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

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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

S. aureus is a master pathogen due to its formidable collection of virulence factors. These are tightly controlled by a diverse group of regulators that titrate their abundance to adapt to unique infectious niches. The role of regulatory RNAs in stress adaptation and pathogenesis is becoming increasingly more relevant in *S. aureus*. In this study, we provide the most comprehensive global analysis to date of just such a factor, SSR42. Specifically, we uncover

that SSR42 is required for mediating cytotoxicity - one of the pillars of infection - in response to phagocytosis by human neutrophils. We find that SSR42 is induced by components of the host immune system and facilitates downstream activation of cytotoxic factors via RNA-RNA interactions. This illustrates that SSR42 forms a pivotal link between sensing the external environment and mediating resistance to oxidative stress while promoting virulence, solidifying it as a major global regulator in *S. aureus.*

Introduction

Over the last few decades, more than 300 regulatory RNAs have been identified in *Staphylococcus aureus* strains(1–6). While progress has been made exploring these elements mechanistically, there is still a dearth of knowledge on the role of regulatory RNAs in complex regulatory systems, as compared to classical transcription factors. Commonly referred to as small RNAs (sRNAs), these factors range from *trans*-encoded- to antisense- transcripts, typically acting in a posttranscriptional manner to allow for rapid adaptation to environmental cues (6–13). The most well-studied example of an sRNA in *S. aureus* is RNAIII, which is the effector for the Agr system. It is known to modulate mRNA abundance by relieving inhibitory secondary structures at the Shine-Dalgarno sequence (e.g. *hla*), or by occluding this site to block access of the ribosome (e.g. *spa, rot, sbi*) ultimately promoting transcript degradation (14–17).

Many other virulence-related regulatory RNAs have also been documented in *S. aureus*, such as the Teg family of transcripts, which include Teg41 that impacts hemolytic ability through engagement with the PSMs, and Teg58 which monitors arginine dysbiosis and modulates biofilm production (18–20). The Spr-group of regulators form another family of regulatory RNAs, and includes SprC, which plays a role in controlling metabolism and immune evasion, SprY, which works to titrate RNAIII activity through direct ‘sponge’ interactions, and SprD, which works

69 in consort with RNAIII to repress *sbi* (21–24) . RsaC is a ~1,100-nt transcript that is expressed
70 during manganese starvation and enhances the oxidative stress response, leading to increased
71 survival during confrontation with the host immune system; thus forming a link between metal
72 homeostasis and pathogenesis (25). From a broader perspective, a recent global study utilizing
73 CLASH technology to uncover RNA-RNA interactions in host-like conditions identified hundreds
74 of binding events, occurring not only between regulatory RNAs and mRNAs, but between
75 regulatory RNAs themselves as well. Some of these interactions linked the metabolic status of
76 the cell to the expression of membrane-permeabilizing toxins, while furthermore uncovering an
77 RNA-sponge relationship between RsaE and RsaI (13)

78 The largest regulatory RNA in *S. aureus* is SSR42, which is 1,233 nt long, contains no functional
79 ORFs, and bears 98% sequence conservation across strains, with only two single nucleotide
80 variants found in UAMS-1 (26). It was first characterized by Morrison et al. from a larger group
81 of Small Stable RNAs (SSRs), where it was shown that the SSR42 transcript exhibits enhanced
82 stability during stationary phase growth, with a half-life of over 30 minutes, compared to a ~5
83 minutes in exponential phase. Using microarray analysis, 80 mRNA species were found to be
84 altered in an SSR42 mutant strain, with key virulence factors such as *hla, lukF, hlgC, aur,* and
85 *sraP* found to be differentially expressed. Phenotypically, an SSR42 mutant demonstrated an
86 abrogated ability to lyse human erythrocytes and impaired virulence in a murine model of skin
87 abscess formation (26). Others have shown that SSR42 is tightly controlled via the Repressor of
88 Surface Proteins (Rsp), and in turn may control expression of the two-component system
89 SaeRS, potentially explaining the ahemolytic nature of SSR42 mutant strains (27). Work by our
90 group identified that SSR42 is unique to *S. aureus* and does not exist in other Staphylococcal
91 species (5). We also demonstrate that expression of SSR42 is profoundly increased during the
92 growth of *S. aureus* in human serum (1).

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

101 **Methods**

102 (Additional methods can be found in the supplemental methods file.)

103 **Bacterial strains and growth conditions:** All bacterial strains and plasmids used for this study
104 are listed in **Table 1**. Overnight cultures were routinely grown at 37°C with shaking at 250 rpm in
105 5mL of tryptic soy broth (TSB) for *S. aureus* or lysogeny broth (LB) for *E. coli.* When required,
106 media was supplemented with the following antibiotics: for *E. coli*: 100µg/mL ampicillin; for *S.*
107 *aureus*: 10µg/mL chloramphenicol, 5µg/mL erythromycin, 25µg/mL lincomycin, and 5µg/mL
108 tetracycline. To obtain synchronous growth of *S. aureus,* overnight cultures were diluted 1:100
109 into fresh TSB and grown for 3h before being standardized to a uniform OD_{600} of 0.05 in fresh
110 TSB. Unless otherwise described, cell pellets were harvested via centrifugation at 4000 x *g* for
111 10 minutes.

