



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

Emma Desgranges, Laura Barrientos, Lucas Herrgott, Stefano Marzi, Alejandro Toledo-Arana, Karen Moreau, François Vandenesch, Pascale Romby, Isabelle Caldelari

► To cite this version:

Emma Desgranges, Laura Barrientos, Lucas Herrgott, Stefano Marzi, Alejandro Toledo-Arana, et al.. The 3'UTR-derived sRNA RsaG coordinates redox homeostasis and metabolism adaptation in response to glucose-6-phosphate uptake in *Staphylococcus aureus*. *Molecular Microbiology*, 2022, 117 (1), pp.193-214. 10.1111/mmi.14845 . hal-03836042

HAL Id: hal-03836042

<https://hal.science/hal-03836042>

Submitted on 2 Nov 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Journal:	<i>Molecular Microbiology</i>
Manuscript ID	MMI-2021-18579.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	08-Nov-2021
Complete List of Authors:	Desgranges, Emma; Centre National de la Recherche Scientifique, UPR9002 Barrientos, Laura; Centre National de la Recherche Scientifique, UPR9002 Herrgott, Lucas; Centre National de la Recherche Scientifique, UPR9002 Marzi, Stefano; Centre National de la Recherche Scientifique, UPR 9002 Toledo-Arana, Alejandro; Spanish Scientific Research Council, Instituto de Agrobiotecnología (IdAB) Moreau, Karen; Centre International de Recherche en Infectiologie, U1111 Vandenesch, François; Centre International de Recherche en Infectiologie, U1111 Romby, Pascale; Centre National de la Recherche Scientifique, UPR 9002 Caldelari, Isabelle; Centre National de la Recherche Scientifique, UPR9002
Key Words:	3'UTR derived sRNA, <i>Staphylococcus aureus</i> , redox homeostasis

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

Emma Desgranges¹, Laura Barrientos¹, Lucas Herrgott¹, Stefano Marzi¹, Alejandro Toledo-Arana², Karen Moreau³, François Vandenesch³, Pascale Romby¹ and Isabelle Caldelari^{1*}

¹Université de Strasbourg, CNRS, Architecture et Réactivité de l'ARN, UPR9002, F-67000 Strasbourg, France.

²Instituto de Agrobiotecnología (IdAB). CSIC-UPNA-GN, 31192-Mutilva, Navarra, Spain.

³CIRI, Centre international de Recherche en Infectiologie, Inserm, U1111, Université Claude Bernard Lyon 1, CNRS, UMR5308, École Normale Supérieure de Lyon, Hospices Civils de Lyon, Univ Lyon, F-69008, Lyon, France.

Running title: A 3' untranslated region-derived sRNA responds to Glucose 6-phosphate

Keywords: 3'UTR derived sRNA, *Staphylococcus aureus*, redox homeostasis

*To whom correspondence should be addressed. Tel: 33(0) 388417068; Fax: 33(0) 388602218; Email: i.caldelari@ibmc-cnrs.unistra.fr

SUMMARY (199/200 words)

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* are induced when bacteria are internalized into host cells or in 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, to degrade, or to repress translation of its mRNA targets. While RsaG is conserved only in closely related species, the *uhpT* 3'UTR of the ape pathogen *S. simiae* harbors a 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.

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 non-phagocytic host cells (Hamza and Li, 2014). To be successful as a pathogen, this bacterium needs to adapt to the hostile environments of the host and must acquire imposed nutriment for its survival. Consequently, 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 (Villanueva *et al.*, 2018; Richardson, 2019). *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 expression of genes, whose products act in 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 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 (Crooke *et al.*, 2013; Christmas *et al.*, 2019). While 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 defences. 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 to various metabolites or small compounds (Villanueva *et al.*, 2018). In glucose-limiting conditions, when *S. aureus* penetrates host cells, the bacteria could utilise 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 is 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 and Vanderpool, 2013; Wagner and Romby, 2015; Desgranges *et al.*, 2019). They often act at the post-transcriptional 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 (Miyakoshi *et al.*, 2015; Desgranges *et al.*, 2019). Their transcription is often induced by sensing of external stimuli via TCS or TF (Brosse and 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 *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, 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 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, presence of NAD⁺ enhances binding of Rex to RsaE promoter region to repress its expression (for a review, 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 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.

RESULTS

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 rich medium (Geissmann *et al.*, 2009) or in presence of G6P (Bronsky *et al.*, 2019). Because no obvious predicted promoter sequence was detected for *rsaG* and because *uhpT* is induced in response to extracellular G6P (Bronsky *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 a 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 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 HG001 Δ *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 analysed 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.

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, HG001 Δ *rsaG*, and HG001 Δ *hptRS* strains in glucose depleted LB medium or in 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 presence of G6P than the HG001 Δ *hptRS* strain under identical conditions (Figure 2A). These data indicated that RsaG is not required for G6P catabolism in contrast to the TCS HptRS. We next performed internalization assays with HG001 and HG001 Δ *hptRS* strains in (non-phagocytic) myoblasts and (phagocytic) macrophages cell lines cultured in K-MEM or 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 of HptRS, which allows the expression of the *uhpT*-RsaG co-transcript (Figures 2B and 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 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 HG001 Δ *hptRS* strains. Bacteria were gently recovered, and total RNA was extracted. Northern blot experiments were performed with a probe specific for RsaG. The data showed that 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 since no significant signal was observed for RsaG in the mutant strain HG001 Δ *hptRS*. We also compared the accumulation of RsaG when staphylococci were incubated in presence of colon cell lines HT-29 or HT-29MTX cultivated for 4 days and for 14 days to allow differentiation and production of mucus (Figure S1B, right panel) (von Kleist *et al.*, 1975; Behrens *et al.*, 2001). HT-29 cells are heterogeneous cells as they contain less than 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. A strong induction of RsaG was observed when HG001 strains were incubated with either HT-29 or HT-29 MTX cells cultivated for 14 days as compared to 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 HG001 Δ *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 adapt the cells in new environments during the infection, i.e. presence of mucus and/or internalization into host cells.

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 h (when RsaG is present) was treated with or without Tex and run on an agarose gel. Surprisingly, RsaG level was only slightly reduced in 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 (Figures 3C and S2B, C and 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 and Altman, 1984). The data showed that the co-transcript was not cleaved in contrast to the pre-mature 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 (HG001 Δrnc), RNase Y (HG001 Δrny), and RNases J1 or J2 (RN4220 $\Delta j1$, Sa624 $\Delta j1$ or Sa624 $\Delta j2$), 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. While 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 ($\Delta j1$) or RNase J2 ($\Delta j2$), and concomitantly an accumulation of *uhpT*-RsaG was visualized (Figures 3C and S2D). Because the growth of the double mutant strain ($\Delta j1\Delta j2$) was strongly impaired, we did not manage to obtain enough bacteria to extract sufficient amount of RNAs for further analysis (Linder *et al.*, 2014). In the mutant $\Delta j1$ and $\Delta j2$ 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 (Figures 3C and 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.

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.*, 2018a). 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 h in BHI medium and then G6P 0,5 % was added for 1 h 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 analysed 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 comparison of the number of reads obtained from the MS2-RsaG purification and the MS2 alone as 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 two-fold 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, RpiR). In addition, the PTS operon (phosphoenolpyruvate (PEP)-dependent phosphotransferase system) encoded the genes *ptsH* and *ptsI*, allows 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, Ndh, Hom). Besides, we have pulled out several polycistronic transcripts encoding transporters including the mechanosensitive ion channel MscL, the ABC transporters for K⁺ and Mn²⁺, KdpBAF and MntABC, respectively (Xue *et al.*, 2011; Ando *et al.*, 2003), the *Pmt* operon that exports the PSM toxins (phenol soluble modulins) causing host cell lysis (Chatterjee *et al.*, 2013), and the multi-drug efflux

pumps SrdM-SepA. Finally, although RsaI is weakly expressed in 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, while *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 co-purified 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 nM to 600 nM (Table S1, Figures 4A and 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_02010, 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 since 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, Figures 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 trans-acting 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.

