SSR42 is a Novel Regulator of Cytolytic Activity in *Staphylococcus aureus*

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Running title: SSR42 controls *Staphylococcus aureus* cytotoxicity.

Abstract

SSR42 is the longest noncoding RNA in the *S. aureus* cell and the second-most abundant transcript in the stationary phase transcriptome, second only to RNAIII. It is highly conserved across strains and exhibits pronounced stability in stationary phase, however the mechanism behind its regulatory role has yet to be fully elucidated. Herein, we used transcriptomic and proteomic approaches to probe the role of SSR42, revealing that it is a powerful, novel activator of the primary leukocidin LukAB. SSR42 is required for cytotoxicity towards, and escape from within, human neutrophils, and also mediates survival within human blood. We show that SSR42 wields this role via derepression by the peroxide repressor PerR in response to the presence of human neutrophils and governs *lukAB* induction in this niche. Importantly, this regulation is driven by direct RNA-RNA interaction, as we show binding of the 5' UTR of the *lukAB* transcript with the 3' end of SSR42, which ultimately modulates transcript stability as well as translational activity. Finally, we demonstrate that this behavior is absolutely required for full virulence of *S. aureus* in murine models of both pneumonia and sepsis. Collectively, we present SSR42 as a pleiotropic regulatory RNA that acts as a nexus between environmental sensing and the regulation of pathogenesis, responding to environmental stimuli and host immune factors to bolster cytotoxic behavior and facilitate infection in *S. aureus.*

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Importance

41 *S. aureus* is a master pathogen due to its formidable collection of virulence factors. These
42 are tightly controlled by a diverse group of regulators that titrate their abundance to adapt
43 to unique infectious niches. The role of regulatory RNAs in stress adaptation and
44 pathogenesis is becoming increasingly more relevant in *S. aureus*. In this study, we
45 provide the most comprehensive global analysis to date of just such a factor, SSR42.
46 Specifically, we uncover that SSR42 is required for mediating cytotoxicity - one of the
47 pillars of infection - in response to phagocytosis by human neutrophils. We find that
48 SSR42 is induced by components of the host immune system and facilitates downstream
49 activation of cytotoxic factors via RNA-RNA interactions. This illustrates that SSR42 forms
50 a pivotal link between sensing the external environment and mediating resistance to
51 oxidative stress while promoting virulence, solidifying it as a major global regulator in *S.*
52 *aureus.*

53 **Introduction**

54 Over the last few decades, more than 300 regulatory RNAs have been identified in
55 *Staphylococcus aureus* strains (1–6). While progress has been made exploring these
56 elements mechanistically, there is still a dearth of knowledge on the role of regulatory
57 RNAs in complex regulatory systems, as compared to classical transcription factors.
58 Commonly referred to as small RNAs (sRNAs), these factors range from *trans*-encoded-
59 to antisense- transcripts, typically acting in a posttranscriptional manner to allow for rapid
60 adaptation to environmental cues (6–13). The most well-studied example of an sRNA in
61 *S. aureus* is RNAIII, which is the effector for the Agr system. It is known to modulate
62 mRNA abundance by relieving inhibitory secondary structures at the Shine-Dalgarno

63 sequence (e.g. *hla*), or by occluding this site to block access of the ribosome (e.g. *spa,*
64 *rot, sbi*) ultimately promoting transcript degradation (14–17).

65 Many other virulence-related regulatory RNAs have also been documented in *S. aureus*,
66 such as the Teg family of transcripts, which include Teg41 that impacts hemolytic ability
67 through engagement with the PSMs, and Teg58 which monitors arginine dysbiosis and
68 modulates biofilm production (18–20). The Spr-group of regulators form another family of
69 regulatory RNAs, and includes SprC, which plays a role in controlling metabolism and
70 immune evasion, SprY, which works to titrate RNAIII activity through direct 'sponge'
71 interactions, and SprD, which works in consort with RNAIII to repress *sbi* (21–24) . RsaC
72 is a ~1,100-nt transcript that is expressed during manganese starvation and enhances
73 the oxidative stress response, leading to increased survival during confrontation with the
74 host immune system; thus forming a link between metal homeostasis and pathogenesis
75 (25). From a broader perspective, a recent global study utilizing CLASH technology to
76 uncover RNA-RNA interactions in host-like conditions identified hundreds of binding
77 events, occurring not only between regulatory RNAs and mRNAs, but between regulatory
78 RNAs themselves as well. Some of these interactions linked the metabolic status of the
79 cell to the expression of membrane-permeabilizing toxins, while furthermore uncovering
80 an RNA-sponge relationship between RsaE and RsaI (13)

81 The largest regulatory RNA in *S. aureus* is SSR42, which is 1,233 nt long, contains no
82 functional ORFs, and bears 98% sequence conservation across strains, with only two
83 single nucleotide variants found in UAMS-1 (26). It was first characterized by Morrison et
84 al. from a larger group of Small Stable RNAs (SSRs), where it was shown that the SSR42
85 transcript exhibits enhanced stability during stationary phase growth, with a half-life of

86 over 30 minutes, compared to a ~5 minutes in exponential phase. Using microarray
87 analysis, 80 mRNA species were found to be altered in an SSR42 mutant strain, with key
88 virulence factors such as *hla, lukF, hlgC, aur,* and *sraP* found to be differentially
89 expressed. Phenotypically, an SSR42 mutant demonstrated an abrogated ability to lyse
90 human erythrocytes and impaired virulence in a murine model of skin abscess formation
91 (26). Others have shown that SSR42 is tightly controlled via the Repressor of Surface
92 Proteins (Rsp), and in turn may control expression of the two-component system SaeRS,
93 potentially explaining the ahemolytic nature of SSR42 mutant strains (27). Work by our
94 group identified that SSR42 is unique to *S. aureus* and does not exist in other
95 Staphylococcal species (5). We also demonstrate that expression of SSR42 is profoundly
96 increased during the growth of *S. aureus* in human serum (1).

97 Despite SSR42 being well linked to *S. aureus* pathogenesis, no direct target or
98 mechanism of action has been demonstrated for this molecule. Herein, we use a
99 combination of large-scale omics studies coupled with genetic and biochemical analyses
100 to demonstrate that SSR42 is a novel regulator of *lukAB* expression and cytolytic activity
101 through direct RNA-RNA interaction. We show that this regulation is controlled by the
102 peroxide sensor PerR and is stimulated in response to human neutrophils, mediating
103 escape from the intracellular niche. Finally, the impact of this interaction is illustrated by
104 impaired virulence in murine models of both sepsis and pneumonia.

105 **Methods**

106 **Toxicity towards neutrophils:** Cytotoxicity assays were performed as previously
107 described (28), with some variations. The immortalized HL-60 cell line was used as a
108 model of human neutrophil engagement. Cells were grown to confluence in RPMI

109 supplemented with 10% FBS and 100U Pen/Strep in 37°C and 5% CO_2. Upon reaching
110 sufficient cell density, cells were differentiated into neutrophil-like cells by the addition of
111 1.25% DMSO for 4 days; which was confirmed via observation of cell morphology. For
112 use, $1x10^5$ cells were seeded in 100µl RPMI with 10% FBS into a 96-well plate. Bacterial
113 cultures were grown in biological triplicate overnight and standardized to 0.05 before
114 being grown for 15h. Following incubation, cultures were pelleted via centrifugation and
115 supernatant was diluted two-fold in TSB. Seeded neutrophils were intoxicated in technical
116 duplicate with 5µl of diluted bacterial supernatant (5% total volume) followed by incubation
117 at 37°C, 5% CO_2 for 1h. Cell viability was measured using the CellTiter96 Aqueous One
118 Solution Cell proliferation reagent (Promega). Following 1h incubation, 20µl of the
119 CellTiter reagent was added to each well, and the plate was returned to the incubator for
120 2h. Color development was assessed via OD_{490} using a Biotek Cytation 5 plate reader.
121 Data is reported as percent viability of supernatant-treated cells with respect to
122 neutrophils treated with 5µl TSB only.

123 **Intracellular survival within neutrophils:** To assess intracellular survival within human
124 neutrophils, HL-60 cells were grown and differentiated as described above, and $2x10^6$
125 cells were seeded into 500µl in a 24-well plate. Bacterial cultures were also grown and
126 processed as above, with the following modifications. After centrifugation, pelleted cells
127 were washed 2X with PBS, following which all strains were standardized to 10x the
128 desired final MOI of 30 (OD_{600}=0.135 for *S. aureus*), thus all strains were standardized to
129 an OD_{600}=13.5 in PBS. From these standardized cultures, 5µl was added to each well of
130 neutrophils and incubated for 1h at 37°C and 5% CO_2. Following incubation, cells were
131 removed from wells and pelleted at 2.5 x *g* for 1 minute before being resuspended in pre-

132 warmed PBS. This PBS wash was repeated, and the cells were finally resuspended in
133 pre-warmed RPMI supplemented with 30µg/mL gentamicin and returned to incubate for
134 24h. At this point in time the cells were removed and washed twice with pre-warmed PBS
135 once again, however the final resuspension was in 0.5% Triton X-100 to lyse neutrophils.
136 Lysates were serially diluted and plated to enumerate CFU of internalized bacteria.
137 Viability of the infected neutrophils was also quantified after the 24h infection period by
138 adding 100µl of CellTiter reagent to each well, incubating for 1h at 37°C and 5% CO_2, and
139 measuring OD_{490} using a Biotek Cytation 5 plate reader as described above.

140 **Measuring translational control by SSR42 via GFP reporters:** SSR42 mutant strains
141 containing both the *lukAB* mRNA-GFP fusion plasmid (pCN33) alongside the SSR42
142 expression or empty vector plasmid (pICS) were grown overnight in biological triplicate.
143 Cultures were synchronized for 3h and standardized to an OD_{600} of 0.05 in a black-walled,
144 clear bottom 96-well plate. Strains were then monitored for OD_{600} and fluorescence
145 (excitation:485/20, emission:528/20, Gain:75) measured every 15min for 18h.
146 Fluorescence levels of the mRNA reporter in the presence of SSR42 *in trans* were
147 compared to fluorescence levels of the mRNA reporter in the presence of an empty vector.
148 Strains were normalized by growth via OD_{600}.

149 **Electrophoretic mobility shift assays (EMSAs):** All RNA was generated via *in vitro*
150 transcription using a T7 RNA polymerase promoter and the MAXIscript T7 kit
151 (ThermoFisher). SSR42 was amplified using OL7499/OL7500 while fragments of the *lukA*
152 UTR and *splE* UTR were amplified using primers 7501/7502 and 7523/7524, respectively.
153 These T7-controlled fragments were cloned into pGEM-T Easy and liberated from the
154 plasmid using restriction enzymes NcoI and PstI (Thermo Fisher Scientific). *In vitro*

155 transcription was performed using the MAXIscript T7 kit according to manufacturer
156 instructions and using 1µg of template DNA, ^{32}P-labeled UTP for the mRNA, and
157 unlabeled UTP for SSR42. TurboDNase was added to degrade any remaining DNA.
158 Reactions were cleaned using a Nucleotide Clean-up Kit (Qiagen). RNA-RNA binding
159 reactions were performed with labeled mRNA and SSR42 at varying concentrations,
160 which were heated to 90°C for 2 minutes and then incubated at RT for 30 min. Native
161 acrylamide gels were equilibrated on ice for 30 minutes in 0.5x TBE before loading binding
162 reactions, which were mixed with RNA loading dye and then run at 100V until the dye
163 front has moved ¾ of the way through the gel. Gels were dried and affixed to filter paper
164 using a gel dryer before being visualized via autoradiography.