112 **Toxicity towards neutrophils:** Cytotoxicity assays were performed as previously described
113 (28), with some variations. The immortalized HL-60 cell line was used as a model of human
114 neutrophil engagement. Cells were grown to confluence in RPMI supplemented with 10% FBS
115 and 100U Pen/Strep in 37°C and 5% CO_2. Upon reaching sufficient cell density, cells were
116 differentiated into neutrophil-like cells by the addition of 1.25% DMSO for 4 days; which was

117 confirmed via observation of cell morphology. For use, $1x10^5$ cells were seeded in 100µl RPMI
118 with 10% FBS into a 96-well plate. Bacterial cultures were grown in biological triplicate overnight
119 and standardized to 0.05 before being grown for 15h. Following incubation, cultures were
120 pelleted via centrifugation and supernatant was diluted two-fold in TSB. Seeded neutrophils
121 were intoxicated in technical duplicate with 5µl of diluted bacterial supernatant (5% total volume)
122 followed by incubation at 37°C, 5% CO_2 for 1h. Cell viability was measured using the CellTiter96
123 Aqueous One Solution Cell proliferation reagent (Promega). Following 1h incubation, 20µl of the
124 CellTiter reagent was added to each well, and the plate was returned to the incubator for 2h.
125 Color development was assessed via OD_{490} using a Biotek Cytation 5 plate reader. Data is
126 reported as percent death of supernatant-treated cells with respect to neutrophils treated with
127 5µl TSB only.

128 **Intracellular survival within neutrophils:** To assess intracellular survival within human
129 neutrophils, HL-60 cells were grown and differentiated as described above, and $2x10^6$ cells
130 were seeded into 500µl in a 24-well plate. Bacterial cultures were also grown and processed as
131 above, with the following modifications. After centrifugation, pelleted cells were washed 2X with
132 PBS, following which all strains were standardized to 10x the desired final MOI of 30
133 (OD_{600}=0.135 for *S. aureus*), thus all strains were standardized to an OD_{600}=13.5 in PBS. From
134 these standardized cultures, 5µl was added to each well of neutrophils and incubated for 1h at
135 37°C and 5% CO_2. Following incubation, cells were removed from wells and pelleted at 2.5 x *g*
136 for 1 minute before being resuspended in pre-warmed PBS. This PBS wash was repeated, and
137 the cells were finally resuspended in pre-warmed RPMI supplemented with 30µg/mL gentamicin
138 and returned to incubate for 24h. At this point in time the cells were removed and washed twice
139 with pre-warmed PBS once again, however the final resuspension was in 0.5% Triton X-100 to
140 lyse neutrophils. Lysates were serially diluted and plated to enumerate CFU of internalized
141 bacteria.

142 **Blood survival assays:** Blood survival assays were performed as described previously by our
143 group (29). Briefly, bacterial cultures were grown in biological triplicate overnight before being
144 diluted 1:100 and grown for a further 3h. These synchronized cultures were then used to
145 inoculate 1mL of human, gender-pooled whole blood (BioIVT) at a final OD_{600} of 0.05. Samples
146 were taken immediately after inoculation (T=0) as well as after 4h of rotation at 37°C. Samples
147 were serially diluted and plated onto TSA to calculate CFU/mL. Data is represented as percent
148 survival in comparison to T=0.

149 **Luminescent reporter assays with neutrophil exposure:** HL60 cells were differentiated as
150 described above before being seeded into black-walled, clear bottomed 96-well plates at a
151 concentration of $2x10^5$ in 100µl of phenol red-free RPMI. Bacterial strains were grown overnight
152 as previously described and washed 2x with PBS and standardized to a final OD_{600} of 1.0.
153 Following this, standardized bacterial cells were added to seeded HL-60 cells to reach a final
154 OD_{600} of 0.1. Luminescence and OD_{600} T=0 readings were taken immediately after inoculation
155 (T=0) on a Biotek Cytation 5 plate reader, with additional readings taken hourly, between which
156 plates were incubated at 37°C and 5%CO_2. Luminescence was normalized to OD_{600} for each
157 well.

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

166 **Electrophoretic mobility shift assays (EMSAs):** All RNA was generated via *in vitro*
167 transcription using a T7 RNA polymerase promoter and the MAXIscript T7 kit (ThermoFisher).
168 SSR42 was amplified using OL7499/OL7500 while fragments of the *lukA* UTR and *splE* UTR
169 were amplified using primers 7501/7502 and 7523/7524, respectively. These T7-controlled
170 fragments were cloned into pGEM-T Easy and liberated from the plasmid using restriction
171 enzymes NcoI and PstI (Thermo Fisher Scientific). *In vitro* transcription was performed using the
172 MAXIscript T7 kit, ^{32}P-labeled UTP for the mRNA, and unlabeled UTP for SSR42. TurboDNase
173 was added to degrade any remaining DNA. Reactions were cleaned using a Nucleotide Clean-
174 up Kit (Qiagen). RNA-RNA binding reactions were performed with labeled mRNA and SSR42 at
175 varying concentrations, which were heated to 90°C for 2 minutes and then incubated at RT for
176 30 min. Native acrylamide gels were equilibrated on ice for 30 minutes in 0.5x TBE before
177 loading binding reactions, which were mixed with RNA loading dye and then run at 100V until
178 the dye front has moved ¾ of the way through the gel. Gels were dried and affixed to filter paper
179 using a gel dryer before being visualized via autoradiography.