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), i.e, *ccpA*, *sarA*, *arcR*, *tcaR*, *ndh*. Likewise, RsaG is predicted to interact with the 5'UTR of several mRNAs, i.e, *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), while the nts 126 to 148 of RsaG are complementary to the RBS of *ccpA* mRNA encoding the catabolite control protein A (Seidl *et al.*, 2009) (Figures 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 mut1/2 (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 of 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*.

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* (Figures 5A et 5B). 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 eliminate 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 compensate 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). Significant 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.

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 *lacZ* under the control of the strong promoter *PrpoB* and *rsaG* was cloned under the constitutive *blaZ* promoter. The synthesis of β -galactosidase was analyzed in the Δ *rsaG* mutant strain transformed with the plasmid carrying the *lacZ* reporter alone or with the plasmid containing the *lacZ* and *rsaG*. The β -galactosidase activity was reproducibly increased by 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 analysed 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* (HG001 Δ *rsaG*) grown in presence or absence of G6P, and in the deleted strain of *rsaG* complemented with a plasmid over-expressing RsaG under the P3 promoter (HG001 Δ *rsaG* pCN51::P3*rsaG*). In absence of G6P, the half-life of *rex* mRNA was less than 30 sec in the WT and Δ *rsaG* mutant strains while the overexpression of RsaG in the mutant strain enhanced the half-life to 90 sec (Figure 5C). In presence of G6P, the half-life of *rex* reached values above 1 min in the WT strain (77.9 sec) and in the mutant strain overexpressing RsaG while *rex* was poorly detected in the mutant Δ *rsaG* strain (Figures 5C and 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 as

compared to the WT strain, and this effect was independent of the accumulation of RsaG (Figure S6E).

Interestingly the main repressed target of Rex, the lactate deshydrogenase *Ldh1* was found in MAPS albeit with a threshold below 2-fold. Therefore, RsaG would act on *ldh1* expression either directly or indirectly through the stabilisation 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 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 minor effect on the formation of the ternary ribosomal complex (Figure S7A). The stability of *ldh1* mRNA was measured using rifampicin treatment after 4 h of growth in absence or 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 post-transcriptional regulator to modulate positively or negatively the stability of target mRNAs. Through the regulation of *rex* and *ldh1*, RsaG would avoid glycolytic fermentation in favour of amino acid catabolism, when G6P is consumed.

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). 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* (Figures 7A, 7B). 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 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 (Figures 7D, 7E). These data are well correlated with the fact that *sarA* and RsaI bind to two distinct regions of RsaG while 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, *i.e.* *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 h of growth. Total RNAs were extracted from HG001 strain in absence or 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 min 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.

Differential evolution of the *uhpT* 3'UTRs containing RsaG in *Staphylococcaceae*.

The *uhpT* gene sequence is highly conserved in the *Staphylococcaceae* family (between 83 to 91% similarity). However, previous studies indicated that RsaG was only conserved in *S. aureus* (Geissmann et al., 2009). Since 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 *S. schweitzeri*, which are closely related to *S. aureus*, while 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 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 to *S. aureus* (294 nts). As the HptR box is rather conserved, the induction of the orthologous *S. simiae* RsaG in absence or 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.

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 solely carbon source. We also showed that *uhpT* mRNA and RsaG are highly induced by G6P when *S. aureus* is internalised into host cytosol and in 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 (Richard *et al.*, 2013). Interestingly, a hierarchical regulation mediated by SgrS has been demonstrated (Bobrovskyy *et al.*, 2019; Poddar *et al.*, 2021). At 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 regulation (Figure 9).

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 (Kim *et al.*, 2014; Eisenhardt *et al.*, 2018; 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, while 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 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 towards 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 exonuclease activity (Figure 3D).

Intriguingly, RsaG is the **fifth** example of a 3'UTR-derived sRNA issued from a 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 cofactor (Lalaouna 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 presence of maltose (Bar et al, 2021; Iosub et al, 2021).** Finally, in *Vibrio cholerae* OppZ is issued from the OppA oligopeptide transporter, and binds to the second gene of the *oppABCDF* operon mediating repression of OppB synthesis (Lalaouna et al., 2019; Wang et al., 2020; Hoyos 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. While 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 and 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 presence of mucus-secreting cells.

RsaG expands the regulon of HptRS.

In order to scrutinize the functional impact of RsaG, we performed MAPS in 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 illustrate a possible hierarchical regulation. However, binding affinities *in vivo* might be different as the sRNA is expected to bind the mRNA co-transcriptionally and trans-acting 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 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 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 favour G6P uptake in detrimental to glycolysis and carbon catabolite repression (Figure 9). Such a 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). While CcpA inhibits transcription of *ald1*, *adh*, *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, acetoin 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 status 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*, *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 activator 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 the oxidative stress resistance in presence of methyl viologen or diamide (Seidl *et al.*, 2009; Ballal and Manna, 2009). Additionally, when staphylococcal cultures were starved in Mn, the atypical sRNA RsaC blocked translation of *sodA*, a Mn-dependent superoxide dismutase, while SodM was enhanced most probably because RsaC inhibited *sarA* translation (Lalaouna *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).

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 *et al.*, 2018b). Nevertheless, the first C-rich motif was demonstrated as the main site for RsaI, a sRNA which is repressed by CcpA, and which is activated when glucose is consumed (Bronesky *et al.*, 2019). RsaI favoured 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 absence of glucose. At least *in silico*, the binding site 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).

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*, while *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 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 different locus of the genome might play similar functions as RsaG.

Another level of complexity was added by a recent re-analysis of ribosome profiling data identifies several sRNAs with 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 analyse 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 independent pathway as *S. aureus* RNAIII (Raina et al., 2018).

In conclusion, acquisition of glucose transporters such as GlcC transporters was described as an adaptative advantage for *S. aureus* to survive within host tissue when O₂ 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 presence of mucus-producing cells.

EXPERIMENTAL PROCEDURE

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 DH5 α 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 201739 to 201936 and nucleotides 200154 to 200282 (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 non-permissive temperature (44°C), followed by several subcultures at 28°C and 37°C to favour 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 mut1/2 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. 1585, 1456, 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 template to amplify *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 template for a PCR

with oligonucleotides *Pst*I-pBlaZ-pES for/ *Pst*I-RsaG rev and ligated into the *Pst*I-digested pLUG220::*rpoB*::*rex*::*lacZ*.

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).

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 (Whayman 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).

Western blot and microscopy

Total proteins were extracted from cultures grown for 4 h 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 1:5000 followed by an incubation with a goat anti-mouse peroxidase (HRP) (Biorad) at the final dilution 1:5000. 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.

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 h in BHI and then 0.5% G6P was added for another hour of incubation before harvesting and lysis as previously described (Lalaouna *et al.*, 2018a). 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.*, 2018a). The enrichment values were calculated by DEseq2 (P -adj < 0.05; fold change > 2) as previously published (Tomasini *et al.*, 2017).

Preparation of RNAs for *in vitro* experiments

Transcription of RsaG or RsaI was achieved with linearized pUC18 vectors (Geissmann *et al.*, 2009; Bronesky *et al.*, 2019). PCR fragments containing RsaG mut1, RsaG mut2 and RsaG mut1/2, 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 ³²P] ATP.

