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1 **A multifaceted small RNA modulates gene expression upon glucose limitation**
2 **in *Staphylococcus aureus*.**

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36 **ABSTRACT**

37 Pathogenic bacteria must rapidly adapt to ever-changing environmental signals resulting in
38 metabolism remodeling. The carbon catabolite repression, mediated by the catabolite control
39 protein A (CcpA), is used to express genes involved in utilization and metabolism of the
40 preferred carbon source. Here, we have identified a CcpA-dependent small non-coding RNA,
41 RsaI that is inhibited by high glucose concentrations. When glucose is consumed, RsaI
42 represses translation initiation of mRNAs encoding a permease of glucose uptake and the
43 FN3K enzyme that protects proteins against damages caused by high glucose
44 concentrations. RsaI also binds to the 3' untranslated region of *icaR* mRNA encoding the
45 transcriptional repressor of exopolysaccharide production, and to sRNAs responding to the
46 uptake of glucose-6 phosphate or to nitric oxide. Furthermore, RsaI expression is
47 accompanied by a decreased synthesis of genes involved in carbone catabolism pathway and
48 an activation of genes involved in energy production, fermentation, and nitric oxide
49 detoxification. This multifaceted RNA can be considered as a metabolic signature when
50 glucose becomes scarce and growth is arrested.

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53

54 INTRODUCTION

55 All bacteria require a carbon source, providing energy for their growth and for the synthesis of
56 all macromolecules. Besides, pathogenic bacteria during the host infectious process must
57 cope with hostile conditions such as nutrient deficiency, temperature, oxidative and osmotic
58 shocks, and must overcome innate immune responses. For instance, *Staphylococcus aureus*
59 uses carbohydrates to grow under high nitric oxide (NO) and anaerobiosis (Vitko et al., 2016).
60 To survive in these complex environments and to counteract the host defense, *S. aureus*
61 produces a plethora of virulence factors. The synthesis of these factors is fine-tuned by
62 intricate interactions between multiple regulators involving both proteins and RNAs (Novick,
63 2003). Additionally, biosynthetic intermediates, generated from the central metabolism of *S.*
64 *aureus*, have strong impacts on the synthesis of virulence factors. Besides, several
65 metabolite-sensing regulatory proteins (CcpA, CodY, Rex and RpiR) act as key regulatory
66 factors to coordinate the synthesis of genes involved in metabolic pathways, in stress
67 responses and in pathogenesis (Somerville & Proctor, 2009; Richardson et al., 2015). Through
68 the adaptation of the metabolism of the bacteria to specific host microenvironment, these
69 proteins contribute to *S. aureus* pathogenesis (Richardson et al., 2015).

70 Among these proteins, the catabolite control protein A (CcpA) acts as the main global
71 regulator of carbon catabolite repression, allowing the bacteria to use the preferred carbon
72 source (i.e., glucose) in a hierarchical manner (Seidl et al., 2008a; Seidl et al., 2009). CcpA
73 belongs to the LacI repressor family and binds to a specific DNA sequence, called the *cre*
74 (catabolite responsive element) sequence, which is conserved in many Gram-positive bacteria.
75 Transcription of CcpA is constitutive and the protein is activated through the binding of its co-
76 regulator histidine-containing phosphocarrier protein HPr in the presence of glucose.
77 Inactivation of *ccpA* gene decreases transiently the growth rate and yield as long as glucose
78 is present in the medium (Seidl et al., 2006), and affects the expression of a large number of
79 metabolic encoding genes in a glucose-dependent and -independent manner (Seidl et al.,
80 2008a). Additionally, CcpA has a strong impact on the expression of *S. aureus* virulon. It
81 enhances the yield of the quorum-sensing induced RNAIII, which represses Protein A and
82 various adhesion factors at the post-transcriptional level, and conversely activates the
83 synthesis of many exotoxins. However, CcpA also modulates the transcription of mRNAs
84 encoding Protein A, α -hemolysin (*hla*) and Toxic Shock Syndrome Toxin (TSST) (Seidl et al.,
85 2008b ; Seidl et al., 2006), represses capsule formation, and activates biofilm formation in a
86 glucose-rich environment (Seidl et al., 2008a). Indeed a *S. aureus ccpA* deletion mutant strain
87 was less pathogenic than the wild-type strain in a murine abscess model (Li et al., 2010) and
88 *ccpA* inactivation increased the susceptibility of hyperglycemic animals to acute pneumonia
89 infections (Bischoff et al., 2017). Nevertheless, the mechanism by which CcpA affected *S.*

90 *aureus* pathogenesis cannot be simply resumed as a modulation of the RNAIII-dependent
91 regulatory networks. Therefore, it has been suggested that CcpA can also act indirectly on
92 gene expression through the action of other regulatory proteins or small non-coding RNAs
93 (sRNAs) (Somerville & Proctor, 2009; Richardson et al., 2015).

94 In Enterobacteriaceae, several sRNAs have been shown as key actors of the uptake
95 and the metabolism of carbohydrates (reviewed in Bobrovskyy & Vanderpool, 2016). For
96 instance, they participate in the regulation of the galactose operon and carbon catabolite
97 repression, metabolism of amino acids, and contribute to bacterial survival during phospho-
98 sugar stress. The importance of sRNAs in regulatory networks is now well recognized to
99 rapidly adjust cell growth to various stresses and changes in the environment. Thus, they are
100 obvious candidates creating the links between virulence and metabolism. One example is
101 RsaE, a sRNA conserved in *S. aureus* and *Bacillus subtilis*, that controls enzymes involved in
102 the TCA cycle (Geissmann et al., 2009; Bohn et al., 2010) and in arginine degradation pathway
103 (Rochat et al., 2018). In *B. subtilis* the transcriptional repressor ResD represses RoxS
104 (homologous to *S. aureus* RsaE) to readjust the pool of NAD⁺/NADH in responses to various
105 stress and stimuli (Durand et al., 2017). Its promoter is also highly conserved among
106 *Staphylococceae* and is recognized by the orthologous response regulator SrrA in *S. aureus*.
107 Responding to reactive oxygen species through SrrAB, *S. aureus* RsaE may also intervene in
108 the survival of cells against host immune reactions (Durand et al., 2015; Durand et al., 2017).

109 Here, we have identified a signaling pathway responding to glucose uptake, which
110 involves a sRNA, called RsaI (or RsaOG). This 144 nucleotides-long sRNA is highly
111 conserved among *Staphylococceae*, and carries two conserved regions including two G-
112 track sequences and a long unpaired interhelical region rich in pyrimidines (Geissmann et al.,
113 2009; Marchais et al., 2010). The expression of RsaI is observed exclusively at the stationary
114 phase of growth in rich medium (Geissmann et al., 2009) and is enhanced after vancomycin
115 exposure (Howden et al., 2013). In this study, we revealed that CcpA is the main repressor of
116 RsaI expression in the presence of glucose, and that this inhibition is alleviated after the
117 utilization of glucose. The identification of the targetome of RsaI using the MS2-affinity
118 purification approach coupled with RNA sequencing (MAPS), unexpectedly showed two
119 classes of RNA targets, including mRNAs involved in glucose uptake, sugar metabolism, and
120 biofilm formation, as well as various sRNAs. Using site-directed mutagenesis, we identified
121 two distinct and functional regions of RsaI. All in all, our data showed the existence of two
122 sRNAs involved in essential pathways responding to either glucose or glucose 6-phosphate
123 uptake, with RsaI acting as a signature for a metabolic switch when the preferred carbon
124 source is metabolized.

125 We will discuss the importance of sRNA-mediated regulation in *S. aureus* to fine-tune
126 the expression of genes according to essential nutrient availability, and their possible
127 consequences on metabolism adaptation and virulence.

128

129 RESULTS

130

131 **The expression of RsaI is inhibited by glucose and by the catabolite control protein A.**

132 We have previously shown that the synthesis of RsaI is high at the stationary phase of growth
133 in BHI medium while its expression was constitutive in MHB medium (Geissmann et al., 2009).

134 These data suggested that RsaI expression is regulated in a manner dependent on nutrient
135 or biosynthetic intermediate availability. A major difference between BHI and MHB
136 composition is their carbon source, glucose and starch, respectively. We wondered if the
137 expression of RsaI might be dependent on the available carbon source. For this purpose,
138 Northern blot experiments were performed on total RNAs prepared from HG001 (wild-type)
139 strain grown in MHB medium at various time points (Fig 1A). In MHB, where the glucose is
140 not immediately available as the carbon source, RsaI was constitutively and highly expressed
141 (Fig 1A). Conversely, when glucose was added, either at the beginning of the culture or after
142 3h of growth, the steady state level of RsaI was immediately dropped, indicating the repressing
143 effect of this sugar (Fig 1A).

144 As CcpA sensed the intracellular concentration of glucose (Seidl et al., 2006), we
145 analyzed if the expression of RsaI was CcpA-dependent. Northern blot analysis was
146 performed on total RNA extracts prepared from HG001 strain and an isogenic mutant deleted
147 of *ccpA* gene ($\Delta ccpA$). In parallel, we have also tested CodY, another global regulator of
148 metabolism and virulence, which was shown as a direct regulator of amino acid biosynthesis
149 and transport of macromolecules (Majerczyk et al., 2010; Pohl et al., 2009). The data showed
150 that in the absence of glucose, the yield of RsaI was similar in all strains. However, in MHB
151 medium supplemented with glucose, the yield of RsaI declined dramatically in the WT and
152 $\Delta codY$ strains, whereas it was still high in $\Delta ccpA$ strain (Fig 1B). In BHI medium, RsaI was
153 expressed after 4h of growth in the WT strain, while its expression became constitutive in the
154 mutant $\Delta ccpA$ strain (Fig 1C, EV1A).

155 We also analyzed the expression of RsaI in MHB medium supplemented with various
156 sugars such as fructose and xylose (Fig 1D). The data showed that expression of RsaI was
157 very low at the beginning of growth in MHB supplemented with either glucose or fructose but
158 not with xylose. These data shows that CcpA senses both glucose and fructose (Fig 1).

159 Overall, we showed that CcpA represses RsaI expression in the presence of glucose.
160 In accordance with this observation, a conserved *cre* (GGAAAcGcTTACAT) sequence was
161 found at position -30 upstream the transcriptional start site of RsaI (Fig EV1B). This region

162 was sufficient to confer repression by glucose in the complemented strain containing
163 pCN51::*Prsal* (Fig EV1C).

164

165 **The targetome of *RsaI* as revealed by the MAPS approach.**

166 The MAPS approach (“MS2 affinity purification coupled with RNA sequencing”) was used to
167 purify *in vivo* regulatory complexes involving *RsaI*. MAPS has been successful to identify the
168 RNA targets of any sRNAs in *E. coli* (Lalaouna et al., 2015), and more recently of *RsaA* sRNA
169 in *S. aureus* (Tomasini et al., 2017). Briefly, the MS2 tagged version of *RsaI* was expressed
170 from a plasmid under the control of the *agr*-dependent P3 promoter, allowing an accumulation
171 of *RsaI* at the stationary phase of growth in the Δ *rsaI* mutant strain. *RsaI* was detected by
172 Northern blot using total RNAs extracted at 2, 4 and 6h of growth in BHI medium. Using a
173 DIG-labeled *RsaI* probe, we showed that the steady state levels of MS2-*RsaI* were very similar
174 to the wild-type (WT) *RsaI*, and that MS2-*RsaI* was specifically and strongly retained by the
175 MS2-MBP fusion protein after the affinity chromatography (Fig EV1D). The RNAs were then
176 extracted from the eluted fraction to be sequenced. The data were analyzed using Galaxy
177 (Afgan et al., 2016) and the sequencing reads were mapped, counted per feature, and
178 normalized using the HG001 genome as reference (Caldelari et al., 2017; Herbert et al., 2010).
179 The enrichment of putative targets was measured by comparing the number of reads obtained
180 from the MS2-*RsaI* purification and the MS2 alone as control. Since the MS2 tag alone was
181 produced in the WT HG001 background, the untagged *RsaI* was also expressed under the
182 conditions of growth but was poorly retained on the affinity chromatography (Fig EV1D). The
183 data were sorted by a decreasing fold-change. In the following study, we have considered as
184 *RsaI* targets, the RNAs that were enriched at least four-fold and were reproducibly detected
185 in two independent experiments (Table EV1, Table EV2).