180 **Murine models:** All animal studies in this work were performed in accordance with and
181 approved by the Institutional Animal Care and Use Committee of the University of South Florida.
182 To prepare inocula for a model of bacterial sepsis, bacteria was washed 2x with PBS before
183 being adjusted to $5x10^8$ CFU/mL in PBS. Female, six-week-old CD-1 mice (Charles River
184 Laboratories) were inoculated with 100µl of bacteria, resulting in a final dose of $5x10^7$. Infection
185 was allowed to progress for 7 days, or until mice reach a premoribund state, at which point they
186 were euthanized. After the infection period, all mice were euthanized and the brain, heart, liver,
187 spleen, lungs, and kidneys were harvested. For murine models of pneumonia, inocula were
188 prepared in a similar manner, however the bacterial density was instead adjusted to $1x10^9$.
189 Female and male 6-week old C57BL/6 mice were intranasally inoculated with 100µl of bacteria,
190 resulting in a final dose of $1x10^8$. Infections were allowed to proceed for 24h, after which mice

191 were euthanized and lungs harvested. Organs for both models were homogenized in 2mL PBS
192 and serially diluted and plated for CFU/mL. All infection experiments were performed with 8
193 mice per strain and a Mann-Whitney test was used to measure statistical significance.

194 **Data Availability:** RNAseq data from this study is available under the GEO accession
195 #GSE237701. Proteomics data is available via proteomeXchange under the ID PXD043724.

196 ## Results

197 **SSR42 is global regulator of virulence factor expression**: Although previous studies exist
198 exploring SSR42, the only large-scale omics examination of this factor is a microarray
199 performed over a decade ago (26). Thus, to bring new light to the role of SSR42, we herein
200 performed RNA-sequencing on an SSR42 mutant in the USA300 background. Strikingly, in the
201 wild-type strain, SSR42 was the second most abundant transcript in the cell, second only to
202 RNAIII **(Supplemental figure 1).** Moreover, 20% of all RNA in stationary phases cells (absent
203 rRNA) belongs to SSR42, and 45% to SSR42 and RNAIII combined. Beyond this, we found a
204 total of 220 genes were significantly differentially expressed between wild-type and mutant (**Fig.
205 1A)**, greatly surpassing the 80 genes previously shown to be controlled by SSR42 via
206 microarray. These included those involved in metabolism, transport, signal transduction and, by
207 far the largest functional category (33 such factors), human disease. Regarding this latter group,
208 SSR42 primarily serves as an activator, with 83% of transcripts decreased in abundance in the
209 mutant strain **(Fig. 1B,C).**

210 Key virulence factors regulated by SSR42 included secreted toxins, such as alpha hemolysin
211 (*hla,* -3.44), gamma hemolysin (*hlgA* -5.39, *hlgB* -4.77), the Panton-Valentine leukocidin (*lukS* -
212 3.62, *lukF* -3.86), and the *lukAB* leukocidin (-5.49, -4.29). Secreted proteases were also
213 impacted, with all serine protease (*spl*) operon genes downregulated in the mutant (~-3-fold),
214 whilst aureolysin (*aur)* and the V8 protease (*sspA)* were both increased in abundance (+4.5-fold

215 and +2.81-fold, respectively). Moreover, SSR42 appears to activate immune evasion factors,
216 including igG-binding protein *sbi* (-5.36), extracellular fibrinogen-binding protein *efb* (-2.85), and
217 staphylokinase *sak* (-2.48). In the same vein, we identified a profound change in the secreted
218 extracellular matrix and plasma binding protein *emp* (-34.6). Other virulence factors repressed
219 by SSR42 include the serine-rich adhesin for platelets *sraP* (+4.12) and the Immunodominant
220 Antigen B *isaB* (+2.92).

221 We also uncovered alterations in known transcriptional regulators, the most striking being the
222 SarA-family proteins *sarZ* and *sarR*, which were downregulated 9.6-fold and 62.3-fold,
223 respectively, in the mutant. Interestingly, the Repressor of Surface Proteins (*rsp*), which is
224 divergently transcribed from SSR42 and has been shown to be a primary activator of its
225 expression (27), was found to be upregulated 4.31-fold upon SSR42 deletion. Finally, we
226 identified changes in expression of the metalloregulators *fur* (+3.14) and *mntR* (-2.13), as well
227 as the alternative sigma factor *sigS* (-2.75), in the mutant strain. A random subset of these fold
228 changes were explored and confirmed by qPCR analysis (**Supplemental Figure 2**).

229 We next used proteomic analysis to validate these changes, revealing strong correlation to our
230 RNAseq data **(Fig 1 A,B,C, and Table S1).** Notably, while leukocidins LukSF and LukAB exhibit
231 decreased protein abundance in the mutant that matches RNAseq trends, LukAB in particular
232 exhibits a profound decrease in fold-changes at the protein level (RNAseq – *lukA* = -5.51, *lukB* =
233 -4.3; Proteomics - LukA = -9.87, LukB = -7.83).

234 **SSR42 is a novel regulator of cytolytic activity:** Given that LukAB had such profound
235 changes in our omic studies we next set out to explore if these had an impact of leukocidin
236 activity. As LukAB is the primary effector for the lysis of human neutrophils (30), human
237 promyeloblast (HL-60) cells were differentiated into neutrophils before being treated with
238 bacterial supernatant from the wild type, mutant, and complemented strains. Strikingly, after a
239 1h incubation, supernatant from the SSR42 mutant elicited a 5-fold reduction in killing compared

240 to the wild-type and complement **(Fig. 2A).** To see how this translated into bacterial viability, we
241 next explored how deletion of SSR42 impacted *S. aureus* survival during exposure to whole
242 human blood. Here we noted the mutant exhibited a marked 7-fold increase in survival
243 compared to the wild type and complementing strains **(Fig. 2B).** Of note, *lukAB* mutants of *S.*
244 *aureus* have been shown to have increased intracellular survival within neutrophils (a key
245 component of whole blood), due to an inability to use the toxin to escape the phagosome (31).
246 To determine if this was the case for the SSR42 mutant, we next measured intracellular survival
247 of our strains within HL-60 cells. Here we determined that, after 24h incubation, the mutant
248 displayed a dramatic increase in survival in comparison to the wild type and complement within
249 this niche **(Fig. 2C).** As such, it appears that SSR42 is a major new regulator of LukAB activity
250 in *S. aureus*.

