candidates, four mRNAs (*mscL*, *cspA*, *slyA_2*, and *arcC2*) did not interact efficiently with RsaG in vitro (Table S1 and Figure S4). Most probably that *arcC2* was pulled down together with *arcR* as both genes are located on the same operon. For the other mRNAs, either the structure of the full-length mRNAs was not correctly folded in vitro or that an unknown transacting factor might be required in vivo to promote complex formation.

The MAPS data did not reveal any mRNAs involved directly in the G6P catabolism supporting the hypothesis that RsaG would adapt the bacterial metabolism in response to G6P uptake.

2.5 | RsaG hinders ribosome binding on several mRNA targets

Among the top 20 candidates, stable basepairing interactions between RsaG and the ribosome-binding site (RBS) of several mRNAs were predicted using CopraRNA and IntaRNA (Wright et al., 2014), that is, *ccpA*, *sarA*, *arcR*, *tcaR*, and *ndh*. Likewise, RsaG is predicted to interact with the 5′UTR of several mRNAs, that is, *sarX*. The C-rich sequence in the interhelical region (C90–C92) of RsaG is complementary to the *SD* sequences of mRNAs encoding the biofilm repressor TcaR (Brandenberger et al., 2000; Jefferson et al., 2004) and the accessory regulator SarA (Cheung et al., 2008), whereas the nts 126–148 of RsaG are complementary to the RBS of *ccpA* mRNA encoding the catabolite control protein A (Seidl et al., 2009) (Figure 4a). RsaG binds efficiently to *tcaR*, *sarA*, and *ccpA* mRNAs (Figure 4b). Base substitutions in one of the C-rich motifs of RsaG (RsaG mut1, RsaG mut2) did not significantly alter the binding of RsaG mutants to *tcaR* and *sarA* mRNAs, but a noticeable effect on binding efficiency was observed with the double-mutant RsaG mut1/mut2 (Figure S5). Due to the presence of three redundant C-rich sequence motifs in RsaG, alternative pairings are possible (Figure 4a).

The consequence of the *SD* sequestration by RsaG on the formation of the initiation ribosomal complex was then addressed by toe-printing assays with *tcaR*, *sarA*, and *ccpA* mRNAs (Figure 4c). For the three mRNAs, the addition of the initiator $tRNA^{fMet}$ and of the *S. aureus* 30S subunits causes a pause of the reverse transcriptase at position +16, the so-called toe-print. The addition of increasing concentrations of RsaG strongly decreased the toe-print signal showing that RsaG is able to prevent the formation of the initiation ribosomal complexes. Hence, RsaG might act as a translational repressor. However, using antibodies against CcpA or against a flag-tagged SarA, we did not detect significant changes in the protein yields in the WT and mutant Δ*rsaG* strains grew in the presence of G6P (results not shown). The discrepancy between the in vitro and in vivo data suggested a possible hierarchical regulation of the targets in vivo.

2.6 | RsaG differentially alters the mRNA stabilities of *rex* and its regulated target *ldh1*

The mRNA encoding the redox transcriptional repressor Rex was highly enriched with RsaG in MAPS. Basepairings were predicted between RsaG and several distant regions of *rex* (Figure 5a,b). The 5′-end of *rex* (nts −105 to −98) can form several basepairings with nts 117 to 138 of RsaG (C site, Figure 5a). Besides, alternative interactions are predicted to occur between either the apical loop (nts 41 to 49, A site) or nts 102 to 117 (B site) of RsaG with the *rex* coding sequence (nts 185–194) (Figure 5a). Based on these predictions, we have constructed two RsaG mutants carrying base substitution (RsaG mut3, RsaG mut4) to alter the A and B binding sites, respectively. In vitro binding assays showed that RsaG formed a stable complex with *rex* mRNA (around 150 nM), and that only mutation in the B region of RsaG completely eliminates the binding to *rex* mRNA (Figure 5b). We also introduced R2 substitutions (Figure 5a) in the *rex* coding region, which partially restore basepairing complementarity with RsaG mut3 or RsaG mut4. As expected, RsaG cannot bind to *rex* containing the R2 substitution, but surprisingly, neither RsaG mut3 nor RsaG mut4 compensates the R2 mutations in *rex* mRNA (Figure 5b). Lead-induced cleavage was performed to monitor the effect of *rex* mRNA binding on 5′-end-labeled RsaG (Figure S6b). Significantly reduced cleavages were observed in the 5′ hairpin loop (nts 40 to 51) and in the interhelical region (nts 98 to 110) of RsaG (Figure S6b). Although we did not manage to find appropriate mutations to fully characterize the basepairing schemes, our data strongly suggested that complex formation involves two distant regions of both RNAs.

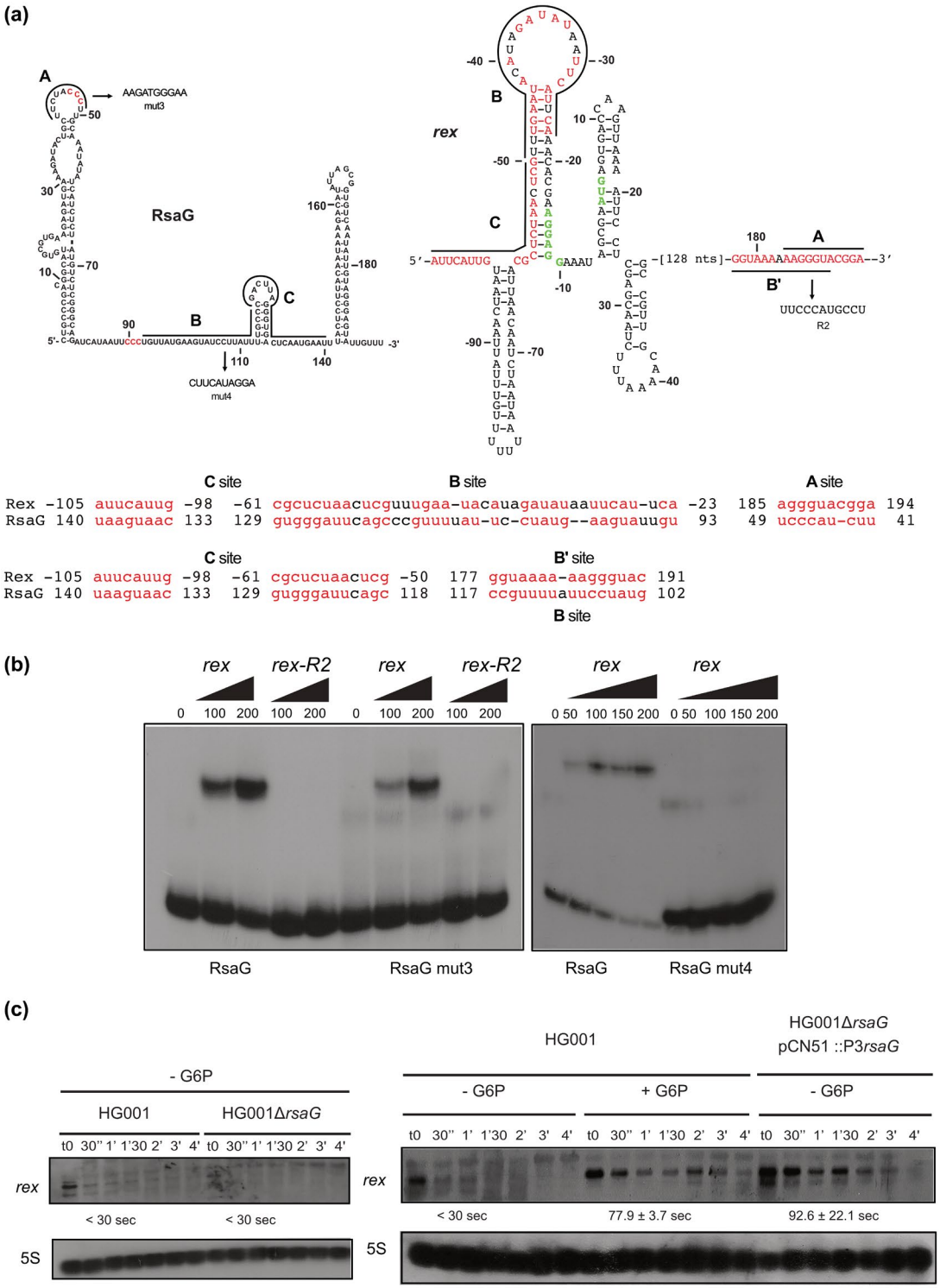


FIGURE 5 Prediction of interaction sites between RsaG and *rex* mRNA. (a) Secondary structures of RsaG (left) and *rex* mRNA (right). The potential interaction sites between *rex* and RsaG are named A, B, B′, and C. The Shine and Dalgarno sequence of *rex* and the translational start (AUG) are depicted in green. Substituted nucleotides in RsaG mut3, mut4, and *rex*-R2 are indicated. Below, two possibilities of interaction schemes between RsaG and *rex* are given with the different sites. (b) Electrophoretic mobility shift assay showing the formation of the complex between RsaG, RsaG mut3, RsaG mut4, or RsaG mut3/4 and *rex* mRNA. The 5′-end-labeled RsaG or its mutated forms were incubated with increasing concentrations of cold wild-type *rex* mRNA (*rex*) or of the cold mutant *rex* mRNA carrying R2 substitution (*rex*-R2) (given in nM). (c) Measurements of the half-lives of *rex* mRNA in HG001, HG001Δ*rsaG* (deletion of rsaG) and HG001Δ*rsaG*::pCN51::P3*rsaG* (overexpressing RsaG) strains. Bacterial cultures were grown in an MHB medium containing or not glucose-6-phosphate (±G6P) and treated with rifampicin at 4 hr of growth at 37°C. Total RNA was extracted at various times (from 30 s to 4 min). RsaG and 5S rRNA were probed to quantify the yield of RNAs in each lane. Calculated half-lives (normalized to 5S rRNA) are shown beneath the autoradiography and are the average of two experiments