186 Two classes of RNAs were co-purified with *RsaI*, including mRNAs and sRNAs. On
187 the one hand, the most enriched mRNA encodes *IcaR*, the repressor of the *icaADBC* operon,
188 which encodes the enzymes required for the synthesis of the poly- β -1,6-N-acetylglucosamine
189 polymer (PIA-PNAG), the main staphylococcal exopolysaccharide constituting biofilms. In
190 addition, several mRNAs encoding proteins linked to sugar utilization and transport, such as
191 *glcU_2* encoding a major transporter of glucose, and *fn3K* encoding fructosamine 3-kinase
192 were significantly enriched. It is noteworthy that other less enriched mRNAs (< 4-fold) encode
193 proteins related to sugar metabolism such as the trehalose-specific PTS transporter (TreB), a
194 sugar phosphatase (YidA), and a maltose transport system permease (Table EV2). Finally, in
195 addition to *icaR*, several mRNAs express transcriptional regulators (Xre type, the maltose
196 regulatory protein GlvR, SlyA, SigS). On the other hand, we found the *RsaD*, *RsaE*, and *RsaG*
197 sRNAs as putative *RsaI* targets (Table EV1), which all contained at least one conserved C-

198 rich motif (Geissmann et al., 2009). RsaH could be also included in this group though it was
199 less enriched (< 4-fold) (Table EV2).

200 The MAPS data suggested that RsaI is involved in networks of RNA pairings.

201

202 **RsaI contains two distinct regulatory domains.**

203 Following the MAPS experiments, we searched for intermolecular base-pairing interactions
204 between RsaI and its potential RNA targets using IntaRNA. Stable interactions were predicted
205 for most of the enriched RNAs (Table EV1). The CU-rich unpaired region of RsaI was
206 predicted to form base-pairings with most of the mRNAs. They were located close or at the
207 ribosome binding site of most of the mRNAs except for *icaR* and *isaA*, which involves
208 nucleotides in their 3' untranslated regions (Table EV1). A second domain of interaction
209 corresponds to the G-track sequences located in the first hairpin domain of RsaI and the C-
210 rich sequences of the sRNAs RsaD, RsaE, RsaG, and RsaH (Fig 2A).

211 These results suggested that RsaI contains at least two functional domains, the
212 unpaired CU-rich region dedicated to mRNA pairing and the G-track used to interact with other
213 sRNAs. To validate whether RsaI effectively binds to the RNA candidates identified by MAPS
214 through these motifs, we performed gel retardation assays using WT and mutated RsaI
215 molecules (Fig 2B, Fig 2C and Fig EV2). For that, *in vitro* 5' end-labeled RsaI was first
216 incubated with increasing concentrations of various mRNAs encoding proteins involved in
217 biofilm formation (*IcaR*), sugar uptake and metabolism (*GlcU_2* and *Fn3K*), and several
218 sRNAs (*RsaG*, *RsaE*, and *RsaD*). For these experiments, we used the full-length mRNAs and
219 sRNAs (Table EV3). Complex formation was performed with RNAs, which were renatured
220 separately in a buffer containing magnesium and salt. As expected, the data showed that RsaI
221 formed complexes with high affinity (between 20-100 nM, Table 1) with many RNAs such as
222 *icaR*, *glcU_2*, and *fn3K* mRNAs, and *RsaG* sRNA (Fig 2B). The stability of other complexes
223 (e.g. *treB* mRNA and *RsaD* sRNA) was significantly lowered (> 250 nM) (Fig EV2).

224 Based on the base-pairing predictions, mutations have been introduced into *rsaI* to
225 map its functional regulatory regions. The two conserved G-track sequences were mutated
226 separately (*RsaI* mut1: Δ G7-G10, *RsaI* mut2: Δ G26-G29) or together (*RsaI* mut3: Δ G7-
227 G10/ Δ G26-G29), while several nucleotides (*RsaI* mut4: Δ U81-U107) were deleted in the
228 conserved interhelical unpaired sequence (Fig 2A). We analyzed the ability of mutated RsaI
229 derivatives to form complexes with *glcU_2*, *fn3K*, and *icaR* mRNAs, and *RsaG* sRNA (Fig 2B).
230 Quantification of the data showed that *RsaI* mut3 bind to all three mRNAs similarly to the WT
231 *RsaI*, while complex formation was completely abolished with *RsaI* mut4. Only the mutation in
232 the second G-track sequences (*RsaI* mut2) strongly altered the binding to *RsaG*, while the two
233 other mutated *RsaI* (mut1 and mut4) recognized *RsaG* with an equivalent binding affinity as
234 the WT *RsaI* (Fig 2C).

235 To demonstrate that the RNA-RNA interactions were also occurring *in vivo*, MAPS
236 approach was used to monitor the effect of the RsaI mutations on its target RNAs. The RsaI
237 mut2 and mut4, which independently affected *in vitro* binding of sRNAs and mRNAs,
238 respectively, were tagged with MS2 and expressed in the HG001 Δ rsaI mutant strain. As
239 described above, the enrichment of putative targets was calculated using a similar procedure
240 (Table EV4). For this analysis, we have used the data coming from three independent
241 experiments performed with the WT MS2-RsaI, the two MS2 controls and the MS2-RsaI
242 mutants (mut2 and mut4) taking into account that the MAPS were done in two distinct sets of
243 experiments (see Supplementray Material and Methods). This allowed us to directly compare
244 the fold changes between the WT and the mutant RsaI versions (Table EV5). In the fraction
245 containing MS2-RsaI mut4, most of the mRNA targets including *icaR*, *fn3K*, and *glcU_2* were
246 strongly reduced while RsaG was still significantly enriched. Conversely, we observed that the
247 three mRNAs were still co-purified together with the MS2-RsaI mut2 at a level close to the WT
248 RsaI while RsaG was strongly reduced in the fraction containing MS2-RsaI mut2 (Table EV5).
249 In addition, with the two MS2-RsaI mutants, RsaE was almost lost while the enrichment of the
250 transcriptional regulator XRE was strongly reduced. Finally, *dck* mRNA remained enriched at
251 the same level as for the WT MS2-RsaI (Table EV5). These data strongly suggested that
252 mutations at the G-track motif of RsaI had a major effect on RsaG/RsaE sRNAs binding
253 whereas the deletion of the C/U rich sequence in RsaI mut4 had a major impact on the
254 recognition of many mRNAs. The lost of RsaE in the MAPS performed with MS2-RsaI mut4
255 suggested that the sRNA might also be associated with one of the RsaI-dependent mRNA
256 target. In support to the hypothesis, base-pairings have been recently predicted between
257 RsaE and the 5'UTR of *icaR* mRNA (Rochat et al., 2018).

258 Taken together, our data reveal that RsaI has at least two distinct regulatory domains
259 that directly interact either with mRNAs or with sRNAs.

260

261 **RsaI inhibits ribosome binding by masking the Shine and Dalgarno sequence of** 262 **mRNAs.**

263 Because the C/U unpaired region of RsaI was predicted to form base-pairings with the Shine
264 and Dalgarno (SD) sequence of several mRNAs (Fig 2, Fig 3A), we analyzed whether RsaI
265 would have the ability to compete with the ribosome for binding to *glcU_2* and *fn3K* mRNAs.
266 Using toe-printing assays, we analyzed the effect of RsaI on the formation of the ternary
267 initiation complex constituting of mRNA, the initiator tRNA, and the 30S subunit. Binding of the
268 30S on the two mRNAs is illustrated by the presence of a toe-print signal at position +16 (+1
269 being the initiation codon). For *fn3K* mRNA, two toe-print signals were observed at +16 and
270 at +20, most probably corresponding to the presence of two AUG codons distant of 5
271 nucleotides. However, only the first AUG is used *in vivo* (Gemayel et al., 2007). The addition

272 of increasing concentrations of RsaI together with the 30S strongly decreased the toe-print
273 signals for both mRNAs showing that RsaI is able to form a stable complex with the mRNA
274 sufficient to prevent the binding of the 30S subunit (Fig 3A).

275 The *in vivo* relevance of RsaI-dependent repression of several mRNAs (*glcU_2*, *fn3K*,
276 *treB*, *HG001_01242*, *HG001_02520*) was then analyzed using gene reporter assays. The
277 whole leader regions of the mRNAs (54 nts in *glcU_2*, 34 nts in *fn3K*, 24 nts in *treB*, 35 nts in
278 *HG001_01242*, and 72 nts in *HG001_02520*) including 100 nucleotides of their coding
279 sequences were cloned in frame with *lacZ* under the control of the strong promoter *PrpoB*.
280 The synthesis of β -galactosidase was analyzed in the Δ *rsaI* mutant strain and in the same
281 strain transformed with the plasmid carrying the *lacZ* reporter, and RsaI under its own
282 promoter. For the *fn3K-lacZ* fusion, we also analyzed its expression in the mutant strain
283 expressing RsaI mut4. The β -galactosidase activity was reduced almost 8 times in cells
284 expressing wild-type RsaI for all reporter constructs (Fig 3B). In addition, the expression of
285 RsaI mut4 did not cause any repression of the β -galactosidase expressed from *fn3K-lacZ* (Fig
286 3B). Therefore, disrupting the interaction between RsaI mut4 and *fn3K* alleviated the
287 repression of the reporter gene suggesting that the regulation of *fn3k* is occurring at the
288 translational level through specific RNA-RNA interactions.

289 These data showed that RsaI hinders ribosome binding on *glcU_2* and *fn3K* mRNAs
290 in agreement with the fact that the C/U rich region of RsaI would bind to the SD sequence of
291 the mRNAs. We propose a similar mechanism for *treB*, *HG001_01242* and *HG001_02520*
292 mRNAs considering that the predicted base-pairings include the RBS of these mRNAs (Tables
293 EV1, EV2).

294

295 **RsaI interacts with the 3'UTR of *icaR* mRNA and affects PIA-PNAG synthesis.**

296 The MAPS approach revealed that *icaR* mRNA, which encodes the repressor of the main
297 exopolysaccharidic compound of *S. aureus* biofilm matrix, is the most enriched mRNA co-
298 purified with MS2-RsaI. This mRNA is of particular interest because it contains a large 3'UTR
299 that is able to bind to its own SD sequence through the anti-SD UCCCCUG motif (Ruiz de los
300 Mozos et al., 2013). Consequently, the long-range interaction provokes an inhibitory effect on
301 translation and generates a cleavage site for RNase III (Ruiz de los Mozos et al., 2013). RsaI
302 is predicted to form base-pairings with the 3'UTR of *icaR* downstream of the anti-SD sequence
303 (Table EV1, Fig 4A). We first monitored whether the long-range interaction might be critical
304 for RsaI binding. Previous work showed that the substitution of the anti-SD UCCCCUG
305 sequence by AGGGGAC significantly destabilized the long-range interaction to enhance *icaR*
306 translation (Ruiz de los Mozos et al., 2013). However, gel retardation assays showed that the
307 WT and the mutant *icaR* mRNAs bind to RsaI with an equivalent binding affinity (Fig 4B),
308 suggesting that the anti-SD sequence is not required for RsaI binding. Instead, the 3'UTR of

309 *icaR* retained the full capacity to efficiently bind RsaI (Fig 4B, Table EV1). We then analyzed
310 whether RsaI binding to the 3'UTR would alter the circularization of *icaR* mRNA. Gel
311 retardation assay was performed with radiolabelled 3'UTR in the presence of the 5'UTR at a
312 concentration of 500 nM leading to almost 50% of binding (Fig EV3A, lane 2). The addition of
313 increasing concentrations of RsaI led to the formation of a 3'UTR-RsaI binary complex but
314 also of a ternary complex involving the three RNA species (Fig EV3A, lanes 4-8). Hence, RsaI
315 does not prevent the formation of the interaction between the two UTRs of *icaR* mRNA *in vitro*.