251 **SSR42 is upregulated upon exposure to neutrophils via PerR derepression:** It has
252 previously been shown that *lukAB* expression is induced following exposure of *S. aureus* to
253 neutrophils, and that this is mediated, indirectly, via the peroxide regulator PerR, which is known
254 to derepress its targets in the presence of reactive oxygen species (32–34). Given that SSR42
255 plays an intimate role in intracellular survival and cytotoxicity, we next set out to determine if
256 SSR42 was the missing link in this chain of regulation. To this end, we created a luciferase
257 transcriptional reporter for the SSR42 promoter and exposed it to differentiated neutrophil-like
258 HL-60 cells. In so doing, we determined that P_{SSR42} exhibited a >3-fold increase in activity after
259 just 1h in the presence of neutrophils **(Fig 3A)**. Next, to investigate if this regulation was
260 governed by PerR, we re-assessed SSR42 promoter activity in a *perR* mutant. This time we
261 determined that P_{SSR42} expression is significantly higher in the mutant strain when exposed to
262 neutrophils as compared to wild-type **(Fig 3B)**, which is consistent with PerR derepression.

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

265 engagement with neutrophils. As such, we created a P_{lukAB}-*lux* fusion and assessed its response
266 to neutrophils in the context of SSR42 and PerR. In so doing, we noted that upon addition of
267 neutrophils, there was a significant increase in *lukAB* expression in the wild-type strain that
268 mirrored that of P_{SSR42} **(Fig. 4A).** However, this response is strikingly abrogated in an SSR42
269 mutant, where the luciferase signal is 5-fold lower in comparison to the wild-type after 2h of
270 neutrophil exposure **(Fig. 4B).** To uncouple the effect of SSR42 from PerR, we next assessed
271 *lukAB* expression in a *perR* mutant as well as an SSR42/*perR* double mutant. This revealed
272 that, once again in a similar manner to SSR42, *lukAB* expression was higher in a *perR* mutant,
273 however, *lukAB* expression in the SSR42/*perR* mutant mirrors that of an SSR42 mutant **(Fig. 4**
274 **A-C).** This suggests that the response of *lukAB* to the presence of neutrophils is likely a result of
275 direct regulation by SSR42, which is in turn controlled by PerR. This is further proven upon
276 overexpression of PerR in an SSR42 mutant, where there is no significant difference in P_{lukAB}
277 activity **(Fig S3.)**.

278 **SSR42 regulates *lukAB* post-transcriptionally:** To determine if the changes in *lukAB*
279 expression are mediated by SSR42 binding to, and affecting stability of, the *lukAB* mRNA, we
280 fused a fragment of the *lukA* 5' UTR and a portion of its coding sequence in frame to GFP and
281 expressed it under the control of a constitutive promoter in *S. aureus.* We then quantified GFP
282 fluorescence in an SSR42 mutant vs. wild-type, revealing a significant decrease in the stability
283 of the translated LukA-GFP product in the mutant strain **(Fig 5A).** Next, we used a two-plasmid
284 system in which we expressed SSR42 under its native promoter alongside the LukA-GFP
285 reporter in the SSR42 mutant strain. Here we observed a striking increase in LukA-GFP
286 translation in the presence of SSR42 when compared to empty vector controls **(Fig. 5B).** Taken
287 together, this suggests that SSR42 putatively binds post-transcriptionally to the *lukAB* mRNA to
288 regulate its stability and thus translation.

289 **The 3' end of SSR42 is required for interaction with *lukAB*:** To explore interaction between
290 SSR42 and the *lukAB* mRNA further, we first used IntaRNA to predict possible base pairing
291 interactions between the two RNA molecules (35). This predicted interaction with the 3' end of
292 SSR42, ~380bp from the end of the transcript, and the 5' UTR of *lukAB*, ending 7bp upstream of
293 the ribosome binding site (RBS) **(Fig. 6A)**. To investigate the relevance of this interaction *in*
294 *vivo,* we generated a series of truncated SSR42 variants and introduced these into the SSR42
295 mutant, before reassessing cytotoxicity. In so doing we noted that a 648-nt SSR42 fragment
296 (from the +1 onwards) was unable to restore wild-type levels of cytotoxicity, while a longer 793-
297 nt transcript partially restored toxicity. **(Fig 6B).** However, upon complementation with a 940-nt
298 SSR42 transcript that contains the region identified by IntaRNA, wild-type cytotoxicity was
299 observed. This suggests that the far 3' end of SSR42 is primarily responsible for mediating
300 interaction with *lukAB*, and thus governing cytotoxicity.