Gel retardation assay

The 5' end radioabeled RsaG, the mutants RsaG mut1, RsaG mut2, RsaG mut1/2, RsaG mut3, and RsaG mut4 (10'000 cps/sample, < 1 pM) and cold mRNAs were renatured separately by incubation at 90°C for 1 min in 100 mM Tris-HCl pH 7.5, 300 mM KCl, 200 mM NH₄Cl, cooled down 1 min on ice, and incubated at 20°C 10 min in presence of 10 mM MgCl₂. Complexes were formed at 37°C for 15 min, then 10 μ l of glycerol blue was added and the samples loaded on a native 6% polyacrylamide gel containing 10 mM MgCl₂ (4-6 h, 300 V, 4°C).

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.

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 presence or 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 analysed as previously described (Lalaouna *et al.*, 2019). The sequencing ladder was obtained with a PCR product using oligonucleotides RsaG-like for/RsaG-like rev.

Monitoring of the 5' extremity of RsaG

Terminator™ 5'-Phosphate-dependent exonuclease Terminator (Epicentre) enzyme degrades secondary transcripts carrying a mono-phosphate 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 h culture in BHI with or without G6P 0.5 % and incubated for 1 h at 30°C with Terminator Exonuclease (1U) 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.

Co-culture 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% CO₂ atmosphere. At 4 days or 14 days (HT29 and HT29MTX cells), cells are plated in 100 X 20 mm Petri dishes at 0.75x10⁶ cells for HU-H7, 10⁶ cells for A549 and 1.5x10⁶ cells for HT-29 and HT-29MTX. Then 10 ml of HG001 or HG001 $\Delta hptRS$ grown for 4 h in MHB, when RsaG is poorly expressed (ca.

OD_{600nm} = 1) are added to human cells for 1 h 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 analysed by Northern blot and supernatants were examined for the presence of mucus by dot blot experiments.

Dot Blot assay

1 µl (or 4 µl supernatant of infected HT-29 cells) of co-cultures supernatants or non-infected supernatants was drop 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 1:1000 followed by respectively a goat anti-rabbit and a goat anti-mouse at the final dilution of 1:10000.

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, 0.2 mg/ml dexamethasone at 37°C under 5% CO₂ atmosphere. The murine macrophages RAW 264.7 were cultured in DMEM (Thermofisher) supplemented with 10% FBS at 37°C under 5% CO₂ 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 h, cells were washed twice with 1 mL of PBS and infected at a multiplicity of infection (MOI) of 10:1 with bacterial culture (9 h of growth) diluted in cell medium antibiotic free. The MOI was confirmed by CFU counting upon agar plate inoculation. After 2 h of infection at 37°C, cells were washed twice with 1 mL of PBS and incubated for 1 h in medium containing 200 µg/mL gentamicin and 10 µg/mL lysostaphin to kill extracellular bacteria. After the antibiotic treatment, 3 wells were pooled, cells and bacteria were harvested by trypsin detachment and centrifugation. As controls, bacteria were incubated for 1 h 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). 1 µL of 1/5 diluted cDNA was used as 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 ΔC_t methods using *gyrB* gene as an internal standard and confirmed by *hu* and 16S gene (see Table S3 for oligonucleotides).

DATA AVAILABILITY STATEMENT

MAPS data are openly available in the public repository GEO under accession [GSE176028](#).

SUPPLEMENTARY DATA

Supplementary data are available.

ACKNOWLEDGMENTS

The cell lines (HT-29, HT-29 MTX), the antibodies (anti-MUC-2 and anti-MUC-5CA) were kindly supplied by Dr. Benoît Marteyn (IBMC, Strasbourg), the A549 cell line by Dr. Alain Lescure (IBMC, Strasbourg), and the Hu-H7 cell line by Dr. Catherine Schuster (Institut de virologie, Strasbourg). We are thankful to Delphine Bronesky and David Lalaouna for helpful discussions, and Anne-Catherine Helfer who has performed footprinting experiments.

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. ED and LB were supported by the "Fondation pour la Recherche Médicale" (FDT201904007957 and ECO202006011534).

REFERENCES

906

907 Afgan, E., Baker, D., Beek, M. van den, Blankenberg, D., Bouvier, D., Čech, M., *et al.*
908 (2016) The Galaxy platform for accessible, reproducible and collaborative biomedical
909 analyses: 2016 update. *Nucleic Acids Research* **44**: gkw343.

910

911 Ando, M., Manabe, Y.C., Converse, P.J., Miyazaki, E., Harrison, R., Murphy, J.R., and
912 Bishai, W.R. (2003) Characterization of the Role of the Divalent Metal Ion-Dependent
913 Transcriptional Repressor MntR in the Virulence of *Staphylococcus aureus*. *IAI* **71**:
914 2584–2590.

915

916 Arnaud, M., Chastanet, A., and Débarbouillé, M. (2004) New Vector for Efficient Allelic
917 Replacement in Naturally Nontransformable, Low-GC-Content, Gram-Positive
918 Bacteria. *Appl Environ Microbiol* **70**: 6887–6891.

919

920 Augagneur, Y., King, A.N., Germain-Amiot, N., Sassi, M., Fitzgerald, J.W., Sahukhal,
921 G.S., *et al.* (2020) Analysis of the CodY RNome reveals RsaD as a stress-responsive
922 riboregulator of overflow metabolism in *Staphylococcus aureus*. *Mol Microbiol* **113**:
923 309–325.

924

925 Ballal, A., and Manna, A.C. (2009) Regulation of Superoxide Dismutase (sod) Genes
926 by SarA in *Staphylococcus aureus*. *JB* **191**: 3301–3310.

927

928 Bar, A., Argaman, L., Altuvia, Y., and Margalit, H. (2021). Prediction of novel bacterial small
929 RNAs from RIL-Seq RNA-RNA interaction data. *Front in Microbiol* **12**: 635070.

930

931 Behrens, I., Stenberg, P., Artursson, P., and Kissel, T. (2001) Transport of lipophilic
932 drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells.
933 *Pharmaceutical Research* **18**: 1138–1145.

934

935 Bobrovskyy, M., and Vanderpool, C.K. (2013) Regulation of Bacterial Metabolism by
936 Small RNAs Using Diverse Mechanisms. *Annu Rev Genet* **47**: 209–232.

937

938 Bobrovskyy, M., Azam M.S., Frandsen, J.K., Zhang, J., Poddar, A., Ma, X., Henkin,
939 T.M., Ha, T., and Vanderpool, C. K. (2019) Determinants of target prioritization and
940 regulatory hierarchy for the bacterial small RNA SgrS. *Mol Microbiol* **112**(4): 1199-1218.

941

942 Brandenberger, M., Tschierske, M., Giachino, P., Wada, A., and Berger-Bächi, B.
943 (2000) Inactivation of a novel three-cistronic operon *tcaR-tcaA-tcaB* increases
944 teicoplanin resistance in *Staphylococcus aureus*. *Biochimica et Biophysica Acta (BBA)*
945 - *General Subjects* **1523**: 135–139.

946

947 Bronesky, D., Desgranges, E., Corvaglia, A., François, P., Caballero, C.J., Prado, L.,
948 *et al.* (2019) A multifaceted small RNA modulates gene expression upon glucose
949 limitation in *Staphylococcus aureus*. *The EMBO Journal* e99363.