316 Then, to analyze the *in vivo* effect of the RsaI interaction with the *icaR* mRNA, we
317 monitored the production of PIA-PNAG as a natural reporter, assuming that variations on IcaR
318 expression would be directly reflected on the production of this exopolysaccharide. For that,
319 dot-blot assays were performed with anti PIA-PNAG specific antibodies to monitor the levels
320 of PIA-PNAG in the strain 132, which expresses *rsaI* and produces high levels of this
321 exopolysaccharide compared to HG001 in our experimental conditions. We measure the PIA-
322 PNAG levels in WT 132 strain and the isogenic mutant strains Δ *rsaI* and Δ 3'UTR *icaR*, which
323 carried a deletion of the 3'UTR of *icaR*. Bacteria were grown for 6h in TSB containing NaCl,
324 which is required to enhance PIA-PNAG synthesis in 132 strain (Vergara-Irigaray et al., 2009)
325 (Fig 4C). The results showed that both RsaI and the 3'UTR of *icaR* are required for efficient
326 production of PIA-PNAG because only the WT strain produces significant levels of
327 exopolysaccharides. In addition, the three strains were transformed with a plasmid
328 overexpressing RsaI or RsaI mut5 carrying a substitution of nucleotides 88 to 103
329 (UUAUUACUUACUUUCC to AAUAAUGAAUGAAAGG) under the control of a strong and
330 constitutive promoter. This mutation decreased the stability of RsaI-*icaR* duplex (Fig EV3B).
331 Northern blots confirmed RsaI over-expression in these strains (Fig 4C). Dot-blot results
332 revealed that production of PIA-PNAG could be restored in Δ *rsaI* strain when RsaI is
333 constitutively expressed. However, complementation is observed neither in the Δ *rsaI*
334 expressing the RsaI mut5 nor in the Δ 3'UTR mutant strains overexpressing RsaI or RsaI mut5.
335 These results suggested that the interaction of RsaI with 3'UTR is required to promote PIA-
336 PNAG synthesis (Fig 4C). Interestingly, over-expression of RsaI in the WT strain considerably
337 increased PIA-PNAG production. Although this effect is less pronounced in the WT expressing
338 RsaI mut5, it suggested the presence of additional regulatory pathways.

339 Taken together, these data suggest that RsaI would contribute to PIA-PNAG synthesis
340 by at least reducing the IcaR repressor protein levels through a specific interaction with the
341 3'UTR of *icaR* mRNA.

342

343 **Two sRNAs responded to sugar uptake**

344 The MAPS experiments revealed that RsaG was the most highly copurified sRNA together
345 with RsaI (Table EV1), and the complex formed between RsaI and RsaG is very stable (Fig

346 2C). Interestingly, *rsaG* is localized just downstream *uhpT* encoding the hexose phosphate
347 transporter (Fig EV4A), whose transcription is activated by the two component system HptRS
348 in response to extracellular glucose-6 phosphate (G-6P), another major carbon source
349 produced by host cells (Park et al, 2015). We therefore analyzed whether RsaG expression
350 would also respond to the cellular concentration of G-6P. Northern blot experiments were
351 performed on total RNA extracts produced from the HG001 strain grown in BHI medium
352 supplemented with G-6P. Under these conditions, the synthesis of RsaG is strongly enhanced
353 (Fig EV4B, left panel), in contrast to RsaI, which is completely inhibited under the same
354 conditions (Fig EV4B, right panel). The deletion of *hptRS* considerably reduced the levels of
355 RsaG (Fig EV4C). Therefore, these data indicated that RsaG is activated by HptRS upon G-
356 6P signaling together with *uhpT* and that RsaG sRNA might be derived from the *uhpT* 3'UTR.

357 We then addressed the consequences of RsaI-RsaG pairings on target recognition.
358 Using gel retardation assays, we analyzed whether RsaG is able to form a ternary complex
359 with RsaI and one of its target mRNA, or if RsaG competes with the mRNA for RsaI binding.
360 These experiments were conducted for three mRNA targets (*glcU_2*, *HG001_1242*,
361 *HG001_0942*), which all formed stable complexes with the 5' end labeled RsaI (Fig 2, Fig 5A,
362 Fig EV2, Table EV1). On the opposite, RsaG is not able to interact with *glcU_2* and
363 *HG001_1242* as shown the gel retardation assays performed with 5' end labeled RsaG (Fig
364 5B). We then performed competition experiments between RsaI and RsaG for mRNA binding.
365 The experiments were performed in the presence of a single concentration of unlabeled RsaG
366 (50 nM) sufficient to bind most of 5' end labeled RsaI in the presence of increasing
367 concentrations of mRNA (Fig 5A). For the three mRNAs, the addition of RsaG causes the
368 formation of a high molecular weight complex most likely formed by RsaG, RsaI and the mRNA
369 (Fig 5A). Hence, RsaG does not interfere with the mRNA binding to RsaI.

370 Furthermore, *in vivo* the synthesis of the β -galactosidase expressed from the *fn3K-*
371 *lacZ* fusion was measured in the WT and Δ *rsaG* mutant strains. The expression of the fusion
372 was strictly identical in both strains. The overexpression of RsaI WT from a plasmid causes a
373 weak but specific inhibition of β -galactosidase synthesis because the RsaI-dependent
374 repression was alleviated in the strain transformed with a plasmid expressing RsaI mut4 (Fig
375 EV4D). This experiment showed that the expression of RsaG does not significantly impact the
376 synthesis of one of the RsaI target.

377 Using rifampicin treatment, the half-lives of RsaI and RsaG were measured in the WT
378 and the mutant strains (Fig 5C). Quantification of the autoradiographies showed that the two
379 RNAs are highly stable as it was previously shown (Geissman et al., 2009). However, the
380 stability of RsaI was reproducibly higher in the WT strain (24 min) than in the Δ *rsaG* mutant
381 strain (15 min) (Fig 5C).

382 Taken together, the data show that the synthesis of RsaG and RsaI are regulated in
383 response to different but related carbon sources, G-6P and glucose, respectively. In addition,
384 their binding has no major consequences on the RsaI target recognition and regulation,
385 although RsaG has a slight effect on RsaI stability.

386

387 **RsaD, the other sRNA target candidate of RsaI, responds to nitric oxide**

388 The signaling pathway of RsaD, another potential sRNA partner of RsaI, has been studied.
389 RsaD binds to RsaI *in vitro* albeit with a lower affinity than RsaG. Although RsaD was
390 significantly enriched in the MAPS experiments using the wild-type MS2-RsaI (Table EV2),
391 the data were unfortunately not reliable with the mutant MS2-RsaI mut 2 and mut4. This is
392 most probably due to the fact that RsaD is weakly expressed at the stationary phase of growth
393 in BHI medium as revealed by the transcriptomic analysis (Table EV6).

394 Upstream the *rsaD* gene, we identified a conserved motif AGTGACAA that has been
395 described as a binding site for the response regulator SrrA (Pragman et al., 2004; Kinkel et
396 al., 2013). SrrAB is a major two-component system affecting the temporal expression of the
397 virulence factors (Pragman et al., 2004) while it promotes resistance to nitrosative stress and
398 hypoxia (Richardson et al., 2006; Kinkel et al., 2013). We demonstrated that the expression
399 of RsaD drops considerably in a Δ *srrAB* mutant strain (Fig EV4E) while its expression is not
400 under the control of the two-component system SaeRS, another global regulator of virulence
401 (Fig EV4E; Liu et al., 2016). Because SrrAB is able to sense and respond to nitric oxide (NO)
402 and hypoxia (Kinkel et al., 2013), we tested the effect of NO on RsaD synthesis by adding
403 diethylamine-NONOate, as it was previously described for RsaE (Durand et al., 2015). We
404 observed a significant and reproducible enhanced expression of RsaD in the WT HG001 strain
405 about 10 min after the addition of diethylamine-NONOate to BHI medium (Fig EV4E). RsaD
406 expression decreased after 20 min due to the short half-life of diethylamine-NONOate.
407 Noteworthy, RsaE, which was significantly enriched together with MS2-RsaI, was also shown
408 to be under the control of SrrAB (Durand et al., 2015).

409 These data suggested that through the binding of sRNAs, a network of sRNAs would
410 coordinate sugar metabolism pathways, energy production, and NO stress responses.

411

412

413 **The effect of RsaI on global gene expression in *S. aureus*.**

414 Before to perform the comparative transcriptomic analysis, we first analyzed whether the
415 deletion or the overexpression of RsaI might cause growth phenotype. Unexpectedly, we did
416 not succeed to overexpress RsaI from a strong promoter in the WT HG001 strain. This result
417 was not due to technical problems as we managed to express RsaI under the control of its

418 own promoter on the same plasmid. Hence, the growth phenotypic experiment was performed
419 in the 132 strain. The WT 132 and the Δ rsal mutant strains harbor a similar growth curve in
420 BHI medium (Fig EV5A). However, when RsaI was expressed under the control of a strong
421 promoter, a slight but reproducible delay in growth was observed only in the WT 132 strain
422 (Fig EV5A). Northern blot analysis was performed to measure the steady state levels of RsaI
423 during growth in the various strains (Fig EV5B). Quantification of the autoradiographies and
424 normalization with 5S rRNA showed that the highest yields of RsaI were observed in the WT
425 132 strain expressing RsaI from the strong promoter, and only in this context a significant
426 amount of RsaI was detected at the beginning of growth (Fig EV5B).

427 In order to get a global overview of RsaI impact on gene regulation, a comparative
428 transcriptomic analysis was performed on total RNAs extracted from the WT HG001 strain,
429 the isogenic HG001 Δ rsal mutant strain, and the same mutant strain complemented with a
430 plasmid expressing RsaI under the control of its own promoter in order to avoid side effects of
431 a too strong expression of RsaI on growth (Tables EV6-7, and Fig EV5C). The cultures were
432 done in triplicates with high reproducibility in BHI medium at 37°C until 6h, under the conditions
433 allowing the expression of RsaI (Fig 1). We have considered a gene to be regulated by RsaI
434 if the ratio between two strains is at least two-fold. Changes in gene expression were more
435 pronounced between the mutant Δ rsal versus the same strain expressing RsaI from a plasmid
436 rather than between the mutant Δ rsal versus the WT strain (Table EV7). This effect is most
437 likely due to the higher expression of RsaI in the complemented strain in comparison to the
438 WT strain (Fig EV5C). The levels of 26 and 50 mRNAs were significantly decreased and
439 enhanced, respectively, when the complemented strain was compared to the mutant Δ rsal
440 (Table EV7). The complete data set is presented in Table EV6. Beside genes encoding phage
441 related proteins, most of the RsaI-dependent activation was observed for genes involved in
442 fermentation processes, in NO resistance and in energy-generating processing (in red in Table
443 EV7). In addition, weaker effects were also observed for mRNAs encoding proteins involved
444 in iron-sulfur cluster repair (*ScdA*), in NO detoxification (*qoxABCD*, *hmp*), and in various
445 metabolic pathways. The level of *RsaG* was also enhanced in the complemented strain.
446 Conversely, the overexpression of RsaI caused a reduced expression of genes that are
447 functionally related. Several of them are involved in glycolysis (*fba1*) and pentose phosphate
448 pathway (*gnd*, *tkt*), in thiamine co-factor synthesis (*thiW*, *tenA*), and in arginine catabolism
449 (*arcABCD*, *arcR*) (in blue in Table EV7). Additionally, significant repression was also observed
450 for *miaB* encoding tRNA specific modification enzyme, *tyrS* encoding tryptophanyl-tRNA
451 synthetase, *ebpS* encoding the cell surface elastin binding protein, and to a weaker extent for
452 *rex* encoding redox-sensing transcriptional regulator. Interestingly, the MAPS approach
453 revealed that several of these mRNAs (i.e., *qoxABCD* operon, *tyrS*, *plfA-plfB*) were also
454 enriched together with RsaI (Table EV2). Surprisingly, the most enriched RsaI targets

455 identified by MAPS (i.e, *icaR*, *glcU_2*, *fn3K*) did not show significant mRNA level variations
456 when RsaI was deleted or overexpressed suggesting that RsaI would primarily regulate their
457 translation (Table EV1).