Based on these data, we cloned *rex* mRNA including the whole 5′UTR (103 nts) and 207 nts of its coding sequence in frame with *lac*Z under the control of the strong promoter P*rpo*B and *rsaG* was cloned under the constitutive *bla*Z promoter. The synthesis of ß-galactosidase was analyzed in the Δ*rsaG*-mutant strain transformed with the plasmid carrying the *lac*Z reporter alone or with the plasmid containing the *lacZ* and *rsaG*. The ß-galactosidase activity was reproducibly increased two-fold in cells expressing RsaG (Figure S6c). Because RsaG binds to the 5′-end of *rex* and to a hairpin region, which partially sequesters the *SD* sequence, we wondered whether RsaG might facilitate ribosome recruitment on *rex* mRNA. However, using toe-printing assays, the addition of increasing concentrations of RsaG did not enhance the formation of the ternary ribosomal complex including *rex* mRNA, the initiator tRNA, and the 30S subunit (Figure S6d). We then analyzed whether RsaG might impact *rex* mRNA stability. Using rifampicin assays, the half-life of *rex* mRNA was measured in the WT strain HG001, the deleted strain of *rsaG* (HG001Δ*rsaG*) grown in the presence or absence of G6P, and in the deleted strain of *rsaG* complemented with a plasmid overexpressing RsaG under the P3 promoter (HG001Δ*rsaG* pCN51::P3*rsaG*). In the absence of G6P, the half-life of *rex* mRNA was <30 s in the WT and Δ*rsaG*-mutant strains while the overexpression of RsaG in the mutant strain enhanced the half-life to 90 s (Figure 5c). In the presence of G6P, the half-life of *rex* reached values above 1 min in the WT strain (77.9 s) and in the mutant strain overexpressing RsaG, whereas *rex* was poorly detected in the mutant Δ*rsaG* strain (Figure 5c and Figure S6a). These data suggested that enhancing the yield of RsaG stabilizes *rex* mRNA. Because *rex* mRNA presented several hairpin structures in the 5′-end, we analyzed whether the endoribonuclease III might be involved in the degradation pathway of the mRNA. In the absence of G6P, the half-life of *rex* mRNA was indeed enhanced two-fold compared with the WT strain, and this effect was independent of the accumulation of RsaG (Figure S6e).

Interestingly, the main repressed target of Rex, the lactate dehydrogenase Ldh1 was found in MAPS although with a threshold below two-fold. Therefore, RsaG would act on *ldh1* expression either directly or indirectly through the stabilization of *rex* mRNA. Prediction of pairing between *ldh1* and RsaG indicated that the large interhelical unpaired region (nts 88 to 109) of RsaG might form basepairings with the RBS of *ldh1* mRNA with the *SD* (−14 to +2) and with its coding region (+15 to +20) (Figure 6a). Base substitution of the second C-rich motif of RsaG (RsaG mut2) did not impair binding with *ldh1* most probably due to the formation of alternative pairings involving the other C-rich motifs. Because we failed to design mutations that disrupt *ldh1* mRNA-RsaG pairings, an indirect approach based on a competition experiment was performed using a single-strand DNA oligonucleotide complementary to 34 nucleotides of *ldh1* mRNA (from −14 to +20). Using gel retardation assay, this probe was sufficient to compete efficiently with the mRNA (Figure 6b). Surprisingly, using toe-printing assays, RsaG binding to *ldh1* mRNA had only a minor effect on the formation of the ternary ribosomal complex (Figure S7a). The stability of *ldh1* mRNA was measured using rifampicin treatment after 4 hr of growth in the absence or the presence of G6P from HG001 strain and from the Δ*rsaG* deleted strain complemented with a vector expressing RsaG. Quantification of the data revealed that the half-life of *ldh1* was strongly decreased from around 10 min to 2–3 min when RsaG expression was strongly induced (Figure 6c).

Our data suggested that RsaG acts as a posttranscriptional regulator to modulate positively or negatively the stability of target mRNAs. Through the regulation of *rex* and *ldh1*, RsaG would avoid glycolytic fermentation in favor of amino acid catabolism, when G6P is consumed.

2.7 | RsaI interferes with the binding of specific targets of RsaG

We previously identified that the second G-rich (nts 20–23) motif of RsaI binds to RsaG (Figure 7). The binding of RsaG did not prevent RsaI to interact with its target mRNA *glcU_2* (Bronesky et al., 2019). Using base substitutions in RsaG, we could decipher that only the C-rich motif in the apical loop of RsaG (nts 46–48) forms stable basepairings with RsaI in vitro (Figure 7a,b). The consequence of RsaG-RsaI interaction on several RsaG targets recognition was then monitored by gel retardation assays. Radiolabeled RsaG was mixed together with *sarA* mRNA at a concentration sufficient to bind most of RsaG molecules. The addition of an increasing amount of RsaI induces the appearance of a supershift indicating the formation of a ternary complex RsaI–RsaG–*sarA* (Figure 7c). Conversely, when the same experiment was performed between RsaG, RsaI, and *rex* or *steT* mRNAs, a competition was clearly observed (Figure 7d,e). These data are well correlated with the fact that *sarA* and RsaI bind to two distinct regions of RsaG, whereas RsaI, *steT*, and *rex* have overlapping interaction sites.

In the list of targets enriched by MAPS, we identified several potential targets common to both RsaI and RsaG, that is, *glcU_2*, *icaR*, HG001_02210 and HG001_02520, *tdcB*, *treB*, and *fn3K* (Table S1). Most of them did not bind directly to RsaG in vitro (except *fn3k*, *treB*, and *tdcB*), and no significant basepairing sites were predicted suggesting that they were purified through RsaI (Figure S4). For *fn3K*, RsaI was described as a translational repressor since the interaction site covered the RBS (Bronesky et al., 2019). In contrast, the second C-rich motif (nts 90 to 93) of RsaG interacted within the *fn3K* coding region (Figure S7b). Indeed, gel retardation assays showed that the 5′-end-labeled RsaG did not hybridize to an in vitro transcribed RNA corresponding to the 5′UTR of *fn3K* but was able to form a stable complex with an in vitro transcribed RNA including the open-reading frame of *fn3K* (Figure S7b). The result was further validated using competition experiments with an oligonucleotide of 17 nucleotides (nts 268 to 285 complementary to *fn3k*), which efficiently competes with *fn3K* to bind RsaG in vitro (Figure S7b). The stability of *fn3k* mRNA was measured using rifampicin treatment after 4 hr of growth. Total RNAs were