950

951 Brosse, A., and Guillier, M. (2018) Bacterial Small RNAs in Mixed Regulatory Networks.
952 *Microbiol Spectr* **6**.

953

954 Caldelari, I., Chane-Woon-Ming, B., Noirot, C., Moreau, K., Romby, P., Gaspin, C.,
955 and Marzi, S. (2017) Complete Genome Sequence and Annotation of the

- 956 *Staphylococcus aureus* Strain HG001. *Genome Announc* **5**: e00783-17,
957 /ga/5/32/e00783-17.atom.
- 958
- 959 Chatterjee, S.S., Joo, H.-S., Duong, A.C., Dieringer, T.D., Tan, V.Y., Song, Y., *et al.*
960 (2013) Essential *Staphylococcus aureus* toxin export system. *Nat Med* **19**: 364–367.
- 961
- 962 Cheng, C., Bhardwaj, N., and Gerstein, M. (2009) The relationship between the
963 evolution of microRNA targets and the length of their UTRs. *BMC Genomics* **10**: 431.
- 964
- 965 Cheung, A.L., Nishina, K.A., Trottonda, M.P., and Tamber, S. (2008) The SarA protein
966 family of *Staphylococcus aureus*. *The international journal of biochemistry & cell*
967 *biology* **40**: 355–361.
- 968
- 969 Chico-Calero, I., Suarez, M., Gonzalez-Zorn, B., Scotti, M., Slaghuis, J., Goebel, W.,
970 *et al.* (2002) Hpt, a bacterial homolog of the microsomal glucose- 6-phosphate
971 translocase, mediates rapid intracellular proliferation in *Listeria*. *Proceedings of the*
972 *National Academy of Sciences* **99**: 431–436.
- 973
- 974 Christmas, B.A.F., Rolfe, M.D., Rose, M., and Green, J. (2019) *Staphylococcus aureus*
975 adaptation to aerobic low-redox-potential environments: implications for an
976 intracellular lifestyle. *Microbiology* **165**: 779–791.
- 977
- 978 Crooke, A.K., Fuller, J.R., Obrist, M.W., Tomkovich, S.E., Vitko, N.P., and Richardson,
979 A.R. (2013) CcpA-Independent Glucose Regulation of Lactate Dehydrogenase 1 in
980 *Staphylococcus aureus*. *PLoS ONE* **8**: e54293.
- 981
- 982 Desgranges, E., Marzi, S., Moreau, K., Romby, P., and Caldelari, I. (2019) Noncoding
983 RNA. *Microbiology Spectrum* **7**
984 [http://www.asmscience.org/content/journal/microbiolspec/10.1128/](http://www.asmscience.org/content/journal/microbiolspec/10.1128/microbiolspec.GPP3-0038-2018)
985 [microbiolspec.GPP3-0038-2018](http://www.asmscience.org/content/journal/microbiolspec/10.1128/microbiolspec.GPP3-0038-2018). Accessed June 23, 2020.
- 986
- 987 Deutscher, J., Küster, E., Bergstedt, U., Charrier, V., and Hillen, W. (1995) Protein kinase-
988 dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in
989 Gram-positive bacteria. *Mol. Microbiol.* **15**:1049–1053.
- 990
- 991 Eisenhardt, K.M.H., Reuscher, C.M., and Klug, G. (2018) PcrX, an sRNA derived from
992 the 3'- UTR of the *Rhodobacter sphaeroides* *puf* operon modulates expression of *puf*
993 genes encoding proteins of the bacterial photosynthetic apparatus. *Molecular*
994 *Microbiology* **110**: 325–334.
- 995
- 996 Fechter, P., Chevalier, C., Yusupova, G., Yusupov, M., Romby, P., and Marzi, S. (2009)
997 Ribosomal initiation complexes probed by toeprinting and effect of trans-acting
998 translational regulators in bacteria. *Methods Mol Biol* **540**: 247–263.
- 999
- 1000 Garzoni, C., Francois, P., Huyghe, A., Couzinet, S., Tapparel, C., Charbonnier, Y., *et*
1001 *al.* (2007) A global view of *Staphylococcus aureus* whole genome expression upon
1002 internalization in human epithelial cells. *BMC Genomics* **8**: 171.
- 1003
- 1004 Geissmann, T., Chevalier, C., Cros, M.-J., Boisset, S., Fechter, P., Noirot, C., *et al.*
1005 (2009) A search for small noncoding RNAs in *Staphylococcus aureus* reveals a

conserved sequence motif for regulation. *Nucl Acids Res* **37**: 7239–7257.

Gemayel, R., Fortpied, J., Rzem, R., Vertommen, D., Veiga-da-Cunha, M., and Van Schaftingen, E. (2007) Many fructosamine 3-kinase homologues in bacteria are ribulosamine/erythrulosamine 3-kinases potentially involved in protein deglycation: Bacterial fructosamine 3-kinase homologues. *FEBS Journal* **274**: 4360–4374.

Guerrier-Takada, C., and Altman, S. (1984) Catalytic activity of an RNA molecule prepared by transcription in vitro. *Science* **223**: 285–286.

Hamza, T., and Li, B. (2014) Differential responses of osteoblasts and macrophages upon *Staphylococcus aureus* infection. *BMC Microbiol* **14**: 207.

Hoyos, M., Huber, M., Förstner, K.U., and Papenfort, K. (2020) Gene autoregulation by 3' UTR-derived bacterial small RNAs. *eLife* **9**: e58836.

Iosub, I.A., Marchiorretto, M., van Nues, R.W., McKellaar, S., Viero, G., and Granneman, S. (2021) The mRNA derived MalH sRNA contributes to alternative carbon source utilization by tuning maltoporin expression in *E. coli*. *RNA Biology* **18**:6, 914-931.

Jefferson, K.K., Pier, D.B., Goldmann, D.A., and Pier, G.B. (2004) The Teicoplanin-Associated Locus Regulator (TcaR) and the Intercellular Adhesin Locus Regulator (IcaR) Are Transcriptional Inhibitors of the ica Locus in *Staphylococcus aureus*. *Journal of Bacteriology* **186**: 2449–2456.

Khusainov, I., Vicens, Q., Ayupov, R., Usachev, K., Myasnikov, A., Simonetti, A., et al. (2017) Structures and dynamics of hibernating ribosomes from *Staphylococcus aureus* mediated by intermolecular interactions of HPF. *EMBO J* **36**: 2073–2087.

Kim, H.M., Shin, J.-H., Cho, Y.-B., and Roe, J.-H. (2014) Inverse regulation of Fe- and Ni-containing SOD genes by a Fur family regulator Nur through small RNA processed from 3'UTR of the sodF mRNA. *Nucleic Acids Research* **42**: 2003–2014.

Kleist, S. von, Chany, E., Burtin, P., King, M., and Fogh, J. (1975) Immunohistology of the Antigenic Pattern of a Continuous Cell Line From a Human Colon Tumor. *JNCI: Journal of the National Cancer Institute* **55**: 555–560.

Lalaouna, D., Baude, J., Wu, Z., Tomasini, A., Chicher, J., Marzi, S., et al. (2019) RsaC sRNA modulates the oxidative stress response of *Staphylococcus aureus* during manganese starvation. *Nucleic Acids Research* **47**: 9871–9887.

Lalaouna, D., Desgranges, E., Caldelari, I., and Marzi, S. (2018a) MS2-Affinity Purification Coupled With RNA Sequencing Approach in the Human Pathogen *Staphylococcus aureus*. In *Methods in Enzymology*. Elsevier, pp. 393–411 <https://linkinghub.elsevier.com/retrieve/pii/S007668791830301X>. Accessed January 15, 2019.

Lalaouna, D., Eyraud, A., Devinck, A., Prévost, K., and Massé, E. (2018b) GcvB small RNA uses two distinct seed regions to regulate an extensive targetome. *Mol Microbiol* **141**:168.