458 These data showed that high concentrations of RsaI affected the mRNA levels of
459 several enzymes involved in sugar metabolism, in arginine catabolism, in the pentose
460 phosphate pathway, and in various processes linked to NO detoxification, energy production
461 and fermentation. However, the small overlap between the two approaches suggested that
462 many of the RsaI-dependent effects as revealed in the transcriptomic data resulted from
463 indirect effects.

464

465 **DISCUSSION**

466 In this work, we have investigated the cellular functions of one abundant sRNA, called RsaI
467 (or RsaOG), which is highly conserved among *Staphylococcaeae* (Geissmann et al., 2009;
468 Marchais et al., 2010). In contrast to many sRNAs that contained C-track motifs, this RNA has
469 the particularity to carry two conserved G-rich sequences and a large unpaired CU-rich
470 interhelical region (Fig 2). This RNA was proposed to fold into a pseudoknot structure involving
471 the two conserved regions limiting the access of regulatory regions (Marchais et al., 2010).
472 Combining several global approaches (MAPS, transcriptomic analysis), our data show that
473 RsaI is linked to a large regulon involved in sugar uptake and metabolism, biosynthetic and
474 co-factor synthesis, cytochrome biosynthesis, anaerobic metabolism, as well as iron-sulfur
475 cluster repair, and NO detoxification (Fig 6).

476

477 **The expression of RsaI is derepressed when glucose concentration decreases.**

478 The cellular level of RsaI is tightly controlled during growth in rich BHI medium. In this study,
479 we showed that RsaI expression is strongly and rapidly repressed at the transcriptional level
480 through the activity of CcpA in response to glucose availability. Carbon catabolite repression
481 is a universal regulatory phenomenon that allows the cells to use the preferred carbon source
482 to produce energy, and to provide the building blocks for macromolecules. Concomitantly, it
483 represses genes that are involved in the metabolism of less-preferred carbon sources. To do
484 so, CcpA has to be activated through a cascade of events involving its co-regulator histidine-
485 containing phosphocarrier protein (HPr), which has been phosphorylated by its cognate
486 kinase/phosphorylase HprK/P activated in the presence of glycolytic intermediates. It is
487 thought that binding of the phosphorylated HPr to CcpA enhances its DNA binding affinity to
488 the *cre* binding site to repress or activate the target genes. RsaI repression requires the
489 presence of CcpA, most probably through the binding at a *cre* motif located at -45/-30
490 (GGAAAACGCTTACAT) from the *rsal* transcriptional start site (Fig 1, Fig EV1B). Interestingly,
491 deletion of *ccpA* gene affected vancomycin resistance (Seidl et al., 2009) and recent

492 observations showed that sub-inhibitory treatment of cells with vancomycin led to an
493 enhanced expression of RsaI (Howden et al., 2013). We showed that the repression of RsaI
494 is alleviated as soon as the concentration of glucose is strongly reduced. Therefore, RsaI
495 might be a signature of a metabolic switch of the bacterial population. Using the MAPS
496 approach and gel retardation assays, we could identify several mRNAs that strongly bind to
497 RsaI with its long unpaired and conserved CU-rich region. Binding of RsaI to *glcU_2* encoding
498 a glucose permease and to *fn3K* encoding the fructosamine 3 kinase hindered the formation
499 of the initiation ribosomal complex. Because their steady state yields are not affected, we
500 suggest that both mRNAs are regulated at the translational level. We propose that this
501 mechanism is most likely common to all mRNAs that could form base-pairings between their
502 ribosome binding sites and the CU-rich region of RsaI. This is the case for mRNAs encoding
503 a transcriptional regulator of the XRE family, the PTS system trehalose-specific EIIBC
504 component TreB, a peptidase, a cell wall binding lipoprotein, and DegV containing protein
505 (Table EV2). Noteworthy, *in vivo* most of these mRNAs were no more found enriched by using
506 MS2-RsaI mut 4 in which the CU-rich region has been deleted (Table EV5). Among these
507 proteins, two of them are directly involved in sugar uptake and metabolism (*GlcU_2*, TreB). In
508 *S. aureus*, the PTS (phosphotransferase system)-dependent and independent transport
509 systems ensure efficient glucose transport (reviewed in Götz et al., 2006). If a rapidly
510 metabolizable sugar (such as glucose) is used during growth at a rather low concentration,
511 the transport will occur via the PTS system and concomitantly, the carbon catabolite
512 repression system will be activated through CcpA protein. At high concentration of glucose, it
513 is assumed that the sugar will be transported by the PTS system and in addition by the
514 permease *GlcU_2*. The glucose transported by the permease will be phosphorylated within
515 the cell by the glucose kinase GlkA. Hence, it is reasonable to propose that RsaI would repress
516 the synthesis of *GlcU_2* when the glucose concentration dropped. In addition, RsaI represses
517 the expression of *treB* mRNA most probably through base-pairings with its SD sequence. TreB
518 is the enzyme specific for trehalose, a diholoside which is transported exclusively by PTS
519 (Bassias & Brückner, 1998). Transcriptomic analysis also revealed that RsaI strongly
520 repressed various key enzymes involved in glucose catabolism pathway such as fructose-
521 biphosphate aldolase (*fba1*), 6-phosphogluconate dehydrogenase 2C decarboxylase (*gnd*),
522 and the thiamine-dependent enzyme transketolase (*tkt*). Additionally, we showed that RsaI
523 represses the synthesis of fructosamine 3-kinase (Fn3K), which deglycated products of
524 glycation formed from ribose 5-phosphate or erythrose 4-phosphate produced by the pentose
525 phosphate pathway (Gemayel et al., 2007). This enzyme is part of a repair machinery to
526 protect the cells from damages caused by glycation as the results of high glucose
527 concentrations (Deppe et al., 2011). The pentose phosphate pathway is also an alternative
528 route for glucose metabolism, and provides the source of pentose phosphates necessary for

529 nucleotide synthesis. Although it is not known whether RsaI regulated their expression in a
530 direct or indirect manner, base-pairings were predicted between RsaI and the ribosome
531 binding site of *fbpA* and *tkt* mRNAs. The transketolase is a key enzyme of the pentose
532 phosphate pathway, which requires thiamine diphosphate as a co-factor. Interestingly, the
533 thiamine pathway is also repressed in strain expressing RsaI (Table EV6). Although, we have
534 observed very similar pathways that are deregulated by RsaI using the MAPS and RNA-seq
535 approaches, not so many overlaps were identified. It is possible that the conditions of the
536 MAPS approach performed on the WT strain expressing all the ribonucleases has
537 preferentially enriched the mRNAs that are regulated at the translational level. Therefore, it is
538 tempting to deduct that RsaI would inhibit the synthesis of the major permease of glucose
539 uptake, of enzymes involved in the glycolysis, of unnecessary enzymes involved in
540 detoxification of high glucose concentration, and of the pentose phosphate pathway when
541 glucose concentration decreases (Fig 6).

542

543 **RsaI interacts with sRNA containing C-rich motifs**

544 The MAPS approach revealed that several sRNAs (RsaG, RsaD, RsaE) were enriched
545 significantly with RsaI. We demonstrated here that the second G-track sequence located in
546 the first hairpin domain of RsaI is able to form a highly stable complex with RsaG, and to a
547 lesser extent with RsaD. Additionally, mRNA targets interacting with the CU-rich domain of
548 RsaI did not disturb the binding of RsaG suggesting that the two functional domains of RsaI
549 are independent. Conversely, preliminary data suggested that the apical loop of the first
550 hairpin of RsaG contain the C-rich motif that is recognized by RsaI, but which is also used to
551 regulate the expression of several mRNAs (Desgranges *et al.*, unpublished data). RsaG is
552 part of the 3'UTR of *uhpT* mRNA, and its expression was strongly enhanced by the
553 extracellular concentration of G-6P. Under these conditions, the levels of RsaI are much lower
554 than for RsaG (Fig EV4B). These two sRNAs are thus involved in pathways related to the use
555 of the preferred carbon sources. Indeed, RsaI negatively controls glucose uptake when
556 glucose is consumed or absent from the medium while RsaG responds to the extracellular
557 concentration of G-6P. Although the functions of RsaG remains to be addressed, we
558 hypothesized that the sRNA might regulate either the expression of unnecessary genes, or of
559 genes involved in sugar metabolism, or of genes required to protect cells against damages
560 linked to sugar-phosphate uptake and metabolism (Bobrovskyy & Vanderpool, 2016).
561 Noteworthy, RsaI sequence is conserved in the *Staphylococcus* genus while RsaG is only
562 conserved in the *S. aureus* species. Our data suggested that RsaG has no major impact on
563 the regulation of RsaI targets. Its slight stabilizing effect on RsaI strongly supports the MAPS
564 data showing that the two sRNAs interact *in vivo* and potentially would form ternary complexes
565 in the presence of the RsaI targets. Whether RsaI might regulate the functions of RsaG

566 remained to be addressed.

567 In addition, the overproduction of RsaI induced several changes into the transcriptome
568 of *S. aureus* that resembled the regulon of the two-component system SrrAB, which was
569 demonstrated as the essential system responding to both NO and hypoxia (Kinkel et al., 2013).
570 The SrrAB regulon has also been shown to confer to the cells the ability to maintain energy
571 production, to promote repair damages, and NO detoxification (Richardson et al., 2006; Kinkel
572 et al., 2013). We observed here that RsaI enhances the expression of genes encoding
573 cytochrome biosynthesis (*qoxABCD*), as well as genes involved in anaerobic metabolism
574 (*pflAB*, *ldh1*, *focA*, *adh*), in iron sulfur cluster repair (*scdA*), and most importantly in NO
575 detoxification (*qoxABCD*, *hmp*) and NO resistance (*ldh1*). These effects are most likely indirect
576 and might be due to the interaction between RsaI and the SrrAB-dependent sRNAs RsaD and
577 RsaE, which both contained a typical *srrA* site upstream their genes. These two sRNAs
578 present a C-rich sequence that can potentially form base-pairings with the G-track sequences
579 of RsaI (Table EV1), but the formation of complexes with RsaI is less efficient than with RsaG.
580 It cannot be excluded that an RNA-binding protein might be required in these cases to stabilize
581 the base-pairings. We also do not exclude that the two sRNAs were pooled down because
582 they might share similar mRNA targets with RsaI. RsaD and RsaE are both upregulated by
583 the presence of NO in the cellular medium (Durand et al., 2015; Fig EV4D). Although the
584 functions of RsaD requires additional studies, *S. aureus* RsaE was previously shown to
585 coordinate the downregulation of numerous metabolic enzymes involved in the TCA cycle and
586 the folate-dependent one-carbon metabolism (Bohn et al., 2010; Geissmann et al., 2009), and
587 of enzymes involved in arginine degradation pathway (Rochat et al., 2018). Additionally, in *B.*
588 *subtilis*, the homologue of RsaE called RoxS is under the control of the NADH-sensitive
589 transcription factor Rex, and the Rex binding site is also conserved in *S. aureus* *rsaE* gene
590 (Durand et al., 2017). Hence, it was proposed that RsaE downregulated several enzymes of
591 the central metabolism under non favorable conditions, and in addition would contribute to
592 readjust the cellular NAD⁺/NADH balance under stress conditions (Bohn et al., 2010; Durand
593 et al., 2015; 2017).

594 Many of the RsaI-dependent effects, which have been monitored by the transcriptomic
595 analysis, are indirect, and we propose that some of these effects might result from the
596 interaction of RsaI with sRNAs when the preferred carbon source became scarce.

