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6S RNA-dependent antibiotic susceptibility

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§ This work is dedicated to the memory of our colleague Brice Felden who passed away unexpectedly on March 5, 2021

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6S RNA / rifampicin / *Staphylococcus aureus* / RNAP / antibiotic resistance

23

24 **Abstract**

25

26 Bacterial small RNAs (sRNAs) contribute to a variety of regulatory mechanisms that
27 modulate wide ranging pathways, including metabolism, virulence, and antibiotic resistance.
28 We investigated the involvement of sRNAs in rifampicin resistance in the opportunistic
29 pathogen *Staphylococcus aureus*. Using a competition assay with an sRNA mutant library,
30 we identified 6S RNA as being required for protection against low concentrations of
31 rifampicin, an RNA polymerase (RNAP) inhibitor. This effect applied to rifabutin and
32 fidaxomicin, two other RNAP-targeting antibiotics. 6S RNA is highly conserved in bacteria
33 and its absence in two other major pathogens, *Salmonella enterica* and *Clostridioides*
34 *difficile*, also impaired susceptibility to RNAP inhibitors. In *S. aureus*, 6S RNA is produced
35 from an autonomous gene and accumulates in stationary phase. In contrast to what was
36 reported in *Escherichia coli*, *S. aureus* 6S RNA does not appear to play a critical role in the
37 transition from exponential to stationary phase, but affects σ^B -regulated expression in
38 prolonged stationary phase. Nevertheless, its protective effect against rifampicin is
39 independent of alternative sigma factor σ^B activity. Our results suggest that 6S RNA helps
40 maintain RNAP- σ^A integrity in *S. aureus*, which could in turn help bacteria withstand low
41 concentrations of RNAP inhibitors.

42

43 Introduction

44 *Staphylococcus aureus* is a commensal Gram-positive bacterium but also an
45 opportunistic pathogen responsible for diseases ranging from benign (mostly cutaneous
46 forms) to life-threatening (visceral or osteoarticular forms) infections (reviewed in (1, 2)). Due
47 to the emergence of resistant strains, mainly methicillin-resistant (MRSA) and vancomycin-
48 intermediate *S. aureus* (VISA), *S. aureus* has become a high priority target for the discovery
49 of new antibiotics (3).

50 In standard antibiotic treatment regimens, if penicillin M and glycopeptides give
51 unsatisfactory results, combination therapy with rifampicin may be considered, particularly in
52 complicated prosthetic device-associated infections (4). Rifampicin, a rifamycin derivative, is
53 an inhibitor of bacterial RNA polymerase (RNAP) (5-7). The molecule binds to the RNAP β -
54 subunit in the DNA/RNA channel to prevent transcription by steric hindrance. This effect
55 occurs during a narrow window, just after the synthesis of the first ribonucleotides; rifampicin
56 is ineffective on transcripts once they are elongated (7).

57 Highly conserved among bacteria, the core RNA polymerase contains four essential
58 subunits (two α , β and β') and one accessory subunit (ω) (8-10). Among Gram-positive
59 bacteria, RNA polymerase includes two other accessory subunits: δ and ϵ (ϵ is specific to
60 Firmicutes). These accessory subunits may enhance transcriptional specificity and recycle
61 RNAP. A sigma (σ) factor subunit completes the core enzyme: when present, the complex is
62 called RNAP holoenzyme. σ factors recognize bacterial promoters and participate in
63 adaptation to changing growth conditions (11). The σ factors are associated with specific
64 transcriptional programs whose function and features may differ among species (11). The
65 number of σ factors varies between species. For example, seven σ factors were described in
66 *Escherichia coli* and *Salmonella enterica* (12), and twenty-two in the spore-forming bacterium
67 *Clostridioides difficile* (13). In contrast, *S. aureus* possesses only four σ factors, σ^A , σ^B , σ^H
68 and σ^S . σ^A is the vegetative factor responsible for transcription of housekeeping genes (14),
69 σ^B is the main alternative sigma factor contributing to stress adaptation (15-17). The last two
70 factors are expressed only in response to specific conditions: σ^H is involved in the regulation
71 of competence (18, 19) and σ^S in response to miscellaneous environmental stresses (20). A
72 number of transcriptional factors participate together with σ factors in modulating bacterial
73 transcription (21).

74 Small RNAs (sRNAs) are recognized as ubiquitous elements that fine-tune gene
75 expression at transcriptional and post-transcriptional levels (22, 23). sRNAs are well studied
76 in Gram-negative bacteria. However, in Gram-positive bacteria, including *S. aureus*, their
77 roles in virulence, metabolism and antibiotic resistance are less understood, although there is

78 no doubt about their involvement in these processes (24-26). The majority of characterized
79 sRNAs interact with mRNAs. However, some sRNAs interact directly with protein complexes.
80 This is the case for 6S RNA, one of the first described sRNAs, identified in *Escherichia coli* in
81 1967 (27) and sequenced in 1970 (28). In *E. coli*, 6S RNA binds preferentially to RNAP
82 associated with the σ^{70} factor. 6S RNA accumulates during exponential growth, and reaches
83 its maximum levels in stationary phase (29). The 6S RNA / RNAP interaction leads to
84 inhibition of numerous *E. coli* σ^{70} -dependent promoters and consequently re-orientates the
85 transcription dependent of alternative sigma factors, allowing adaptation to many
86 environmental conditions (reviewed in (30, 31)). Although conserved among bacteria (32),
87 the 6S RNA role(s) and function(s) in many of them remain unknown.

88 We recently developed a platform to assess *S. aureus* sRNAs required for fitness
89 based on an sRNA mutant library (33). Using this platform, we identified a rifampicin
90 susceptibility phenotype associated with the lack of 6S RNA, pointing to a possible new
91 mechanism of resistance against low rifampicin concentrations. We showed that this
92 phenotype is neither restricted to rifampicin nor to *S. aureus* but extends to other RNAP
93 inhibitors and bacterial species. Characterization of 6S RNA in *S. aureus* indicates its partial
94 involvement in σ^B -dependent transcription regulation at late stationary phase, rather than
95 during transition from exponential to stationary phase. Additional experimental evidence
96 suggests that *S. aureus* 6S RNA has a role in RNAP holoenzyme cohesion.

97

98 **Results**

99 **Absence of 6S RNA confers increased susceptibility to rifampicin in *S. aureus* and**
100 ***S. enterica*.**

101 At the beginning of this study we examined the possible involvement of sRNAs in
102 processes underlying *S. aureus* susceptibility to antibiotics. To uncover sRNA-associated
103 phenotypes, our laboratory has previously developed a fitness assay based on competition
104 between sRNA-tagged mutants within a library that includes mutants of most *S. aureus bona*
105 *fide* sRNAs, defined as expressed by an autonomous gene without antisense transcription
106 (33, 34). Briefly, the fitness of individual sRNA deletion mutants growing within a collection of
107 mutants is tested by comparing their proportion in the presence or absence of different
108 compounds. The accumulation or reduction of individual strains are identified by monitoring
109 the tagged sequences. This method distinguishes strains showing even subtle growth
110 differences. Three identical libraries containing 74 putative sRNA mutants and 3 control
111 mutants were challenged with rifampicin at a sub-lethal concentration ($6 \mu\text{g.L}^{-1}$). After three
112 days of growth, one mutant was under-represented ≈ 100 -fold compared to the other mutants
113 when normalized to the same libraries grown in the same medium, without rifampicin (Fig. 1).
114 The mutant with reduced fitness due to the presence of rifampicin carried a deletion of the
115 *ssrS* gene (referred to here as *ssrS^{Sa}*), which encodes 6S RNA, an sRNA known to interact
116 with RNAP, the rifampicin target (5-7).

117 We asked whether *ssrS^{Sa}* mutant susceptibility to rifampicin observed in the fitness
118 experiment is detectable in monocultures. Serial dilutions of WT and *ssrS^{Sa}* overnight
119 cultures were spotted on solid medium containing low levels of rifampicin ($5 \mu\text{g.L}^{-1}$); in this
120 condition, the *ssrS^{Sa}* mutant was 100-fold more sensitive to rifampicin than the parental strain
121 (Fig. 2A). This susceptibility was reversed by insertion of an *ssrS^{Sa}* copy at an ectopic
122 chromosomal locus ($\Delta\textit{ssrS}^{\text{Sa}}$ ecto-*ssrS^{Sa}*) (Fig. S1; Fig. 2A, left panel). A longer growth lag in
123 rifampicin-containing liquid medium was also observed for the *ssrS^{Sa}* mutant compared to the
124 wild-type or complemented strains (Fig. 2C). These two tests indicated that the rifampicin
125 susceptibility phenotype was solely due the absence of 6S RNA. This phenotype is observed
126 within a narrow window of rifampicin concentrations, below the minimal inhibitory
127 concentration (MIC, $12 \mu\text{g.L}^{-1}$). We conclude that 6S RNA protects *S. aureus* cells against
128 sub-lethal concentrations of rifampicin.

129 As 6S RNA is widely conserved in the bacterial kingdom, we examined its protective
130 role against rifampicin in the enteric pathogen *Salmonella enterica*, a Gram-negative species.
131 The *ssrS* gene of *S. enterica* (*ssrS^{LT2}*) was deleted (Fig. S2). As in *S. aureus*, *ssrS^{LT2}* deletion
132 led to a rifampicin susceptibility phenotype when compared to its parental strain (Fig. 2B, left

133 panel). However, this phenotype was only partly complemented by insertion of *ssrS*^{LT2} wild-
134 type gene at a chromosomal ectopic position (Fig. 2B, left panel).