301 **SSR42 directly binds to the *lukAB* mRNA and enhances its stability:** To explore further the
302 direct interaction between SSR42 and the *lukAB* mRNA, electrophoretic mobility shift assays
303 (EMSAs) were performed. A portion of the *lukA* UTR that included the region identified in
304 IntaRNA was radiolabeled using *in vitro* transcription and incubated with increasing
305 concentrations of *in vitro* transcribed SSR42. When these samples were analyzed, we observed
306 a concentration-dependent interaction between the two RNA molecules (**Fig. 7A).** By
307 comparison, no binding was seen with radiolabeled *splE* mRNA under identical conditions,
308 supporting the specificity of the SSR42-*lukAB* interaction. We next set out to investigate whether
309 the observed binding had any impact on transcript stability. To that end, transcriptional arrest
310 studies were performed on the wild type, mutant, and complemented strains grown for 15h.
311 Samples were taken at t=0 before the addition of Rifampin, and then again at 5, 10, 30, and 45
312 minutes posttreatment. RNA abundance was then quantified via qPCR, revealing that the *lukA*
313 transcript degraded far more quickly, and to a greater overall degree, in the mutant strain when

314 compared to the wild type and complement **(Fig 7B).** When the mRNA half-life was calculated
315 using a one phase decay curve, we found that the *lukAB* mRNA $t_{1/2}$ was 2.9 minutes in the
316 SSR42 mutant, but 4.5 minutes in the wild type. This suggests SSR42 is required for
317 maintaining stability of the *lukAB* mRNA and facilitating translation of its protein products.

318 **SSR42 is required to cause invasive *S. aureus* infections*:*** Previous work has shown that
319 SSR42 is important for localized infections using a murine model of abscess formation (26). To
320 assess the role SSR42 plays in invasive disease we assessed the pathogenic capacity of the
321 mutant in a murine model of septic infection. Mice were infected with the SSR42 mutant, or wild-
322 type strain and the bacterial burden was determined across multiple organ systems after 7 days.
323 Here we found that the SSR42 mutant exhibited an abrogated ability to disseminate to the liver,
324 heart, and spleen **(Fig. 8A).** Specifically, in the heart, ΔSSR42 exhibits a 4-log decrease in
325 bacterial burden compared to WT, with 1-2-log decreases found in the liver and spleen. To
326 explore this virulence defect more fully, we next used an acute model of murine pneumonia,
327 intranasally inoculating mice with the wild type and SSR42 mutant strains. After 24h of infection
328 we assessed bacterial recovery from the lungs and once again found a major defect in the
329 mutant's ability to survive, with the ΔSSR42 strain exhibiting a 3-log decrease in CFU/mL in
330 comparison to the wild type **(Fig. 8B)**. These findings, alongside those from others demonstrate
331 that SSR42 is required for *S. aureus* infection in three different animal models, representing
332 both localized and invasive forms of disease.

333 Discussion

334 Recent years have seen an abundance of new discoveries regarding the complex nature of
335 RNA-based regulation, revealing that many of these elements exhibit pleiotropic behavior and
336 nuanced roles in pathogenesis. Herein, we explore just such a factor, broadening the known
337 regulome of the long noncoding RNA SSR42 from *S. aureus*. Using both transcriptomic and

338 proteomic approaches, with high sensitivity technology, we expand the SSR42 network of
339 control almost three-fold from previous studies.

340 Unraveling the SSR42 regulome reveals a far more complex network than was previously
341 ascertained. Of note, we identified no significant changes in any component relating to the
342 SaeRS TCS, which previous studies suggested was responsible for SSR42-related regulation of
343 at least some virulence factors (27). Instead, we note the strong downregulation of two SarA-
344 homologs, *sarZ* and *sarR*, at -9.6-fold and -62.6-fold, respectively, in our SSR42 mutant. Both
345 transcription factors have been characterized to play a role in promoting virulence factor
346 expression in various *S. aureus* strains (36–39). Specifically, SarZ upregulates the expression of
347 *aur, sspAB,* and *hla* while SarR activates *sspAB* and the *spl* proteases. Upon assessment of our
348 own transcriptomics, the regulation of the proteases *aur* (via SarZ) and *sspAB* (via SarR) are
349 consistent with the trends seen in our data, however the changes in *hla* (via SarZ) and *spl* (via
350 SarR) expression are not. It is intriguing that these transcripts are so strikingly decreased in
351 abundance, suggesting that SSR42 plays a strong role in controlling their expression or stability.
352 Indeed, bioinformatic searches for RNA-RNA binding do predict very strong interactions
353 between SSR42 and the mRNAs of both transcription factors **(Fig. S4)**. Thus, it is possible that
354 changes in *sarR* and *sarZ* expression may be causative for some, but not all, of the alterations
355 in virulence factor transcription seen in the absence of SSR42.

356 It is known that the Repressor of Surface Proteins, Rsp, is absolutely required for SSR42
357 expression (27). Both are transcribed divergently from each other, sharing a putative promoter
358 region, and yet in an SSR42 mutant *rsp* transcript levels are increased ~4-fold. This suggests
359 some element of negative feedback by which SSR42 effectively represses its own expression
360 during stationary phase by repressing *rsp*. It is well-established that SSR42 exhibits profound
361 transcript stability during stationary phase (>30min half-life) (26), thus this auto-repression via
362 *rsp* could be a method to titrate SSR42 abundance in the cell when it is not necessary to

363 produce new transcript. More importantly, however, is the fact that *rsp* has previously been
364 shown to be induced in the presence of hydrogen peroxide and is required for full cytotoxicity in
365 an Agr-independent manner (40). We posit that these phenotypes are at least partially SSR42-
366 mediated. To explore this, we assessed expression from the *rsp* promoter in the presence of
367 neutrophils and found that it too is induced by their presence **(Fig S5)**. Importantly, however, P_{rsp}
368 is not derepressed by PerR in this context, suggesting that the PerR-mediated control of *lukAB*
369 by SSR42 is independent of Rsp. This demonstrates that, depending on the environment, Rsp
370 is necessary, but not sufficient, for SSR42 expression.