165 **Murine models:** All animal studies in this work were performed in accordance with and
166 approved by the Institutional Animal Care and Use Committee of the University of South
167 Florida. To prepare inocula for a model of bacterial sepsis, bacteria was washed 2x with
168 PBS before being adjusted to $5x10^8$ CFU/mL in PBS. Female, six-week-old CD-1 mice
169 (Charles River Laboratories) were inoculated with 100µl of bacteria, resulting in a final
170 dose of $5x10^7$. Infection was allowed to progress for 7 days, or until mice reach a
171 premoribund state, at which point they were euthanized. After the infection period, all mice
172 were euthanized and the brain, heart, liver, spleen, lungs, and kidneys were harvested.
173 For murine models of pneumonia, inocula were prepared in a similar manner, however
174 the bacterial density was instead adjusted to equal a final dose of $1x10^8$ in 30 µl. Female
175 and male 6-week old C57BL/6 mice were intranasally inoculated and infections were
176 allowed to proceed for 24h, after which mice were euthanized and lungs harvested.
177 Organs for both models were homogenized in 2mL PBS and serially diluted and plated

178 for CFU/mL. All infection experiments were performed with 8 mice per strain and a Mann-
179 Whitney test was used to measure statistical significance.

180 **Data Availability & Additional Methods:** RNAseq data from this study is available under
181 the GEO accession #GSE237701. Proteomics data is available via proteomeXchange
182 under the ID PXD043724. Additional methods can be found in the supplemental file.

183 **Results**

184 **SSR42 is global regulator of virulence factor expression**: Although previous studies
185 exist exploring SSR42, the only large-scale omics examination of this factor is a
186 microarray performed over a decade ago (26). Thus, to bring new insight to the role of
187 SSR42, we created a complete SSR42 deletion in the USA300 background and
188 performed RNA-sequencing of the WT and mutant strains. To confirm validity of our
189 mutant strain, an analysis of RNAseq reads revealed the expected deletion of SSR42
190 **(Fig. S1A),** with concomitant downregulation of this gene observed (-2,955-fold compared
191 to WT)**.** We also determined that this deletion had no impact on growth kinetics **(Fig.**
192 **S1B).** When assessing reads from the wild-type dataset, we noted that SSR42 was the
193 second most abundant transcript in the cell, second only to RNAIII **(Fig. S2).** Moreover,
194 20% of all RNA in stationary phase cells under these conditions (absent rRNA) belongs
195 to SSR42, and 45% to SSR42 and RNAIII combined. Beyond this, we found a total of 220
196 genes were significantly differentially expressed between wild-type and ΔSSR42 (**Fig.**
197 **1A)**, greatly surpassing the 80 genes previously shown to be controlled by SSR42 via
198 microarray. These included those involved in metabolism, transport, signal transduction
199 and, by far the largest functional category (33 such factors), human disease. Regarding

200 this latter group, SSR42 primarily serves as an activator, with 83% of transcripts
201 decreased in abundance in the mutant strain **(Fig. 1BC).**

202 Key virulence factors regulated by SSR42 included secreted toxins, such as alpha
203 hemolysin (*hla,* -3.44-fold in ΔSSR42 versus WT), gamma hemolysin (*hlgA* -5.39, *hlgB* -
204 4.77), the Panton-Valentine leukocidin (*lukS* -3.62, *lukF* -3.86), and the *lukAB* leukocidin
205 (-5.49, -4.29). Secreted proteases were also impacted, with all serine protease (*spl*)
206 operon genes downregulated in the mutant (~-3-fold), whilst aureolysin (*aur*, +4.5) and
207 the V8 protease (*sspA*, +2.81) were both increased in abundance. Moreover, SSR42
208 appears to activate immune evasion factors, including igG-binding protein *sbi* (-5.36),
209 extracellular fibrinogen-binding protein *efb* (-2.85), and staphylokinase *sak* (-2.48). In the
210 same vein, we identified a profound change in the secreted extracellular matrix and
211 plasma binding protein *emp* (-34.6). Other virulence factors increased in abundance in
212 ΔSSR42 include the serine-rich adhesin for platelets *sraP* (+4.12) and the
213 Immunodominant Antigen B *isaB* (+2.92).

214 We also uncovered alterations in known transcriptional regulators, the most striking being
215 the SarA-family proteins *sarZ* (-9.6) and *sarR* (-62.3), which were downregulated in
216 ΔSSR42 compared to wild-type. Interestingly, the Repressor of Surface Proteins (*rsp*),
217 which is divergently transcribed from SSR42 and has been shown to be a primary
218 activator of its expression (27), was found to be upregulated 4.31-fold upon SSR42
219 deletion. Finally, we identified changes in expression of the metalloregulators *fur* (+3.14)
220 and *mntR* (-2.13), as well as the alternative sigma factor *sigS* (-2.75), in the mutant strain.
221 A random subset of these fold changes were explored and confirmed by qPCR analysis
222 (**Fig S3A**).

223 We next used proteomic analysis to validate these changes, revealing strong correlation
224 to our RNAseq data **(Fig. 1 A-C).** Notably, while leukocidins LukSF and LukAB exhibit
225 decreased protein abundance in the mutant that matches RNAseq trends, LukAB in
226 particular exhibits an even more profound decrease in fold-changes at the protein level
227 (RNAseq: *lukA* = -5.51, *lukB* = -4.3; Proteomics - LukA = -9.87, LukB = -7.83). A complete
228 list of all fold changes for both omic studies can be found in **Table S1** and a comparison
229 between the two datasets can be found in **Fig. S3B.**

230 **SSR42 is a novel regulator of cytolytic activity:** Given that LukAB levels had such
231 profound changes in our -omic studies, we next set out to explore if this had a
232 physiological relevant impact on leukocidin activity. As LukAB is the primary effector for
233 the lysis of human neutrophils (29), human promyeloblast (HL-60) cells were
234 differentiated into neutrophils before being treated with bacterial supernatant from the wild
235 type, mutant, and complemented strains. Strikingly, after a 1h incubation, supernatant
236 from ΔSSR42 elicited markedly reduced killing compared to the wild-type and
237 complement **(Fig. 2A).** To see how this translated into bacterial viability, we next explored
238 how deletion of SSR42 impacted *S. aureus* survival during exposure to whole human
239 blood. Here we noted the mutant exhibited a 7-fold increase in survival compared to the
240 wild type and complementing strains **(Fig. 2B).** Of note, *lukAB* mutants of *S. aureus* have
241 been shown to have increased intracellular abundance within neutrophils (a key
242 component of whole blood), due to an inability to use the toxin to escape the phagosome
243 (30). To determine if this was the case for ΔSSR42, we next measured intracellular loads
244 of our strains within HL-60 cells. Here we determined that, after 24h incubation, the
245 mutant displayed a dramatic increase in intracellular abundance compared to the wild

246 type and complement within this niche **(Fig. 2C).** We also measured the viability of
247 infected neutrophils following the 24h infection period, revealing increased viability in cells
248 infected with ΔSSR42 in comparison to the wild-type; likely due to decreased cytotoxicity
249 of the mutant strain **(Fig. S4).** Although this trend is not statistically significant,
250 complementation of SSR42 on a multi-copy plasmid causes a profound decrease in cell
251 viability in comparison to ΔSSR42. As such, we conclude that SSR42 is a major new
252 regulator of LukAB activity in *S. aureus*, mediating exoenzyme-based cytotoxicity as well
253 as intracellular escape from the phagosome.

254 **SSR42 is upregulated upon exposure to neutrophils via PerR derepression:** It has
255 previously been shown that *lukAB* expression is induced following exposure of *S. aureus*
256 to neutrophils, and that this is mediated, indirectly, via the peroxide regulator PerR, which
257 is known to derepress its targets in the presence of reactive oxygen species (31–33).
258 Given that SSR42 plays an intimate role in intracellular survival and cytotoxicity, we next
259 set out to determine if SSR42 was the missing link in this chain of regulation. To this end,
260 we created a luciferase transcriptional reporter for the SSR42 promoter and exposed it to
261 differentiated neutrophil-like HL-60 cells. In so doing, we determined that P_{SSR42} exhibited
262 a >3-fold increase in activity after just 1h in the presence of neutrophils **(Fig. 3A)**. Next,
263 to investigate if this regulation was governed by PerR, we re-assessed SSR42 promoter
264 activity in a *perR* mutant. This time we determined that P_{SSR42} expression is significantly
265 higher in the mutant strain when exposed to neutrophils as compared to wild-type **(Fig.
266 3B)**, which is consistent with PerR derepression.

267 **SSR42 controls *lukAB* expression in response to neutrophils:** We next set out to
268 investigate whether SSR42 might mediate the impact of PerR on *lukAB* expression during

269 engagement with neutrophils. As such, we created a P_{lukAB}-*lux* fusion and assessed its
270 response to neutrophils in the context of SSR42 and PerR. In so doing, we noted that
271 upon addition of neutrophils, there was a significant increase in *lukAB* expression in the
272 wild-type strain that mirrored that of P_{SSR42} **(Fig. 4A).** However, this response is ablated
273 in ΔSSR42, where the luciferase signal is 5-fold lower in comparison to the wild-type after
274 2h of neutrophil exposure **(Fig. 4B).** To uncouple the effect of SSR42 from PerR, we next
275 assessed *lukAB* expression in a *perR* mutant as well as an SSR42/*perR* double mutant.
276 This revealed that, once again in a similar manner to SSR42, *lukAB* expression was
277 higher in a *perR* mutant, however, *lukAB* expression in the SSR42/*perR* mutant mirrors
278 that of ΔSSR42 **(Fig. 4 A-C).** Native RNA levels were also quantified under these
279 conditions, and identical induction patterns were observed **(Fig. S5**). This suggests that
280 the response of *lukAB* to the presence of neutrophils is likely a result of direct regulation
281 by SSR42, which is in turn controlled by PerR. This is further demonstrated upon
282 overexpression of PerR in an SSR42 mutant, where there is no significant difference in
283 P_{lukAB} activity **(Fig. S6.)**.

284 **SSR42 regulates *lukAB* post-transcriptionally:** To determine if the changes in *lukAB*
285 expression are mediated by SSR42 binding to, and affecting stability of, the *lukAB* mRNA,
286 we fused a fragment of the *lukA* 5' UTR and a portion of its coding sequence in frame to
287 GFP and expressed it under the control of a constitutive promoter in *S. aureus.* We then
288 quantified GFP fluorescence in ΔSSR42 vs. wild-type, revealing a significant decrease in
289 the stability of the translated LukA-GFP product in the mutant strain **(Fig. 5A).** Next, we
290 used a two-plasmid system in which we expressed SSR42 under its native promoter
291 alongside the LukA-GFP reporter in ΔSSR42. Here we observed a striking increase in

292 LukA-GFP translation in the presence of SSR42 when compared to empty vector controls
293 **(Fig. 5B).** Taken together, this suggests that SSR42 may bind post-transcriptionally to the
294 *lukAB* mRNA to regulate its stability and thus translation.

295 **The 3' end of SSR42 is required for interaction with the *lukAB* 5' UTR:** To explore
296 interaction between SSR42 and the *lukAB* mRNA further, we first used IntaRNA to predict
297 possible base pairing interactions between the two RNA molecules (34). This predicted
298 an interaction with the 3' end of SSR42, ~153bp from the end of the transcript, and the 5'
299 UTR of *lukAB*, ending 7bp upstream of the ribosome binding site (RBS) **(Fig. 6A)**. To
300 investigate the relevance of this interaction *in vivo,* we first generated a series of truncated
301 SSR42 variants and introduced these into ΔSSR42, before reassessing LukAB translation
302 using the dual plasmid expression system. In so doing, we noted that shorter variants of
303 SSR42 excluding the last 150-bp were unable to elicit wild-type levels of LukAB
304 translation **(Fig. 6B).** This suggests that consistent with *in silico* predictions, the far 3' end
305 of SSR42 is primarily responsible for mediating interaction with *lukAB.* To next validate
306 the binding region identified in *lukAB,* we performed mutagenesis on the predicted binding
307 site within our translational LukAB-GFP fusion and assessed the impact of this on
308 fluorescence **(Fig. 6C).** Upon disruption of the predicted binding sequence, we identify no
309 difference between GFP levels in the wild-type and ΔSSR42, indicating that this region is
310 indeed important for interaction with SSR42.