- Linder, P., Lemeille, S., and Redder, P. (2014) Transcriptome-Wide Analyses of 5'-Ends in RNase J Mutants of a Gram-Positive Pathogen Reveal a Role in RNA Maturation, Regulation and Degradation. *PLoS Genet* **10**: e1004207.
- Makhlin, J., Kofman, T., Borovok, I., Kohler, C., Engelmann, S., Cohen, G., and Aharonowitz, Y. (2007) *Staphylococcus aureus* ArcR Controls Expression of the Arginine Deiminase Operon. *JB* **189**: 5976–5986.
- Marincola, G., Wencker, F.D.R., and Ziebuhr, W. (2019) The Many Facets of the Small Non-coding RNA RsaE (RoxS) in Metabolic Niche Adaptation of Gram-Positive Bacteria. *Journal of Molecular Biology* **431**: 4684–4698.
- Menendez-Gil, P., Caballero, C.J., Catalan-Moreno, A., Irurzun, N., Barrio-Hernandez, I., Caldelari, I., and Toledo-Arana, A. (2020) Differential evolution in 3'UTRs leads to specific gene expression in *Staphylococcus*. *Nucleic Acids Research* **48**: 2544–2563.
- Meydan, S., Marks, J., Klepacki, D., Sharma, V., Baranov, P.V., Firth, A.E., Margus, T., Kefi, A., Vázquez-Laslop, N., and Mankin, A.S. (2019) Retapamulin-Assisted Ribosome Profiling Reveals the Alternative Bacterial Proteome. *Mol Cell* **74**(3):481-493.
- Michalik, S., Depke, M., Murr, A., Gesell Salazar, M., Kusebauch, U., Sun, Z., *et al.* (2017) A global *Staphylococcus aureus* proteome resource applied to the in vivo characterization of host-pathogen interactions. *Sci Rep* **7**: 9718.
- Miyakoshi, M., Chao, Y., and Vogel, J. (2015) Regulatory small RNAs from the 3' regions of bacterial mRNAs. *Current Opinion in Microbiology* **24**: 132–139.
- Pagels, M., Fuchs, S., Pané-Farré, J., Kohler, C., Menschner, L., Hecker, M., *et al.* (2010) Redox sensing by a Rex-family repressor is involved in the regulation of anaerobic gene expression in *Staphylococcus aureus*: Redox sensitive gene regulation in *S. aureus*. *Molecular Microbiology* **76**: 1142–1161.
- Park, J.Y., Kim, J.W., Moon, B.Y., Lee, J., Fortin, Y.J., Austin, F.W., *et al.* (2015) Characterization of a Novel Two-Component Regulatory System, HptRS, the Regulator for the Hexose Phosphate Transport System in *Staphylococcus aureus*. *Infection and Immunity* **83**: 1620–1628.
- Poddar A., Azam M.S., Kayikcioglu T., Bobrovskyy M., Zhang J., Ma X., *et al.* (2021) Effects of individual base-pairs on in vivo target search and destruction kinetics of bacterial small RNA. *Nat Commun* **8**;12(1):874. doi: 10.1038/s41467-021-21144-0.
- Raina, M., King, A., Bianco, C., and Vanderpool, C.K. (2018) Dual-Function RNAs. In *Regulating with RNA in Bacteria and Archaea*. Storz, G., and Papenfort, K. (eds). ASM Press, Washington, DC, USA. pp. 471–485
<http://doi.wiley.com/10.1128/9781683670247.ch27>. Accessed May 18, 2021.

- Reed, J.M., Olson, S., Brees, D.F., Griffin, C.E., Grove, R.A., Davis, P.J., *et al.* (2018) Coordinated regulation of transcription by CcpA and the *Staphylococcus aureus* two-component system HptRS. *PLoS ONE* **13**: e0207161.
- Reyer M.A., Chennakesavalu S., Heideman E.M., Ma X., Bujnowska M., Hong L., Dinner A.R., Vanderpool C.K., Fei J. (2021) Kinetic modeling reveals additional regulation at co-transcriptional level by post-transcriptional sRNA regulators. *Cell Rep.* 2021 Sep 28;36(13):109764. doi: 10.1016/j.celrep.2021.109764.
- Richards, G.R., Patel, M.V., Lloyd, C.R. and Vanderpool, C.K. (2013) Depletion of glycolytic intermediates plays a key role in glucose-phosphate stress in *Escherichia coli*. *J Bacteriol* 195(21):4816-25.
- Richardson, A.R. (2019) Virulence and Metabolism. *Microbiology Spectrum* **7** <http://www.asmscience.org/content/journal/microbiolspec/10.1128/microbiolspec.GP-P3-0011-2018>. Accessed June 23, 2020.
- Rochat, T., Bohn, C., Morvan, C., Le Lam, T.N., Razvi, F., Pain, A., *et al.* (2018) The conserved regulatory RNA RsaE down-regulates the arginine degradation pathway in *Staphylococcus aureus*. *Nucleic Acids Research* **46**: 8803–8816.
- Romilly, C., Lays, C., Tomasini, A., Caldelari, I., Benito, Y., Hammann, P., *et al.* (2014) A Non-Coding RNA Promotes Bacterial Persistence and Decreases Virulence by Regulating a Regulator in *Staphylococcus aureus*. *PLoS Pathog* **10** <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3961350/>. Accessed August 1, 2016.
- Runyen-Janecky, L.J., and Payne, S.M. (2002) Identification of Chromosomal *Shigella flexneri* Genes Induced by the Eukaryotic Intracellular Environment. *IAI* **70**: 4379–4388.
- Seidl, K., Müller, S., François, P., Kriebitzsch, C., Schrenzel, J., Engelmann, S., *et al.* (2009) Effect of a glucose impulse on the CcpA regulon in *Staphylococcus aureus*. *BMC Microbiol* **9**: 95.
- Sit, B., Crowley, S.M., Bhullar, K., Lai, C.C.-L., Tang, C., Hooda, Y., *et al.* (2015) Active Transport of Phosphorylated Carbohydrates Promotes Intestinal Colonization and Transmission of a Bacterial Pathogen. *PLoS Pathog* **11**: e1005107.
- Sorensen, H.M., Keogh, R.A., Wittekind, M.A., Caillet, A.R., Wiemels, R.E., Laner, E.A., and Carroll, R.K. (2020) Reading between the Lines: Utilizing RNA-Seq Data for Global Analysis of sRNAs in *Staphylococcus aureus*. *mSphere* **5**: e00439-20, [/msphere/5/4/mSphere439-20.atom](https://doi.org/10.1128/mSphere.00439-20).
- Tomasini, A., Moreau, K., Chicher, J., Geissmann, T., Vandenesch, F., Romby, P., *et al.* (2017) The RNA targetome of *Staphylococcus aureus* non-coding RNA RsaA: impact on cell surface properties and defense mechanisms. *Nucleic Acids Research* **45**: 6746–6760.
- Trouillet, S., Rasigade, J.-P., Lhoste, Y., Ferry, T., Vandenesch, F., Etienne, J., and Laurent, F. (2011) A novel flow cytometry-based assay for the quantification of *Staphylococcus aureus* adhesion to and invasion of eukaryotic cells. *Journal of Microbiological Methods* **86**: 145–149.