597

598 **Physiological consequences of RsaI regulation.**

599 Our data suggested that several sRNAs in *S. aureus* are part of intricate regulatory networks
600 to interconnect in a dynamic manner various metabolic pathways following sugar metabolism
601 and uptake. The concept that sRNAs are key actors to coordinate the regulation of metabolic
602 enzymes has been largely demonstrated for *Enterobacteriaceae* (reviewed in Papenfort and

603 Vogel, 2014). These sRNA-dependent regulations often induced significant growth
604 phenotypes in response to the availability of carbon sources and nutrient. For instance, *E. coli*
605 and *Salmonella* SgrS contribute to stress resistance and growth during glucose-phosphate
606 stress by inhibiting synthesis of sugar transporters or activating dephosphorylation and efflux
607 of sugars (e.g., Vanderpool & Gottesman, 2004; 2007; Kawamoto et al., 2005; Rice et al.,
608 2012; Papenfort et al., 2013). The repertoire of SgrS targets has been recently expanded to
609 mRNAs involved in key metabolic pathways, which allow restoring metabolic homeostasis
610 during sugar-phosphate stress and growth recovery (Richards et al., 2013; Bobrovskyy &
611 Vanderpool, 2016). *Salmonella* GcvB regulon is required for growth if peptides represent the
612 unique carbon source (Miyakoshi et al., 2015), while *E. coli* Spot42 is important for optimal
613 utilization of carbon sources and its overproduction inhibited growth on several nonpreferred
614 carbon sources (Møller et al., 2002, Beisel & Storz, 2011). In *S. aureus*, the overproduction of
615 RsaE induces a growth defect, which was partially alleviated by acetate (Bohn et al., 2010).
616 These examples illustrate how the yields of these sRNAs should be tightly controlled during
617 growth in order to optimize the use of the preferred carbon sources, to restore metabolic
618 homeostasis during stress, and to avoid cell damages or metabolites depletion caused by the
619 intracellular production of glucose-phosphate. Interestingly, in *Bacillus subtilis*, the sRNA SR1
620 was found repressed under glycolytic conditions mainly by CcpN and to a lesser extent by
621 CcpA (Licht et al., 2005; Heidrich et al., 2007; Gimpel et al., 2012). SR1 expression is induced
622 by L-arginine and acts as a main regulator of arginine catabolism through the translational
623 repression of *ahrC* mRNA (Heidrich et al., 2007). In this case, the deletion or the
624 overexpression of SR1 did not affect growth (Licht et al., 2005). The deletion of *rsal* gene does
625 not cause a growth phenotype and only high levels of RsaI constitutively expressed from a
626 plasmid in the WT strain causes a weak growth delay (Fig EV5A). Additional works will be
627 required to identify the growth conditions under which the function of RsaI can be studied in a
628 more relevant way.

629 One possible track resides in the fact that RsaI interconnects various metabolic
630 pathways. By acting as a post-transcriptional regulator, it could play an important role during
631 the infection process. *S. aureus* has the ability to generate infections through the colonization
632 of many different metabolic host niches. Several studies have shown that both glycolysis and
633 gluconeogenesis systems are mandatory for the infection process, and moreover *S. aureus*
634 appears to be resistant to NO radicals that are heavily produced by the macrophages.
635 Interestingly, glycolysis is an important process that contributes to persist within the
636 macrophages, and to protect the intracellular bacteria against NO (Vitko et al., 2015). However,
637 if the bacteria escape the macrophages or lyse the host cells, *S. aureus* is thought to form
638 aggregates at the centre of highly inflamed and hypoxic abscesses. Under these conditions,
639 the host cells consumed a large amount of glucose to fight the inflammation. Hence, glucose

640 will become scarce for *S. aureus* suggesting that lactate and amino acids derived from the
641 host might be used as the major sources of carbon to enter gluconeogenesis (Richardson et
642 al., 2015). These conditions favored the activation of the two-component system SrrAB, which
643 in turn activates genes required for anaerobic metabolism, cytochrome and heme biosynthesis,
644 and NO radicals detoxification should play an essential role in the survival of the bacteria
645 (Kinkel et al., 2013). Because the CcpA-dependent repression of RsaI is alleviated under
646 conditions where the glucose is strongly reduced, and because many SrrAB-dependent genes
647 are also induced when RsaI is expressed at high levels, it is thus expected that RsaI might
648 also contribute to metabolic adaptations of the cells to the dynamic nature of the host immune
649 environment. Besides, it is also tempting to propose that RsaI might be involved in the dormant
650 state of bacterial cells while environmental conditions are unfavorable (Lennon & Jones, 2011).
651 Interestingly enough, we also observed a RsaI-dependent activation of the expression of the
652 mRNA encoding EsxA, a type VII secreted virulence factor required for the release of the
653 intracellular *S. aureus* during infection (Korea et al., 2014). We also showed here that RsaI
654 activates the synthesis of the PIA-PNAG exopolysaccharide most probably by decreasing the
655 translation of IcaR, which is the transcriptional repressor of *icaADBC* operon (Fig 4, Fig 6).
656 We demonstrated that RsaI base-pairs *icaR* mRNA without interfering the interaction between
657 *icaR* 3'UTR and 5'UTR (Fig EV3). Although the precise molecular mechanism is not yet
658 defined, we propose that RsaI would inhibit the synthesis of IcaR by stabilizing the
659 circularization of the mRNA and/or by counteracting with the binding of an activation factor of
660 *icaR* mRNA translation (Fig 6). Interestingly, RsaE, which is another RsaI target, also binds
661 *icaR* mRNA at two distinct positions close to the SD sequence and in the coding sequence
662 through C-rich motifs (Rochat et al., 2018). These motifs are almost identical to the anti-SD
663 sequence (UCCCC) comprised in the *icaR* 3'UTR (Ruiz de los Mozos et al., 2013). This opens
664 the possibility of a competition for hindering the *icaR* SD by RsaE or the 3'UTR. Thus, RsaI
665 and RsaE might interact individually or simultaneously with *icaR* mRNA to repress its
666 expression in response to changes in cellular medium of glucose and NO. Though further
667 investigations are needed to decipher the physiological consequences of such complex
668 interactions, it indicates that the regulation of the synthesis of the PIA-PNAG is rather
669 complexed and multifactorial and is tightly controlled according to the metabolic state of the
670 bacterial population (Cue et al., 2012). The signals modifying the yield of these RNAs would
671 be crucial to determine the levels of IcaR repressor and PIA-PNAG synthesis. We also do not
672 exclude that RsaI might regulate another regulator of the synthesis of PIA-PNAG. For instance,
673 inactivation of the TCA cycle resulted in a massive derepression of the PIA-PNAG biosynthetic
674 enzymes to produce the exopolysaccharide (Sadykov et al., 2008), and glucose enhances
675 PIA-PNAG dependent biofilm formation (You et al., 2014), while SrrAB appears as an inducer
676 of PIA-PNAG dependent biofilm (Ulrich et al., 2007). It remains to be elucidated if the biofilm

677 induction mediated by SrrAB occurs through activation of RsaE that might promote interaction
678 with *icaR* mRNA and/or RsaI. Hence, *icaR* can be considered as a hub gene, integrating both
679 transcriptional regulatory networks and sRNA-mediated post-transcriptional regulatory signals
680 to control biofilm formation in *S. aureus*.

681 Although our study shed light on the regulatory activities of this multifaceted sRNA,
682 there is still much to be learned on how RsaI and other sRNAs can be integrated into the
683 networks regulating the metabolic pathways that are essential for *S. aureus* biofilm formation,
684 survival, persistence and invasion within the host.

685

686 **MATERIAL AND METHODS**

687

688 **Plasmids and Strains Constructions**

689 All strains and plasmids constructed in this study are described in Table EV8. The
690 oligonucleotides designed for cloning and for mutagenesis are given in Table EV3.
691 *Escherichia coli* strain DC10B was used as a host strain for plasmid amplification before
692 electroporation in *S. aureus*. Plasmids were prepared from transformed *E. coli* pellets following
693 the Nucleospin Plasmid kit protocol (Macherey-Nagel). Transformation of both *E. coli* and *S.*
694 *aureus* strains was performed by electroporation (Bio-Rad Gene Pulser).

695 The *rsal* deletion mutant was constructed by homologous recombination using plasmid
696 pMAD in *S. aureus* HG001 and 132 (Arnaud et al., 2004). The deletion comprises nucleotides
697 2376101 to 2376306 according to HG001 genome (Caldelari et al., 2017). Experimental
698 details are given in supplementary materials.

699 The vector pCN51::*Prsal* was constructed by ligating a PCR-amplified fragment (Table
700 EV3) containing *rsal* with 166 pb of its promoter region and digested by *SphI* and *PstI* into
701 pCN51 vector digested with the same enzymes. The vector pUC::*T7rsal* was constructed by
702 ligating a PCR-amplified fragment (Table EV3) containing *rsal* with the T7 promoter sequence
703 and digested by *EcoRI* and *PstI* into pUC18 vector digested with the same enzymes.
704 Mutagenesis of pUC::*T7rsal* was performed with Quikchange XL Site-directed mutagenesis
705 (Stratagene) leading to pUC::*T7rsal* mut1, 2, 3 and 4 (Table EV3). To obtain the plasmid
706 pCN51::*P3::MS2-rsal*, a PCR product containing the MS2 tag fused to the 5' end of *rsal* was
707 cloned into pCN51::*P3* by digestion of both PCR fragments and of the plasmid by *PstI/BamHI*
708 (Table EV3). Plasmids from positive clones were sequenced (GATC Biotech) before being
709 transformed in DC10B, extracted and electroporated into *S. aureus* strains. The plasmids
710 pCN51::*P3::MS2-rsal* mut2 and mut4 were generated by Quikchange mutagenesis as above.
711 Construction of plasmids pES::*rsal* and pES::*rsal* mut5 is described in supplementary
712 materials.

713

714 **Growth conditions**

715 *E. coli* strains were cultivated in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract,
716 1% NaCl) supplemented with ampicillin (100 µg/mL) when necessary. *S. aureus* strains were
717 grown in Brain-Heart Infusion (BHI), Tryptic Soy Broth (TSB) or Muller Hinton Broth (MHB)
718 media (Sigma-Aldrich) supplemented with erythromycin (10 µg/mL) for plasmid selection.
719 When needed, MHB was complemented with either 5 g/L of glucose 6-phosphate, or 1 g/L of
720 glucose, fructose or xylose (Sigma-Aldrich). NO production was induced by addition of 100 µM
721 Na-diethylamine NONOate (Sigma-Aldrich) as previously described (Durand et al., 2015).

722

723 **Preparation of total RNA extracts**

724 Total RNAs were prepared from *S. aureus* cultures taken at different times of growth. After
725 centrifugation, bacterial pellets were resuspended in 1 mL of RNA Pro Solution (MP
726 Biomedicals). Lysis was performed with FastPrep and RNA purification followed strictly the
727 procedure described for the FastRNA Pro Blue Kit (MP Biomedicals). Electrophoresis of either
728 total RNA (10 µg) or MS2-eluted RNA (500 ng) was performed on 1,5% agarose gel containing
729 25 mM guanidium thiocyanate. After migration, RNAs were vacuum transferred on
730 nitrocellulose membrane. Hybridization with specific digoxigenin (DIG)-labeled probes
731 complementary to RsaI, RsaG, RsaD, 5S, *ccpA* sequences, followed by luminescent detection
732 was carried out as previously described (Tomasini et al., 2017).

733

734 **MAPS experiments, transcriptomic and RNA-seq analysis**

735 Crude bacterial extract were prepared and purified by affinity chromatography as previously
736 described (Tomasini et al., 2017). The eluted RNA samples were either used for Northern blot
737 or treated with DNase I prior to RNA-seq analysis. Isolation of tagged sRNAs and the co-
738 purified RNAs was performed in duplicates. The experiments were carried out with the tagged
739 wild-type RsaI and two mutant forms (mut2 and mut4). RNAs were treated to deplete abundant
740 rRNAs, and the cDNA libraries were performed using the ScriptSeq complete kit (bacteria)
741 from Illumina. The libraries were sequenced using Illumina MiSeq with a V3 Reagent kit
742 (Illumina), which preserves the information about the orientation of the transcripts and
743 produces reads of 150 nts, which map on the complementary strand. Each RNA-seq was
744 performed at least in duplicates. The reads were then processed to remove adapter
745 sequences and poor quality reads by Trimmomatic (Bolger et al., 2014), converted to the
746 FASTQ format with FASTQ Groomer (Blankenberg et al., 2010), and aligned on the HG001
747 genome (Caldelari et al., 2017) using BOWTIE2 (Langmead et al., 2009). Finally, the number
748 of reads mapping to each annotated feature has been counted with HTSeq (Anders et al.,
749 2015) using the interception non-empty protocol. To estimate the enrichment values for the
750 MAPS experiment or the differential expression analysis for the transcriptomic experiment, we

751 used DEseq2 (Varet et al., 2016). The statistical analysis process includes data normalization,
752 graphical exploration of raw and normalized data, test for differential expression for each
753 feature between the conditions, raw p-value adjustment, and export of lists of features having
754 a significant differential expression (threshold p-value=0.05; fold change threshold=2)
755 between the conditions. All processing steps were performed using the Galaxy platform (Afgan
756 et al., 2016).