311 **SSR42 directly binds to the *lukAB* mRNA and enhances its stability:** To explore
312 further the direct interaction between SSR42 and the *lukAB* mRNA, electrophoretic
313 mobility shift assays (EMSAs) were performed. A portion of the *lukA* 5' UTR that included
314 the region identified by IntaRNA was radiolabeled using *in vitro* transcription and

315 incubated with increasing concentrations of *in vitro* transcribed SSR42. When these
316 samples were co-incubated, we observed a concentration-dependent interaction between
317 the two RNA molecules (**Fig. 7A).** By comparison, no binding was seen with radiolabeled
318 *splE* mRNA under identical conditions, supporting the specificity of the SSR42-*lukAB*
319 interaction. We next set out to investigate whether the observed binding had any impact
320 on transcript stability. To this end, transcriptional arrest studies were performed on the
321 wild type, mutant, and complemented strains grown for 15h. Samples were taken at t=0
322 before the addition of Rifampin, and then again at 5-, 10-, 30-, and 45-minutes
323 posttreatment. RNA abundance was then quantified via qPCR, revealing that the *lukA*
324 transcript degraded far more quickly, and to a greater overall degree, in the mutant strain
325 when compared to the wild type and complement **(Fig. 7B).** When the mRNA half-life was
326 calculated using a one phase decay curve, we found that the *lukAB* mRNA $t_{1/2}$ was 2.9
327 minutes in ΔSSR42, but 4.5 minutes in the wild type. This suggests SSR42 is required for
328 maintaining stability of the *lukAB* mRNA and facilitating translation of its protein products.

329 **SSR42 is required to cause invasive *S. aureus* infections*:*** Previous work has shown
330 that SSR42 is important for localized infections using a murine model of abscess
331 formation (26). To assess the role SSR42 plays in invasive disease we assessed the
332 pathogenic capacity of the mutant in a murine model of septic infection. Mice were
333 infected with ΔSSR42 or wild-type and the bacterial burden was determined across
334 multiple organ systems after 7 days. Here we found that the mutant exhibited an
335 abrogated ability to disseminate to the liver, heart, and spleen **(Fig. 8A).** Specifically, in
336 the heart, ΔSSR42 exhibits a 4-log decrease in bacterial burden compared to WT, with 1-
337 2-log decreases found in the liver and spleen. To explore this virulence defect more fully,

338 we next used an acute model of murine pneumonia, intranasally inoculating mice with the
339 wild type and ΔSSR42. After 24h of infection we assessed bacterial recovery from the
340 lungs and once again found a major defect in the mutant's ability to survive, with ΔSSR42
341 exhibiting a 3-log decrease in CFU/mL in comparison to the wild-type **(Fig. 8B)**. These
342 findings, alongside those from others demonstrate that SSR42 is required for *S. aureus*
343 infection in three different animal models, representing both localized and invasive forms
344 of disease.

345 **Discussion**

346 Recent years have seen an abundance of new discoveries regarding the complex nature
347 of RNA-based regulation, revealing that many of these elements exhibit complex and
348 nuanced roles in pathogenesis. Herein, we explore just such a factor, broadening the
349 known regulome of the long noncoding RNA SSR42 from *S. aureus*. Using both
350 transcriptomic and proteomic approaches we expand the SSR42 network of control
351 almost three-fold from previous studies. From this, it is clear that the SSR42 regulome is
352 far more complex than was previously ascertained. Of note, we identified no significant
353 changes in any component relating to the SaeRS TCS, which previous studies suggested
354 was responsible for SSR42-related regulation of at least some virulence factors (27).
355 Instead, we note the strong downregulation of two SarA-homologs, *sarZ* and *sarR*, at -
356 9.6-fold and -62.6-fold, respectively, in ΔSSR42 compared to the wild-type. Both
357 transcription factors have been characterized to play a role in promoting virulence factor
358 expression in various *S. aureus* strains (35–38). Specifically, SarZ upregulates the
359 expression of *aur, sspAB,* and *hla* while SarR activates *sspAB* and the *spl* proteases.
360 Upon assessment of our own transcriptomics, the regulation of the proteases *aur* (via

361 SarZ) and *sspAB* (via SarR) are consistent with the trends seen in our data, however the
362 changes in *hla* (via SarZ) and *spl* (via SarR) expression are not. It is intriguing that these
363 transcripts are so strikingly decreased in abundance in ΔSSR42, suggesting that SSR42
364 plays a key role in controlling their expression or stability. Indeed, bioinformatic searches
365 for RNA-RNA binding do predict very strong interactions between SSR42 and the mRNAs
366 of both transcription factors **(Fig. S7)**. Thus, it is possible that changes in *sarR* and *sarZ*
367 expression may be causative for some, but not all, of the alterations in virulence factor
368 transcription seen in the absence of SSR42.

369 When homing in on SSR42's impact on the leukocidin LukAB, we show that SSR42
370 binding to the *lukAB* mRNA affects its stability, producing a concomitant impact on protein
371 production. The latter effect, we posit, is reflective of this interaction altering the secondary
372 structure of the *lukAB* mRNA to facilitate translation. Indeed, when predicting secondary
373 structure for the *lukAB* mRNA we find that the RBS is predicted to lie locked within a
374 hairpin, and thus may be inaccessible to the ribosome **(Fig. S8).** Just upstream of this
375 lies the region where SSR42 appears to bind. Therefore, it is entirely plausible that binding
376 of SSR42 5' to the *lukA* RBS could alter the secondary structure such that the RBS is
377 revealed and becomes accessible for translation. Of note, a very similar binding
378 mechanism is seen for the well-studied interaction between RNAIII and the *hla* mRNA,
379 whereby the former binds directly upstream of the latter's RBS, liberating this sequence
380 and activating translation (17). In the case of the *lukAB* mRNA, SSR42 binding also
381 results in enhanced transcript stability, as in its absence we not only see decreased
382 LukAB translation but also a significant decrease in *lukAB* mRNA abundance and half-life
383 as well. Interestingly, in addition to the impact of SSR42 on *lukAB* mRNA stability and/or

384 translational abundance, we also observe alterations in P_{lukAB} promoter activity. This, at
385 first glance, is contradictory to typical sRNA behavior, which usually only manifest post-
386 transcriptionally. However, the P_{lukAB} fragment used in our luciferase studies also contains
387 the 5' UTR region of the transcript, including the SSR42 binding site. Thus, as the
388 absence of SSR42 destabilizes the *lukAB* mRNA, the promoter fusion transcript will
389 inevitably be destabilized in our luciferase assays as well.

390 Recent work by Savin et al. identified the peroxide sensor PerR as a regulator of LukAB
391 activity that responds to the presence of hPMNs and leads to enhanced leukocidin
392 expression (33). Their study suggested no direct regulation between PerR and *lukAB* as
393 there are no PerR binding boxes detected in the leukocidin promoter. In our study, we
394 observed that the SSR42 promoter is also induced in the presence of human neutrophil-
395 like cells and, importantly, is required for the *lukAB* promoter to respond under these
396 conditions. Investigation of P_{SSR42} architecture revealed 3 putative PerR binding boxes
397 (sequence identified by Horsburgh et. al (32)) upstream and/or within the SSR42
398 transcript **(Fig. S9)**. It has previously been shown that known PerR targets such as *katA,*
399 *ahpC,* and *ftnA* can possess multiple binding boxes within their promoters and are de-
400 repressed during oxidative stress (31–33). When we mutated each of these binding
401 sequences individually, we identified no changes in promoter induction (data not shown),
402 however, we were unable to combinatorially mutate more than one box within the
403 promoter sequence at the same time. It is possible that profound derepression in the
404 absence of these PerR binding sites results in toxic over production of SSR42. Beyond
405 this, there are likely multiple factors controlling the expression of SSR42 as its production
406 is still modulated in response to neutrophils in the absence of PerR. We should note that

407 these findings were observed using differentiated HL-60 cells rather than primary human
408 neutrophils, thus, additional studies using primary *ex vivo* samples would be highly
409 valuable for corroborating our findings. Moreover, to further test for the full nuance of
410 SSR42 induction within the host, it would be intriguing to investigate how inhibition of the
411 oxidative burst of neutrophils within murine systems impacts SSR42 production.

412 The promoter of SSR42, as well as its primary regulator Rsp, have previously been shown
413 to be induced in response to the presence of hydrogen peroxide (39). Moreover, previous
414 work identified that Rsp is required for full cytotoxicity towards epithelial cells and
415 neutrophils in an Agr-independent manner (39). We posit that these phenotypes are at
416 least partially SSR42-mediated. To explore this, we assessed expression from the *rsp*
417 promoter in the presence of neutrophils and found that it too is induced by their presence
418 **(Fig. S10)**. Importantly, however, P_{rsp} is not derepressed by PerR in this context,
419 suggesting that the PerR-mediated control of *lukAB* by SSR42 is independent of Rsp.
420 This demonstrates that, depending on the environment, Rsp is necessary, but not
421 sufficient, for SSR42 expression. Thus, we propose that SSR42 is the missing link
422 between PerR-mediated ROS sensing and subsequent activation of LukAB activity to
423 mediate survival and escape from phagocytic cells. Further evidence for PerR-mediated
424 regulation of SSR42 also arises from a recent study by our group. Here, upon assessing
425 transcriptomic changes in response to treatment with Calprotectin (i.e. metal starved),
426 SSR42 was found to be downregulated ~2-fold. When samples were treated with a
427 mutated form of Calprotectin that only sequesters Zn and Cu, SSR42 was upregulated
428 >3-fold (40). In *S. aureus,* PerR requires Fe and Mn for oxidation in response to
429 endogenous H_2O_2 (31); thus, it follows that derepression of SSR42 would be detected

430 only in conditions including these metals. Finally, it is intriguing to consider that P_{SSR42}
431 may also be activated in response to bacterial-intrinsic ROS, as SSR42 is highly abundant
432 in stationary phase without the presence of host cells. Indeed, PerR derepression has
433 been shown to occur in response to intrinsic H_2O_2 levels, which are highest in stationary
434 phase *S. aureus* cells (32).

435 Metal homeostasis and the PerR-mediated oxidative stress response are intimately linked
436 through a network of metalloregulators: Fur, Zur, and MntR (32, 41–44). Interestingly, the
437 *fur* transcript is upregulated 3.14-fold in our SSR42 mutant while *mntR* expression is
438 decreased -2-fold. It is important to note that in addition to regulating SSR42, PerR plays
439 a role in regulating Fur and MntR expression, while all three players act in a coordinated
440 manner to induce overlapping regulons contributing to oxidative stress resistance,
441 including factors such as catalase (KatA), thioredoxin reductase (TrxB), and
442 alkylhydroperoxide reductase (AhpCF) (41, 44). While these elements were not altered
443 in the SSR42 mutant transcriptome, we did uncover three alternative antioxidant-like
444 factors that were decreased in expression in the SSR42 mutant. These include a
445 nitroreductase linked to iron acquisition (*ntrA,* -3.41-fold), a nitroreductase with
446 glutathione antioxidant behavior (SAUSA300_0790, -2.26-fold), and a thioredoxin-like
447 protein (SAUSA300_0795, -2.27-fold) (45–47). Thus, we posit that SSR42 weaves a
448 common thread connecting these metallo-oxidative regulators and subsequently impacts
449 virulence factor abundance in response to ROS-generating niches. Key examples of this
450 behavior in *S. aureus* have been shown in the case of IsrR and RsaC, two sRNAs that
451 are expressed in low iron or manganese environments, respectively, and function to
452 enhance the oxidative stress response and pathogenesis upon activation (25, 48–50).

453 When considering the wider context of *lukAB* regulation, it is strikingly diverse, and not
454 limited merely to induction in the presence of neutrophils and/or reactive oxygen species.
455 For example, as with numerous other exotoxins, alterations in LukAB abundance have
456 been linked to the Agr system through toxin suppression via Rot; although there is
457 currently no evidence to show any direct interaction between Rot and P_{lukAB} (51). SaeRS,
458 which has been shown to be crucial for pathogenesis and survival within neutrophils,
459 serves as a primary regulator of LukAB abundance through proposed binding at the
460 promoter region (52–54). Additionally, the ArlRS system has been shown to impact LukAB
461 abundance likely through its effector MgrA; although once again no direct interactions
462 have been identified (55). Finally, the autolysin Atl indirectly controls LukAB trafficking and
463 release through modulation of cell wall architecture via cleavage of peptidoglycan (56).
464 Collectively, despite the fact that LukAB has been shown to be vital for pathogenesis and
465 interaction with components of host immunity, the full regulatory network that controls its
466 expression has yet to be fully elucidated.