- Villanueva, M., García, B., Valle, J., Rapún, B., Ruiz de los Mozos, I., Solano, C., *et al.* (2018) Sensory deprivation in *Staphylococcus aureus*. *Nat Commun* **9**: 523.
- Vitko, N.P., Grosser, M.R., Khatri, D., Lance, T.R., and Richardson, A.R. (2016) Expanded Glucose Import Capability Affords *Staphylococcus aureus* Optimized Glycolytic Flux during Infection. *mBio* **7**: e00296-16, /mbio/7/3/e00296-16.atom.
- Wagner, E.G.H., and Romby, P. (2015) Chapter Three - Small RNAs in Bacteria and Archaea: Who They Are, What They Do, and How They Do It. In *Advances in Genetics*. Theodore Friedmann, J.C.D. and S.F.G. (ed.). Academic Press, pp. 133–208 <http://www.sciencedirect.com/science/article/pii/S0065266015000036>. Accessed August 1, 2016.
- Wang, C., Chao, Y., Matera, G., Gao, Q., and Vogel, J. (2020) The conserved 3' UTR-derived small RNA NarS mediates mRNA crossregulation during nitrate respiration. *Nucleic Acids Research* **48**: 2126–2143.
- Wencker, F.D.R., Marincola, G., Schoenfelder, S.M.K., Maaß, S., Becher, D., and Ziebuhr, W. (2021) Another layer of complexity in *Staphylococcus aureus* methionine biosynthesis control: unusual RNase III-driven T-box riboswitch cleavage determines *met* operon mRNA stability and decay. *Nucleic Acids Research* **49**: 2192–2212.
- Wright P.R., Georg J., Mann M., Sorescu D.A., Richter A.S., Lott S., Kleinkauf R., Hess W.R., Backofen R. (2014) CopraRNA and IntaRNA: predicting small RNA targets, networks and interaction domains. *Nucleic Acids Res.* **42**: W119-23.
- Xue, T., You, Y., Hong, D., Sun, H., and Sun, B. (2011) *The Staphylococcus aureus* KdpDE Two-Component System Couples Extracellular K⁺ Sensing and Agr Signaling to Infection Programming. *Infect Immun* **79**: 2154–2167.
- Yang, Y., Sun, H., Liu, X., Wang, M., Xue, T., and Sun, B. (2016) Regulatory mechanism of the three-component system HptRSA in glucose-6-phosphate uptake in *Staphylococcus aureus*. *Med Microbiol Immunol* **205**: 241–253.

FIGURES LEGENDS

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, HG001 Δ *hptRS* and HG001 Δ *PuhpT*. Total RNA was extracted at 2, 4 and 6 h of growth in MHB medium with or without the addition of 0.5% G6P. 5S rRNA (5S) was used as 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 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 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, HG001 Δ *rsaG* and HG001 Δ *hptRS* strains in LB medium with or without the addition of 0.5% glucose-6-phosphate (G6P). ns is for non significant, statistical analysis with Anova (**** $p < 0,0005$). B. Levels of RsaG in HG001 and HG001 Δ *hptRS* strains as determined with qRT-PCR. Samples were taken after 1 h of *S. aureus* internalization into RAW 264.7 macrophages or CTi400 myoblasts. As control, the yield of RsaG was also quantified for HG001 wild-type or HG001 Δ *hptRS* cultured for 1 h in K-MEM or D-MEM medium. Data were normalized to *gyrB* and represent the mean of at least three independent experiments, **statistical analysis with T-test (* $p < 0,05$)**. C. Left panel, Northern blot analysis of RsaG in HG001 (pink bars) and HG001 Δ *hptRS* (green bars). Total RNA was extracted after 1 h incubation with liver cell line Hu-H7 and lung cell line A549 at days 4 of culture. As control, RNA was extracted from HG001 and

HG001 Δ *hptRS* incubated 1 h in 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. 1 μ l of supernatant from the Hu-H7 or A549 cells incubated with HG001 (pink bars) and HG001 Δ *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: non-infected Hu-H7 or A549 cells.

Figure 3: RsaG is matured from *uhpT* by 5'-3'exoribonucleases J1 and J2. A, B. Determination of the 5' end status of various RNAs. 10 μ g of total RNA extracted after 4 h of growth in MHB medium (+/- glucose-6-phosphate, G6P) was treated with the Terminator™ 5'-Phosphate-Dependent Exonuclease (+/- **Tex**). **RsaG**, **5S (A)** and **RsaI (B)** were revealed by Northern blot analysis using specific probes while 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 h of growth in MHB medium supplemented with 10 mg/L uracil and 0.5% glucose-6-phosphate. 5S rRNA (5S) was used as 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.

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 to 8 : formation of the initiation complex in the presence of increasing concentrations of RsaG: 50 nM (lane 5), 100 nM (lane 6), 200 nM (lane 7), 400 nM (lane 8) for *tcaR* and *ccpA*, and 25 nM (lane 5), 100 nM (lane 6), 200 nM (lane 7), 300 nM (lane 8) for *sarA*. Lanes T, A, C, G: sequencing ladders. The Shine and Dalgarno (SD) sequence, the start site of translation (ATG) and the toe-printing signals (+16) are indicated.

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 MHB medium containing or not glucose-6-phosphate (+/- G6P) and treated with rifampicin at 4 h of growth at 37°C. Total RNA was extracted at various times (from 30 sec 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.

Figure 6: RsaG modulates stability of *ldh1* and *fn3K* mRNAs. A. EMSA showing 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 HG001 Δ rsaG::pCN51::P3rsaG strains. Bacterial cultures were grown in MHB medium containing or not glucose-6-phosphate (+/- G6P) treated with rifampicin at 4 h 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).

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 is 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*. D. 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*.

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 to *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 h of growth in 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, while 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 loading control, and was revealed using a 5' 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 h of growth in BHI 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, A correspond to sequencing ladders. D. Northern blot analysis of RsaG in *S. simiae*. Total RNA was extracted at 2, 4 and 6 h of growth in 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 to 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 post-transcriptional 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.

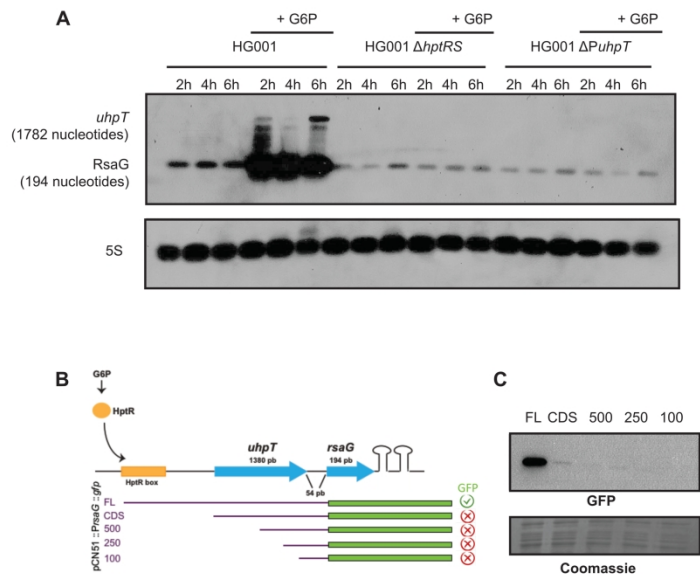


Figure 1

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, HG001 Δ hptRS and HG001 Δ PuhpT. Total RNA was extracted at 2, 4 and 6 h of growth in MHB medium with or without the addition of 0.5% G6P. 5S rRNA (5S) was used as 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 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 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.

209x296mm (300 x 300 DPI)