135 **6S RNA protection against rifampicin is partially interchangeable between *S. aureus*** 136 **and *S. enterica***

137 Since *S. aureus* and *S. enterica* *ssrS* mutants show a similar rifampicin susceptibility
138 phenotype, we investigated whether the 6S RNA genes would be functional in heterologous
139 backgrounds. For this, gene swaps were performed, replacing i) the native *S. enterica* LT2
140 *ssrS*^{LT2} gene by the *S. aureus* *ssrS*^{Sa} homolog (*S. enterica* Δ *ssrS*^{LT2}::*ssrS*^{Sa}; Fig S2), and ii)
141 the native *S. aureus* *ssrS* gene (*ssrS*^{Sa}) by the *S. enterica* *ssrS*^{LT2} homolog (*S. aureus*
142 Δ *ssrS*^{Sa}::*ssrS*^{LT2}; Fig. S1).

143 The *ssrS*^{Sa} gene failed to compensate the *S. enterica* Δ *ssrS*^{LT2} strain rifampicin
144 susceptibility (Fig. 2B, left panel). This is possibly due reduced synthesis of staphylococcal
145 6S RNA in the *S. enterica* background as suggested by the results of Northern blot analysis
146 (Fig. S3A). Interestingly, however, the reverse swap in *S. aureus* Δ *ssrS*^{Sa} partially restored
147 growth in rifampicin (Fig. 2A, left panel). Complementation of *ssrS*^{Sa} by *ssrS* from an
148 evolutionary distant species suggests that different 6S RNAs shield against rifampicin using
149 similar mechanisms.

150 **6S RNA protects RNAP against different RNA polymerase inhibitors**

151 The family of RNAP inhibitors comprises molecules with different mechanisms of
152 action. We chose two RNAP inhibitors, rifabutin, fidaxomicin and a putative RNAP inhibitor,
153 aureothricin, to test the impact of *ssrS*^{Sa} on drug susceptibility.

154 Rifabutin, a spiro-piperidyl-rifamycin, is a rifampicin analog (35, 36). Fidaxomicin (also
155 known as lipiarmycin (37, 38) and tiacumicin B (39)) is a narrow spectrum antibiotic (40) that
156 inhibits transcription initiation by locking RNAP through an open-clamp state that prevents an
157 efficient interaction with the promoter (41-44). Aureothricin is a member of the
158 dithiopyrrolones group and has broad-spectrum activity (45). However, the mechanism of
159 action of this molecule remains unclear. For each drug, the appropriate sub-lethal
160 concentrations to use were first established using the HG003 *S. aureus* strain. The *ssrS*^{Sa}
161 mutant showed a ~4 log-fold greater susceptibility to rifabutin than the HG003 strain (Fig.
162 3A). The *ssrS*^{Sa} mutant was also negatively affected by fidaxomicin compared to the parental
163 strain, with visibly smaller colonies (Fig. 3A). As seen with rifabutin, the susceptibility
164 phenotype was not fully complemented by ectopic expression of *ssrS*^{Sa} with either drug.
165 Contrary to rifabutin and fidaxomicin, no aureothricin phenotype was associated with the
166 absence of 6S RNA (Fig. 3A), possibly reflecting its poorly characterized mode of action.

167 Fidaxomicin is mainly active against *C. difficile*, a major human intestinal pathogen
168 (40). We decided to test whether *ssrS* deletion also impacts RNAP inhibitor susceptibility in
169 this species. For this, we constructed a $\Delta ssrS$ derivative ($\Delta ssrS^{Cd}$) of *C. difficile* 630 Δerm .
170 *C. difficile* $\Delta ssrS^{Cd}$ was 1000-fold more susceptible to fidaxomicin compared to its parental
171 strain (Fig. 3B, left panel). A plasmid carrying the *ssrS*^{Cd} gene introduced in the $\Delta ssrS^{Cd}$
172 strain complemented the phenotype by restoring wild-type level of susceptibility to
173 fidaxomicin (Fig. 3B, left panel).

174 We conclude that *ssrS*-related susceptibility to antibiotics is a common feature of
175 different RNAP inhibitors in evolutionary distant bacterial species. The mechanism
176 associated with this susceptibility phenotype is likely the same for different RNAP-targeted
177 antibiotics and different species, one possibility being 6S RNA protection of RNAP via steric
178 occlusion.

179 **Growth phase-dependent expression of *ssrS* in *S. aureus***

180 The expression profile of 6S RNA differs according to species (30). We performed
181 Northern experiments to evaluate 6S RNA expression in *S. aureus* (Fig. 4A). 6S RNA was
182 strongly expressed and accumulates to 20-fold higher levels in stationary phase, as
183 determined in *S. aureus* HG003. The expression profile of *S. aureus* 6S RNA was similar to
184 that reported in *Salmonella* (46), *E. coli* (29) and *B. subtilis* 6S-1 RNA, which carries a
185 second 6S RNA (47-50).

186 In overnight (O/N) samples where 6S RNA is the most abundant, a second, faster-
187 migrating band was also observed (Fig. 4A and S3A). A second band was reported in
188 *C. difficile* even during exponential phase (51). To determine a potential alternative 6S RNA
189 form in *S. aureus*, also previously suggested (52), we performed 5'-3' RACE on samples
190 collected at different time points during growth in rich medium: OD₆₀₀=7 (OD7, corresponding
191 to entry into stationary phase), O/N, and on day 4 (D4). At all sampling points, the major
192 Transcription Start Site (TSS) is the same (Fig. 4B) and in agreement with the site
193 determined by a global TSS mapping (53). Concerning the 3'-end, the longest form ending
194 with a T is the most abundant in OD7 samples, representing 28.6% of analyzed sequences.
195 These data confirm that the size of the longest most abundant form is 231 nucleotides
196 (predicted at 230 nt (52)). Samples from overnight or day 4 cultures exhibited shorter forms
197 that may result from processing or degradation by 3' exonucleases.

198 **Moderate impact of 6S RNA on the global *S. aureus* transcription profile.**

199 The role of 6S RNA in transcriptional regulation was suggested early (29) and then
200 validated by transcriptomic analysis in *E. coli* (54-56); in RNA-seq data, 35 genes were at
201 least 2-fold differentially expressed in a 6S RNA deficient strain compared to the parental at

202 the onset of stationary phase. To determine whether *S. aureus* 6S RNA could play a similar
203 role, the transcriptional profile of the $\Delta ssrS^{Sa}$ mutant was compared with that of its parental
204 strain by RNA-seq on samples collected at $OD_{600}=7$, which corresponds to the entry into
205 stationary phase of *S. aureus* (Table 1). Transcriptome analyses were performed on
206 biological triplicates and features with a p-value < 0.05 were retained for interpretation.
207 Surprisingly, the transcriptional profiles of parental and $\Delta ssrS^{Sa}$ strains were highly similar.
208 Only three genes were more than 2-fold down-regulated in $\Delta ssrS^{Sa}$ (FC < 0.5; Table 1 top
209 panel). They encode a hypothetical epoxyqueuosine reductase (QueH/SAOUHSC_02911), a
210 hemin transporter (HrtA/SAOUHSC_02640 (its co-functional partner HrtB/SAOUHSC_02641
211 is also down-regulated), and a 30 amino-acid peptide (SAOUHSC_01817) of unknown
212 function. Other genes related to transporters, cell wall metabolism and redox state were also
213 significantly reduced but with a lower-fold change. All of these genes are regulated by σ^A ,
214 except *bstA*, a σ^B DNA-damage-induced gene coding a putative DinB superfamily protein.
215 Taken together, these results suggest that 6S RNA does not redirect transcription during the
216 stationary phase transition in a sigma-dependent manner.

217 Transcriptome results, as performed in the early period of stationary phase, did not
218 provide evidence linking σ^B transcriptional activity to the presence of 6S RNA. We used a
219 reporter fusion strategy to pursue this question: the gene encoding the fluorescent protein
220 mAmetrine was placed under the transcriptional control of the σ^B -regulated
221 SAOUHSC_00624 promoter (57) (pPsigB-mAmetrine; Fig. 5A). No significant difference of
222 fluorescence was observed between *ssrS^{Sa}* and parental strains when measured for the first
223 18h of growth in rich liquid medium. Thus, in keeping with transcriptomic findings, we
224 conclude that 6S RNA does not appear to redirect transcription during transition from
225 exponential to stationary phase in *S. aureus*, which differs from what was reported in *E. coli*
226 (56-58). Interestingly, however, after 18h of culture, mAmetrine expression continues to
227 increase in the parental strain, compared to markedly lower expression in $\Delta ssrS^{Sa}$. This
228 result, suggesting that 6S RNA could be important for efficient σ^B -dependent gene
229 expression during starvation, remains to be investigated.