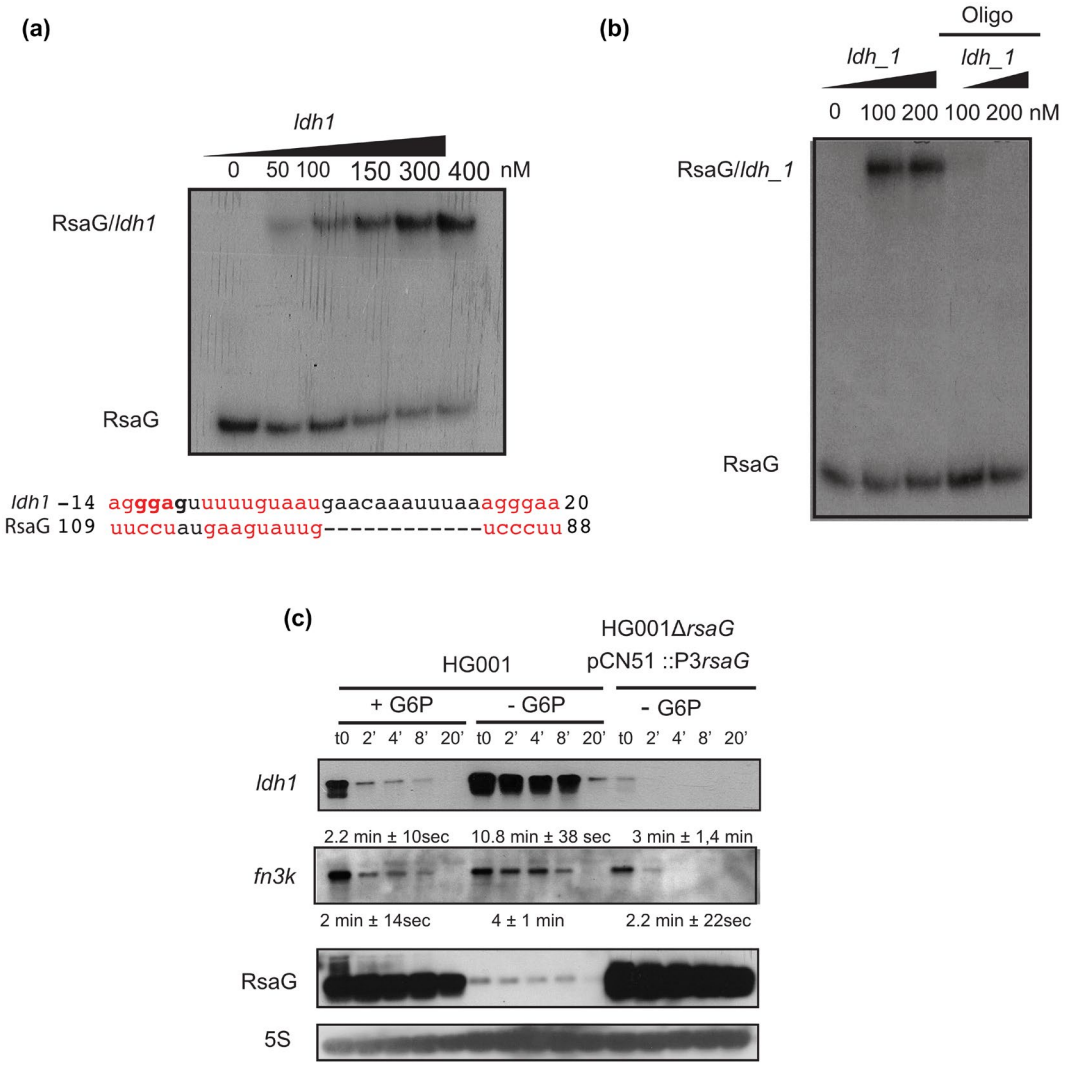


FIGURE 6 RsaG modulates the stability of *ldh1* and *fn3K* mRNAs. (a) EMSA shows the formation of the complex between RsaG and *ldh1* mRNA. The 5′-end-labeled RsaG was incubated with increasing concentrations of cold mRNA (given in nM). Below the gels, the predicted interaction site is depicted. The Shine and Dalgarno (*SD*) sequence is in bold characters. (b) Electrophoretic mobility shift assay showing the formation of the complex between RsaG and *ldh_1* and competition experiment performed with an oligonucleotide encompassing the region from −14 to +20 of *ldh_1*. The 5′-end-labeled RsaG was incubated with increasing concentrations of cold mRNA (in nM) or with 200 nM of oligonucleotide (Oligo). (c) Measurements of the half-lives of *ldh1* and *fn3K* in wild-type HG001 and mutant HG001Δ*rsaG*::pCN51::P3*rsaG* strains. Bacterial cultures were grown in an MHB medium containing or not glucose-6-phosphate (±G6P) treated with rifampicin at 4 hr of growth at 37°C. Total RNA was extracted after 2, 4, 8, and 20 min. Calculated half-lives are shown beneath the autoradiography and are the average of two experiments (see legend above)

extracted from HG001 strain in the absence or the presence of G6P, and the Δ*rsaG* deleted strain complemented with a vector expressing RsaG. The data showed that the expression of RsaG significantly destabilizes *fn3K* as the half-life decreases from 4 to 2 min (Figure 6c). Another common target between RsaG and RsaI is *tdcB* mRNA whose predicted binding sites are different. Gel retardation assays performed with 5′-end-labeled RsaG mixed together with *tdcB* mRNA and an increasing amount of RsaI showed a supershift indicating the formation of a ternary complex RsaI–RsaG–*tdcB* (Figure 7f).

In summary, RsaI affects the binding of only part of RsaG mRNA targets.

2.8 | Differential evolution of the *uhpT* 3′UTRs containing RsaG in *Staphylococcaceae*

The *uhpT* gene sequence is highly conserved in the *Staphylococcaceae* family (between 83% and 91% similarity). However, previous studies indicated that RsaG was only conserved in *S. aureus* (Geissmann et al., 2009). As we have demonstrated that RsaG is derived from the long *uhpT* 3′UTR, we analyzed the *uhpT-rsaG-hptRS* locus conservation among *Staphylococcus* species using Blastn. The results showed that *hptRS* genes on the complementary strand were conserved as well as *uhpT* (Figure 8a). In contrast, the *uhpT* 3′UTR containing RsaG appears to be only conserved in *S. argenteus* and