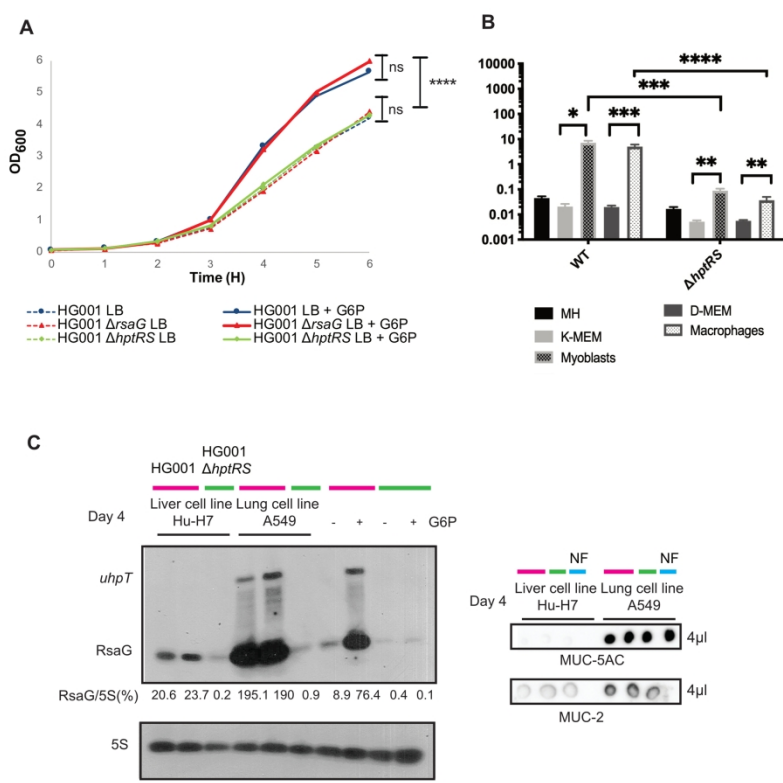


Figure 2

Figure 2: The *uhpT* mRNA and RsaG are enhanced under conditions mimicking the infection. A. Growth curve of HG001 wild-type strain, HG001 \square rsaG and HG001 \square hptRS strains in LB medium with or without the addition of 0.5% glucose-6-phosphate (G6P). ns is for non significant, statistical analysis with Anova (****p<0,0005). B. Levels of RsaG in HG001 and HG001 Δ hptRS strains as determined with qRT-PCR. Samples were taken after 1 h of *S. aureus* internalization into RAW 264.7 macrophages or CTi400 myoblasts. As control, the yield of RsaG was also quantified for HG001 wild-type or HG001 \square hptRS cultured for 1 h in K-MEM or D-MEM medium. Data were normalized to *gyrB* and represent the mean of at least three independent experiments, statistical analysis with T-test (*p<0,05). C. Left panel, Northern blot analysis of RsaG in HG001 (pink bars) and HG001 \square hptRS (green bars). Total RNA was extracted after 1 h incubation with liver cell line Hu-H7 and lung cell line A549 at days 4 of culture. As control, RNA was extracted from HG001 and HG001 \square hptRS incubated 1 h in MHB medium supplemented or not with 0.5% glucose-6-phosphate (-/+ G6P). Same legend as in Figure 2C. Right panel, Mucin 2 (MUC-2) and Mucin 5AC (MUC-5AC) proteins were quantified by dot blot assay. 1 μ l of supernatant from the Hu-H7 or A549 cells incubated with HG001 (pink bars) and HG001 \square 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: non-infected Hu-H7 or A549 cells.

210x297mm (300 x 300 DPI)

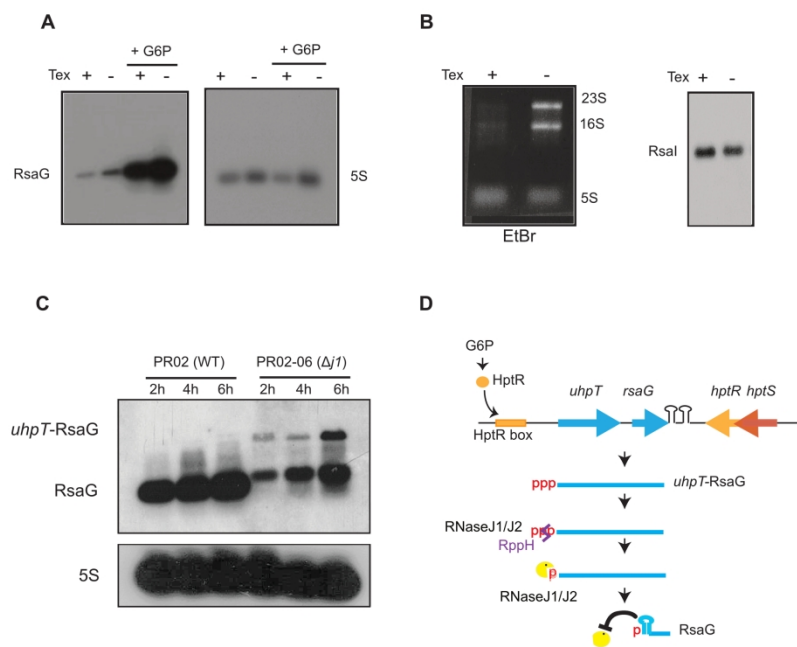


Figure 3

Figure 3: RsaG is matured from uhpT by 5'-3'exoribonucleases J1 and J2. A, B. Determination of the 5' end status of various RNAs. 10 μ g of total RNA extracted after 4 h of growth in MHB medium (+/- glucose-6-phosphate, G6P) was treated with the Terminator™ 5'-Phosphate-Dependent Exonuclease (+/- Tex). RsaG, 5S (A) and RsaI (B) were revealed by Northern blot analysis using specific probes while 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 ($\Delta j1$). Total RNA was extracted at 2, 4 and 6 h of growth in MHB medium supplemented with 10 mg/L uracil and 0.5% glucose-6-phosphate. 5S rRNA (5S) was used as loading control (see above). 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 involves most probably the RppH enzyme to remove the pyrophosphate subsequently followed by the attack of RNases J1/J2. The 5' hairpin of RsaG blocked the progression of the two exoribonucleases leading to its accumulation.



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 targets are depicted. On the right side, three examples of basepairing interactions are given for *tcaR*, *sarA*, and *ccpA*. The translation start codon sequence is underlined, and Shine and Dalgarno (SD) 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^{Met} (tRNAⁱ); lanes 5 to 8 : formation of the initiation complex in the presence of increasing concentrations of RsaG,: 50 nM (lane 5), 100 nM (lane 6), 200 nM (lane 7), 400 nM (lane 8) for *tcaR* and *ccpA*, and 150 nM (lane 6), 300 nM (lane 7) for *sarA*. Lanes T, A, C, G: sequencing ladders. The Shine and Dalgarno (SD) sequence, the start site of translation (ATG)

and the toe-printing signals (+16) are indicated.

209x296mm (300 x 300 DPI)