467 Taken together, our findings expand the role of SSR42 beyond a direct virulence-related
468 pathway and illustrate a much more complex role for this sRNA as it pertains to sensing
469 oxidative stress, nutritional immunity, and other exogenous signals. A proposed
470 mechanism of SSR42's involvement in the regulation of these processes as well as its
471 role in the broader regulation of LukAB is summarized in **Figure 9**. To our knowledge, this
472 is the first direct RNA-based regulation of *lukAB* characterized to date in *S. aureus*. Not
473 only do we uncover a novel regulator or leukocidin activity, but we begin to unravel the
474 labyrinthine network that controls the response to changing environments, particularly
475 within the host. Indeed, past work on SSR42 demonstrated its role in murine abscess

476 formation, while our own study broadens this to include complex and disseminated
477 infections including sepsis and pneumonia. Thus, we present SSR42 as a global
478 regulatory RNA that responds to pathophysiologically relevant conditions to bolster
479 virulence factor abundance and facilitate pathogenesis in *S. aureus.*

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482 **References**

483 1. Carroll RK, Weiss A, Broach WH, Wiemels RE, Mogen AB, Rice KC, Shaw LN.
484 2016. Genome-wide annotation, identification, and global transcriptomic analysis
485 of regulatory or small RNA gene expression in *Staphylococcus aureus*. mBio 7.

486

487 2. Liu W, Rochat T, Toffano-Nioche C, Le Lam TN, Bouloc P, Morvan C. 2018.
488 Assessment of bona fide sRNAs in *Staphylococcus aureus*. Front Microbiol 9:1–
489 13.

490

491 3. Pichon C, Felden B. 2005. Small RNA genes expressed from *Staphylococcus*
492 *aureus* genomic and pathogenicity islands with specific expression among
493 pathogenic strains. Proc Natl Acad Sci U S A 102:14249–14254.

494

495 4. Laner EA, Carroll K. 2020. Reading between the Lines: Utilizing RNA-Seq Data
496 for Global Analysis of sRNAs in *Staphylococcus aureus*. mSphere 5(4):e00439-
497 20.

498

499 5. Broach WH, Weiss A, Shaw LN. 2016. Transcriptomic analysis of staphylococcal
500 sRNAs: Insights into species-specific adaption and the evolution of pathogenesis.
501 Microb Genomics 2 (7).

502

503 6. Bronsard J, Pascreau G, Sassi M, Mauro T, Augagneur Y, Felden B. 2017. SRNA
504 and cis-Antisense sRNA identification in *Staphylococcus aureus* highlights an
505 unusual sRNA gene cluster with one encoding a secreted peptide. Sci Rep 7:1–
506 17.

507

508 7. Menard G, Silard C, Suriray M, Rouillon A, Augagneur Y. 2022. Thirty Years of
509 sRNA-Mediated Regulation in *Staphylococcus aureus*: From Initial Discoveries to
510 In Vivo Biological Implications. International Journal of Molecular Sciences.
511 23(13), 7346.

512

513 8. Barrientos L, Mercier N, Lalaouna D, Caldelari I. 2021. Assembling the Current
514 Pieces: The Puzzle of RNA-Mediated Regulation in *Staphylococcus aureus*. Front
515 Microbiol 12:1–7.

516

517 9. Bastock RA, Marino EC, Wiemels RE, Holzschu DL, Keogh RA, Zapf RL, Murphy
518 ER, Carroll RK. 2021. *Staphylococcus aureus* Responds to Physiologically
519 Relevant Temperature Changes by Altering Its Global Transcript and Protein
520 Profile. mSphere 6.

521

522 10. Bordeau V, Cady A, Revest M, Rostan O, Sassi M, Tattevin P, Donnio PY, Felden
523 B. 2016. *Staphylococcus aureus* regulatory RNAs as potential biomarkers for
524 bloodstream infections. Emerg Infect Dis 22:1570–1578.

525

526 11. Kim S, Reyes D, Beaume M, Francois P, Cheung A. 2014. Contribution of teg49
527 Small RNA in the 5' Upstream Transcriptional Region of sarA to Virulence in
528 *Staphylococcus aureus*. Infect Immun 82:4369–4379.

529

530 12. Le Huyen KB, Gonzalez CD, Pascreau G, Bordeau V, Cattoir V, Liu W, Bouloc P,
531 Felden B, Chabelskaya S. 2021. A small regulatory RNA alters *Staphylococcus*
532 *aureus* virulence by titrating RNAIII activity. Nucleic Acids Res 49:10644–10656.

533

534 13. McKellar SW, Ivanova I, Arede P, Zapf RL, Mercier N, Chu LC, Mediati DG,
535 Pickering AC, Briaud P, Foster RG, Kudla G, Ross Fitzgerald J, Caldelari I, Carroll

536 RK, Tree JJ, Granneman S. 2022. RNase III CLASH in MRSA uncovers sRNA
537 regulatory networks coupling metabolism to toxin expression. Nat Commun 13.
538
539 14. Gupta RK, Luong TT, Lee CY. 2015. RNAIII of the *Staphylococcus aureus* agr
540 system activates global regulator MgrA by stabilizing mRNA. Proc Natl Acad Sci U
541 S A 112:14036–14041.
542
543 15. Huntzinger E, Boisset S, Saveanu C, Benito Y, Geissmann T, Namane A, Lina G,
544 Etienne J, Ehresmann B, Ehresmann C, Jacquier A, Vandenesch F, Romby P.
545 2005. *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately
546 regulate spa gene expression. EMBO Journal 24:824–835.
547
548 16. Boisset S, Geissmann T, Huntzinger E, Fechter P, Bendridi N, Possedko M,
549 Chevalier C, Helfer AC, Benito Y, Jacquier A, Gaspin C, Vandenesch F, Romby P.
550 2007. *Staphylococcus aureus* RNAIII coordinately represses the synthesis of
551 virulence factors and the transcription regulator Rot by an antisense mechanism.
552 Genes Dev 21:1353–1366.
553
554 17. Morfeldt E, Taylor D, Von Gabain A, Arvidson S. 1995. Activation of alpha-toxin
555 translation in *Staphylococcus aureus* by the trans-encoded antisense RNA,
556 RNAIII. EMBO Journal 14:4569–4577.

557

558 18. Briaud P, Zapf RL, Mayher AD, McReynolds AKG, Frey A, Sudnick EG, Wiemels
559 RE, Keogh RA, Shaw LN, Bose JL, Carroll RK. 2022. The Small RNA Teg41 Is a
560 Pleiotropic Regulator of Virulence in *Staphylococcus aureus*. Infect Immun 19.

561

562 19. Zapf RL, Wiemels RE, Keogh RA, Holzschu DL, Howell KM, Trzeciak E, Caillet
563 AR, King KA, Selhorst SA, Naldrett MJ, Bose JL, Carroll RK. 2019. The small
564 RNA Teg41 regulates expression of the alpha phenol-soluble modulins and is
565 required for virulence *in Staphylococcus aureus*. mBio 10:1–19.

566

567 20. Manna AC, Leo S, Girel S, González-Ruiz V, Rudaz S, Francois P, Cheung AL.
568 2022. Teg58, a small regulatory RNA, is involved in regulating arginine
569 biosynthesis and biofilm formation in *Staphylococcus aureus*. Sci Rep 12:1–18.

570

571 21. Zhou J, Zhao H, Yang H, He C, Shu W, Cui Z, Liu Q. 2022. Insights Into the
572 Impact of Small RNA SprC on the Metabolism and Virulence of *Staphylococcus*
573 *aureus*. Front Cell Infect Microbiol 12:1–13.

574

575 22. Le Huyen KB, Gonzalez CD, Pascreau G, Bordeau V, Cattoir V, Liu W, Bouloc P,
576 Felden B, Chabelskaya S. 2021. A small regulatory RNA alters *Staphylococcus*
577 *aureus* virulence by titrating RNAIII activity. Nucleic Acids Res 49:10644–10656.

578

579 23. Chabelskaya S, Gaillot O, Felden B. 2010. A *Staphylococcus aureus* small RNA is
580 required for bacterial virulence and regulates the expression of an immune-
581 evasion molecule. PLoS Pathog 6:1–11.

582

583 24. Chabelskaya S, Bordeau V, Felden B. 2014. Dual RNA regulatory control of a
584 *Staphylococcus aureus* virulence factor. Nucleic Acids Res 42:4847–4858.

585

586 25. Lalaouna D, Baude J, Wu Z, Tomasini A, Chicher J, Marzi S, Vandenesch F,
587 Romby P, Caldelari I, Moreau K. 2019. RsaC sRNA modulates the oxidative stress
588 response of *Staphylococcus aureus* during manganese starvation. Nucleic Acids
589 Res 47:9871–9887.

590

591 26. Morrison JM, Miller EW, Benson MA, Alonzo F, Yoong P, Torres VJ, Hinrichs SH,
592 Dunmana PM. 2012. Characterization of SSR42, a Novel virulence factor
593 regulatory RNA that contributes to the pathogenesis of a *Staphylococcus aureus*
594 USA300 representative. J Bacteriol 194:2924–2938.

595

596 27. Horn, J., Klepsch, M., Manger, M., Wolz, C., Rudel, T., & Fraunholz, M. (2018).
597 Long Noncoding RNA SSR42 Controls *Staphylococcus aureus* Alpha-Toxin

598 Transcription in Response to Environmental Stimuli. Journal of
599 bacteriology, 200(22), e00252-18.
600
601 28. DuMont AL, Nygaard TK, Watkins RL, Smith A, Kozhaya L, Kreiswirth BN,
602 Shopsin B, Unutmaz D, Voyich JM, Torres VJ. 2011. Characterization of a new
603 cytotoxin that contributes to *Staphylococcus aureus* pathogenesis. Mol Microbiol
604 79:814–825.
605
606 29. DuMont AL, Yoong P, Day CJ, Alonzo F, McDonald WH, Jennings MP, Torres VJ.
607 2013. *Staphylococcus aureus* LukAB cytotoxin kills human neutrophils by
608 targeting the CD11b subunit of the integrin Mac-1. Proc Natl Acad Sci U S A
609 110:10794–10799.
610
611 30. DuMont AL, Yoong P, Surewaard BGJ, Benson MA, Nijland R, Strijp JAG van,
612 Torres VJ. 2013. *Staphylococcus aureus* elaborates leukocidin AB to mediate
613 escape from within human neutrophils. Infect Immun 81:1830–1841.
614
615 31. Ji CJ, Kim JH, Won Y Bin, Lee YE, Choi TW, Ju SY, Youn H, Helmann JD, Lee
616 JW. 2015. *Staphylococcus aureus* PerR is a hypersensitive hydrogen peroxide
617 sensor using iron-mediated histidine oxidation. Journal of Biological Chemistry
618 290:20374–20386.

619

620 32. Horsburgh MJ, Clements MO, Crossley H, Ingham E, Foster SJ. 2001. PerR
621 controls oxidative stress resistance and iron storage proteins and is required for
622 virulence in *Staphylococcus aureus*. Infect Immun 69:3744–3754.

623

624 33. Savin, A., Anderson, E. E., Dyzenhaus, S., Podkowik, M., Shopsin, B., Pironti, A.,
625 & Torres, V. J. 2024. *Staphylococcus aureus* senses human neutrophils via PerR
626 to coordinate the expression of the toxin LukAB. Infection and immunity, 92(2),
627 e0052623.

628

629 34. Mann M, Wright PR, Backofen R. 2017. IntaRNA 2.0: Enhanced and customizable
630 prediction of RNA-RNA interactions. Nucleic Acids Res 45:W435–W439.

631

632 35. Manna A, Cheung AL. 2001. Characterization of sarR, a modulator of sar
633 expression in *Staphylococcus aureus.* Infect Immun 69:885–896.

634

635 36. Reyes D, Andrey DO, Monod A, Kelley WL, Zhang G, Cheung AL. 2011.
636 Coordinated regulation by AgrA, SarA, and SarR to control agr expression in
637 *Staphylococcus aureus*. J Bacteriol 193:6020–6031.

638

639 37. Tamber S, Cheung AL. 2009. SarZ promotes the expression of virulence factors
640 and represses biofilm formation by modulating SarA and agr in *Staphylococcus*
641 *aureus.* Infect Immun 77:419–428.