371 When assessing impact on LukAB production, we show that SSR42 binding to the *lukAB* mRNA
372 affects its stability, producing a concomitant impact on protein production. The latter effect, we
373 posit, is reflective of this interaction altering the secondary structure of the *lukAB* mRNA,
374 perhaps making it more favorable for the ribosome to access and initiate translation. Indeed, a
375 very similar mechanism is seen for the well-studied interaction between RNAIII and the *hla*
376 mRNA, where there is binding directly upstream of the RBS for this same purpose(17).
377 Interestingly, in addition to the impact of SSR42 on *lukAB* mRNA stability and/or translational,
378 we also observe alterations in P_{lukAB} promoter activity. This, at first glance, is contradictory to
379 typical sRNA behavior, which usually only manifest post-transcriptionally. However, the P_{lukAB}
380 fragment used in our luciferase studies also contains the 5'UTR region of the transcript,
381 including the SSR42 binding site. Thus, as the absence of SSR42 is proven to destabilize the
382 *lukAB* mRNA, the promoter fusion transcript will inevitably be destabilized in our luciferase
383 assays as well.

384 That said, it is a cornerstone of regulatory RNA behavior to exert multiple methods of regulation
385 on a single target. This is exemplified by RNAIII, which binds the hla mRNA and opens its
386 secondary structure near the SD sequence to allow translation to initiate, while also binding and
387 inhibiting translation of *rot* to prevent transcriptional inhibition of *hla* (16, 17). Exerting diverse

388 modes of control over mRNA targets can ensure a robust regulatory response, which we readily
389 see in the case of SSR42 and LukAB. At the transcript level, we identified a ~5-fold decrease in
390 the lukAB mRNA, which likely reflects mRNA instability in the absence of SSR42; yet we see a
391 nearly 10-fold decrease in abundance of both LukAB components in secretomes, indicating how
392 lost translation can have an amplified effect. However, LukAB is somewhat unique in that it is
393 not only secreted but also stored within the cell envelope, where it can exert proximity-based
394 toxicity when in confrontation with host immune cells (41, 42). In our own proteomic analysis,
395 the cell-associated fraction has a striking -27-fold decrease in protein abundance for LukA and a
396 -6.6-fold decrease for LukB in an SSR42 mutant. This suggests that SSR42 influences not only
397 the canonically secreted variant of this toxin, but also the sorted and stored, yet active, form of
398 LukAB. Thus, in an SSR42 mutant, this weapon is markedly decreased in availability, and thus it
399 follows that the SSR42 mutant is unable to escape the phagocytic niche.

400 Recent work by Savin et al. identified the peroxide sensor PerR as a regulator of LukAB activity
401 that responds to the presence of hPMNs and leads to enhanced leukocidin expression (34).
402 Their study suggested no direct regulation between PerR and *lukAB* as there are no PerR
403 binding boxes detected in the leukocidin promoter. In our study, we observed that the SSR42
404 promoter is also induced in the presence of human neutrophils and, importantly, is required for
405 the *lukAB* promoter to respond under these conditions. Investigating P_{SSR42} architecture
406 revealed that there are 3 putative PerR binding boxes (sequence identified by Horsburgh et. al
407 (33)) upstream and/or within the SSR42 transcript **(Fig S6)**. It has previously been shown that
408 known PerR targets such as *katA, ahpC,* and *ftnA* can possess multiple binding boxes within
409 their promoters and are de-repressed during oxidative stress (32–34). When we mutated each
410 of these binding sequences individually, we identified no changes in promoter induction (data
411 not shown), however, we were unable to combinatorially mutate more than one box within the

412 promoter sequence at the same time. It is possible that profound derepression in the absence of
413 these PerR binding sites results in toxic over production of SSR42.

414 Importantly, the SSR42 promoter has previously been shown to be induced in response to the
415 presence of hydrogen peroxide (40). Thus, we propose that SSR42 is the missing link between
416 PerR-mediated ROS sensing and subsequent activation of LukAB activity to mediate survival
417 and escape from phagocytic cells. Indeed, there is precedence for regulatory RNA involvement
418 in oxidative stress resistance across bacterial species. First, in *E. coli,* the PerR homolog OxyR
419 derepresses the pleiotropic sRNA regulator OxyS in response to hydrogen peroxide (43–45). In
420 *Pseudomonas stutzeri*, the sRNA NfiS regulates catalase production and interacts with OxyR in
421 response to H_2O_2-induced conditions (46). Finally, in *S. aureus,* RsaC is an sRNA that inhibits
422 the translation of *sodA and* promotes oxidative stress resistance through SodM activity (25).
423 Evidence for PerR-mediated regulation of SSR42 also arises from a recent study by our group.
424 Here, upon assessing transcriptomic changes in response to treatment with Calprotectin (i.e.
425 metal starved), SSR42 was found to be downregulated 2-fold. When samples were treated with
426 a mutated form of Calprotectin that only sequesters Zn and Cu, SSR42 was upregulated >3-fold
427 (47). In *S. aureus,* PerR requires Fe and Mn for oxidation in response to endogenous H_2O_2 (32);
428 thus, it follows that derepression of SSR42 would be detected only in conditions including these
429 metals.