230 Since the absence of 6S RNA in *S. aureus* leads to an increased susceptibility to
231 rifampicin, we questioned if this phenotype was related to σ^B regulation. The HG002 strain is
232 a HG003 isogenic strain containing an 11 bp-deletion in the *rsbU* gene encoding a σ^B
233 activator (59). Consequently, HG002 is deficient in σ^B activity, illustrated by the absence of
234 yellow pigmentation (60). We first noticed a greater susceptibility to rifampicin of HG002
235 compared to HG003 (Fig. 5). This observation indicates that lower σ^B activity *per se* confers
236 increased rifampicin susceptibility, as described in *C. difficile sigB* mutant (61). This effect is
237 probably due to a stress adaptation deficiency related to the absence of σ^B regulation. To

238 determine the effect of 6S RNA in this genetic context, the *ssrS^{Sa}* deletion was introduced in
239 HG002. The resulting strain (HG002 Δ *ssrS^{Sa}*) was considerably more susceptible to
240 rifampicin than the parental strain HG002 (Fig. 5B). This observation indicates that the
241 absence of 6S RNA leads to increased rifampicin susceptibility through a pathway that is
242 independent of σ^B activity.

243 **6S RNA plays a role in RNAP stability in *S. aureus***

244 6S RNA binds to RNAP- σ^{70} in *E. coli* (29, 47). As RNAP holoenzyme is a protein
245 complex with accessory subunits (especially σ factors), an element binding to this complex
246 could directly influence its stability or composition. We first performed an electrophoretic
247 mobility shift assay (EMSA) in *S. aureus* with radiolabeled 6S RNA (P^{32} -6S RNA), purified
248 sigma factors (σ^A -His and σ^B -His) and RNAP (with His-tagged RpoC) to assess the
249 interaction with 6S RNA (Fig. 6A). A P^{32} -labelled unrelated sRNA, SprB (62), was used as a
250 control. No interactions were detected with SprB. In contrast, our results showed interaction
251 between 6S RNA and RNAP coupled to the vegetative sigma factor, σ^A , to a lesser extent
252 with RNAP- σ^B .

253 We questioned if the absence of 6S RNA could alter RNAP holoenzyme composition.
254 The amounts of σ^A and β/β' subunits were evaluated in *ssrS^{Sa}* and parental strain cultures at
255 different time points. Western blots were performed with antibodies raised against σ^A and
256 RNAP (Fig. 6B). Interestingly, σ^A pools were lower in the *ssrS^{Sa}* mutant compared to the
257 parental strain and complemented strains in all tested conditions. At day 3 (D3), σ^A pools
258 were decreased by nearly 2-fold in the *ssrS^{Sa}* mutant compared to the parental strain. These
259 results suggest that 6S RNA plays a role in RNAP holoenzyme stability and could act as a
260 protective belt for RNAP- σ^A . We hypothesized that a reduced amount of σ^A could modify
261 strain outgrowth. Growth from HG003 (parental strain), HG003 Δ *ssrS^{Sa}* and its
262 complemented mutant HG003 Δ *ssrS^{Sa}* ecto-*ssrS^{Sa}* from pre-cultures that had grown O/N, 2
263 days and 3 days were compared (Fig. 6C). Surprisingly, no growth difference was observed
264 between the three strains in BHI, regardless of the pre-culture age. Despite the significant
265 effect on σ^A levels, 6S RNA is not an essential factor for *S. aureus* growth in rich medium.

266

267 Discussion

268 Here we demonstrated that the absence of 6S RNA in *S. aureus* leads to a fitness loss
269 in the presence of low rifampicin concentrations. This marked phenotype was associated
270 with only one sRNA gene (*ssrS^{Sa}*) out of 77 tested mutants in a competition experiment. This
271 phenotype is conserved from Gram-positive to Gram-negative bacteria suggesting a
272 common protective effect.

273 In *S. aureus*, the rifampicin phenotype was fully restored by ectopic gene
274 complementation, indicating that it was solely due to the absence of 6S RNA. In *S. enterica*
275 however, similarly done complementation of the *ssrS^{LT2}* deletion was only partial, while the
276 native and ectopic copies had similar expression levels (Fig. S3A). In *E. coli*, the *ssrS* and
277 *ygfA* genes are in an operon (48, 63) and mature 6S RNA results from a 5'- and 3'-transcript
278 ends processing (64, 65); similar organisation and regulation is expected in *S. enterica*. Two
279 hypotheses may explain the incomplete complementation of Δ *ssrS^{LT2}* rifampicin phenotype:
280 (i) The ectopic *ssrS^{LT2}* copy is subjected to a slightly different processing pathway (not
281 detected in the gel in Fig. S3A) and (ii) Δ *ssrS^{LT2}* could exert a polar effect on *ygfA*
282 expression, however, no growth defect has been observed for the mutant so far.

283 Despite weak similarity between *S. aureus ssrS^{Sa}* and the cognate *S. enterica ssrS^{LT2}*
284 gene, we observed partial complementation of the rifampicin phenotype in *S. aureus* Δ *ssrS^{Sa}*
285 by the Δ *ssrS^{LT2}* allele. The lack of the reverse complementation (*ssrS^{Sa}* into *S. enterica*
286 Δ *ssrS^{LT2}*) might be ascribable to the lower expression of *ssrS^{Sa}* in *S. enterica* (Fig. S3A)
287 and/or to any of the hypothesis raised above for the *S. enterica* Δ *ssrS^{LT2}* ecto-*ssrS^{LT2}*
288 phenotype.

289 Susceptibility of the *ssrS* mutants was not observed for all the compounds tested.
290 Differences in the mechanisms of action, binding sites and drug entry efficiencies could
291 explain this observation (42, 66). The Δ *ssrS^{Sa}* mutant showed increased susceptibility of *S.*
292 *aureus* to rifampicin, rifabutin, and fidaxomicin. Similarly, cognate Δ *ssrS* *S. enterica* and *C.*
293 *difficile* mutants showed marked sensitivities to rifampicin and fidaxomicin, respectively.
294 These drugs bind RNAP close to sites interacting with DNA, suggesting that 6S RNA
295 interaction with the enzyme may, at least partially, prevent antibiotic access to their sites.
296 Based on this reasoning, our results suggest that aureothricin, for which toxicity was
297 unaffected by *ssrS^{Sa}* deletion, does not bind RNAP at the interface with DNA.

298 Our findings suggest differences in the regulatory roles of 6S RNA in *S. aureus*
299 compared to those reported in *E. coli* (54). In the latter species, 6S RNA interaction with the
300 RNAP holoenzyme is proposed to coordinate transcriptional regulation with growth (29).
301 Accordingly, numerous transcriptome analyses performed under different conditions,

302 indicated that in *E. coli*, many of 6S RNA-regulated genes were related to
303 translational/transcriptional machinery or amino acid metabolism (29, 54-56, 67-70). In
304 contrast, our *S. aureus* transcriptomic analysis and promoter assay reveal no obvious 6S
305 RNA-related differential expression during transition to stationary phase. Two major features
306 of *S. aureus* could explain this phenomenon: (i) lower diversity of sigma factors in *S. aureus*
307 with only four σ factors, among which σ^A and σ^B control the majority of transcribed genes.
308 Knowing that the main alternative sigma factor σ^B is involved in stress response and not only
309 in stationary phase adaptation, σ^B -promoters could be less sensitive to 6S RNA during the
310 transition phase; (ii) the compensatory effect of a co-regulator. The levels of the alarmone
311 ppGpp increases in *E. coli* *ssrS* mutants, and might compensate the lack of 6S RNA (55, 56,
312 58). This possibility provides an attractive explanation in *S. aureus*, as ppGpp is synthesized
313 in response to nutrient starvation, and drives growth adaptation (71). Further experiments are
314 needed to explore this pathway in *S. aureus*.

315 Our promoter assay (Fig. 5A) suggests a 6S RNA-dependent expression of σ^B -
316 promoters in late stationary phase, after 18h of culture. Among its known roles in *E. coli*, 6S
317 RNA also influences transcription during long-term starvation (54, 67). Whether *S. aureus* 6S
318 RNA interacts with σ^B for alternative promoter expression during late stationary phase
319 remains unclear. In particular, in the absence of a functional σ^B , the absence of 6S RNA still
320 generates rifampicin inhibition indicating that this phenotype was not due to a lack of
321 reprogramming transcription from σ^A to σ^B . The relationship between 6S RNA and σ^B remains
322 to be characterized.

323 We showed that *S. aureus* 6S RNA interacts directly with RNAP- σ^A , raising the
324 question of whether this could directly affect the holoenzyme stability. Of note, the Δ *sau60*
325 mutant exhibits a moderate reduction in fitness in the presence of rifampicin (Fig. 1B).
326 Δ *sau60* corresponds to a deletion within the intergenic sequence upstream of *rpoB* encoding
327 the β subunit of RNA polymerase; this deletion may alter the ratio of RNAP subunits and
328 possibly the RNAP stability, leading to a rifampicin phenotype. However, this attenuated
329 phenotype was not detected by a spot test. In *E. coli*, the majority of 6S RNA is coupled to
330 RNAP- σ^{70} (29). In *Streptococcus pneumoniae*, 6S RNA bound to RNAP was recently
331 proposed to be a stockpile for inactive RNAP (72). In *S. aureus*, the absence of 6S RNA
332 leads to a reduced amount of σ^A in prolonged stationary phase cultures. A similar effect was
333 observed with σ^{70} in *E. coli* (29) and in soluble sigma fraction of *Synechocystis* sp. (73). In *S.*
334 *aureus*, σ^A is unstable (74); our results indicate that it is probably stabilized by RNAP core
335 enzyme and 6S RNA. Knowing that σ^A is the vegetative sigma factor in *S. aureus*, decreased
336 levels in the Δ *ssrS*^{Sa} strain could have a negative impact on growth, and particularly on
337 outgrowth recovery. As comparison, in *B. subtilis*, which expresses two different 6S RNAs,

338 outgrowth is delayed in cells lacking 6S-1 RNA (75), whereas no extended lag phase was
339 noticed in *E. coli* 6S RNA deficient cells (29). Similar to *E. coli* (29), no lag linked to *ssrS* was
340 observed during *S. aureus* outgrowth from stationary phase, suggesting that reduced σ^A
341 pools in the *ssrS*^{Sa} mutant are enough to manage growth restart and that 6S RNA is not
342 essential for growth in rich medium.