757 For total RNA extracts and MS2-eluted RNAs, DNase I (0.1 U/ μ L) treatment was
758 performed 1h at 37°C. The reactions mixtures were then purified by phenol::chloroform::
759 isoamylalcohol 25:24:1 (v/v) and subsequent ethanol precipitation. RNA pellets were re-
760 suspended in sterile milliQ water. RNA was quantified by Qubit (Life Technologies) and the
761 integrity was assessed with the Bioanalyzer (Agilent Technologies). For transcriptomic, 1 μ g
762 of total RNA was ribo-depleted with the bacterial RiboZero kit from Illumina. The TruSeq total
763 RNA stranded kit from Illumina was used for the library preparation. Library quantity was
764 measured by Qubit and its quality was assessed with a TapeStation on a DNA High sensitivity
765 chip (Agilent Technologies). Libraries were pooled at equimolarity and loaded at 7 pM for
766 clustering. The 50 bases oriented single-read sequencing was performed using TruSeq SBS
767 HS v3 chemistry on an Illumina HiSeq 2500 sequencer.

768

769 **Preparation of RNAs for *in vitro* experiments**

770 Transcription of RsaI, RsaI mutants and RsaG was performed using linearized pUC18 vectors.
771 PCR fragments containing the 5'UTR of the coding region of selected mRNA targets were
772 directly used as templates for *in vitro* transcription using T7 RNA polymerase. The RNAs were
773 then purified using a 6% or 8% polyacrylamide-8 M urea gel electrophoresis. After elution with
774 0.5 M ammonium acetate pH 6.5 containing 1 mM EDTA, the RNAs were precipitated in cold
775 absolute ethanol, washed with 85% ethanol and vacuum-dried. The labeling of the 5' end of
776 dephosphorylated RNAs (RsaI/RsaI mutants) and DNA oligonucleotides were performed with
777 T4 polynucleotide kinase (Fermentas) and [γ ³²P] ATP. Before use, cold or labeled RNAs
778 were renatured by incubation at 90°C for 1 min in 20 mM Tris-HCl pH 7.5, cooled 1 min on
779 ice, and incubated 10 min at 20°C in ToeP+ buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 60
780 mM KCl, 1 mM DTT).

781

782 **Gel Retardation Assays**

783 Radiolabeled purified RsaI or RsaI mutants (50000 cps/sample, concentration < 1 pM) and
784 cold mRNAs were renatured separately as described above. For each experiment,
785 increasing concentrations of cold mRNAs were added to the 5' end labeled wild-type or RsaI
786 mutants in a total volume of 10 μ L containing the ToeP+ buffer. Complex formation was

787 performed at 37°C during 15 min. After incubation, 10 µL of glycerol blue was added and the
788 samples were loaded on a 6% or 8 % polyacrylamide gel under non-denaturing conditions
789 (300 V, 4°C). Under these conditions where the concentration of the labeled RNA is negligible,
790 the K_D dissociation constant can be estimated as the concentration of the cold RNA that
791 showed 50% of binding.

792

793 **Toe-printing assays**

794 The preparation of *E. coli* 30S subunits, the formation of a simplified translational initiation
795 complex with mRNA, and the extension inhibition conditions were performed as previously
796 described (Fechter et al., 2009). Increasing concentrations of RsaI were used to monitor their
797 effects on the formation of the initiation complex with *glcU_2* and *fn3K* mRNAs.

798

799 ***In vivo* β-galactosidase assays**

800 Translation fusions were constructed with plasmid pLUG220, a derivative of pTCV-*lac*, a low-
801 copy-number promoter-less *lacZ* vector, containing the constitutive *rpoB* promoter (Table
802 EV8). The whole leader regions of *glcU_2* (-54/+99), *fn3K* (-33/+99), *treB* (-23/+99),
803 HG001_01242 (-34/+99), and HG001_02520 (-71/+99) (Table EV3) were cloned downstream
804 the *PrpoB* in frame with *lacZ*. The whole gene encoding RsaI or RsaI mut4 (obtained
805 synthetically from IDT) with 166 bp of its promoter region was digested with *Pst*I and ligated
806 at the unique *Pst*I of pLUG220::*PrpoB* vector. The final constructs were transformed into the
807 strain HG001Δ*rsal*. β-galactosidase activity was measured four times as previously described
808 (Tomasini et al., 2017).

809

810 **PIA-PNAG quantification.**

811 Cell surface PIA-PNAG exopolysaccharide levels were monitored according to (Cramton et
812 al., 1999). Overnight cultures were diluted 1:50 in TSB-3% NaCl and bacteria were grown at
813 37°C. Samples were extracted at 6h after inoculation. The same number of cells of each strain
814 was resuspended in 50 µL of 0.5 M EDTA (pH 8.0). Then, cells were incubated for 5 min at
815 100°C and centrifuged 17,000 g for 5 min. Supernatants (40 µL) was incubated with 10 µL of
816 proteinase K (20 mg/mL) (Sigma) for 30 min at 37°C. After the addition of 10 µL of the buffer
817 (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.01% bromophenol blue), serial dilutions 1:25 were
818 performed in the same buffer. Then, 10 µL were spotted on a nitrocellulose membrane using
819 a Bio-Dot microfiltration apparatus (Bio-Rad). The membrane was blocked overnight with 5%
820 skimmed milk in phosphate-buffered saline (PBS) with 0.1% Tween 20, and incubated for 2h
821 with specific anti-PNAG antibodies diluted 1:20,000 (Maira-Litrán et al., 2005). Bound
822 antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G

823 antibodies (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA) diluted 1:10,000,
824 using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

825

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847

848 **AUTHOR CONTRIBUTIONS**

849 DB, ED, and IC performed the MAPS experiments and the validation of the data. CJC, LP,
850 ATA, and IL constructed several mutant strains and performed the biofilm assays. AC and PF
851 have done the transcriptomic analysis. SM analyzed all the RNA-Seq analysis, and
852 quantification of the data. KM and FV contributed to phenotypic analysis of some of the mutant
853 strains. PR and IC were responsible of the project, and wrote the manuscript. All authors
854 contributed to the analysis, interpretation of the experiments, and writing of the manuscript.

855

856 **CONFLICT OF INTEREST.** The authors have declared no conflict of interest.

857

858 **DATA AVAILABILITY.** The transcriptomics and MAPS data have been deposited in NCBI's

859 Gene Expression Omnibus (Barrett et al., 2013) and are accessible through the GEO Series
860 accession number
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1051
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1053

1054 **FIGURES LEGENDS**

1055

1056 **Figure 1: RsaI responds to glucose through the transcriptional factor CcpA.**

1057 **(A)** Northern blot experiments show the expression of RsaI during growth phase in the HG001
1058 strain in MHB medium with or without the addition of 1,5 g/L of D-glucose. Glucose was added
1059 either at the beginning of growth (+ glucose, left panel) or after 3h of growth (right panel).

1060 **(B)** Northern blot experiment shows the expression of RsaI during growth phase in the HG001,
1061 $\Delta ccpA$ mutant strain, and $\Delta codY$ mutant strain, in MHB medium with (+) or without (-) the
1062 addition of 1,5 g/L of D-glucose.

1063 **(C)** Northern blot experiment shows the expression of RsaI in the HG001 and $\Delta ccpA$ mutant
1064 strains. Total RNA was prepared after 2, 3, 4, 5 and 6h of culture in BHI medium at 37°C.

1065 **(D)** Northern blot analysis of RsaI in the HG001 strain grown in MHB medium with or without
1066 the addition of 1 g/L of glucose, fructose or xylose. For all the experiments, loading controls
1067 were done using the expression of 5S rRNA (5S) as revealed after hybridization of the
1068 membranes with a specific probe. However for these controls, we used aliquots of the same
1069 RNA preparations but the migration of the samples was performed in parallel to the
1070 experiments on a separate agarose gel because RsaI and 5S rRNA have very similar sizes.

1071

1072 **Figure 2: RsaI binds to *icaR*, *glcU_2*, *fn3K* mRNA and the sRNA RsaG.**

1073 **(A)** Secondary structure model of RsaI. In red, are the nucleotides deleted in the RsaI mutants
1074 (mut1 to mut4), and the nucleotides, which were substituted (mut5). The potential base-
1075 pairings between RsaI and RsaG are shown. Squared nucleotides are conserved sequences
1076 in RsaI.

1077 **(B)** Gel retardation assays show the formation of the complex between RsaI and *icaR*, *glcU_2*
1078 and *fn3K* mRNAs. The 5' end-labeled wild-type RsaI (RsaI), RsaI mutant 3 (RsaI mut3,
1079 deletion of the two G-track motifs), and RsaI mutant 4 (RsaI mut4, deletion of the C/U rich
1080 unpaired interhelical region) were incubated with increasing concentrations of mRNAs: lane 1,
1081 0 nM; lane 2, 25 nM; lane 3, 50 nM; lane 4, 100 nM and lane 5, 200 nM. Below the gels, the
1082 predicted interactions between RsaI and its targets are shown. Translation start codons are in
1083 green and SD is for Shine Dalgarno sequence. Graphs represented the % of complex formed
1084 between either RsaI or its two mutant forms (RsaI mut3 and RsaI mut4) and the target mRNA
1085 (*icaR*, *glcU_2*, *fn3K*) as the function of mRNA concentrations.

1086 **(C)** Gel retardation assays show the formation of the complex between RsaI and RsaG. The
1087 5' end-labeled wild-type RsaI (RsaI), RsaI mutant 2 (RsaI mut2), RsaI mutant 4 (RsaI mut4),
1088 and RsaI mutant 1 (RsaI mut1) were incubated with increasing concentrations of RsaG given
1089 in nM on the top of the autoradiographies.

1090

1091 **Figure 3: RsaI inhibits *glcU_2* and *fn3K* mRNA translation.**

1092 **(A)** Toe-print assays showing the effect of RsaI on the formation of the ribosomal initiation
1093 complex of *glcU_2* and *fn3K* mRNAs, respectively. Lane 1 : incubation control of mRNA alone;
1094 lane 2 : incubation control of mRNA with 30S subunits; lane 3: incubation control of mRNA
1095 with RsaI; lane 4 : formation of the ribosomal initiation complex containing mRNA, 30S and
1096 the initiator tRNA^{Met} (tRNA_i); lanes 5 to 9 : formation of the initiation complex in the presence
1097 of increasing concentrations of RsaI, respectively : 50 nM (lane 5), 100 nM (lane 6), 150 nM
1098 (lane 7), 300 nM (lane 8), and 400 nM (lane 9). Lanes T, A, C, G: sequencing ladders. The
1099 Shine and Dalgarno (SD) sequence, the start site of translation (START) and the toe-printing
1100 signals (+16) are indicated. At the bottom of the gels are shown the predicted interactions
1101 between RsaI and its targets. Translation start codons are in green, and the Shine and
1102 Dalgarno (SD) sequence is underlined, the arrowheads depict the toe-printing signals.