430 Metal homeostasis and the PerR-mediated oxidative stress response are intimately linked
431 through a network of metalloregulators: Fur, Zur, and MntR (33, 48–51). Interestingly, the *fur*
432 transcript is upregulated 3.14-fold in our SSR42 mutant while *mntR* expression is decreased -2-
433 fold. It is important to note that in addition to regulating SSR42, PerR plays a role in regulating
434 Fur and MntR expression, while all three players act in a coordinated manner to induce
435 overlapping regulons contributing to oxidative stress resistance, including factors such as
436 catalase (KatA*)*, thioredoxin reductase (TrxB), and alkylhydroperoxide reductase (AhpCF) (48,

437 51). While these elements were not altered in the SSR42 mutant transcriptome, we did uncover
438 three alternative antioxidant-like factors that were decreased in expression in the SSR42
439 mutant. These include a nitroreductase linked to iron acquisition (*ntrA,* -3.41-fold), a
440 nitroreductase with glutathione antioxidant behavior (SAUSA300_0790, -2.26-fold), and a
441 thioredoxin-like protein (SAUSA300_0795, -2.27-fold) (52–54). Thus, we posit that SSR42
442 weaves a common thread connecting these metallo-oxidative regulators and subsequently
443 impacts virulence factor abundance in response to ROS-generating niches. Key examples of
444 this behavior in *S. aureus* have been shown in the case of IsrR and RsaC, two sRNAs that are
445 expressed in low iron or manganese environments, respectively, and function to enhance the
446 oxidative stress response and pathogenesis upon activation (25, 55–57).

447 Another regulator, the alternative sigma factor *sigS*, was downregulated -2.75-fold in the SSR42
448 mutant. σ^S has previously been shown by our group to play a role in stress adaptation, immune
449 evasion, and pathogenesis, and has recently been shown to have a regulon that includes
450 various noncoding RNAs (58, 59). Although SSR42 was not found to be differentially expressed
451 in a *sigS* mutant, our findings suggest that SSR42 is instead responsible for full σ^S abundance.
452 A similar phenomenon is observed with the *E. coli* sRNA OxyS, which senses hydrogen
453 peroxide and in turn activates expression of the alternative sigma factor, RpoS (44). Moreover,
454 we found that *prli42*, which encodes a membrane protein that is required for the formation of the
455 σ^B controlling stressosome in *Listeria monocytogenes*, but has not yet received characterization
456 in *S. aureus,* was decreased in expression ~ 3-fold in the SSR42 mutant (60–62). In addition to
457 our data showing H_2O_2/neutrophil-mediated SSR42 induction and subsequent stimulation of
458 LukAB activity, these findings demonstrate that SSR42 may also play a role in activating
459 components of other stress-responsive systems. Taken together, these findings expand the role
460 of SSR42 beyond a direct virulence-related pathway and illustrates a much more complex role
461 for this sRNA as it pertains to sensing oxidative stress, nutritional immunity, and other

462 exogenous signals. A proposed mechanism of SSR42's involvement in the regulation of these
463 processes is summarized in **Fig 9.**

464 To our knowledge, this is the first direct RNA-based regulation of *lukAB* characterized to date in
465 *S. aureus*. Not only do we uncover a novel regulator or leukocidin activity, but we begin to
466 unravel the labyrinthine network that controls the response to changing environments,
467 particularly within the host. Indeed, past work on SSR42 demonstrated its role in murine
468 abscess formation, while our own study broadens this to include complex and disseminated
469 infections including sepsis and pneumonia. Thus, we present SSR42 as a pleiotropic regulatory
470 RNA that responds to pathophysiologically relevant conditions to bolster virulence factor
471 abundance and facilitate pathogenesis in *S. aureus.*

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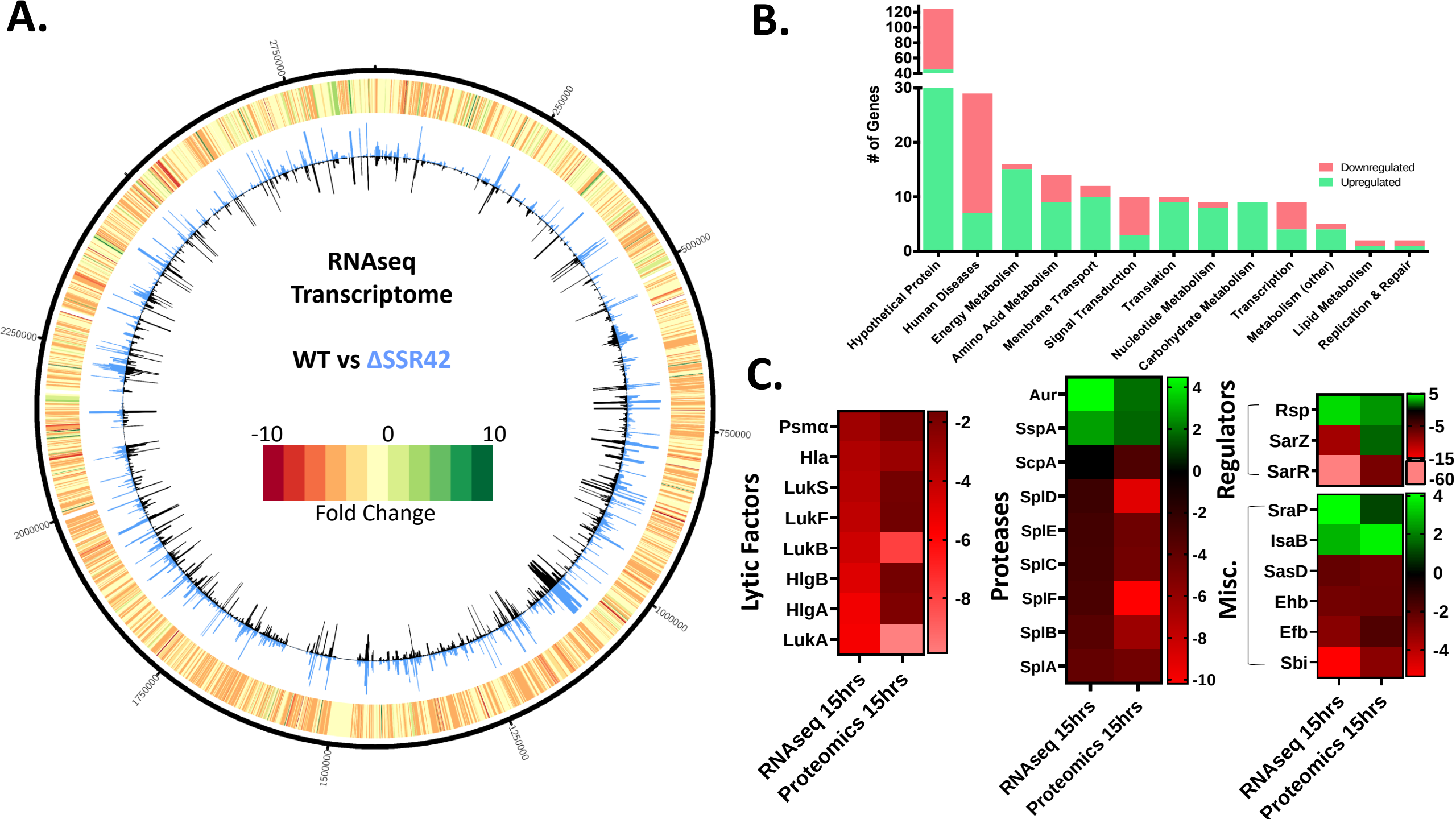
A.
RNAseq
Transcriptome
WT vs ΔSSR42
-10
0
10
Fold Change
2750000
250000
500000
750000
1000000
1250000
1500000
1750000
2000000
2250000
B.
# of Genes
Downregulated
Upregulated
Hypothetical Protein
Human Diseases
Energy Metabolism
Amino Acid Metabolism
Membrane Transport
Signal Transduction
Translation
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.