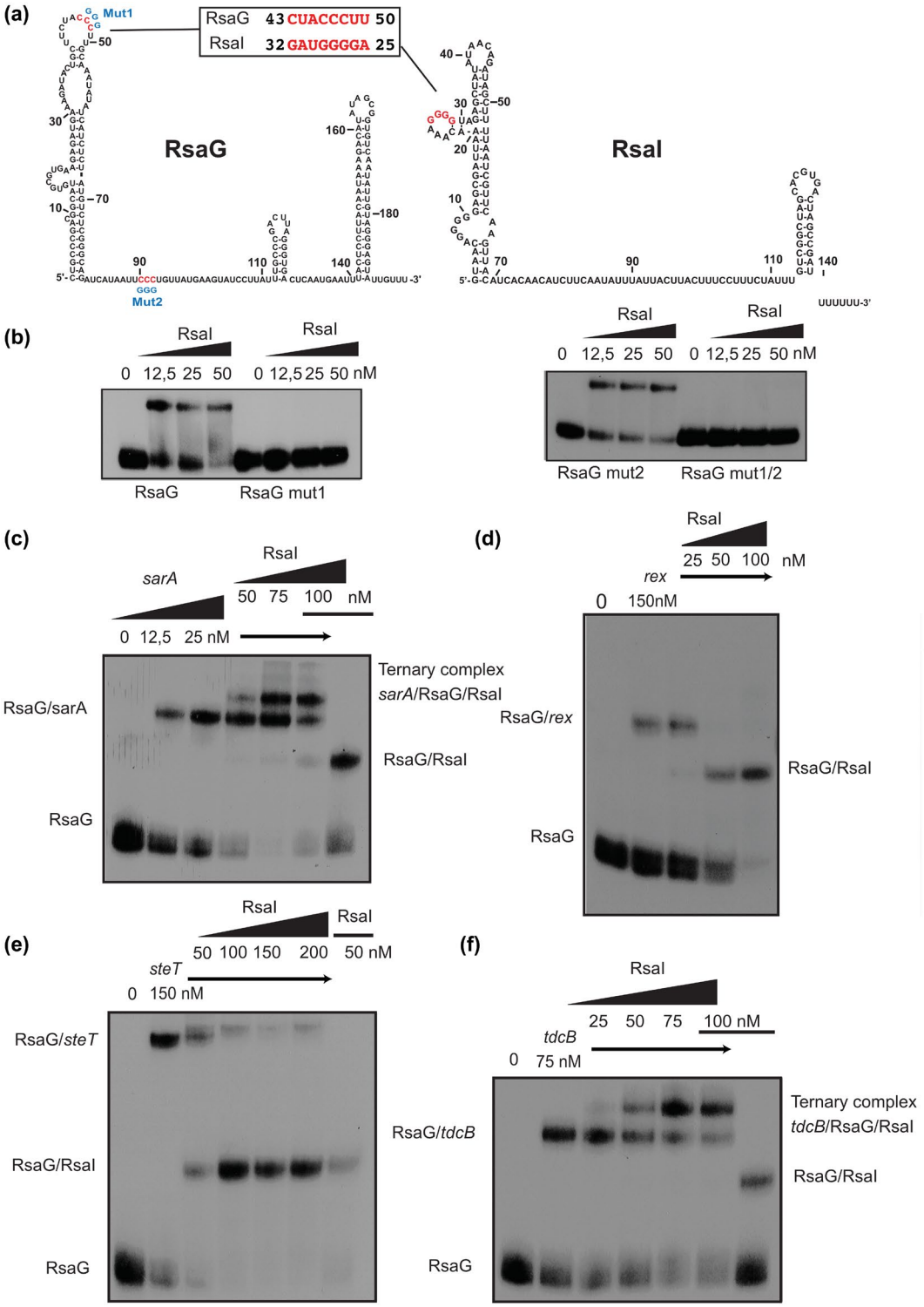


FIGURE 7 RsaI affects the binding of several targets to RsaG. (a) Secondary structure models of RsaG and RsaI. The cytosines in red have been substituted by guanines (in blue) in the RsaG mutants (mut1 or mut2). The basepairing interactions between RsaI and RsaG are depicted in the insert. (b) Gel retardation assays showing the formation of the complex between RsaG and RsaI. The 5′-end-labeled wild-type RsaG (RsaG), RsaG mutant 1 (RsaG mut1), RsaG mutant 2 (RsaI mut2), and the RsaG double mutant 1 and 2 (RsaG mut1/2) were incubated with increasing concentrations of RsaI (given in nM). (c) Ternary complex formation between RsaG, *sarA*, and RsaI. The 5′-end-labeled RsaG was incubated with increasing concentrations of *sarA* mRNA alone or with increasing concentrations of RsaI in the presence of 25 nM of *sarA*. The various complexes are notified on the side of the autoradiography. (d) Complex formation between RsaG, *rex*, and RsaI. The 5′-end-labeled RsaG was incubated with increasing concentrations of *rex* mRNA alone or with increasing concentrations of RsaI in the presence of 150 nM of *rex*. (e) Complexes formation between RsaG, *stet*, and RsaI. The 5′-end-labeled RsaG was incubated with increasing concentrations of *steT* mRNA alone or with increasing concentrations of RsaI in the presence of 150 nM of *steT*. (f) Ternary complex formation between RsaG, *tdcB*, and RsaI. The 5′-end-labeled RsaG was incubated with increasing concentrations of *tdcB* mRNA alone or with increasing concentrations of RsaI in the presence of 75 nM of *tdcB*

S. schweitzeri, which are closely related to *S. aureus*, whereas the sequence and the size considerably diverged in *S. epidermidis* and *S. simiae* (Figure 8a). This was in agreement with previous results that showed an evolutionary bias within *Staphylococcus* 3′UTRs (Menendez-Gil et al., 2020).

Using a specific probe against *S. aureus* RsaG sequence, we identified a transcript in *S. argenteus* and *S. schweitzeri* from total RNAs extracted from bacteria cultures performed in the presence of G6P. Hence, RsaG is also produced in these species despite some nucleotide differences (Figure 8b). In contrast, using a specific *S. epidermidis* RsaG probe, we were unable to detect a processed 3′UTR from *uhpT* mRNA in *S. epidermidis*, even if *uhpT* mRNA was weakly expressed in the presence of G6P since the mRNA was only detected by RT-PCR. Interestingly, when using a specific probe against *S. simiae* 3′UTR, a signal corresponding to a larger processed 3′UTR was visualized (Figure 8b). The 5′-end of the processed band was then determined using reverse transcription assays with total RNAs extracted from *S. simiae* cultures (Figure 8c). The 5′-end localized 195 bp downstream of the stop codon of *uhpT* resulting to an apparent 3′UTR-derived sRNA of 726 nts in *S. simiae*, which was significantly larger in comparison with *S. aureus* (294 nts). As the HptR box is rather conserved, the induction of the orthologous *S. simiae* RsaG in the absence or the presence of G6P was analyzed (Figure 8d). Unexpectedly, the accumulation of the 3′UTR was independent of the presence of G6P. Although the sequence of the 3′UTR of *S. simiae uhpT* has diverged, the maturation process might be conserved. Prediction of the secondary structure of *S. simiae* RsaG revealed a hairpin structure at its 5′-end, which might block the progression of RNases J1/J2.

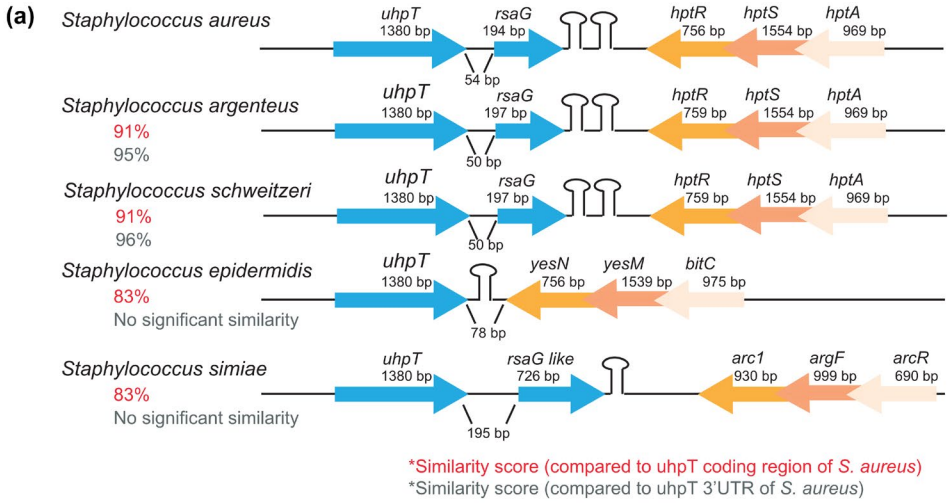
3 | DISCUSSION

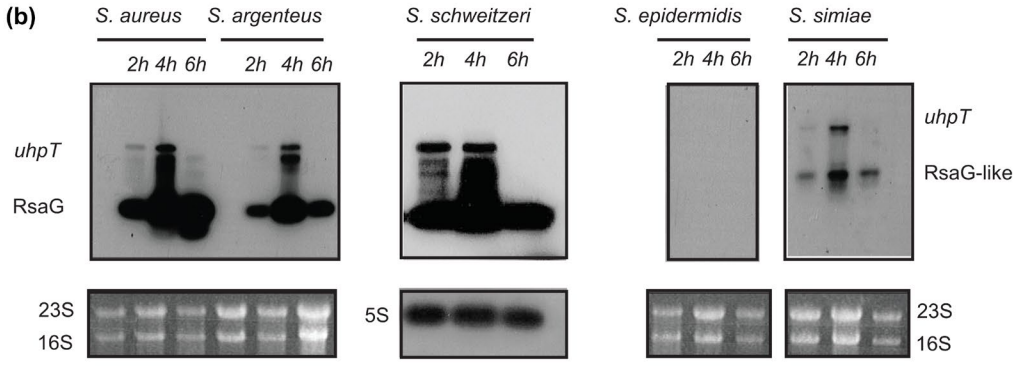
In this study, we have identified RsaG as a new 3′UTR-derived sRNA processed from *uhpT* mRNA encoding the transporter of G6P. The transcription of *uhpT* is activated by the TCS HptRS, which senses the external concentration of G6P. In *S. aureus*, G6P is critical when the bacteria is internalized into host cells because a mutant strain deprived of *hptRS* is not able to survive (Park et al., 2015). However, RsaG is not essential for growth when G6P is used as the sole carbon source. We also showed that *uhpT* mRNA and RsaG are highly induced by G6P when *S. aureus* is internalized into host cytosol and in the presence of pulmonary or intestinal mucus (Figure 2; Garzoni et al., 2007). Our data suggested that RsaG might expand the action of HptRS/UhpT by fine-tuning the cell metabolism in response to G6P uptake. RsaG is different from *E. coli* SgrS, which protects the cell against glucose-phosphate stress and from the depletion of glycolytic intermediates (Richards et al., 2013). Interestingly, a hierarchical regulation mediated by SgrS has been demonstrated (Bobrovskyy et al., 2019; Poddar et al., 2021). At the low level, SgrS represses *ptsG*-encoded glucose transporter and activates a sugar phosphatase, which promotes dephosphorylation and efflux of phosphosugars. At high levels, SgrS regulates other less essential targets to switch the cellular metabolism and to use other available carbon sources. Our study suggests that *S. aureus* RsaG inactivates the lactate fermentation pathway by activating Rex synthesis and by repressing CcpA synthesis using different mechanisms of the regulation (Figure 9).