343 RNAP inhibitors remain in use in combination therapies against difficult-to-treat
344 infections (4). Antibiotic concentrations below the MIC are encountered by bacteria in many
345 environmental conditions including hosts undergoing antimicrobial treatments (76). We
346 demonstrated that 6S RNA provides protection against low concentrations of RNAP
347 inhibitors. 6S RNA is highly conserved, and the effects of *ssrS* deletion on RNAP inhibitor
348 susceptibility were observed in unrelated pathogens. 6S RNA may significantly enhanced
349 fitness to RNAP inhibitors under these conditions. Our studies implicate the importance of 6S
350 RNA in stabilizing RNAP interactions with σ^A , and suggest that it exerts its main roles in
351 prolonged stationary phase. Our findings give insight into the mode of action of 6S RNA in an
352 important pathogen, and suggest the need to develop strategies that prevent low level
353 rifampicin from persisting in the antibiotic-treated host.

354 This protective effect is possibly due to steric hindrance, as the presence of 6S RNA
355 would reduce the accessibility of the RNAP to its inhibitors. A second non-exclusive proposal
356 is that the destabilization of σ^A associated with the absence of 6S RNA affects the
357 transcriptional program to adapt to low concentrations of RNAP inhibitors. Note that in
358 *S. aureus*, this shielding effect is not associated with the sigma stress factor σ^B .

359

360 **Material and methods**

361 **Bacterial strains and culture.** All strains used in this study and their genotypes are listed in
362 Table S1. Strains were cultured at 37°C, with 180 rpm agitation for liquid cultures except for
363 *C. difficile*. The latter was cultured in anaerobic conditions (5% H₂, 5% CO₂, 90% N₂) with 7.5
364 µg.mL⁻¹ (precultures) or 15 µg.mL⁻¹ thiamphenicol (plates) for plasmid selection. *E. coli* and
365 *S. enterica* (serovar Typhimurium) strains were cultured in lysogeny broth (LB), *S. aureus*
366 strains in brain heart infusion (BHI) or tryptic soy broth (TSB) and *C. difficile* in BHI. When
367 necessary, media were supplemented with antibiotics.

368 *S. aureus* mutants were constructed in HG003 or HG002 background (59) by allelic
369 exchange using pIMAY (77) derivatives (except strains for RNAP purification) as described
370 (33). Plasmids used in this study are described in Table S2. Most plasmids constructed for
371 this study were obtained by Gibson assembly (78) as described using primers from Table S3,
372 and cloned in *E. coli* IM08B (79). Genetic features of the main *S. aureus* mutants used in this
373 study are represented in Fig.S1.

374 To purify *S. aureus* σ factors, *sigA* and *sigB* from HG003 were PCR amplified using
375 primers F-SigA/R-SigA-His and F-SigB/R-SigB-His respectively, and cloned into the
376 NdeI/XhoI restriction sites of pET-21C vector. The resulting pET-21C-*sigA* and pET-21C-*sigB*
377 plasmids were transformed into *E. coli* strain BL21(DE3) pLysS leading to strains producing
378 σ^A and σ^B (His)₆-tagged in their C-terminal portions upon IPTG induction.

379 A HG003 strain expressing a chromosomally encoded His-tagged RpoC for the
380 purification of the RNAP core enzyme was constructed as followed. The recombinational
381 transfer of the Histidine sequence into *rpoC* gene was achieved by two-steps PCR. A
382 sequence encoding for ten Histidines was added upstream from the termination codon, in
383 frame with RpoC (β' subunit of RNA polymerase). The *rpoC-His* fragment was generated by
384 long-flanking homology PCR using the primers listed in table S3 and cloned between the
385 BamH1 and PstI restriction sites of temperature-sensitive pBT2 vector (80) to obtain pBT2-
386 *rpoC-His* plasmid. The resulting plasmid was electroporated into *S. aureus* RN4220 and then
387 transferred to HG003 strain. The gene encoding for His-tagged RpoC protein was integrated
388 in the *S. aureus* HG003 chromosome by double cross-over recombination as described (81)
389 to obtain the HG003 *rpoC-his* strain.

390 *S. enterica* serovar Typhimurium mutants were constructed in the background of strain
391 LT2-derived MA7455 (82) using λ Red recombineering (83). See Fig.S2 for constructions.

392 *C. difficile* mutants were constructed in 630 Δ *erm* background (84). The knock out
393 mutant was obtained using an allelic chromosomal exchange following the published method
394 (85) with primers CM57/CM58 and CM59/CM60 and pMSR vector pDIA7052. To

395 complement, *ssrS^{Cd}* sequence and its promoter region were PCR amplified using the primer
396 pair CM77/CM78 and cloned into pMTL84121 to produce pDIA7065. Two *E. coli* strains were
397 used as intermediate: NEB 10-Beta for plasmid construction (Table S2) and HB101 RP4 for
398 conjugation.

399 **MIC determination.** Antimicrobial susceptibility testing by broth microdilution for rifampicin,
400 rifabutin, fidaxomicin and aureothricin MIC determination in *S. aureus* were performed as
401 described (86). *S. enterica* serovar Typhimurium rifampicin MIC was determined as
402 described (87).

403 **Fitness experiment.** The experiment was performed as described (33) with three
404 independent sRNA tagged-mutant libraries grown simultaneously in TSB +/- 6 $\mu\text{g.L}^{-1}$
405 rifampicin and sampled at four points: $\text{OD}_{600}=1$, O/N, $\text{OD}_{600}=1$ after the first dilution and
406 $\text{OD}_{600}=1$ after the second dilution. All the mutants were tag-sequenced with an adapted
407 Illumina protocol. The amount of each mutant was normalized to the total amount of bacteria
408 with/without rifampicin and to the inoculum (Fig. 1A). Totally, three mutants (*locus1*,
409 *rsaD(tag26)* and *teg146*) were discarded from the analysis because of experimental
410 troubleshooting. Concerning *sprD* and *sau5949*, only two values were taken in account in the
411 3rd dilution sampling.

412 **Spot test.** Overnight cultures were 10-fold serial diluted in NaCl 0.9% (*S. aureus* and *S.*
413 *enterica*) or BHI (*C. difficile*) until 10^{-8} and spotted on agar plates containing different
414 sublethal antibiotic concentrations, namely less than MIC (rifampicin $< 12 \mu\text{g.L}^{-1}$ for *S. aureus*
415 or $< 12 \mu\text{g.mL}^{-1}$ for *S. enterica*, rifabutin $< 15.6 \mu\text{g.L}^{-1}$, aureothricin $< 6.25 \mu\text{g.mL}^{-1}$,
416 fidaxomicin $< 4 \text{mg.L}^{-1}$ for *S. aureus* or $< 30 \mu\text{g.L}^{-1}$ for *C. difficile*). Pictures were taken after
417 O/N growth or 24h for *C. difficile*.

418 **Growth curves.** *S. aureus* strains were cultured in microplates from O/N triplicates cultures
419 1/1000 diluted in BHI +/- $5\mu\text{g.L}^{-1}$ rifampicin. Two and three day-cultures were also used as
420 pre-cultures in Fig. 6C. Absorbance at 600 nm (OD_{600}) was measured overtime with a plate
421 reader (Clariostar).

422 **Fluorescence measurement.** mAmetrine expression ($\lambda_{\text{exc}} = 425 \pm 15 \text{ nm}$, $\lambda_{\text{em}} = 525 \pm 15$
423 nm) was monitored overtime in microplates by a plate reader (Clariostar), simultaneously to
424 absorbance measurement of overnight triplicates cultures diluted 1:1000 in TSB to limit auto-
425 fluorescence.

426 **RNA extraction.** Strains were cultured until the desired OD_{600} . After centrifugation, pellets
427 were frozen in dry-ice ethanol. RNAs were then extracted by phenol-chloroform treatment as
428 described (88). When necessary, RNAs were incubated with Turbo DNase treatment
429 (Thermo Fisher Scientific) prior to a second phenol-chloroform extraction.

430 **Northern Blot.** 10 μ g total RNAs per well were separated on 1.3% agarose or 10% TBE-
431 urea polyacrylamide gel (Criterion Precast gel) as described (89). For polyacrylamide gels,
432 electrophoresis in TBE 1X was followed by transfer to Hybond-N⁺ membrane in TBE 0.5X
433 using TE70 ECL Semi-Dry Transfer Unit (Amersham Pharmacia Biotech). Probes (Table S3)
434 were [α -³²P]dCTP-labelled.