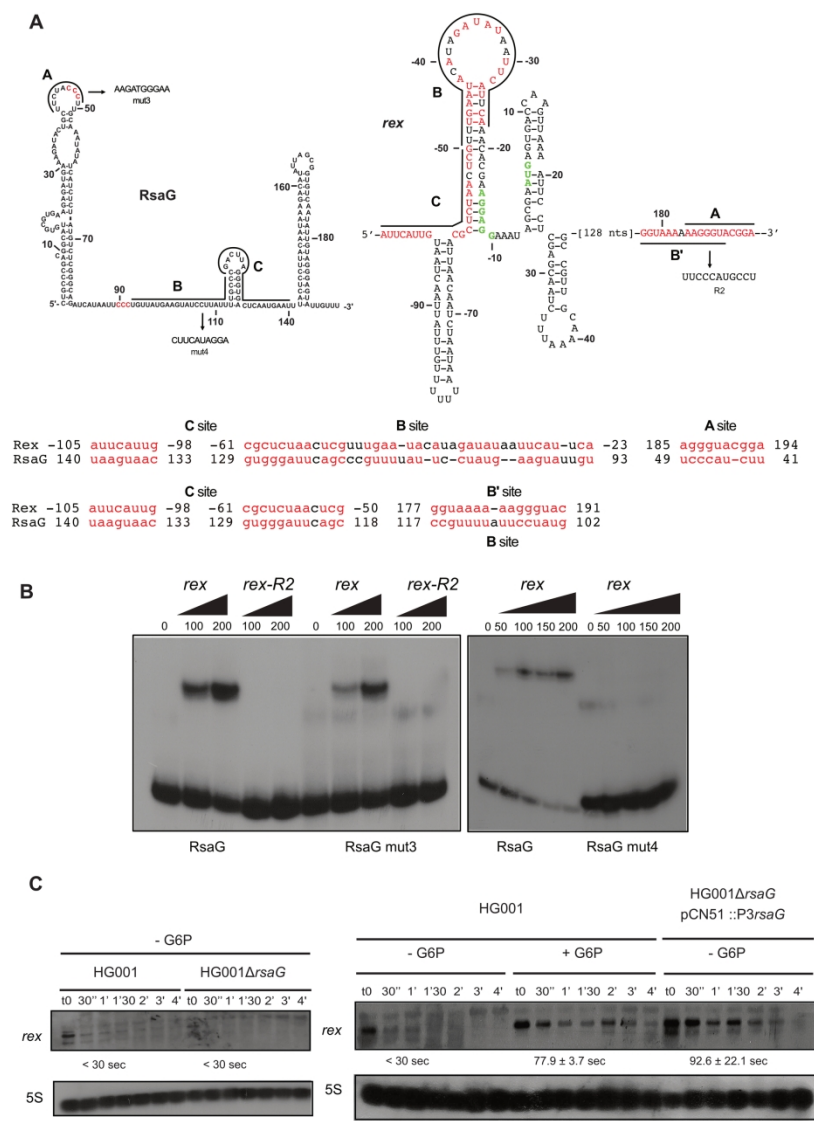


Figure 5

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, HG001ΔrsaG (deletion of rsaG) and HG001ΔrsaG::pCN51::P3rsaG (overexpressing RsaG) strains. Bacterial cultures were grown in MHB medium containing or not glucose-6-phosphate (+/- G6P) and treated with rifampicin at 4 h of growth at 37°C. Total RNA was extracted at various times (from 30 sec 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.

209x296mm (300 x 300 DPI)

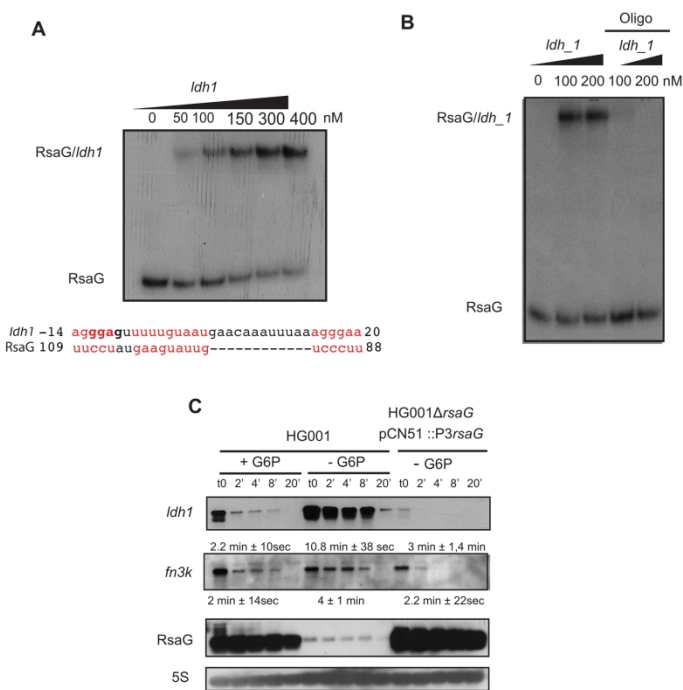


Figure 6

Figure 6: RsaG modulates stability of *ldh1* and *fn3K* mRNAs.

A. EMSA showing 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. EMSA 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 HG001 and HG001Δ*rsaG*::pCN51::P3*rsaG* strains. Bacterial cultures were grown in MHB medium containing or not glucose-6-phosphate (+/- G6P) treated with rifampicin at 4 h 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.

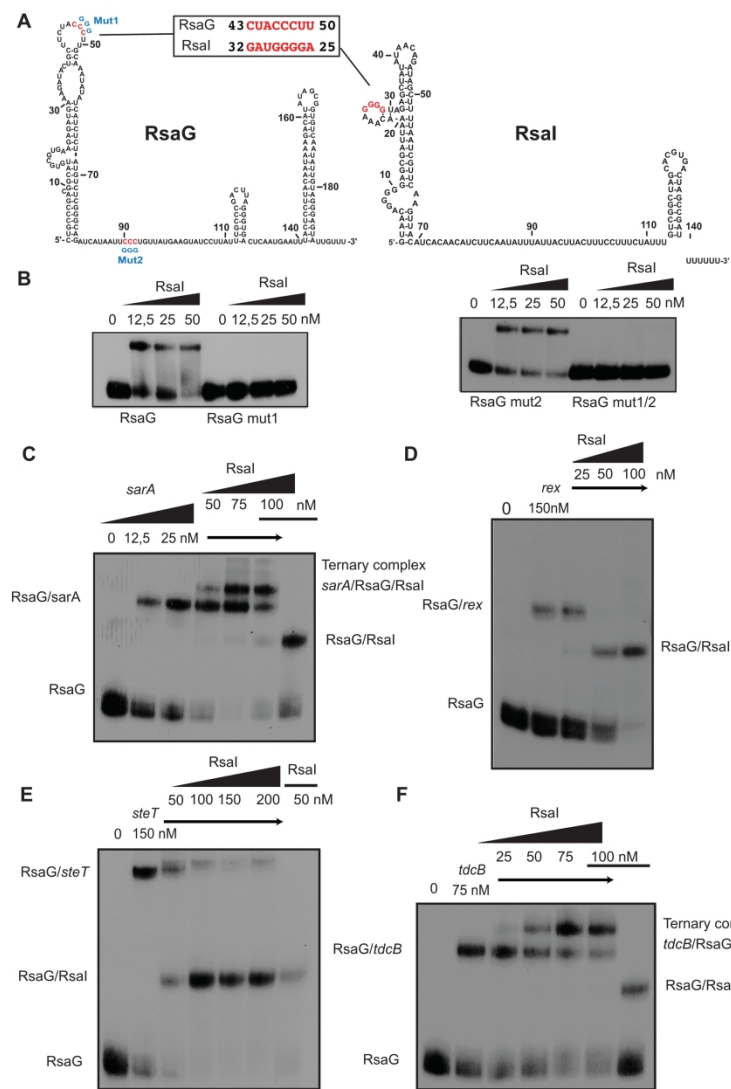


Figure 7

Figure 7: RsaI affects only part of the regulatory functions of 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 is depicted. 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. D. 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.

209x296mm (300 x 300 DPI)



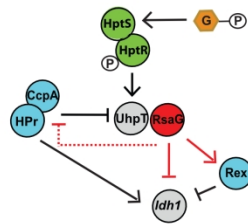
Figure 8: The 3'UTR of uhpT mRNA is not conserved in all staphylococcaceae. A. Genomic context of uhpT locus in *Staphylococcus aureus* HG001, *S. argenteus* MSHR11, *S. schweitzeri* FSA084, *S. epidermidis* ATCC35983 and *S. simiae* CCM7213T. Percentages given in black represent the similarity score of uhpT coding region and in red the similarity score of uhpT 3'UTR compared to *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 h of growth in 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, while 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 loading control, and was revealed using a 5' 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 h of growth in BHI 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, A correspond to sequencing ladders. D. Northern blot analysis of RsaG in *S. simiae*. Total RNA was extracted at 2, 4 and 6 h of growth in 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 to the consensus sequence underlined in yellow (Yang et al., 2016) using blast (NCBI) with default settings.

209x296mm (300 x 300 DPI)



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 post-transcriptional 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.



RsaG is a 3'UTR derived regulatory RNA issued from the degradation of the *uhpT* transcript encoding the glucose-6-phosphate (G6P) transporter in *Staphylococcus aureus*. In presence of G6P, sensed by HptRS, RsaG would transiently favour G6P entry by repressing CcpA-Hpr synthesis. Accordingly, it modulates the redox homeostasis by stabilizing *rex* and degrading *ldh1* mRNAs. The regulation is particularly important when *S. aureus* is in contact of mucus-secreting cells or when it is internalised into host cells.

For Peer Review