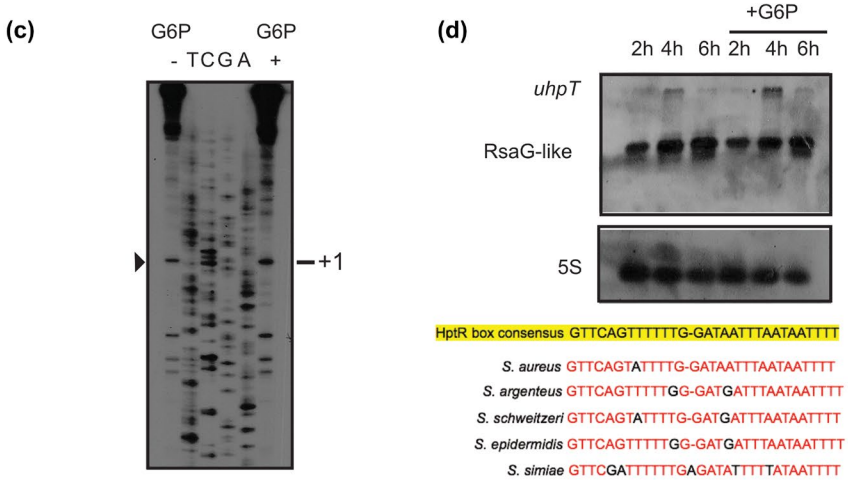
3.1 | The dual ribonuclease J1/J2 generates the 3′UTR-derived RsaG sRNA

In Gram-negative bacteria, 3′UTRs are reservoirs of sRNAs acting in trans after RNase E cleavage (Eisenhardt et al., 2018; Kim et al., 2014; Wang et al., 2020). In *S. aureus*, RsaC accumulates in response to Mn starvation and is processed by RNase III from the polycistronic mRNA encoding the MntABC transporter. Here, we describe the first example of an sRNA for which RNases J1/J2 are required for its processing from the *uhpT* mRNA, whereas the mRNA encoding *uhpT* is rapidly degraded. Hence, RNases J1/J2 are not only essential for stress responses by controlling mRNA degradation but they also contribute to generate sRNA from mRNAs. Very recently, a rather similar degradation mechanism was described in the maturation of the T-box riboswitch in the 5′UTR of the *metICFE-mdh* operon (Wencker et al., 2021). Indeed, in the absence of methionine and after cleavage of the met leader by RNase III, the RNases J1/J2 mediate degradation of the mRNA from the 5′-end generating more stable transcripts toward the 3′-end. We postulate that specific structural features of the RNAs may protect them from degradation. Most likely, the 5′ hairpin structure of RsaG might certainly block the action of the exoribonuclease activity (Figure 3d).

Intriguingly, RsaG is the fifth example of a 3′UTR-derived sRNA issued from an mRNA encoding metabolically active transporters. In *S. aureus* RsaC is processed from the staphylococcal MntABC transporter of manganese and represses SodA activity, which needs Mn as a cofactor (Lalaouna, Baude, et al., 2019). In *E. coli*, NarS is generated from the NarK transporter of nitrate in anaerobiosis. Nitrate is then reduced in nitrite by NarG, and to limit nitrite toxicity, NarS is transcribed together with *narK* to reduce the expression of *nirC* encoding the nitrite transporter (Wang et al., 2020). The sRNA MalH derived from the processing of the 3′UTR of the polycistron *malEFG* encoding the maltose ABC transporter stimulates the use of alternative carbon sources in the presence of maltose (Bar et al., 2021; Iosub et al., 2021). Finally, in *Vibrio cholera*, OppZ is issued from the OppA oligopeptide transporter and binds to the second gene of the *oppABCDF* operon-mediating repression of OppB synthesis (Hoyos et al., 2020; Lalaouna, Baude, et al., 2019; Wang et al., 2020). Overall, sRNAs generated from mRNAs contribute to the same metabolic pathway or help bacteria to choose the most appropriate way to adapt or protect the bacteria to different metabolic niches in response to the effector entry. It is tempting to propose that such dual partners (transporter and sRNA) are more widespread than expected. Although RsaG is cotranscribed with the *uhpT* transcript, we were unable to show its direct role in G6P catabolism. In other pathogenic bacteria, such as *Shigella flexneri* and *Listeria monocytogenes*, their corresponding *uhpT* mRNAs do not possess large 3′UTRs but are highly overexpressed during internalization







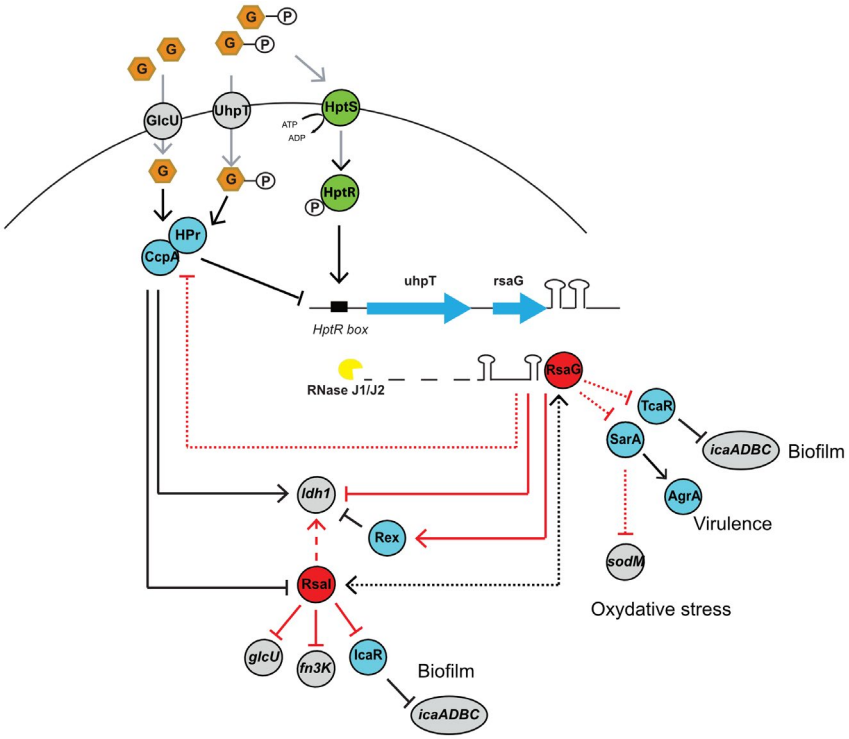
(Chico-Calero et al., 2002; Runyen-Janecky & Payne, 2002). In Listeria, *uhpT* is regulated by the major regulator PrfA, and the deletion mutant of *uhpT* did not proliferate intracellularly or survive in a murine model of infection (Chico-Calero et al., 2002). Although the role of UhpT in staphylococcal virulence requires further investigation, we hypothesized that the accumulation of RsaG would regulate additional metabolic functions when *S. aureus* is internalized into the microaerophilic cytoplasm of host cells or in the presence of mucus-secreting cells.

3.2 | RsaG expands the regulon of HptRS

In order to scrutinize the functional impact of RsaG, we performed MAPS in the presence of G6P to mimic-inducing conditions. Gel retardation assays showed in vitro that RsaG recognized a high number of RNAs with various binding affinities, which illustrates a possible hierarchical regulation. However, binding affinities in vivo might be different as the sRNA is expected to bind the mRNA

FIGURE 8 The 3′UTR of uhpT mRNA is not conserved in all *Staphylococcaceae*. (a) Genomic context of *uhpT* locus in *Staphylococcus aureus* HG001, *S. argenteus* MSHR11, *S. schweitzeri* FSA084, *S. epidermidis* ATCC35983, and *S. simiae* CCM7213T. Percentages given in black represent the similarity score of *uhpT* coding region and in red, the similarity score of *uhpT* 3′UTR compared with *S. aureus*. The similarity score was obtained by Blast (NCBI). (b) Northern blot analysis of RsaG in *S. aureus*, *S. argenteus*, *S. schweitzeri*, *S. epidermidis*, and *S. simiae*. Total RNA was extracted at 2, 4, and 6 hr of growth in a BHI medium containing 0.5% glucose-6-phosphate (G6P). RsaG from *S. aureus*, *S. argenteus*, and *S. schweitzeri* was detected with a specific probe against *S. aureus* RsaG sequence, whereas for *S. epidermidis* and *S. simiae*, the probes were designed against their respective sequences. We used as internal loading controls, 16S and 23S rRNAs, which were revealed by ethidium bromide from the same gel. 5S rRNA (5S) was also used as a loading control and was revealed using a 5′–end-labeled oligonucleotide. However, for this control, we used aliquots of the same RNA preparations but the migration of the samples was performed in parallel to the experiments on a separate agarose gel. (c) Primer extension performed on total RNA using a 5′-radiolabeled oligonucleotide complementary to *S. simiae uhpT* 3′UTR sequence. Total RNA was extracted from *S. simiae* at 4 hr of growth in a BHI medium with (+) or without (–) 0.5% G6P. The primer is located at nucleotides +108 to +125 (from the +1 of the putative RsaG). T, C, G, and A correspond to sequencing ladders. (d) Northern blot analysis of RsaG in *S. simiae*. Total RNA was extracted at 2, 4, and 6 hr of growth in a BHI medium without or with 0.5% G6P. Multiple sequence alignment of the HptR box in *S. aureus*, *S. argenteus*, *S. schweitzeri*, *S. epidermidis*, and *S. simiae* compared with the consensus sequence underlined in yellow (Yang et al., 2016) using Blast (NCBI) with default settings