435 **RNA-seq and transcriptomic analysis.** RNAs (DNA-free) from triplicate cultures sampled at
436 OD₆₀₀ = 7 were sequenced using NextSeq 500/550 High Output Kit v2 (75 cycles).
437 Sequences were aligned to the reference genome (CP000253-1-NCTC8325) with Bowtie2
438 tool and quantified with Feature Counts program. Differential gene expression analysis was
439 performed using DESeq2 algorithm (90). The data for this study have been deposited in the
440 European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB50160
441 (<https://www.ebi.ac.uk/ena/browser/view/PRJEB50160>).

442 **5'-3' RACE.** 5' and 3' ends were determined using circularization method described in (51).
443 10 μ g total RNA of wild-type strain (HG003) were extracted from samplings at OD₆₀₀ 7, O/N
444 and day 4 (D4). Primers used to amplify the 5'/3' junction with Phusion high-fidelity DNA
445 polymerase (Thermo Fisher Scientific) are listed in Table S3. CloneJET PCR cloning kit
446 (Thermo Fisher Scientific) was used to clone the final PCR products.

447 **Purification of σ^A , σ^B and RNAP core enzyme.** For σ purification, *E. coli* strains BL21(DE3)
448 pET-21C-*sigA* and BL21(DE3) pET-21C-*sigB* were grown in 1 L of LB broth at 37°C to OD₆₀₀
449 0.5. After induction with 1 mM isopropyl-1-thio- β -D-galactopyranoside for 3 h, bacteria were
450 collected by centrifugation, resuspended in 10 mL of buffer A (10 mM HEPES, pH 7.5, 200
451 mM NaCl, 1 mM MgCl₂, 20 mM Imidazol, 5% glycerol) with 0.25 mg.mL⁻¹ lysozyme per gram
452 of pellet and frozen and thawed two times. Lysates were treated with DNase I (100 units.mL⁻¹
453 ¹) for 20 min at 30°C, and supernatants containing σ^A -His or σ^B -His proteins were obtained
454 by centrifugation at 8000 g for 15 min. For RNA polymerase purification, a fresh overnight
455 culture of *S. aureus* HG003 *rpoC-his* was used to inoculate 500 mL of BHI at OD₆₀₀ 0.1 and
456 grown 5 hours at 37°C. The culture was harvested by centrifugation at 4000 g for 15 min.
457 The cell-pellet was resuspended in 10 mL of buffer A with 1 mg of lysostaphin and DNase I
458 (100 units.mL⁻¹). After an incubation of 20 min at 37°C, the lysate was clarified by
459 centrifuging 30 min at 40000 g. For affinity purification of σ^A -His, σ^B -His and (His)₁₀-tagged
460 RNAP, HisTRAP™ TALON column (5 mL, GE healthcare) was connected to an AKTA prime
461 chromatography system (GE healthcare) equilibrated with buffer B (10 mM HEPES, pH 7.5,
462 1 M NaCl, 20 mM Imidazol, 5 % glycerol). After loading the lysate containing either σ^A -His,
463 σ^B -His or (His)₁₀-tagged RNAP, the column was washed with 100 mL of buffer B. His-tagged
464 proteins were then eluted using an imidazole gradient, dialyzed in buffer C (10 mM HEPES,
465 pH 7.5, 100 mM NaCl, 1 mM MgCl₂, 5 % glycerol) and then subjected to a second step of

466 purification on heparin column. Proteins were loaded on a HiTrap Heparin HP column (1 mL,
467 GE healthcare) equilibrated with buffer C. After a wash with 20 mL of buffer C, proteins were
468 eluted using a NaCl gradient, dialyzed in buffer C and then concentrated in a centrifugal
469 concentrator with a 10-kDa molecular weight cutoff membrane (Merck Millipore).

470 **Electrophoretic mobility shift assay (EMSA).** *ssrS^{Sa}* and *sprB* were *in vitro* transcribed
471 from a PCR product template T7 promoter-containing (primers listed in Table S3) with
472 MEGAscript T7 transcription kit (Thermo Fisher Scientific). RNAs were separated on 8 %
473 polyacrylamide-7 M urea gel electrophoresis and eluted overnight in G50 elution buffer (20
474 mM Tris-HCl pH7.5, 2 mM EDTA and 0.25 % SDS). RNAs were precipitated in cold ethanol
475 and 0.3 M of sodium acetate and dephosphorylated using Calf-intestinal alkaline
476 phosphatase (New England Biolabs), according to manufacturer protocol. Obtained RNAs
477 were 5'-radiolabelled with T4 polynucleotide kinase (New England Biolabs) and [γ ³²P]
478 adenosine triphosphate (ATP) and purified with MicroSpin G-50 column (Amersham
479 Pharmacia Biotech). 140 nM of RNAP alone or preincubated 10 minutes at 37°C with 420 nM
480 of Sigma factors were mixed with 4 nM of radiolabelled 6S RNA or SprB in buffer D (15 mM
481 Hepes pH 7.5, 100 mM NaCl, 1 mM MgCl₂, 5 % glycerol, 100 μ g.mL⁻¹ BSA, 200 μ g.mL⁻¹ *E.*
482 *coli* tRNA). Complex formation was performed at 37°C during 10 min and samples were
483 loaded on 5 % polyacrylamide-5 % glycerol gel under non-denaturing conditions. Gels were
484 dried and visualized using a Typhoon Phosphorimager (Molecular Dynamic).

485 **σ^A and β subunits quantification.** *S. aureus* strains were cultured in triplicates until the
486 desired OD₆₀₀. Frozen pellets were lysed in 50 mM TRIS-HCl buffer pH 7.5 with glass beads.
487 Total protein amount in the supernatant was determined by Bradford protein assay. Western-
488 Blot electrophoresis was performed with 3 μ g proteins per well, using 8% Bis-TRIS Plus
489 polyacrylamide gel (Bolt, Invitrogen). Transfer and hybridization followed iBlot and iBind
490 manufacturer instructions (Invitrogen) respectively. Membranes were pre-hybridized at 4°C
491 O/N with human serum 1/10000e to saturate unspecific binding. Rabbit primary antibodies
492 were used for immunodetection of σ^A (anti- σ^A , 1:5000 dilution) and β subunit (anti-RNAP,
493 1:10000 dilution). A HRP-conjugated goat anti-rabbit (Advansta, 1:4000 dilution) was chosen
494 as secondary antibody. Pictures were taken with a CCD Camera.

495

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746 dispersion for RNA-seq data with DESeq2. *Genome Biology* 15.

747

748

749

750 **Table 1**

| Locus tag / gene name | FC | P-adj | Function / relevant data | Classification | Regulation | TU |
|------------------------------|------|---------|--|------------------------|---------------------------------|----|
| SAOUHSC_02911 | 0.48 | 2.2E-28 | <i>Epoxyqueuosine reductase</i> | tRNA modification | σ^A | |
| SAOUHSC_02640 / <i>hrtA</i> | 0.48 | 4.2E-10 | Hemin efflux ATP-binding protein HrtA | transport and binding | σ^A , HssR | b |
| SAOUHSC_01817 | 0.49 | 5.9E-10 | <i>integral component of membrane</i> | | σ^A | c |
| SAOUHSC_01736 | 0.52 | 1.0E-08 | <i>Unknown.</i> Downstream <i>ssrS</i> (34) | | σ^A | a |
| SAOUHSC_02641 / <i>hrtB</i> | 0.53 | 3.7E-08 | Hemin efflux system permease protein HrtB | Transport and binding | σ^A , HssR | b |
| SAOUHSC_01818 / <i>ald2</i> | 0.55 | 8.0E-07 | Alanine dehydrogenase | Energy metabolism | σ^A , CcpA | c |
| SAOUHSC_00874 | 0.56 | 3.5E-10 | <i>Thioredoxin-like protein</i> | Hypothetical protein | σ^A | |
| SAOUHSC_02297 | 0.57 | 3.7E-14 | S1 RNA-binding domain-containing protein | Protein synthesis | σ^A | d |
| SAOUHSC_02590 | 0.60 | 3.7E-09 | <i>Amino acid permease</i> | Transport and binding | σ^A , CcpA, CodY | |
| SAOUHSC_02296 | 0.62 | 1.7E-08 | <i>SprT-like protein</i> | | σ^A | d |
| SAOUHSC_00561 / <i>vraX</i> | 0.64 | 4.2E-10 | <i>VraX</i> | | σ^A | |
| SAOUHSC_00704 | 0.64 | 2.0E-08 | <i>ABC-2 transporter</i> | | σ^A | |
| SAOUHSC_03028 / <i>bstA</i> | 0.64 | 1.2E-03 | <i>DinB-like protein</i> | | σ^B | |
| SAOUHSC_01735 / <i>tcdA</i> | 0.65 | 1.9E-06 | <i>ThiF domain-containing protein</i> | Cofactors biosynthesis | σ^A | a |
| SAOUHSC_02656 | 0.65 | 4.0E-10 | <i>Cytochrome c oxidase-like protein</i> | | σ^A | |
| SAOUHSC_00157 / <i>murQ</i> | 1.51 | 1.6E-03 | N-acetylmuramic acid-6-phosphate etherase | Cell envelope | σ^A , MurR, CcpA | e |
| SAOUHSC_00156 / <i>mupG</i> | 1.52 | 1.2E-03 | <i>6-phospho-N-acetylmuramidase</i> | Cell envelope | σ^A , MurR, CcpA | e |
| SAOUHSC_01121 / <i>hla</i> | 1.56 | 1.6E-05 | Alpha-hemolysin | Virulence / toxin | σ^A , SaeR, CcpA, RNAIII | |
| SAOUHSC_02169 / <i>chp</i> | 1.60 | 1.8E-04 | Chemotaxis-inhibiting protein CHIPS | Virulence | σ^A | |
| SAOUHSC_00961 / <i>comK1</i> | 1.85 | 4.3E-08 | <i>Competence protein</i> | | σ^A , CodY | |

751

752 **Table 1. Transcriptomic analysis of Δ ssrS^{Sa} vs. HG003 in late exponential phase.** Fold
753 change (FC) represents the gene expression ratio between Δ ssrS^{Sa} and its parental strain at
754 OD₆₀₀ of 7. Genes with FC < 0.66 (top panel) or > 1.5 (down panel) are presented.
755 Transcription unit (TU) are indicated by letters. *Italic characters*, hypothetical proteins; P-adj,
756 adjusted p-value.