642

643 38. Chen PR, Nishida S, Poor CB, Cheng A, Bae T, Kuechenmeister L, Dunman PM,
644 Missiakas D, He C. 2009. A new oxidative sensing and regulation pathway
645 mediated by the MgrA homologue SarZ in *Staphylococcus aureus*. Mol Microbiol
646 71:198–211.

647

648 39. Das S, Lindemann C, Young BC, Muller J, Österreich B, Ternette N, Winkler AC,
649 Paprotka K, Reinhardt R, Förstner KU, Allen E, Flaxman A, Yamaguchi Y, Rollier
650 CS, Diemen P Van, Blättner S, Remmele CW, Selle M, Dittrich M, Müller T, Vogel
651 J, Ohlsen K, Crook DW, Massey R, Wilson DJ, Rudel T, Wyllie DH, Fraunholz MJ.
652 2016. Natural mutations in a *Staphylococcus aureus* virulence regulator attenuate
653 cytotoxicity but permit bacteremia and abscess formation. Proc Natl Acad Sci U S
654 A 113:E3101–E3110.

655

656 40.
657 Ruiz VM, Freiberg JA, Weiss A, Green ER, Jobson M, Felton E, Shaw LN, Chazin
658 WJ, Skaar EP. 2024. Coordinated adaptation of *Staphylococcus aureus* to
659 calprotectin-dependent metal sequestration. mBio 15:e01389-24.

660

661 41. Horsburgh MJ, Ingham E, Foster SJ. 2001. In *Staphylococcus aureus*, Fur is an
662 interactive regulator with PerR, contributes to virulence, and is necessary for
663 oxidative stress resistance through positive regulation of catalase and iron
664 homeostasis. J Bacteriol 183:468–475.

665

666 42. Torres VJ, Attia AS, Mason WJ, Hood MI, Corbin BD, Beasley FC, Anderson KL,
667 Stauff DL, McDonald WH, Zimmerman LJ, Friedman DB, Heinrichs DE, Dunman
668 PM, Skaar EP. 2010. *Staphylococcus aureus* fur regulates the expression of
669 virulence factors that contribute to the pathogenesis of pneumonia. Infect Immun
670 78:1618–1628.

671

672 43. Lindsay JA, Foster SJ. 2001. Zur: A Zn2+-responsive regulatory element of
673 *Staphylococcus aureus*. Microbiology (N Y) 147:1259–1266.

674

675 44. Horsburgh MJ, Wharton SJ, Cox AG, Ingham E, Peacock S, Foster SJ. 2002.
676 MntR modulates expression of the PerR regulon and superoxide resistance in
677 *Staphylococcus aureus* through control of manganese uptake. Mol Microbiol
678 44:1269–1286.

679

680 45. Tavares AFN, Nobre LS, Melo AMP, Saraiva LM. 2009. A novel nitroreductase of
681 *Staphylococcus aureus* with S-nitrosoglutathione reductase activity. J Bacteriol
682 191:3403–3406.

683

684 46. Liochev SI, Hausladen A, Fridovich I. 1999. Nitroreductase A is regulated as a
685 member of the soxRS regulon of *Escherichia coli*. Proc Natl Acad Sci U S A
686 96:3537–3539.

687

688 47. Nishinaka Y, Masutani H, Nakamura H, Yodoi J. 2001. Regulatory roles of
689 thioredoxin in oxidative stress-induced cellular responses. Redox Report 6:289–
690 295.

691

692 48. Ganske A, Busch LM, Hentschker C, Reder A, Michalik S, Surmann K, Völker U,
693 Mäder U. 2024. Exploring the targetome of IsrR , an iron-regulated sRNA
694 controlling the synthesis of iron-containing proteins in *Staphylococcus aureus* 1–
695 19.

696

697 49. Coronel-Tellez RH, Pospiech M, Barrault M, Liu W, Bordeau V, Vasnier C, Felden
698 B, Sargueil B, Bouloc P. 2022. sRNA-controlled iron sparing response in
699 Staphylococci. Nucleic Acids Res 50:8529–8546.

700

701 50. Rios-Delgado G, McReynolds AKG, Pagella EA, Norambuena J, Briaud P, Zheng
702 V, Munneke MJ, Kim J, Racine H, Carroll R, Zelzion E, Skaar E, Bose JL, Parker
703 D, Lalaouna D, Boyd JM. 2024. The *Staphylococcus aureus* small non-coding
704 RNA IsrR regulates TCA cycle activity and virulence. bioRxiv 2024.07.03.601953.

705

706 51. Mootz JM, Benson MA, Heim CE, Crosby HA, Kavanaugh JS, Dunman PM,
707 Kielian T, Torres VJ, Horswill AR. 2015. Rot is a key regulator of *Staphylococcus*
708 *aureus* biofilm formation. Mol Microbiol 96:388–404.

709

710 52. Voyich, J. M., Vuong, C., DeWald, M., Nygaard, T. K., Kocianova, S., Griffith, S.,
711 Jones, J., Iverson, C., Sturdevant, D. E., Braughton, K. R., Whitney, A. R., Otto,
712 M., & DeLeo, F. R. 2009. The SaeR/S gene regulatory system is essential for
713 innate immune evasion by *Staphylococcus aureus*. *The Journal of infectious*
714 *diseases*, *199*(11), 1698–1706.

715

716 53. Nygaard, T. K., Pallister, K. B., Ruzevich, P., Griffith, S., Vuong, C., & Voyich, J. M.
717 (2010). SaeR binds a consensus sequence within virulence gene promoters to
718 advance USA300 pathogenesis. *The Journal of infectious diseases*, *201*(2), 241–
719 254.

720

721 54. Flack CE, Zurek OW, Meishery DD, Pallister KB, Malone CL, Horswill AR, Voyich
722 JM. 2014. Differential regulation of staphylococcal virulence by the sensor kinase
723 SaeS in response to neutrophil-derived stimuli. Proc Natl Acad Sci U S A
724 111:2037–2045.

725

726 55. Crosby HA, Tiwari N, Kwiecinski JM, Xu Z, Dykstra A, Jenul C, Fuentes EJ,
727 Horswill AR. 2020. The *Staphylococcus aureus* ArlRS two-component system
728 regulates virulence factor expression through MgrA. Mol Microbiol 113:103–122.

729

730 56. Zheng X, Ma SX, John AS, Torres VJ. 2022. The Major Autolysin Atl Regulates the
731 Virulence of *Staphylococcus aureus* by Controlling the Sorting of LukAB. Infect
732 Immun 90:1–13.

733

734

735

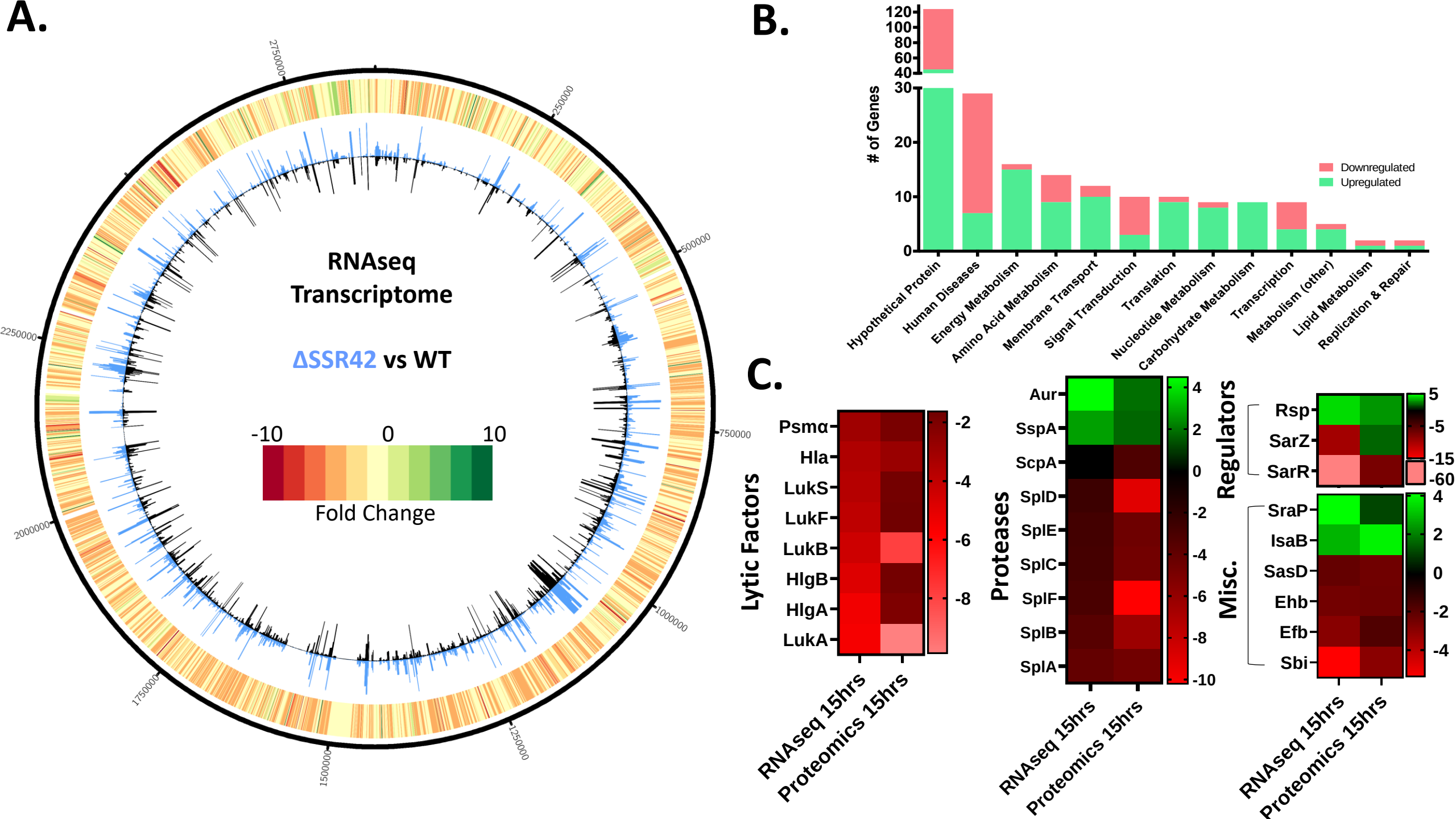

A.
RNAseq
Transcriptome
ΔSSR42 vs WT
-10
0
10
Fold Change
2750000
250000
500000
750000
1000000
1250000
1500000
1750000
2000000
2250000
B.
# of Genes
Downregulated
Upregulated
Hypothetical Protein
Human Diseases
Energy Metabolism
Amino Acid Metabolism
Membrane Transport
Signal Transduction
Translation
Nucleotide Metabolism
Carbohydrate Metabolism
Transcription
Metabolism (other)
Lipid Metabolism
Replication & Repair
C.
Lytic Factors
Psmα
Hla
LukS
LukF
LukB
HlgB
HlgA
LukA
Proteases
Aur
SspA
ScpA
SplD
SplE
SplC
SplF
SplB
SplA
Regulators
Rsp
SarZ
SarR
Misc.
SraP
IsaB
SasD
Ehb
Efb
Sbi
RNAseq 15hrs
Proteomics 15hrs

Figure 1. SSR42 is a global regulator of virulence factor production. (A) CIRCOS file illustrating transcriptional changes between wild-type and ΔSSR42 at 15h of growth. The inner circle exhibits transcript expression represented as a histogram of TPM expression values for the wild type (black bars) and SSR42 mutant (blue bars). Outermost circle is a heatmap describing fold change in expression where green indicates a positive fold change and red indicates a negative fold change in comparison to the wild type. **(B)** The number of genes upregulated (green) or downregulated (pink) $\geq$2-fold in the SSR42 null strain at 15h, sorted by function based on KEGG ontology. **(C)** Heat map illustrating changes in transcript and protein abundance of key virulence factors of interest.