FIGURE 9 Model of RsaG regulation networks in *Staphylococcus aureus*. The two-component system HptRS is shown in green and the transcriptional regulatory proteins are in blue. Red bars and arrows are for repression and activation at the posttranscriptional level, respectively. Black bars and arrows are for repression and activation at the transcriptional level, respectively. The dotted lines are for the regulatory mechanisms, which require additional experimental data. The exoribonuclease activity of RNases J1/J2 is shown in yellow



cotranscriptionally and transacting factors might also modulate the mRNA structure and facilitate the pairings (Reyer et al., 2021). Among the target RNAs, none of them are directly linked to the G6P catabolism per se but some of them are involved in carbohydrate-dependent metabolic pathways (*ccpA*, *ldh1*) and to redox homeostasis (*rex*). It is known that in the presence of glucose, CcpA repressed *uhpT* and the effect was more pronounced in the late exponential phase of growth (Reed et al., 2018). In contrast, HptRS activated *uhpT* in the presence of G6P (Park et al., 2015). Comprehensively, CcpA and HptRS share the same regulon but function in an opposite manner to respond to changes in the availability of glucose or G6P (Reed et al., 2018). Hence, *uhpT* activation by HptRS would be required to alleviate the CcpA inhibition of *uhpT* in the presence of G6P. Remarkably, we also identified *ptsH* as a potential target of RsaG. This mRNA encodes HPr, an activator of CcpA, which is dependent on G6P for its phosphorylation and activation. Through the regulation of HPr and CcpA, RsaG would add another level of regulation to favor G6P uptake in detrimental to glycolysis and carbon catabolite repression (Figure 9). Such regulation is expected to be transient as CcpA is required for glycolysis, which might explain why we could not visualize a significant impact of RsaG on CcpA levels. Interestingly, MAPS analysis also revealed that several CcpA-regulated genes, such as *ldh1*, *ald1*, *adh*, *treB*, *buA1B (alsS/D)*, and *hemC-hemX*, might also be under RsaG control (Table S1 and Seidl et al., 2009). Although CcpA inhibits transcription of *ald1*, *adh*, and *budA1B*, it activates Ldh1 activity. It is tempting to propose that RsaG accumulation reroutes the bacterial metabolism leading to the inhibition of lactate fermentation while amino acid catabolism, acetoine synthesis, and alcohol fermentation would be activated (Figure 9).

Another important target of RsaG is *rex* mRNA, which senses the bacterial redox through changes in the NADH-NAD+ ratio of the bacteria. This ratio can be changed dramatically under different metabolic statuses with or without a change in oxygen viability. For instance, NADH increases during glycolysis, during the activation of the TCA cycle, and under anaerobic conditions. It is known that Rex-NADH dissociates from its promoter allowing transcription of numerous genes involved in electron transport, in nitrogen and anaerobic metabolism (*nirC*, *narG*, *arcA*, *pflB*, *adhE*, *adh*, *ldh1*, and *ald1*) (Pagels et al., 2010). Interestingly, among the potential direct targets, RsaG can also interact with *nirC*, *arcA*, *adh* mRNAs (Table S1) and in addition, induces rapid degradation of *ldh1* mRNA (Figure 6c). Through the activation of *rex* and the repression of *ldh1*, *nirC*, and *adh*, RsaG would modulate the redox status.

Moreover, RsaG binds to several mRNAs encoding transcriptional factors of the Sar family. Particularly, SarA is one of the most important transcriptional activators of the quorum sensing operon *agr* and facilitates the binding of the response regulatory protein AgrA. We showed that RsaG hinders the binding of the ribosome to *sarA* mRNA. Interestingly, SarA also inhibits the transcription of *sodM* which is required for oxidative stress resistance in presence of methyl viologen or diamide (Ballal & Manna, 2009; Seidl et al., 2009). Additionally, when staphylococcal cultures were starved in Mn, the atypical sRNA RsaC blocked translation of *sodA*, an Mn-dependent superoxide dismutase, whereas SodM was enhanced most probably because RsaC inhibited *sarA* translation (Lalaouna, Baude, et al., 2019). Hence, RsaG might be a second sRNA, which links oxidative stress to virulence, when G6P is used as the carbon source. RsaG interacted also with two other mRNAs encoding SarV, an inducer of autolysis, and SarX, which is involved in biofilm formation. Although the mechanism of regulation is awaiting further experimental evidence, in silico predictions suggested that RsaG would bind close to the RBS of these two mRNAs (Table S1).

3.3 | RsaG has two functional domains differently modulated by another sRNA

Our data showed that RsaG contained two functional domains, including the 5′ hairpin loop and the interhelical unpaired region, both containing C-rich motif. However, we had difficulties to design mutations in RsaG to better assign the basepairing schemes with its various targets indicating that alternative pairings were taking place. A similar observation was done in *Enterobacteriaceae* for the regulation of the *cycA* mRNA, which is repressed by the sRNA GcvB. At least five binding sites were predicted and none of them were validated in vivo suggesting redundancy between multiple regions of GcvB to *cycA* (Lalaouna, Eyraud, et al., 2019). Nevertheless, the first C-rich motif was demonstrated as the main site for RsaI, an sRNA which is repressed by CcpA, and which is activated when glucose is consumed (Bronesky et al., 2019). RsaI favored glucose fermentation in lactate by indirectly inducing *ldh1* mRNA (Bronesky et al., 2019). Because RsaI competes with *rex* mRNA for RsaG binding (Figure 7), *rex* activation would thus be impaired leading to Ldh1 activation.

The second C-rich motif located in the interhelical region of RsaG contains the binding sites for *sarA*, *tcaR*, *nirC*, *ldh1* and two common targets of RsaI, *treB* and *fn3K*. In vivo, *fn3K* and *ldh1* mRNAs are degraded faster when RsaG is present at high levels. The fructosamine 3-kinase (*fn3k*) plays a role in protein deglycation by phosphorylating preferentially proteins bound-ribulosamines and erythrulosamines but not fructosamines in bacteria. It has been suggested that bacterial fructosamine kinases inactivate exogenous toxic compounds like the aminoglycosides or macrolides, but the internal substrate remains unknown (Gemayel et al., 2007). The interaction of RsaG or RsaI to *fn3K* mRNA leads, respectively, to mRNA degradation when G6P is present or inhibition of translation in the absence of glucose. At least in silico, the binding sites of RsaI and RsaG on *fn3K* mRNAs are different, suggesting that RsaI would modulate only part of the functions of RsaG. Similarly, in vitro, RsaI does not affect the binding of *tdcB*, another common target, which interacts with the apical loop of RsaG or of *sarA*, which does not interact with RsaI (Figure 7).

Our data suggested that the interaction between RsaG and RsaI might contribute to hierarchical and temporal regulation in order to promote efficient and dynamic cell responses to G6P entry. Quantitative analysis will be certainly required to better decipher the roles of RsaG and the regulatory priority for its targets as it was demonstrated for SgrS during glucose-phosphate stress (Bobrovskyy et al., 2019).

3.4 | RsaG study led to evolutionary considerations

A recent genome-wide comparative analysis of orthologous mRNAs from *Staphylococcus* highlighted a high diversity in the length and sequence of the 3′UTRs (Menendez-Gil et al., 2020). Using chimeric constructs carrying various orthologous 3′UTRs, variation in protein synthesis of the corresponding gene was observed, suggesting that these regions contribute to differentially regulate gene expression by a species-specific mechanism (Menendez-Gil et al., 2020). This hypothesis is also in agreement with the role of 3′UTRs in eukaryotes, which contributed to the divergence of species by evolving regulatory elements (Cheng et al., 2009). It is intriguing that RsaG is conserved only in closely related species as *S. argenteus* and *S. schweitzeri*, whereas *uhpT* is universal. Interestingly, in the ape pathogen *S. simiae*, we detected a long (726 nucleotides) and stable RNA probably processed from the 3′UTR of *uhpT* mRNA. The sequence is not homologous to RsaG and RNA accumulation does not depend on the presence of G6P. As UhpT mediates the exchange of external hexose-6-phosphate, it remains to be explored what is the preferred carbon source that is sensed by UhpT in *S. simiae*. However, the 3′UTR contains several repeats with C-rich motifs, and only interaction sites were predicted between a C-rich motif and a region upstream of the *SD* of *ccpA*. We suggest that the acquisition of RsaG has evolved together with the acquisition of *Staphylococcus* pathogenesis in order to facilitate a better adaptation/protection of the

bacteria during infection. We also do not exclude that in the distant staphylococcal species, other sRNAs encoded from the different locus of the genome might play similar functions as RsaG.