1103 **(B)** The β -galactosidase activity (Miller Units) has been measured from various fusions
1104 expressed from a plasmid which also carries *rsal* gene under its own promoter:
1105 *PrpoB::glcU::lacZ::rsal*, *PrpoB::fn3K::lacZ::rsal*, *PrpoB::treB::lacZ::rsal*,
1106 *PrpoB::HG001_1242::lacZ::rsal*, and *PrpoB::HG001_2520::lacZ::rsal* expressed in the mutant
1107 strain HG001- Δ *rsal*. The same constructs were made in the absence of *rsal* gene. For the
1108 *fn3K-lacZ* fusion, we also used an additional construct *PrpoB::fn3K::lacZ::rsalmut4* expressing
1109 both the fusion FN3K-LacZ protein and RsaI mut4. The β -galactosidase activity was
1110 normalized for bacterial density and the results represented the mean of four independent
1111 experiments. The error bars are standard deviations and the statistical significance was
1112 determined using the Student's t-test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$,
1113 ns is for not significant.

1114

1115 **Figure 4: Interaction of RsaI to *icaR* mRNA induces biofilm production**

1116 **(A)** Secondary structure model of *icaR* mRNA. The nucleotides in red are involved in the long-
1117 range interaction between the 3' and 5'-UTRs or with RsaI. SD and anti-SD are respectively
1118 for the Shine and Dalgarno sequence and for the sequence complementary to the SD
1119 sequence.

1120 **(B)** Gel retardation assays show the formation of the complex between RsaI and *icaR* full
1121 length, *icaR* SUBST, *icaR*-5'UTR, and *icaR*-3'UTR. The 5' end-labeled of RsaI was incubated
1122 with increasing concentrations of the various mRNAs. UTR is for untranslated region, SUBST
1123 stands for the substitution of UCCCCUG sequence by AGGGGAC. This mutation, which is
1124 located in the 3'UTR of *icaR*, significantly destabilizes the long-range interaction, and
1125 enhances *icaR* translation. Lane C represents binding between radiolabelled RsaI and full-

1126 length *icaR* mRNA (50 nM). Due to the rather short migration of the gel, the full-length mRNA
1127 is still observed in the pocket.

1128 **(C)** *In vivo* effect of RsaI on PIA-PNAG synthesis in the *S. aureus* wild-type (WT) 132 strain,
1129 the Δ *rsal* mutant, and the strain carrying a deletion of *icaR* 3'UTR (Δ 3'UTR). This last mutant
1130 strain has been transformed with the pES plasmids expressing *rsal* or *rsal* mut5. The PIA-
1131 PNAG exopolysaccharide biosynthesis was quantified using dot-blot assays. Serial dilutions
1132 (1/5) of the samples were spotted onto nitrocellulose membranes and PIA-PNAG production
1133 was detected with specific anti-PIA-PNAG antibodies. RsaI was detected in the same samples
1134 by Northern blot using a probe directed against RsaI. Ethidium bromide staining of rRNA was
1135 used as loading controls of the same gel.

1136

1137 **Figure 5: Regulatory function of RsaI is not impaired by binding of RsaG.**

1138 **(A)** Ternary complex formation between RsaI, various mRNAs (*glcU_2*, *HG001_00942* and
1139 *HG001_1242*), and RsaG. The 5'-end labeled RsaI was incubated with increasing
1140 concentrations of the target mRNA alone or in the presence of 50 nM of RsaG. The various
1141 complexes are notified on the sides of the autoradiographies.

1142 **(B)** RsaG does not form stable complexes with *glcU_2* and *HG001_01242*. Binding assays
1143 were done in the presence of 5' end-labeled RsaG and either cold RsaI (300 nM) or increasing
1144 concentrations of *glcU_2* and *HG001_01242*.

1145 **(C)** Measurements of the half-lives of RsaI and RsaG in *HG001- Δ rsaG* or *HG001- Δ rsal* mutant
1146 strains using Northern blot experiments. The cells were treated with rifampicin at 4h of growth
1147 and total RNAs were extracted after 2, 4, 8, 15, 30 and 60 min at 37°C in BHI. 5S rRNA was
1148 probed to quantify the yield of RNAs in each lane using the same samples, which were
1149 however run on two different gels. Calculated half-lives are shown beneath the
1150 autoradiographies and are the average of 2 experiments. The data were normalized to 5S
1151 rRNA.

1152

1153 **Figure 6: Model of RsaI regulation networks in *Staphylococcus aureus*.**

1154 In the presence of glucose or glucose 6-phosphate (left panel), the expression of RsaI is
1155 inhibited, in contrast to RsaG. In the absence of glucose or when glucose is metabolized (right
1156 panel), repression of RsaI is alleviated to regulate its RNA targets. In blue, are the
1157 transcriptional protein regulators, and in red the regulatory sRNAs. Grey arrows represent
1158 functional links, black arrows are for activation, bars for repression. Red lines corresponded
1159 to post-transcriptional regulation and black lines to transcriptional regulation. Dotted lines
1160 represented the regulatory events for which the regulation is not yet demonstrated. A
1161 schematic view of the post-transcriptional *icaR* mRNA regulation is represented in the insert.
1162 The 3'UTR of *icaR* contains an anti-Shine and Dalgarno (anti-SD) sequence able to bind the

1163 SD sequence in the 5'UTR (Ruiz de los Mozos et al., 2013). The CU-rich unpaired sequence
1164 of RsaI (in red) binds to the 3'UTR of *icaR* mRNA downstream of the anti-SD, and represses
1165 the translation indirectly either by stabilizing the interaction between the two UTRs or by
1166 preventing the action of trans-acting activators (protein or RNA).

1167

1168 **Figure EV1: Analysis of the RsaI expression.**

1169 **(A)** Northern blot experiments showing the expression of RsaI in HG001 strain during growth
1170 phase. Total RNA was prepared from samples taken after various time points of growth in BHI
1171 medium at 37°C. Hybridization against 5S rRNA was used as loading control on the same
1172 samples which have been migrated on another agarose gel because RsaI and 5S rRNA have
1173 very similar sizes.

1174 **(B)** The CRE-site consensus was defined in *Bacillus cereus* (van der Voort et al., 2008). The
1175 sequence found in the 5' region of RsaI is represented below the graph, the conserved
1176 residues are in red.

1177 **(C)** Northern blot experiments showing the expression of RsaI in HG001 strain, the isogenic
1178 HG001 Δ *rsaI* mutant strain, and the same mutant strain complemented with a plasmid
1179 expressing RsaI under the control of its own promoter (HG001 Δ *rsaI* pCN51::PrsaI). Total RNA
1180 was prepared from samples taken after various time points of growth in MHB medium in the
1181 absence or presence of 1% glucose. For the mutant HG001 Δ *rsaI* strain (used as a negative
1182 control), total RNA was prepared after 4h of growth in MHB medium in the absence or in the
1183 presence of glucose. Hybridization against 5S rRNA was used as loading control on the same
1184 membrane. *Traces of RsaI signal after re-hybridization of the membrane with the 5S probe
1185 were still observed.

1186 **(D)** MS2-RsaI is specifically retained by affinity chromatography containing the MS2-MBP
1187 protein. The Northern blot was performed using a DIG-labeled RsaI probe (Table EV3) to
1188 visualize RsaI and MS2-RsaI following the MS2 chromatography affinity. CE is for crude
1189 extract, FT for Flow-Through, W for Washing, and E for Elution. For CE/FT/W samples, 5 μ g
1190 of total RNA was loaded on a 1,5% agarose gel while for E sample, only 0,5 μ g of total RNA
1191 was used. The two replicates are shown for the RNA purified from the strain
1192 HG001 Δ *rsaI*::MS2-RsaI.

1193

1194 **Figure EV2: RsaI interacts with several RNA species.**

1195 Gel retardation assays showing the formation of complexes between RsaI and several mRNAs,
1196 and the sRNA RsaD. The 5' end-labeled RsaI was incubated with increasing concentrations
1197 of target mRNAs or RsaD as shown on the top of the autoradiographies. Quantification of the
1198 autoradiographies showed that RsaI forms stable complexes with many of the mRNAs (Kd <
1199 50 nM) except for TreB and RsaD (Kd > 200 nM).

1200

1201 **Figure EV3: RsaI interacts with the 3'UTR of *icaR* mRNA.**

1202 **(A)** Analysis of the effect of RsaI on the interaction between the 5'UTR and the 3'UTR of *icaR*.
1203 The 5' end-labeled of the 3'UTR of *icaR* was incubated with 500 nM of the 5'UTR of *icaR* alone
1204 or in the presence of increasing concentrations of RsaI, as shown on the top of the
1205 autoradiography.

1206 **(B)** Gel retardation assays showing the formation of complexes between RsaI or RsaI mut5
1207 and the 3'UTR of *icaR*. RsaI or RsaI mut5 labeled at their 5' ends were incubated with
1208 increasing concentrations of the 3'UTR of *icaR* (10, 25 and 50 nM). In both gels, the various
1209 RNA species present in the different bands are given on one side of the autoradiographies.

1210

1211 **Figure EV4: Analysis of the expression of RsaG and RsaD in response to stress.**

1212 **(A)** Genomic organization of the *uhpT* locus in HG001 strain. This locus is conserved in all *S.*
1213 *aureus* strains (Geissmann et al., 2009). The transcription of *uhpT* containing RsaG is induced
1214 by the two-component system HptRS, which senses the extracellular concentration of
1215 Glucose-6 phosphate (G-6P).

1216 **(B)** Northern blot experiment showing RsaG (left panel) or RsaI (right panel) expression in the
1217 HG001 strain. Total RNA was extracted at 2, 4 and 6h of growth in BHI or MHB with or without
1218 addition of glucose-6 phosphate (+ G-6P). 5S rRNA was used as loading control using the
1219 same samples, which have been migrated on another agarose gel.

1220 **(C)** Northern blot experiment showing RsaG expression in the HG001 Δ *hptRS* deleted mutant.
1221 Total RNA was extracted at 2, 4 and 6h of growth in BHI with or without addition of glucose-6
1222 phosphate (+ G-6P). 5S rRNA was used as loading control using the same samples, which
1223 have been migrated on another agarose gel.

1224 **(D)** The β -galactosidase activity (Miller Units) have been measured from *PrpoB::fn3K::lacZ*,
1225 *PrpoB::fn3K::lacZ::rsal* and *PrpoB::HG001_fn3K::lacZ::rsal* mut4 expressed in HG001 and
1226 HG001 Δ *rsaG* strains. The β -galactosidase activity was normalized for bacterial density and
1227 the results represented the mean of four independent experiments. **** $p < 0.0001$, ns: not
1228 significant.

1229 **(E)** Northern blot experiment showing the steady-state level of RsaD. On the left, RsaD
1230 expression was analyzed at 2, 4 and 6h of growth, in HG001 wild-type strain, and in Δ *saeRS*
1231 and Δ *srrAB* mutant strains. On the right, the expression of RsaD was monitored in response
1232 to nitric oxide (NO) stress. The culture performed in BHI medium until $OD_{600}=0.2$ at 37°C was
1233 then treated with the addition of 100 μ M Na-diethylamine (+ DEA NONOate). 5S rRNA was
1234 used as loading control using the same samples, which have been migrated on another
1235 agarose gel.

1236

1237 **Figure EV5: RsaI represents a signature of a metabolic change as the result of glucose**
1238 **consumption.**

1239 **(A)** Growth curves of the wild-type 132 (132) and mutant Δ *rsaI* (132D) strains containing the
1240 vector pES (empty) or pES::*rsaI* (expressing RsaI from a constitutive promoter) in BHI.

1241 **(B)** Northern blot experiment showing RsaI expression in the wild-type 132 (132) or mutant
1242 Δ *rsaI* (132 Δ *rsaI*) strains containing the vector pES or pES::*rsaI*. Total RNA was extracted at
1243 2, 4 and 6h of growth in BHI. 5S rRNA was used as the loading control using the same samples,
1244 which have been migrated on another agarose gel. Quantification of RsaI normalized to 5S
1245 rRNA was done with ImageQuant TL software (GE Healthcare Life Sciences).