757

758

759 **Figure legends**

760

761 **FIG 1. Fitness loss of the *ssrS*^{Sa} mutant in the presence of sub-lethal concentration of**
762 **rifampicin.**

763 (A) Scheme of the fitness experiment sampling. Three libraries were cultured during three
764 days in Tryptic Soy Broth (TSB) +/- rifampicin. Cultures were diluted 1:1000 at 24h and 48h.
765 After each dilution step (t= 0, 24 and 48h), samples were withdrawn for tag counting in both
766 growth conditions, when optical density at 600 nm (OD₆₀₀) reached the value of 1 (samplings
767 1, 3 and 4) and after the first overnight growth (sampling 2) as indicated. (B) Results of the
768 competition assay between *S. aureus* sRNA mutants in presence of rifampicin 6 µg.L⁻¹. y
769 axis, mutant strain names; x axis, relative proportion of each mutant within the population
770 grown in the presence of rifampicin normalized to the inoculum and to the corresponding
771 sample grown in the absence of rifampicin. For each mutant, four histograms are shown; the
772 color code corresponds to samplings indicated in Fig. 1A. Locus 2 and 3 mutants have tag
773 insertion in loci likely not transcribed and not expected to alter the strain fitness. Error bars
774 represent the experimental standard deviation between the three different libraries.

775

776 **FIG 2. *ssrS* deletions confer a conserved rifampicin susceptibility phenotype from *S.***
777 ***aureus* to *S. enterica***

778 Three independent clones were grown overnight for each indicated strain. (A) Serial dilutions
779 of overnight *S. aureus* cultures were spotted on Brain Heart Infusion (BHI) agar +/- 5 µg.L⁻¹
780 rifampicin (RIF). (B) Serial dilutions of overnight *S. enterica* cultures were spotted on
781 Lysogeny Broth (LB) agar +/- 5 µg.mL⁻¹ rifampicin. (C) Growth kinetics of *S. aureus* strains in
782 BHI +/- 5 µg.L⁻¹ rifampicin. OD₆₀₀ is an arbitrary value due to plate reader conditions, not
783 representative of absorbance measurements of *S. aureus* in flasks. Error bars represent
784 standard deviation of three experiments.

785

786 **FIG 3. Susceptibility to RNAP inhibitors.** (A) Serial dilution of *S. aureus* overnight cultures
787 plated on solid medium containing or not antibiotics as indicated: rifabutin, RB; fidaxomicin,
788 FDX; aureothricin, AUR; numbers (1, 2 and 3), independent clones. The antibiotic
789 concentrations used were below the MIC. (B) Serial dilutions of *C. difficile* overnight cultures
790 plated on solid medium containing or not fidaxomicin (FDX) as indicated. Pictures are
791 representative of four replicates. Thiamphenicol was added in all plates (15 µg.mL⁻¹) to
792 maintain the plasmid. p, empty vector pMTL84121; pssrS^{Cd}, pMTL84121-ssrS^{Cd}.

793

794 **FIG 4. *ssrS* gene expression and 6S RNA sequence in *S. aureus*.**

795 (A) *ssrS*^{Sa} expression. Cultures of HG003 grown in BHI were sampled at OD₆₀₀=1, 4, 7 and
796 O/N. A Northern blot probing for 6S RNA and tmRNA (for normalisation) was performed. A
797 quantification of 6S RNA normalized to tmRNA is presented. The standard deviation is based
798 on biological triplicates. (B) Identification of 6S RNA ends by 5'/3'-RACE mapping.
799 Sequences were analysed separately at different time points (OD7, O/N and day4, D4) and
800 compiled (mix). Coloured letters represent extremities found in analysed sequences. A scale
801 of colours highlights the frequency of each 5' or 3'-end. The highest frequencies are
802 indicated below the corresponding nucleotides.

803

804 **FIG 5. 6S RNA and σ^B interplay in late stationary phase and in rifampicin response in**
805 ***S. aureus*.**

806 (A) Fluorescence and OD₆₀₀ were monitored simultaneously in three strains (HG003
807 [parental], HG003 Δ *ssrS*^{Sa} and HG002) expressing a fluorescent protein (mAmetrine) under
808 the control of the σ^B promoter of *SAOUHSC_00624* from a plasmid (pPsigB-mAmetrine).
809 HG002, *rsbU* strain equivalent to σ^{B-} strain, is a negative control. Error bars represent
810 standard deviation of biological triplicates. (B) Spot test comparing HG003 and HG002
811 (parental and Δ *ssrS*^{Sa} strains, respectively) with sub-lethal concentration of rifampicin (3.13
812 μ g.L⁻¹). Arbitrary values are shown as OD₆₀₀. Experiment was done with independent
813 duplicates.

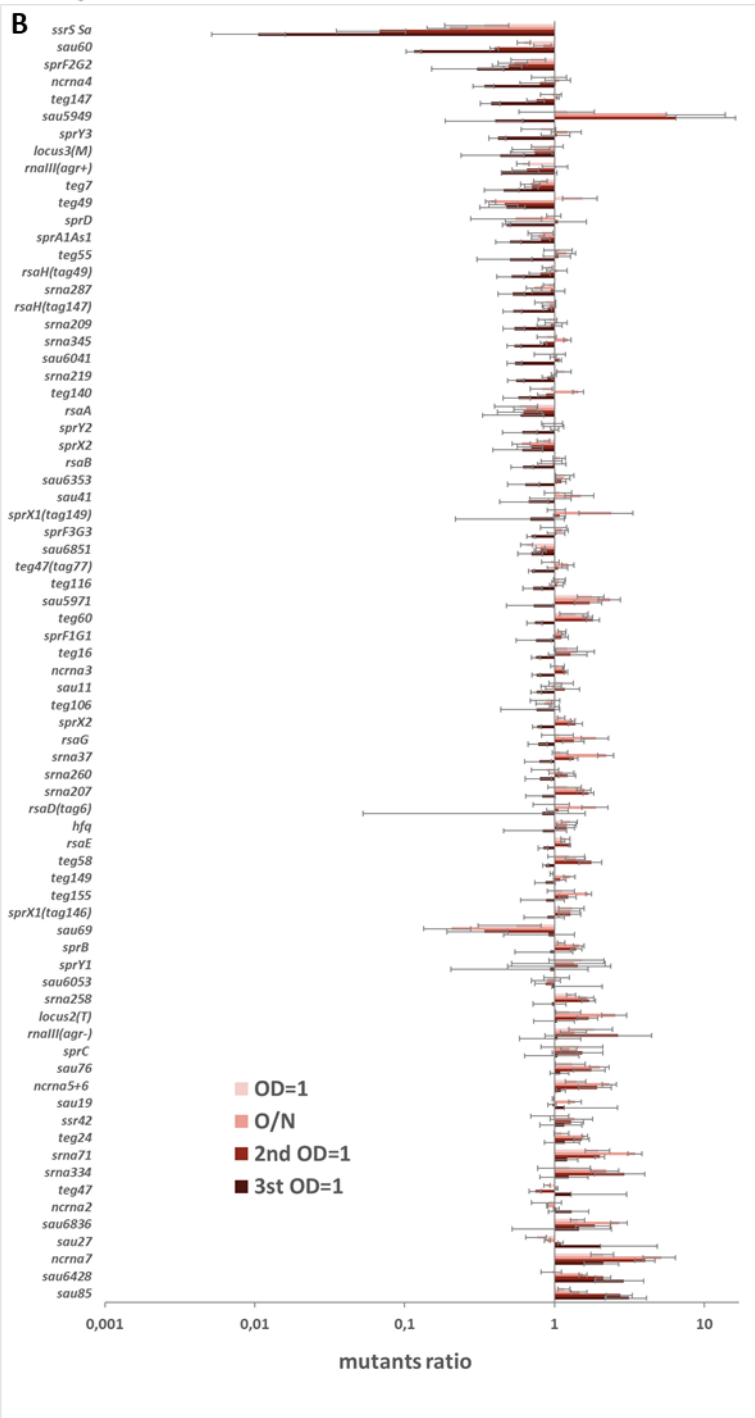
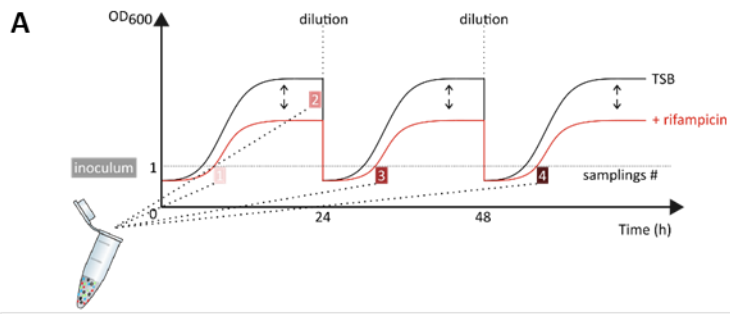
814