Another level of complexity was added by a recent reanalysis of ribosome profiling data that identify several sRNAs with the potential capacity to encode peptides (Sorensen et al., 2020). Among these candidates, RsaG was suspected to contain an open-reading frame leading to a 25 amino acid long peptide although experimental validation is still missing. If RsaG is endowed with coding properties, experiments will be necessary to decipher the function of the peptide, to analyze whether a temporal factor regulates the dual functions of RsaG, and whether the translation event might interfere with its regulatory properties. It will be essential to demonstrate whether the two functions act in the same pathway as SgrS in enterobacteria, which protects the cell against glucose-phosphate stress, or whether the two functions act in an independent pathway as *S. aureus* RNAIII (Raina et al., 2018).

In conclusion, the acquisition of glucose transporters such as GlcC transporters was described as an adaptative advantage for *S. aureus* to survive within host tissue when O_2 is rare (Vitko et al., 2016). By analogy, we could postulate that *uhpT* transporter has evolved a functional 3′UTR (RsaG) because it is necessary to uptake G6P and concomitantly to adapt metabolism and redox homeostasis when *S. aureus* is internalized or in the presence of mucus-producing cells.

4 | EXPERIMENTAL PROCEDURE

4.1 | Strains and plasmids

All strains and plasmids, constructed and used in this study, are described in Table S2. The oligonucleotides sequences are given in Table S3. *E. coli* strain DH5α was used for cloning purposes. *E. coli* strain DC10B, a DNA cytosine methyltransferase negative mutant, and *S. aureus* RN4220 were used for plasmid amplification before *S. aureus* HG001 transformation. Transformation of *E. coli* was performed by heat shock and *S. aureus* by electroporation (Bio-Rad Gene Pulser). The plasmids were extracted from *E. coli* or *S. aureus* with the Nucleospin Plasmid kit (Macherey-Nagel) adding a mechanical breakage of staphylococcal cells with FastPrep in P1 buffer. Deletions of *rsaG* and *uhpT* promoter comprised, respectively, removal of nucleotides 201,739 to 201,936 and nucleotides 200,154 to 200,282 (Caldelari et al., 2017) and were constructed by homologous recombination using the thermosensitive vector pMAD (Arnaud et al., 2004). Chromosomal regions upstream or downstream of *rsaG* or *uhpT* promoter were amplified by PCR (see Table S3 for primers) and cloned into pMAD. The resulting plasmid was electroporated first into RN4220 recipient strain and then into HG001. Transformants were grown at the nonpermissive temperature (44°C), followed by several subcultures at 28°C and 37°C to favor double-crossing over as previously described (Arnaud et al., 2004). To generate the plasmid-expressing *rsaG* for complementation, the entire gene (194 bp) was PCR amplified with the following oligonucleotides: RsaG *PstI* for and RsaG-*BamHI* rev (Table S3) digested by *PstI* and *BamHI* and ligated into pCN51::P3 digested with the same enzymes (Tomasini et al., 2017) to obtain pCN51::P3::*rsaG*. The MS2 tag was fused to the 5′-end of *rsaG* using oligonucleotides *PstI*-MS2-RsaG for and RsaG-*BamHI* rev cloned into *PstI*/*BamHI*-digested pCN51::P3. The pUC-T7::*rsaG* mut1, mut2 and mut1/mut2 vectors were obtained by QuickChange XL Site-directed mutagenesis (Stratagene) from pUC-T7::*rsaG*.

For transcriptional fusions, different length fragments of the *uhpT* upstream region (i.e., 1,585, 1,456, 500, 250, 100 bp before RsaG transcription start site) (see Table S3 for primers sequences et description) were cloned into *SphI*-*BamHI*-digested pEW-GFP plasmid containing the 5′UTR_hly from *Listeria monocytogenes* (Menendez-Gil et al., 2020). Translational fusion to *lacZ* was constructed in pLUG220::*rpoB*::*lacZ* (Romilly et al., 2014). First, a 309 bp fragment containing the 5′UTR region of *rex* mRNA and 69 codons (−103/+207 bp) was amplified with primers *rex* for *BamHI* and *rex* rev *BamHI* and cloned into *BamHI*-digested pLUG220::*rpoB*::*lacZ* to generate pLUG220::*rpoB*::*rex*::*lacZ*. Then an amplicon containing *rsaG* under the *blaZ* promoter was produced by two distinct PCR. The pCN40 plasmid was used as a template to amplify a *blaZ* promoter with primers *PstI*-pBlaZ-pES for/RsaG-pBlaZ-pES3 rev and HG001 genomic DNA to amplify *rsaG* with primers RsaG for/*PstI*-RsaG rev. The two PCR products served as the template for a PCR with oligonucleotides *PstI*-pBlaZ-pES for/PstI-RsaG rev and ligated into the *PstI*-digested pLUG220::*rpoB*::*rex*::*lacZ*.

4.2 | Growth conditions

E. coli strains were grown in Lysogeny-Broth (LB, Roth) medium supplemented with ampicillin (100 μg/ml) or kanamycin (30 μg/ml) when necessary. *S. aureus* strains were cultivated in Brain–Heart infusion (BHI, Sigma) or Muller-Hinton Broth (MHB, Sigma) media containing 10 μg/ml of erythromycin when needed. To induce RsaG, MHB or BHI were complemented with 0.5% of glucose-6-phosphate (G6P) (Sigma).

4.3 | Northern blot

Purification of total RNA extracts was performed strictly following the procedure described for the FastRNA pro blue kit (MP Biomedicals) with the Fastprep apparatus (MP Biomedicals). Electrophoresis of 10 μg of total RNA was run in a 1% TBE-agarose gel, containing 25 mM guanidium thiocyanate (Sigma). After migration, RNAs were transferred on Hybond N+ nitrocellulose membrane (GE Healthcare Life Sciences) by vacuum with the vacuum blot system (Whatman Biometra) or by capillarity and fixed by UV-crosslinking (Stratalinker 1800 Stratagene). RNA detection was performed by hybridization with specific digoxigenin (DIG)-labeled probes complementary to each targeted RNA as described previously (Tomasini et al., 2017).

4.4 | Western blot and microscopy

Total proteins were extracted from cultures grown for 4 hr in BHI before harvesting by centrifugation. Bacterial pellets were resuspended in 1X PBS and lysed using the Fastprep (MP Biomedicals). Quantification of proteins was performed with Bradford reagent following the manufacture protocol (BioRad) and BSA as the standard. Equal amounts (0.5 μg) of total proteins were migrated on 12% polyacrylamide-SDS gels and transferred onto PVDF membranes with a Trans-blot Turbo Transfer system (BioRad). Blots were incubated with anti-GFP at the final dilution 1:5,000 followed by incubation with a goat antimouse peroxidase (HRP) (Biorad) at the final dilution 1:5,000. Gels were stained by Coomassie blue as loading controls of samples.

For fluorescent microscopy, 10 μl of the same cultures were spread between slide and coverslip and observed with Epifluorescence microscope TiE, Nikon (excitation source: LED Spectra X, Lumencor, camera: Orca-Flash IV, objectif: Plan Apo x100 oil, numerical aperture: 1:45) under excitation at 475 nm and emission at 514 ± 24nm.

4.5 | MAPS analyses

Crude bacterial extracts were prepared in duplicates from cultures of Δ*rsaG* strain expressing MS2-RsaG or from WT strain expressing MS2 grown for 5 hr in BHI and then 0.5% G6P was added for another hour of incubation before harvesting and lysis as previously described (Lalaouna et al., 2018). After MS2 affinity chromatography, RNA was purified from the elution fraction and used either for Northern blot or DNase I treated prior to RNA-seq analysis as described (Lalaouna et al., 2018). The enrichment values were calculated by DEseq2 (p-adj <.05; fold change >2) as previously published (Tomasini et al., 2017).

4.6 | Preparation of RNAs for in vitro experiments

Transcription of RsaG or RsaI was achieved with linearized pUC18 vectors (Bronesky et al., 2019; Geissmann et al., 2009). PCR fragments containing RsaG mut1, RsaG mut2, and RsaG mut1/mut2, the 5′UTR of selected mRNAs or the synthetic gene encoding the *rex*-R2 mRNA (Integrated DNA Technologies) downstream the sequence of the T7 promoter were used as templates for in vitro transcription using T7 RNA polymerase (see Table S3 for oligonucleotides and sizes of DNA fragments). RNAs were purified from 6% or 8% polyacrylamide-8 M urea gel, eluted with 0.5 M ammonium acetate pH 6.5, 1 mM EDTA, and 0.1% SDS and finally precipitated in cold absolute ethanol. Dephosphorylated RNAs were labeled with T4 polynucleotide kinase (Fermentas) and [γ^{32}P] ATP.