1246 **(C)** Northern blot experiments showing the expression of RsaI in the wild-type HG001 strain,
1247 the mutant HG001 Δ *rsaI* strain, and the same mutant strain complemented with a plasmid
1248 expressing RsaI from its own promoter (HG001 Δ *rsaI* pCN51::*PrsaI*). All the other strains have
1249 been transformed with the pCN51 plasmid. Total RNA was prepared from samples taken after
1250 6h of growth in BHI medium. Hybridization against 5S rRNA was used as loading control using
1251 the same membrane. Three replicates were carried out (lanes 1 to 3) and the same samples
1252 were used for the differential transcriptomic analysis.

1253 **Table 1: MAPS data revealed the best RNA candidates found enriched with MS2-RsaI.**

1254

1255

Target	Name of the gene and function	Base-pairing interactions prediction	ΔG (kcal/mol)	Fold change* MS2-RsaI/MS2	Binding	Fold change Δ rsal/complemented
HG001_02704	IcaR Biofilm operon <i>icaADBC</i> HTH-type negative transcriptional regulator	949 GAAAUGGAAAGUA AUA AGUAAUAA 973 108 CUUU-CCUUUCAU --- UCAUUUUU 88	-14,92	33,976	i	0,839
HG001_02520	XRE family transcriptional regulator	-21 AUAAACA U UGGAG-UGA -- UGAUUAUG 3 92 UAUUUAUAACUUC U ACAACACUA CAUU 66	-12,10	22,978	i	0,479
HG001_01242	Hypothetical protein	-29 AAGGA AAGUAA UGAAA UAAA--- GGAGAAUG 3 106 UUCUUUCAUU-CAUUUUU AUA ACUUCUACA 76	-16,27	17,369	i	0,818
HG001_02756	RsaG	43 CUACCCUU 50 32 GAUGGGGA 25	-8,82	13,621	i	0,484
HG001_02360	GlvR	602 UACCCUU +608 31 AUGGGGA 25	-6,78	9,961	ni	1,0342
HG001_01537	RsmG Putative O-methyltransferase	-22 GGGGA UCCAGC AGUAGUAAUGUAUGUGAAU 8 106 UUCUUUCAU-UCAUUUUU-UUAU-ACUUC 80	-9,74	7,137	i	1,536
HG001_02609	<i>isaA</i>	741 UAUAUAGCUCU-UCCC 755 41 AUAUAUCGAGAUGGGG 26	-11,36	6,76	ni	1,054
HG001_00847	RsaE	59 CCCCUUUCUU 68 29 GGGGAAACAA 20	-14,21	5,476	ni	0,951
HG001_02545	Hypothetical protein	-2 AAUGGAAAGAAUUUGAAGUAAU 21 113 UUUAUCUUCCUUUCAUUCUUA 91	-11,16	5,262	i	1,103

HG001_00316	Peptidase propeptide and YPEB domain protein	-23 AAAUAGGAAGAGAGAGUGAUUUUUG 3 113 UUUAUCUUUC-CUUUCAUUCAUUAUU 89	-15,55	5,234	i	1,342
HG001_02210	Hypothetical protein	-26 AAAUUUUUAAGGAUGU -10 90 UUUAUAC--UUCUACA 76	-8,33	4,978	ni	1,212
HG001_02293	GlcU_2 putative glucose uptake protein	-25 AAAUAGUAUAGGAGAGUGAGUAGUCUUG 3 113 UUUAUCUU-UCCUUUCAUUCAUUAUUUA 87	-18,25	4,769	i	1,061
HG001_00974	Hypothetical protein	401 CUACCCU 407 32 GAUGGGG 26	-7,18	4,532	ni	1,219
HG001_02628	Fn3K Fructosamine Kinase	-24 UAAGUAGAUUGGAAGGUGUUUGU-AUGAAUG 7 114 GUUUUAUCUUCCUUUCAU--CAUUAUUUAU 86	-16,44	4,37	i	0,935
HG001_00942	Putative cell wall binding lipoprotein	-24 AAAUAGCAAGUGGAGGUACAAGUAUGAAUUGGA 12 113 UUUAUCUUUC-CUUUCAU--UCAUUAUUUAUAAUCUU 81	-16,06	4,091	i	0,676
HG001_00584	RsaD	42 CUCCUUUGU 50 29 GGGGAACA 21	-7,97	4,078	i	1,015

1256

1257 The red bases are involved in base-pairing interactions. In green are shown the Shine and Dalgarno sequence and the translation start codon
1258 AUG (UUG for *glcU_2* mRNA). ΔG for RNA hybrids were predicted with IntaRNA. The enrichment value as deduced from the MAPS data
1259 corresponds to MS2-RsaI versus the MS2 control. *For all assays, the p-value was inferior to 0,00005 (Table S3). Binding of the RNA candidates
1260 to RsaI was monitored by gel retardation assays: n.i, means no interaction, and i for validated interaction. The last column corresponds to the
1261 transcriptomic data (Table S6) and is represented by the ratio between the mutant Δ rsaI strain versus the same strain complemented with a
1262 plasmid expressing RsaI.

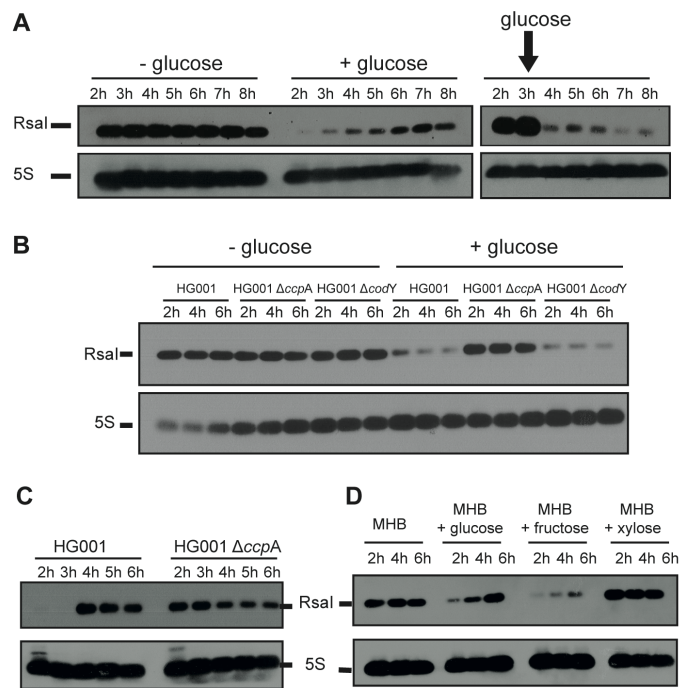


Figure 1- Bronesky et al.

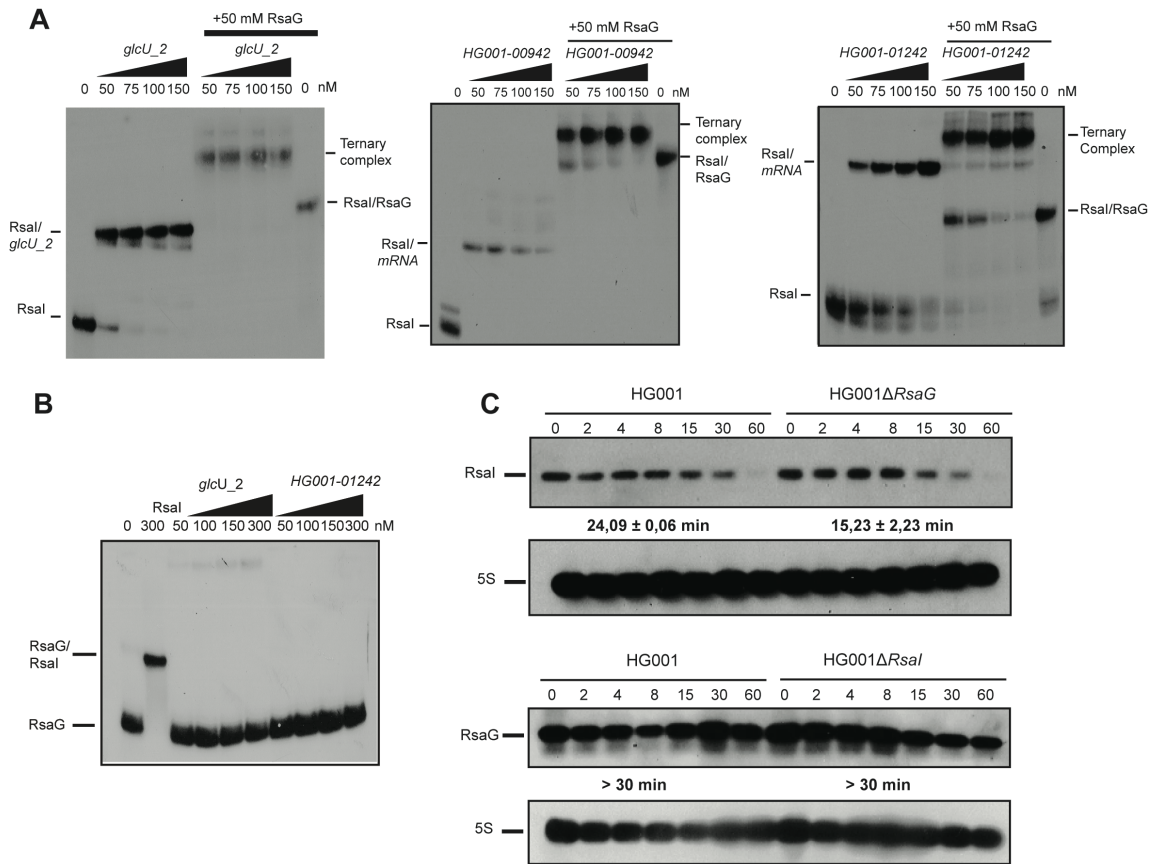


Figure 5-Bronesky et al.

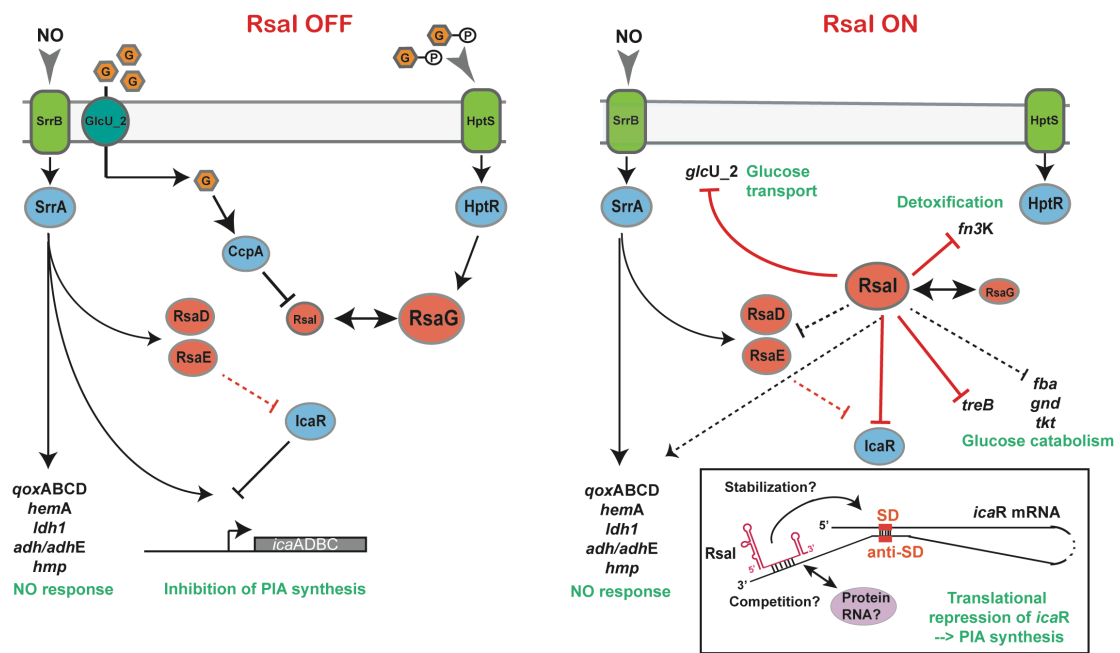


Figure 6- Bronesky et al.