815 **FIG 6. 6S RNA and RNAP holoenzyme interactions in *S. aureus*.**

816 (A) EMSA with P³²-6S RNA (6S RNA), RNAP, σ^A and σ^B . All the proteins are His-tagged
817 purified. P³²-SprB (SprB) is a control RNA. (B) Immunodetection of σ^A performed by Western-
818 blot from samplings at OD₆₀₀=1 and 7, O/N and day 3 (D3). Quantification of σ^A is relative to
819 the amount of RNAP β/β' subunits. Experience realized in biological triplicates. W: wild-type
820 (HG003); Δ : *ssrS*^{Sa} mutant; e: Δ *ssrS*^{Sa} ecto-*ssrS*^{Sa}. (C) Growth curves of HG003, *ssrS*^{Sa}
821 mutant (Δ *ssrS*^{Sa}) and complemented (Δ *ssrS*^{Sa} ecto-*ssrS*^{Sa}) strains in BHI. Strains were
822 cultured in independent triplicates from O/N, two days or three days precultures. Error bars
823 represent standard deviation.

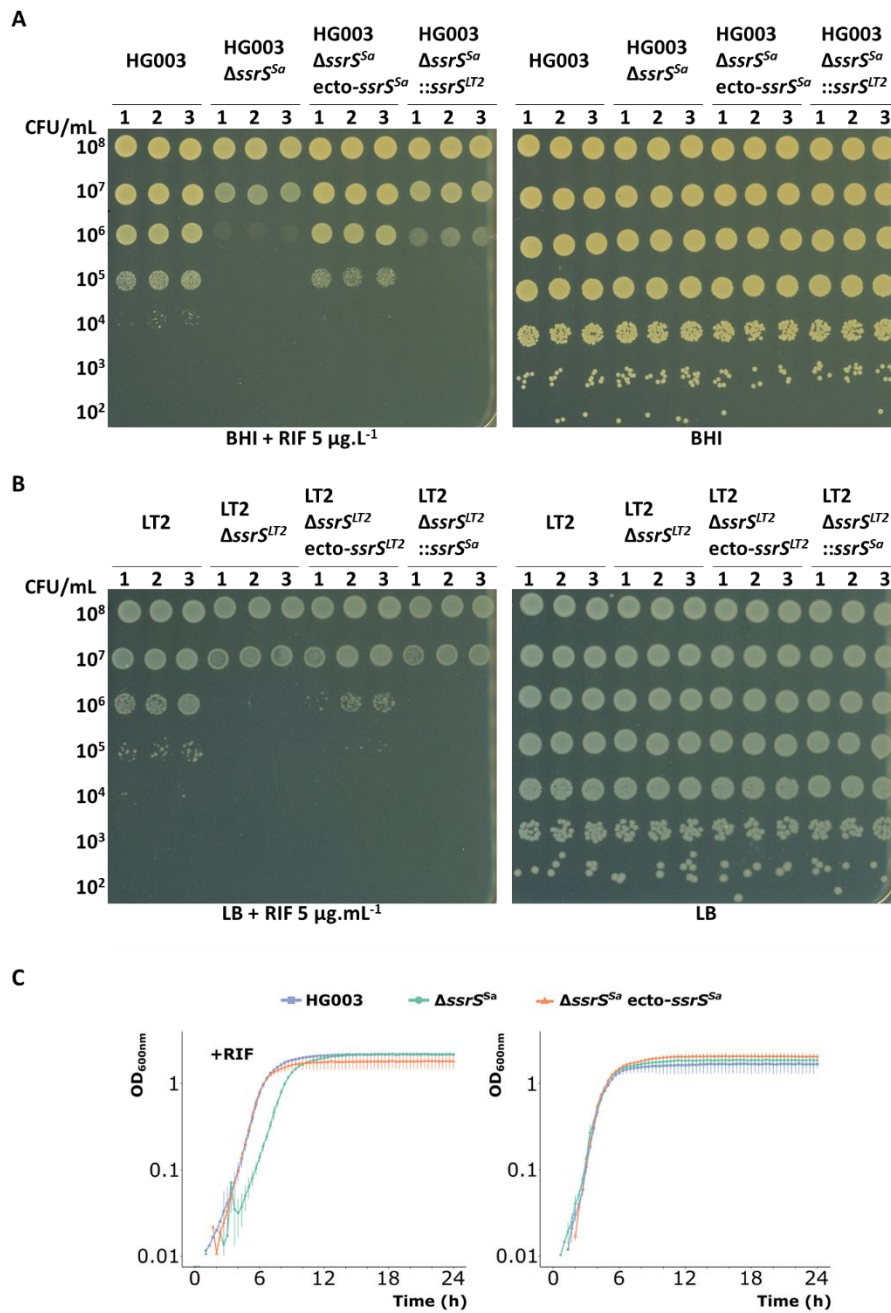
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Figure 2



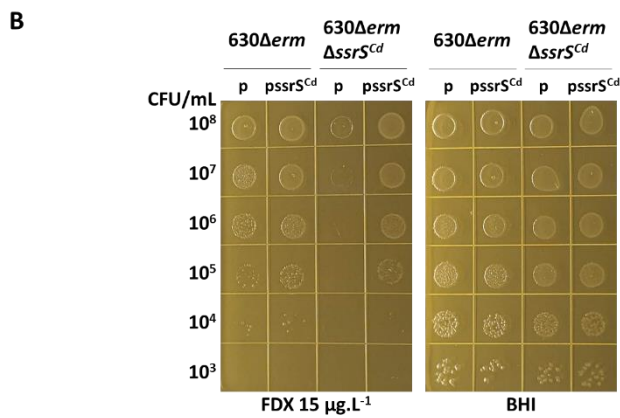
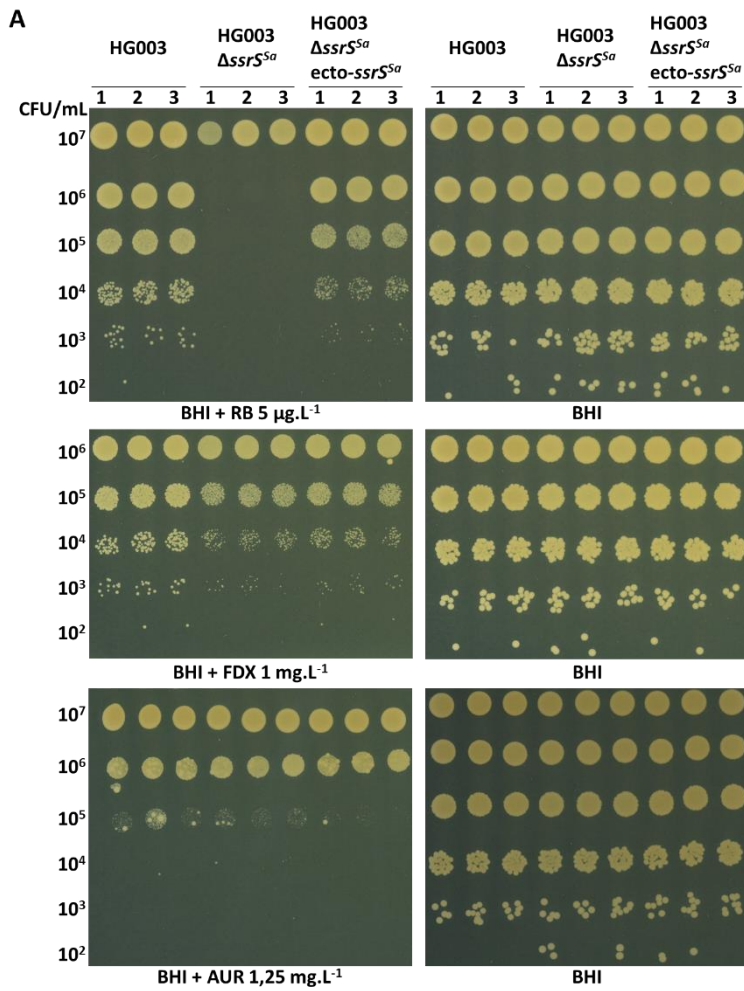
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Figure 3