4.7 | Gel retardation assay

The 5′-end radiolabeled RsaG, the mutants RsaG mut1, RsaG mut2, RsaG mut1/mut2, RsaG mut3, and RsaG mut4 (10,000 cps/sample, <1 pM), and cold mRNAs were renaturated separately by incubation at 90°C for 1 min in 100 mM Tris–HCl pH 7,5, 300 mM KCl, 200 mM NH_4Cl, cooled down 1 min on ice, and incubated at 20°C 10 min in the presence of 10 mM $MgCl_2$. Complexes were formed at 37°C for 15 min, then 10 μl of glycerol blue was added and the samples were loaded on a native 6% polyacrylamide gel containing 10 mM $MgCl_2$ (4–6 hr, 300 V, 4°C).

4.8 | Toe-printing assays

The preparation of 30S subunits of *S. aureus* was described elsewhere (Khusainov et al., 2017), likewise the extension inhibition conditions (Fechter et al., 2009). Increasing concentrations of RsaG were used to monitor its effect on the simplified translational initiation complex formed with *S. aureus* 30S ribosomal subunits, initiator $tRNA^{fMet}$ and mRNAs.

4.9 | Primer extension assays

In order to determine the transcriptional start site of RsaG from *S. simiae*, 15 μg of total RNA from a bacterial culture grown in the presence or the absence of G6P was reversed transcribed with the AMV reverse transcriptase (NEB) and the 5′-radiolabeled oligonucleotide RsaG-like rev. The reaction was performed and analyzed as previously described (Lalaouna, Baude, et al., 2019). The sequencing ladder was obtained with a PCR product using oligonucleotides RsaG-like for/RsaG-like rev.

4.10 | Monitoring of the 5′ extremity of RsaG

Terminator™ 5′-Phosphate-dependent exonuclease Terminator (Epicentre) enzyme degrades secondary transcripts carrying a monophosphate 5′ extremity but has no effect on primary transcript displaying a tri-phosphate 5′ extremity and also on highly structured transcript as 5S rRNA. Total RNA (10 μg) was extracted from HG001 strain grown for 4 hr culture in BHI with or without G6P 0.5% and incubated for 1 hr at 30°C with Terminator Exonuclease (1 U) and Terminator 1X Reaction buffer A. RNAs were then purified with phenol–chloroform alcohol isoamyl extraction and ethanol precipitation. Northern blot experiment was performed on 1% agarose gel containing 25 mM guanidium thiocyanate.

4.11 | Coculture experiments

Human colon epithelial HT29 and HT29MTX, human tumorigenic lung epithelial A549, and human tumorigenic liver HU-H7 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Thermofisher) medium with 10% FCS and 1% penicillin-streptomycin (Gibco) at 37°C under 5% CO_2 atmosphere. At 4 days or

14 days (HT29 and HT29MTX cells), cells are plated in 100 × 20 mm Petri dishes at 0.75x10^6 cells for HU-H7, 10^6 cells for A549, and 1.5x10^6 cells for HT-29 and HT-29MTX. Then 10 ml of HG001 or HG001 Δ*hptRS* grown for 4 hr in MHB, when RsaG is poorly expressed (ca. $OD_{600\ nm}$ 1) are added to human cells for 1 hr at 37°C. As controls, fresh 10 ml MHB was added to cells and 10 ml of HG001 bacterial culture was incubated with or without 0.5% G6P. Then supernatants of human cells were carefully collected and centrifuged. Bacterial pellets were used to extract total RNA, which is analyzed by Northern blot, and supernatants were examined for the presence of mucus by dot blot experiments.

4.12 | Dot blot assay

One microliter (or 4 µl supernatant of infected HT-29 cells) of co-culture supernatants or noninfected supernatants was dropped on a PVDF membrane activated with ethanol 95% and equilibrated in TBS 1X Tween 0.1%. The membranes were incubated with a rabbit anti-Muc2 or a mouse anti-Muc5AC at the final dilution of 1:1,000 followed by, respectively, a goat anti-rabbit and a goat anti-mouse at the final dilution of 1:10,000.

4.13 | Quantification of intracellular expression

The human myoblasts CTi400 were cultured in KMEM medium (1v M199, 4v DMEM), 20% fetal bovine serum (v/v), 25 µg/ml fetuin, 0.5 ng/ml bFGF, 5 ng/ml EGF, 5 µg/ml insulin, and 0.2 mg/ml dexamethasone at 37°C under 5% CO_2 atmosphere. The murine macrophages RAW 264.7 were cultured in DMEM (Thermofisher) supplemented with 10% FBS at 37°C under 5% CO_2 atmosphere.

The intracellular infection of cells was performed using gentamycin protection assay as previously described (Trouillet et al., 2011) with modifications. Cells were seeded at 80,000 cells per well in 24-well tissue culture plates. After 24 hr, cells were washed twice with 1 ml of PBS and infected at a multiplicity of infection (MOI) of 10:1 with bacterial culture (9 hr of growth) diluted in an antibiotic-free cell medium. The MOI was confirmed by CFU counting upon agar plate inoculation. After 2 hr of infection at 37°C, cells were washed twice with 1 ml of PBS and incubated for 1 hr in a medium containing 200 µg/ml gentamicin and 10 µg/ml lysostaphin to kill extracellular bacteria. After the antibiotic treatment, three wells were pooled, cells and bacteria were harvested by trypsin detachment and centrifugation. As controls, bacteria were incubated for 1 hr in KMEM medium or DMEM under the same conditions as above.

To quantify RsaG, pellets were treated with 20 µg lysostaphin, and RNA isolation was performed using the RNeasy Plus mini kit (QIAGEN) according to the manufacturer's instructions. The RNA was quantified using a NanoDrop spectrophotometer, and 150 ng of total RNA was reverse transcribed into cDNA using Reverse Transcriptase System (Promega). One microliter of 1/5 diluted cDNA was used as a template for the real-time PCR amplification using PowerUp SYBR® Green Master Mix and a StepOne Plus system (Applied Biosystem) with specific primers shown in Table S3. Relative RsaG amount analysis was performed by using Δ*Ct* methods using *gyrB* gene as an internal standard and confirmed by *hu* and 16S gene (see Table S3 for oligonucleotides).

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DATA AVAILABILITY STATEMENT

MAPS data are openly available in the public repository GEO under accession GSE176028.

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Le facteur de terminaison de la transcription Rho, un acteur de la virulence et de l'adaptation aux stress chez *Staphylococcus aureus*

Transcription termination factor Rho, a peculiar player in virulence and stress adaptation in *Staphylococcus aureus*

Chez les bactéries à Gram positif comme le pathogène opportuniste *Staphylococcus aureus*, la régulation médiée par les ARN régulateurs ou sARN n'a pas encore été associée à l'action des protéines liant l'ARN, qui jouent un rôle essentiel dans ces réseaux de régulation chez les Enterobacteriaceae. Néanmoins, le facteur de terminaison de la transcription Rho a récemment été décrit comme étant un atténuateur de la virulence de *S. aureus*, suggérant que cette protéine universelle serait un nouvel acteur de la pathophysiologie de cet organisme. Ce facteur est également impliqué dans la répression de la transcription pervasive qui résulte de la transcription au niveau de promoteurs cryptiques ou d'une translecture transcriptionnelle. Ce travail de thèse a permis de montrer que l'ARN messager *rho* est spécifiquement réprimé en phase stationnaire de croissance par l'ARNIII, un sARN dépendant du *quorum sensing* afin de permettre l'expression d'exotoxines permettant la dissémination du pathogène. Les cibles ARN inhibées par Rho qui ont été identifiées, sont souvent impliquées dans l'évasion immunitaire et dans différents processus d'attachement à l'hôte. Ce travail a permis de fournir des explications au niveau moléculaire des fonctions que ce facteur de terminaison bien conservé aurait acquis dans ce pathogène humain.

Mots clés : terminaison de la transcription, Rho, sARN, *Staphylococcus aureus*, virulence

In Gram-positive bacteria, such as the opportunistic pathogen *Staphylococcus aureus*, sRNA-mediated regulation has not been majorly linked to the action of RNA-Binding-Proteins, known to be essential for these regulatory networks in Enterobacteriaceae. However, *S. aureus* transcription termination Rho factor has been described as an attenuator of virulence, making this universal protein a new player in *S. aureus* pathophysiology. The protein is also required for the repression of "pervasive" transcription, a mechanism that results from transcription at cryptic promoters or from transcriptional readthrough. In this work, we have shown that the *rho* transcript is specifically inhibited by the *quorum sensing* dependent RNAIII at stationary phase of growth allowing exotoxins to be activated when the pathogen undergoes dissemination. Several Rho-repressed RNA targets, which have been identified, are involved in adhesion, dissemination, and host-response of the pathogen. Our data provide a molecular explanation for the functions of this well-conserved termination factor that have evolved in this human pathogen.

Keywords : transcription termination, Rho, sRNA, *Staphylococcus aureus*, virulence