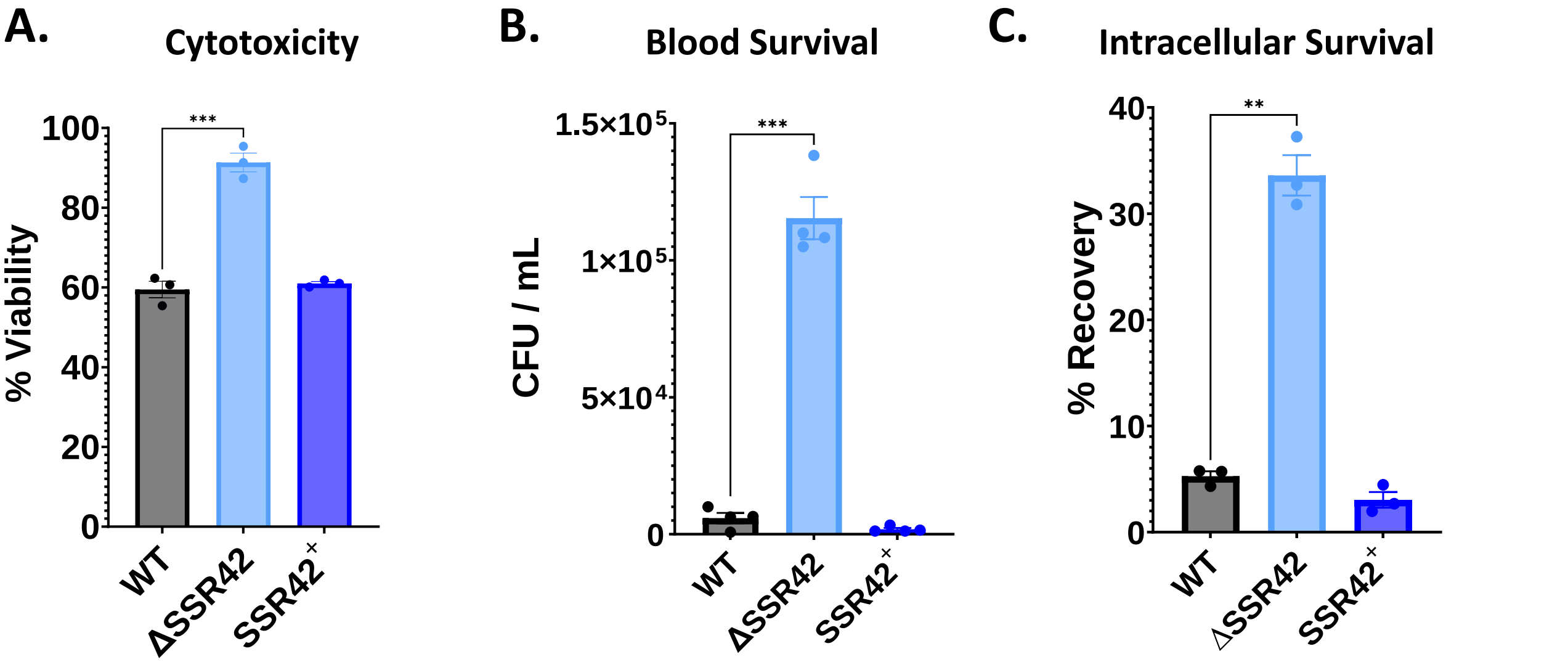


Figure 2. SSR42 plays a central role during engagement with the innate immune system. (A) HL-60 cells were differentiated into neutrophil-like cells with 1.25% DMSO for 4 days before being treated with 5% v/v bacterial supernatant from 15 h cultures of the strains shown. Intoxicated cells were incubated for 1 h before cell viability was measured using the CellTiter reagent. Percent cell viability in comparison to TSB-treated cells is shown. **(B)** Bacterial strains were synchronized and then standardized to an OD_{600} of 0.05 in 1 mL pooled-donor, whole human blood. Bacterial recovery after 4 h was enumerated as CFU/mL. **(C)** Differentiated HL-60 neutrophil-like cells were incubated with the strains shown at an MOI of 30, and intracellular bacterial loads determined after 24h. All experiments were performed in biological triplicate. Error bars represent ±SEM. Student's t test with Welch's correction was used to determine statistical significance relative to wildtype. ** $P \leq 0.01$, ,***, $P \leq 0.001$.

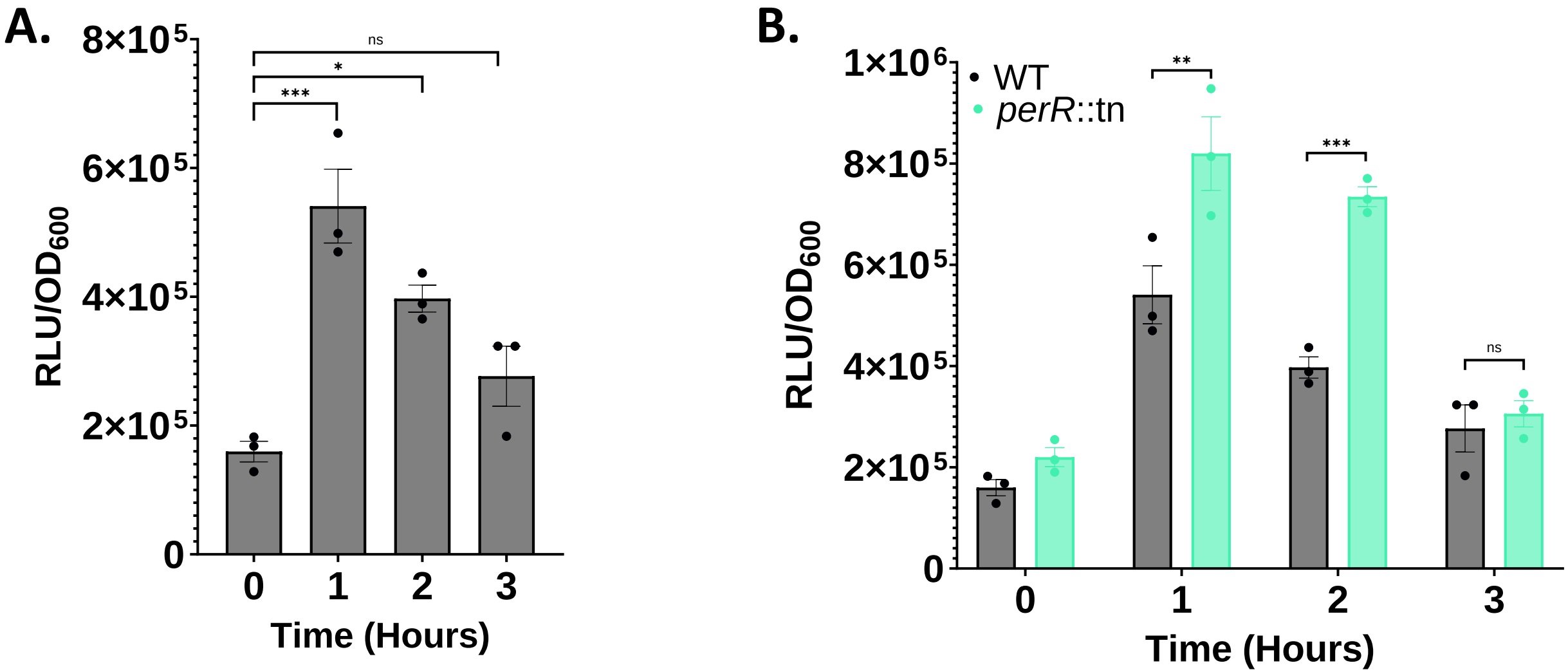


Figure 3. SSR42 is upregulated in response to neutrophils via PerR Derepression. (A) Differentiated HL60 cells were seeded at a concentration of $2x10^5$ before the *S. aureus* WT harboring a P_{SSR42}-lux reporter fusion was added at a final OD_{600} of 0.1. Luminescence and OD_{600} reads were taken at T=0 and then hourly for 3 hours. Luminescence was normalized to OD_{600}. Data is from biological triplicate samples. **(B)** As in A but comparing P_{SSR42} expression between the wild-type and a *perR* mutant strain. A student's t-test with Welch's correction was used to determine significance. Error bars are ±SEM. * $P \leq 0.05$ ** $P \leq 0.01$, ,***, $P \leq 0.001$.

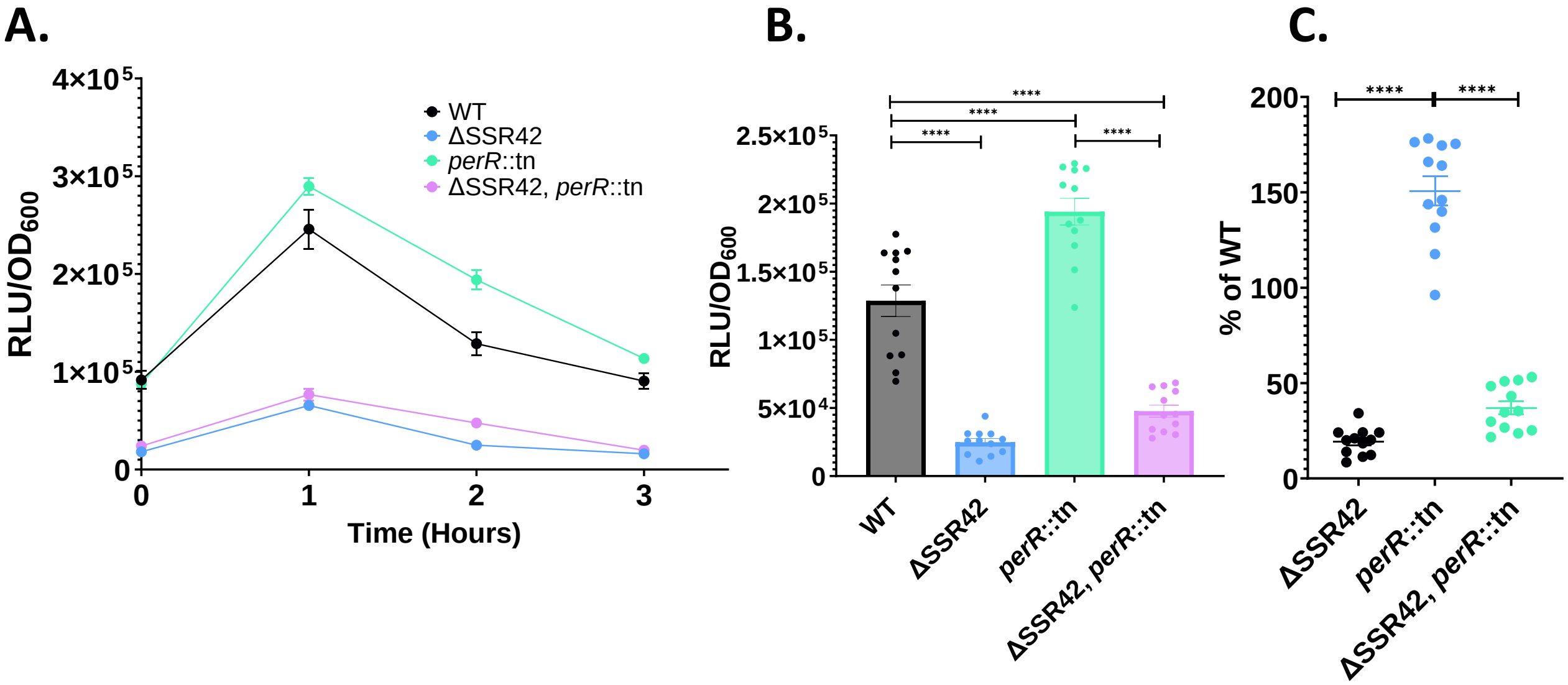


Figure 4. SSR42 controls LukAB expression in response to neutrophils. (A) As in Figure 3A but using the strains shown, all harboring a P_{lukAB}-lux reporter fusion. **(B)** As in A but after 2h of exposure to neutrophils. **(C)** Data from B represented as a percentage of wild-type expression. Experiments were performed with 12 biological replicates. A one-way ANOVA was used to determine statistical significance. Error bars are ±SEM. ****, P<0.0001