832

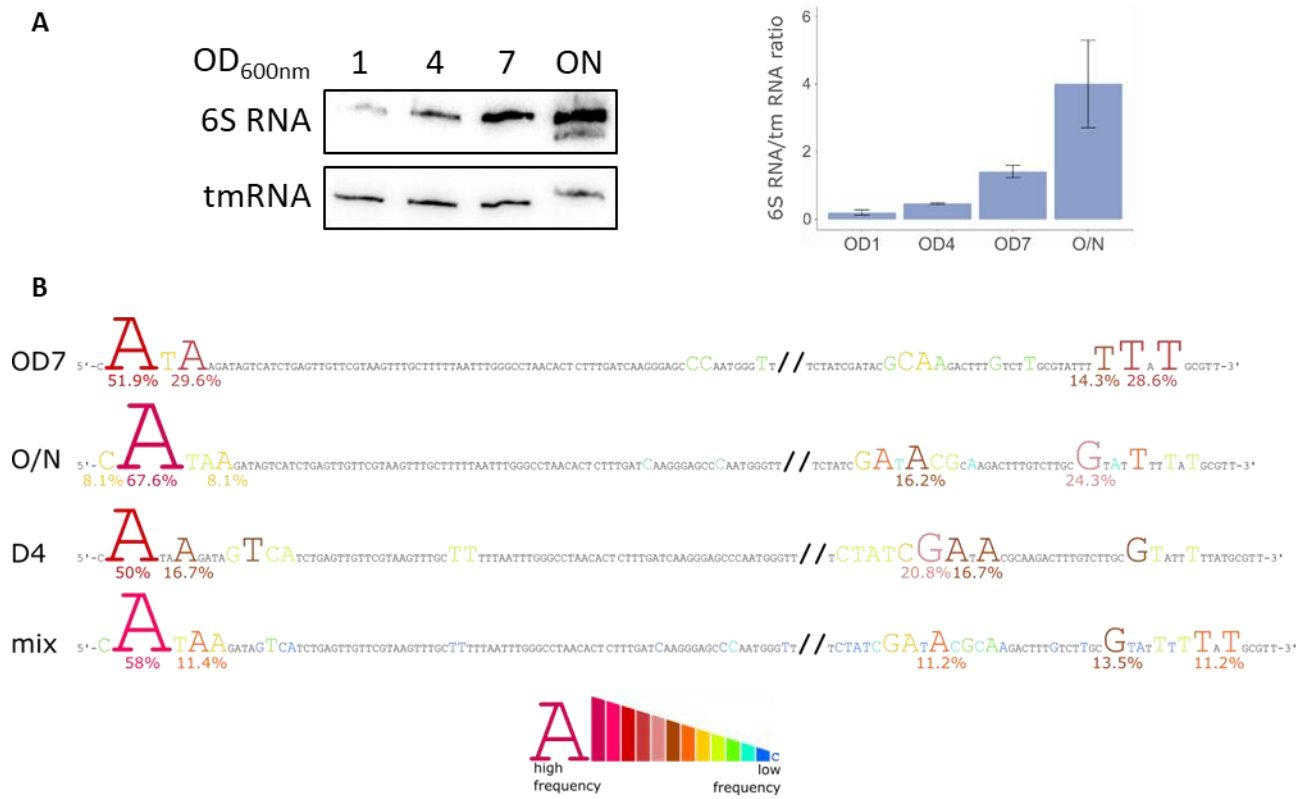


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Figure 4



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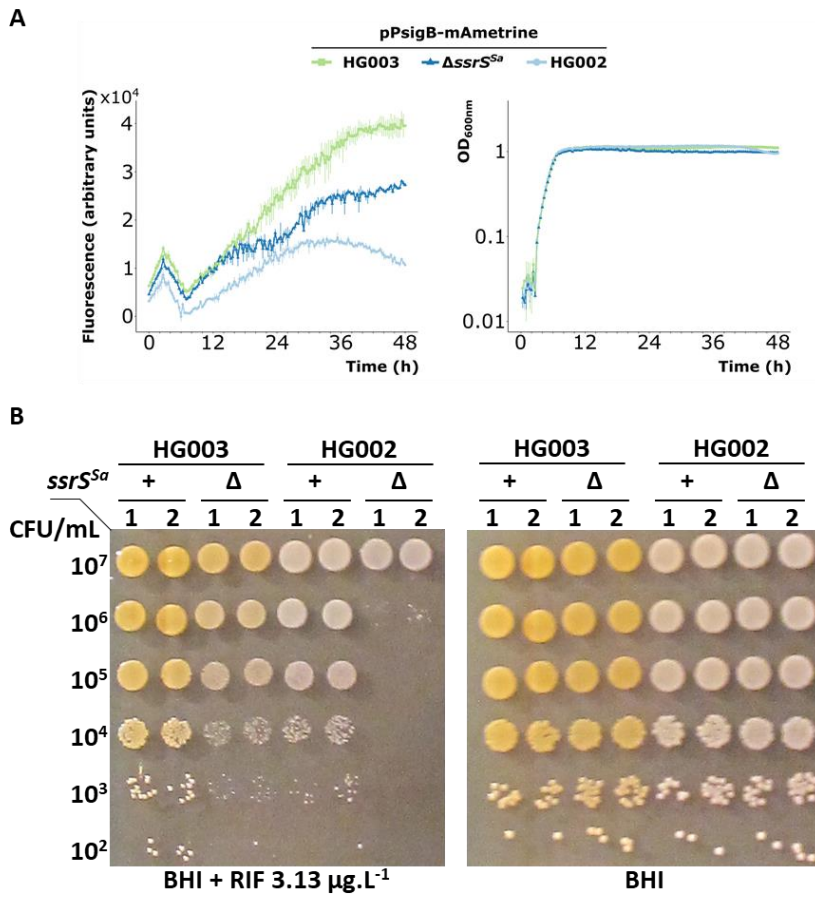
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Figure 5

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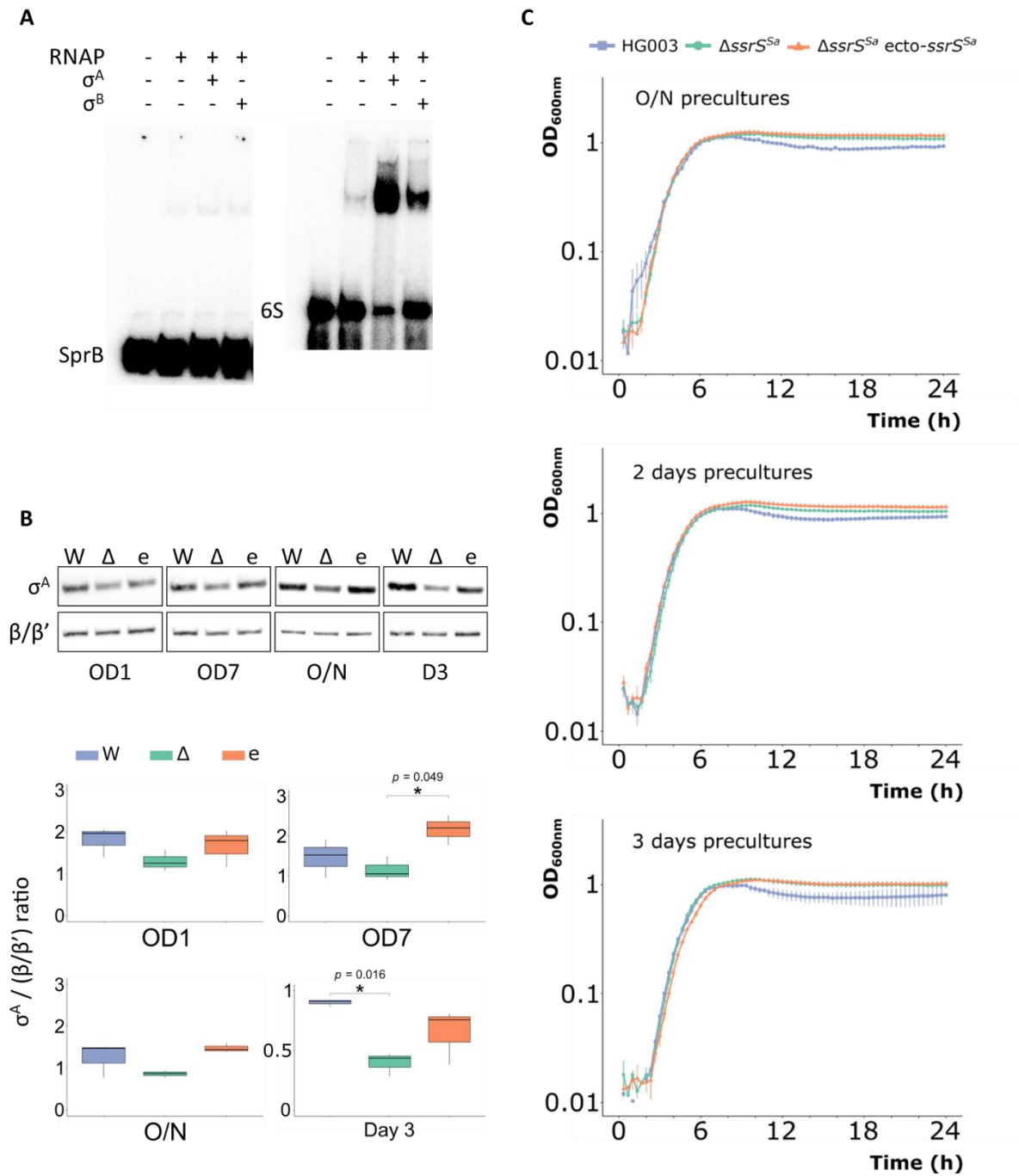
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843

Figure 6

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845