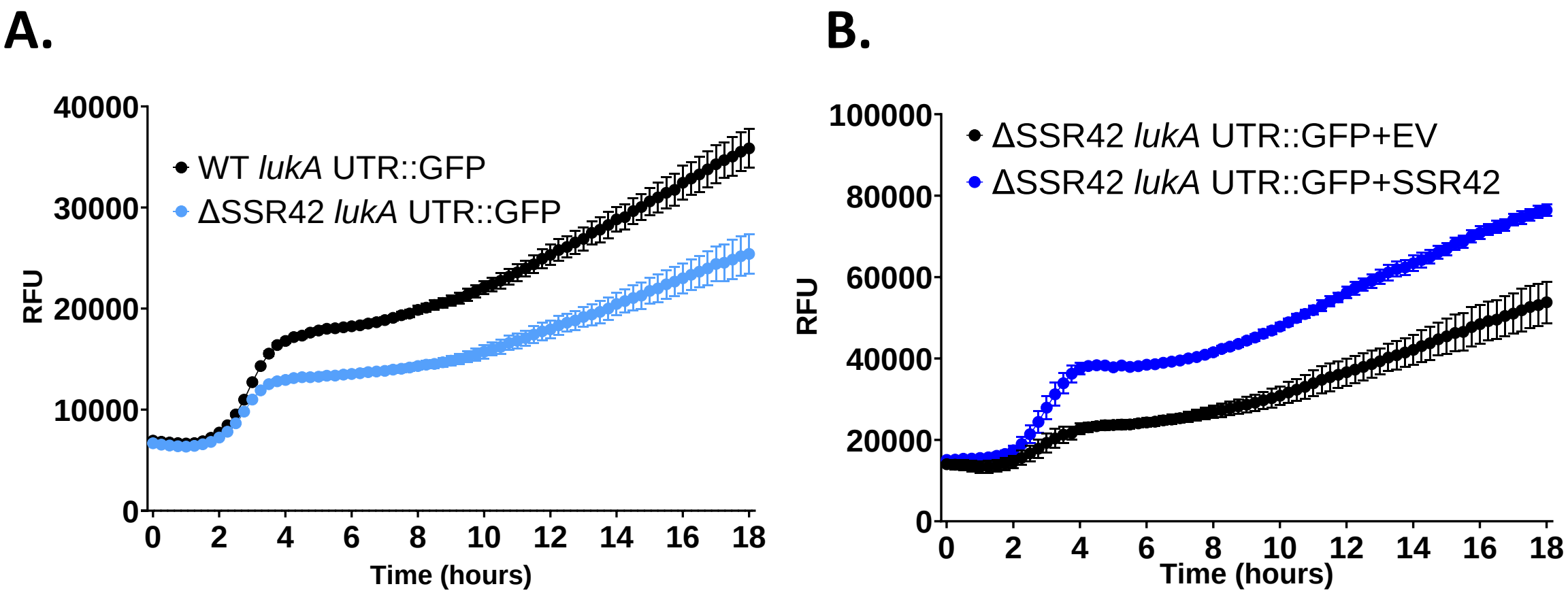
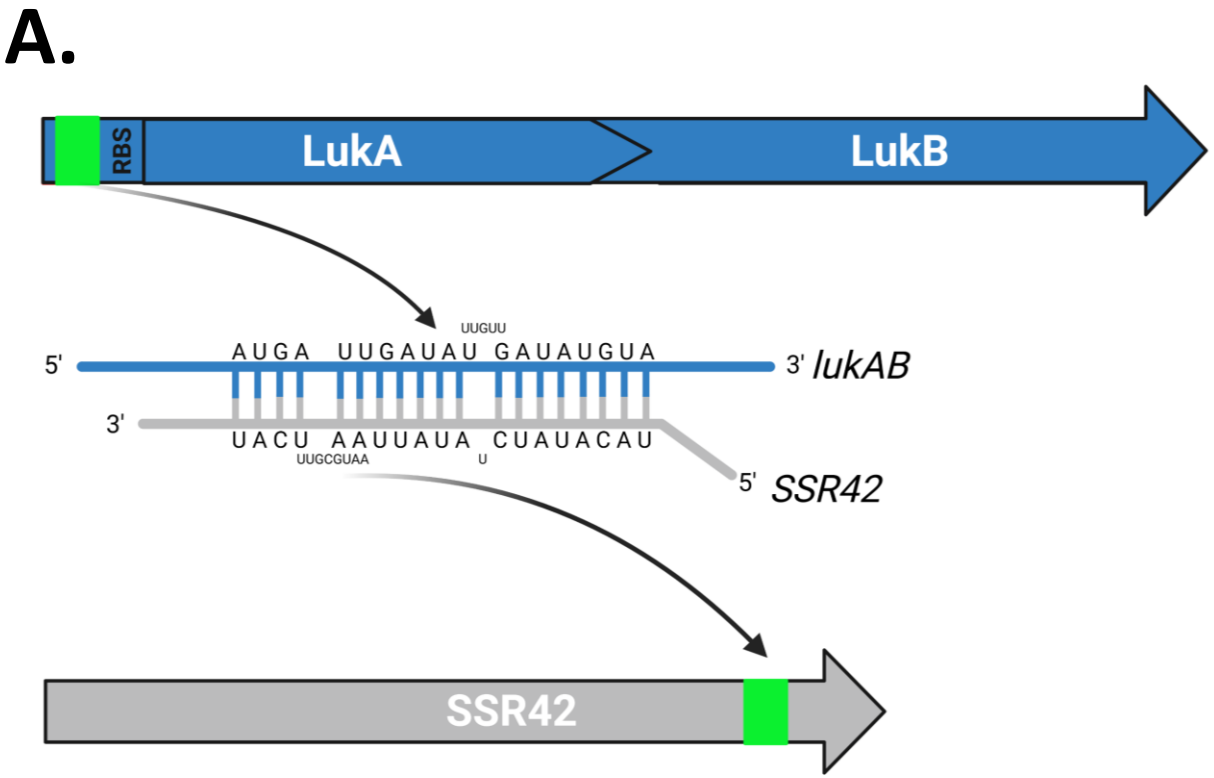
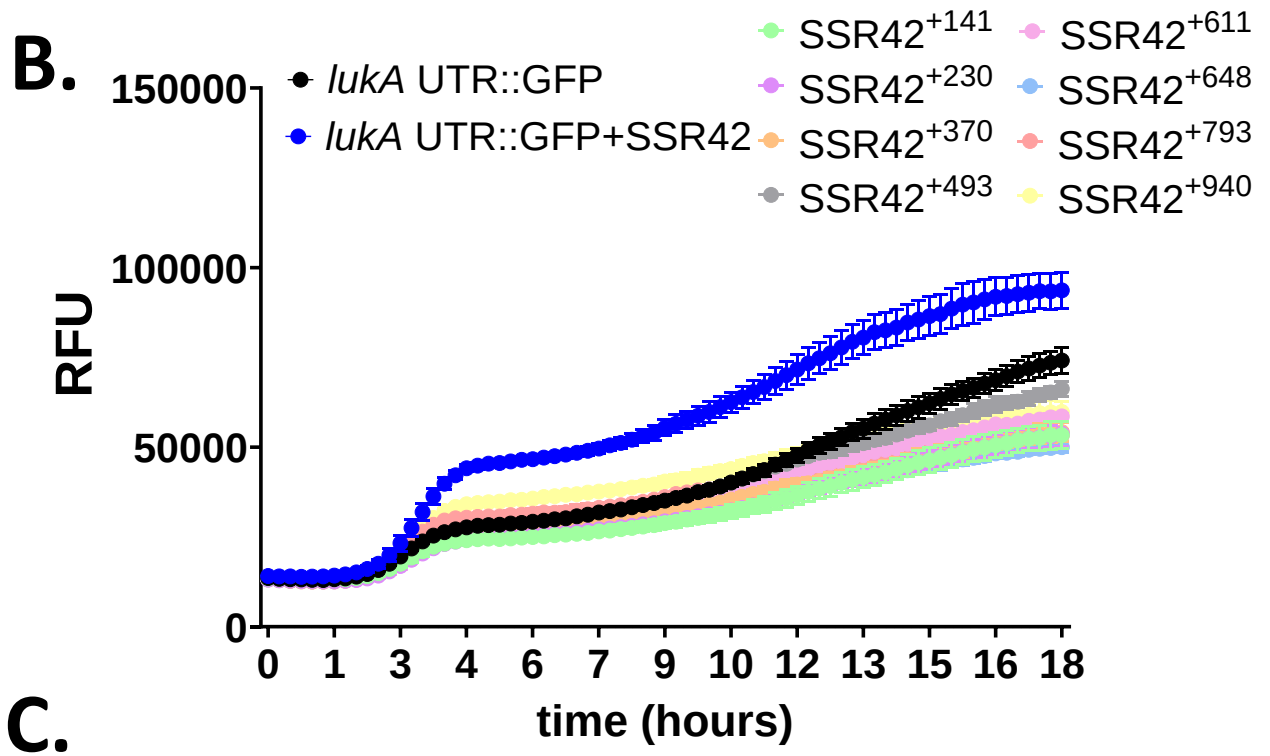


Figure 5. SSR42 post-transcriptionally controls LukAB abundance. (A) A portion of the *lukA* 5' UTR and coding sequence was translationally fused to GFP, cloned into a shuttle vector using a constitutive promoter, and introduced into the wild-type or ΔSSR42 strains. **(B)** The *lukA-GFP* reporter fusion plasmid was co-expressed with either an empty vector (EV) or a plasmid containing SSR42 controlled by its native promoter in ΔSSR42. Fluorescence was measured every 15 minutes. Error bars are ± SEM. All experiments were performed in biological triplicate.


A.
RBS
LukA
LukB
UUGUU
5'
AUGA UUGAUAU GAUAUGUA
3' lukAB
3'
UACU AAUUAUA CUAUACAU
UUGCGUAA
U
5' SSR42
SSR42


B.
lukA UTR::GFP
lukA UTR::GFP+SSR42
SSR42+141
SSR42+230
SSR42+370
SSR42+493
SSR42+611
SSR42+648
SSR42+793
SSR42+940
RFU
150000
100000
50000
0
0 1 3 4 6 7 9 10 12 13 15 16 18
time (hours)

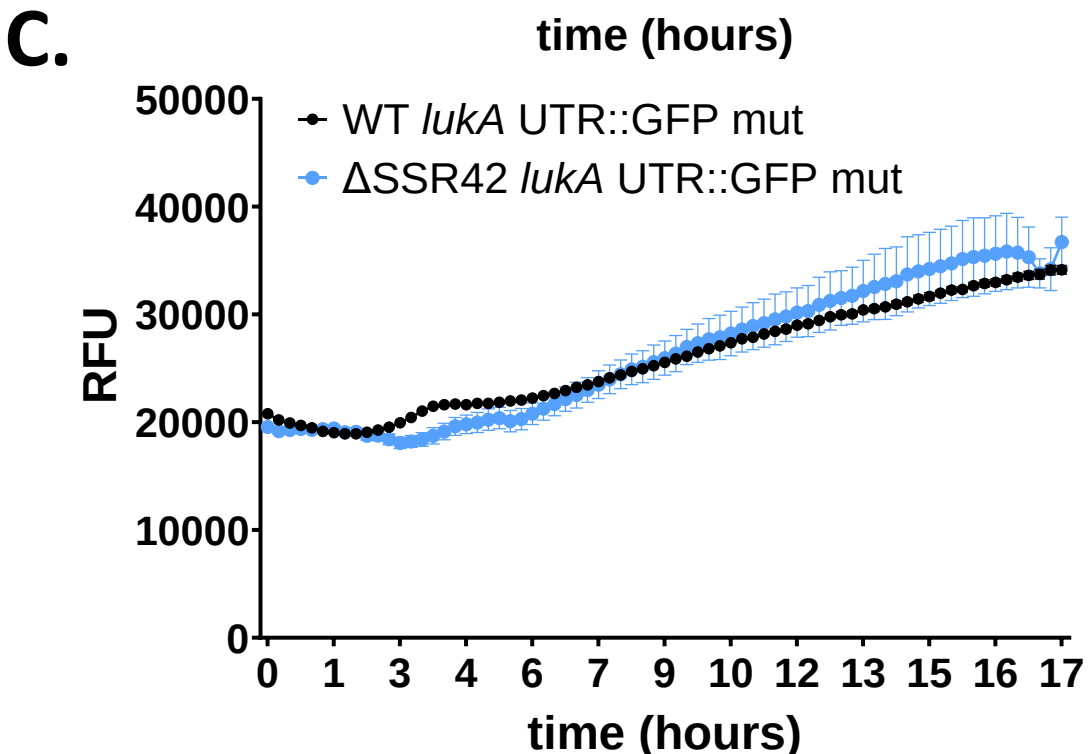

C.
WT lukA UTR::GFP mut
ΔSSR42 lukA UTR::GFP mut
RFU
50000
40000
30000
20000
10000
0
0 1 3 4 6 7 9 10 12 13 15 16 17
time (hours)

Figure 6. The 3’ end of SSR42 mediates binding to the *lukAB* 5’ UTR. (A) IntaRNA was used to identify thermodynamically likely regions of base paring between SSR42 and the *lukAB* mRNA. This identified an interaction region between the 3’ end of SSR42 and the 5’ UTR of *lukAB.* Created using Biorender.com. **(B)** The *lukA* UTR::GFP strain from Figure 5 was co-expressed in ΔSSR42 with either an empty vector (EV) or plasmids containing various truncated variants of SSR42. **(C)** The *lukA* UTR::GFP construct was mutagenized at the predicted site of interaction with SSR42 and luminescence was measured every 15 minutes in the wild-type and ΔSSR42. Error bars are ±SEM.

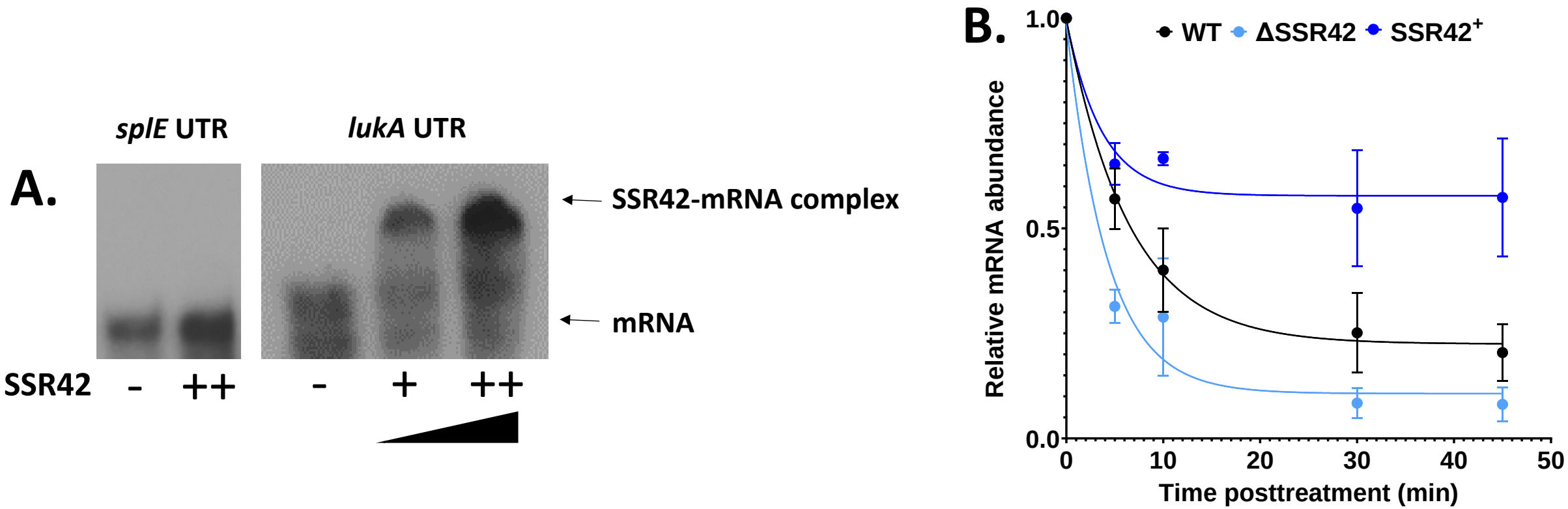


Figure 7. SSR42 directly binds and stabilizes the *lukAB mRNA*. (A) *In vitro* transcribed SSR42 was added in increasing amounts (1,000-fold to 1,500-fold excess) to radiolabeled portions of the *lukAB* mRNA. Samples were heated to 90° C for 2 min and then incubated at room temperature for 30 min before being loaded on to a non-denaturing gel. At identical concentrations, no binding is seen for radiolabeled *splE*. Images shown are representatives of 5 experimental replicates. **(B)** Bacterial cultures were grown in biological triplicate for 15 h before transcription was halted by the addition of 250 µg/mL Rifampin. Samples were collected 0, 5, 10, 30, and 45 minutes post-transcriptional arrest and *lukA* mRNA abundance was assessed via qPCR analysis. Data was compared to initial RNA abundance at t=0 and normalized to 16S rRNA. Shown is a one phase decay curve. Error bars are ±SEM.

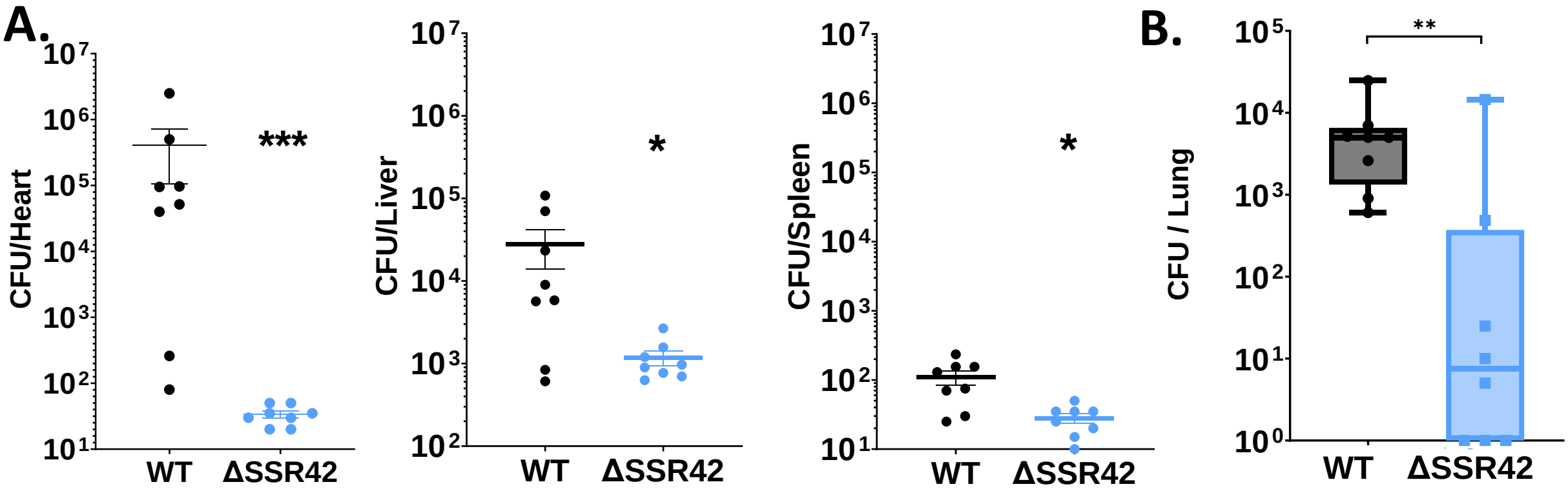


Figure 8. SSR42 is required for full virulence in murine models of invasive disease. (A) CD-1 mice were inoculated with the wild-type or ΔSSR42 at 5 x 10^8 CFU via tail vein injection. Following 7 days of infection, organs were harvested and CFU determined. **(B)** C57BL/6J mice were intranasally inoculated with the wild-type or ΔSSR42 at 1 x 10^8 CFU. After 24h, lungs were harvested and CFU determined. Whiskers represent minimum and maximum values. Solid lines represent median values. Mann Whitney tests were used to determine statistical significance relative to wildtype. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. n=8 for each strain, error bars are ±SEM.

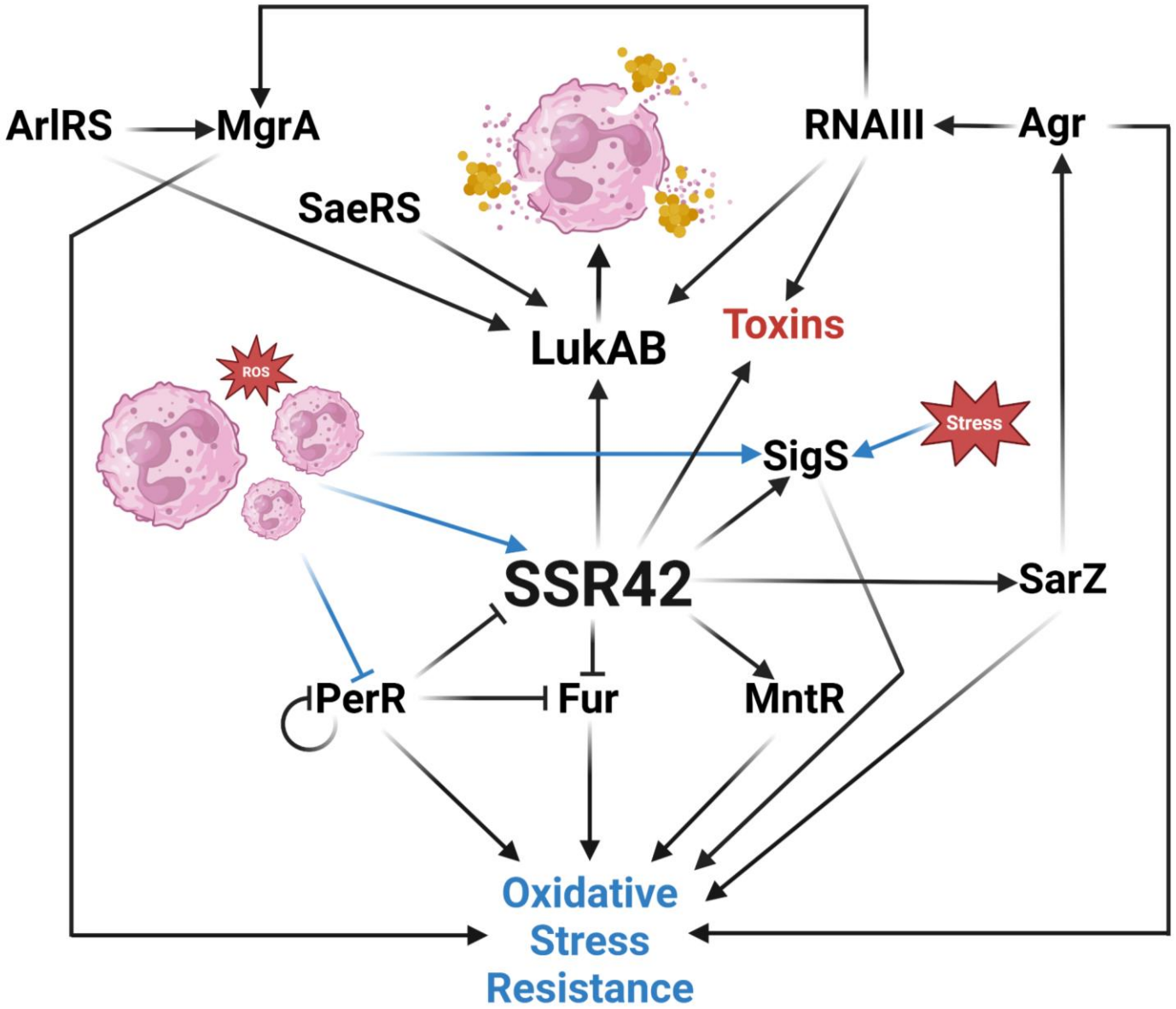


Figure 9. Proposed role of SSR42 in *S. aureus*. The presence of neutrophils and/or reactive oxygen species leads to PerR-mediated derepression of SSR42, which then binds and stabilizes *lukAB* mRNA in order to defend against phagocytosis and/or facilitate escape. The regulatory links between the metalloregulators, stress response, and oxidative stress resistance is represented alongside known regulation of the LukAB and virulence. Created with BioRender.com