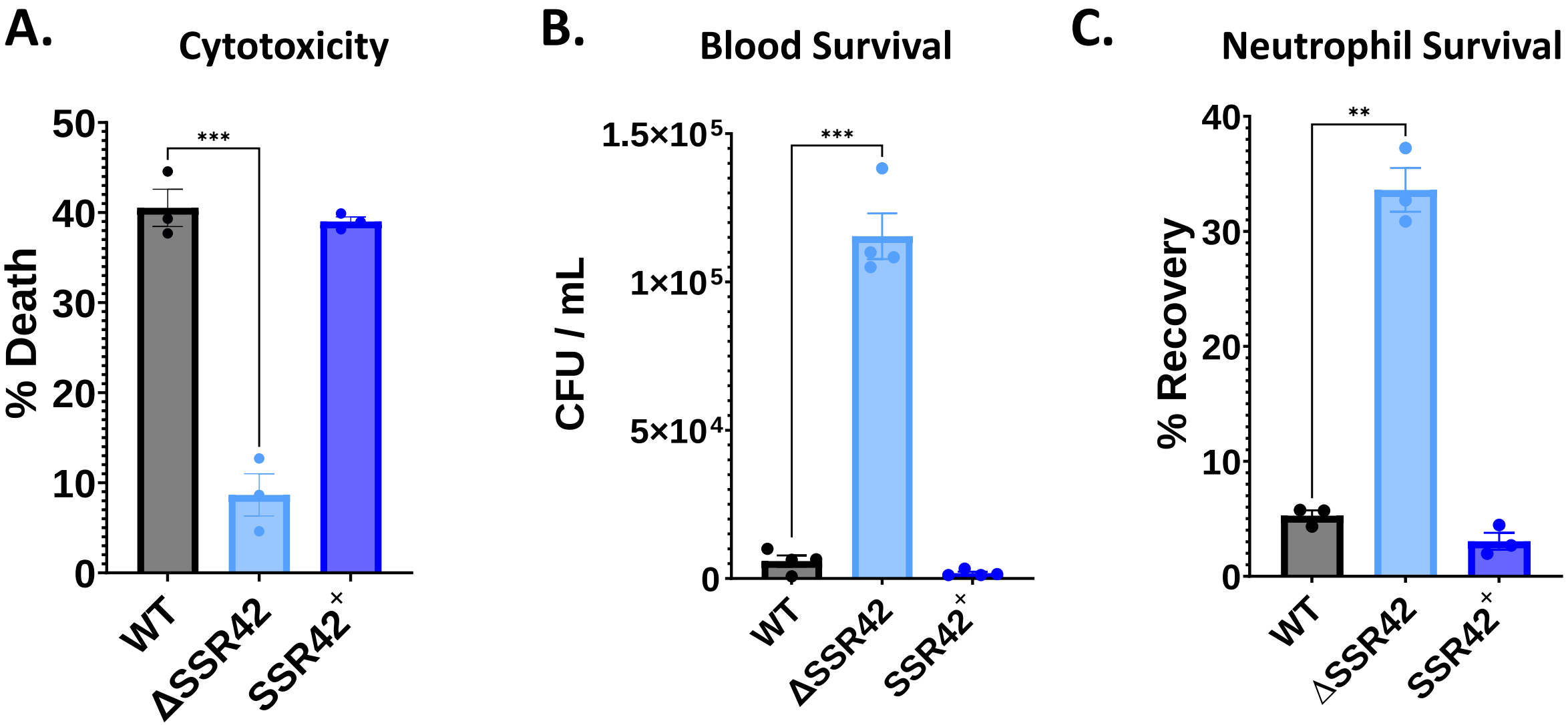

Figure 2. SSR42 plays a central role during engagement with the innate immune system. (A) HL-60 cells were differentiated into neutrophil-like cells with 1.25% DMSO for 4 days before being treated with 5% v/v bacterial supernatant from 15 h cultures of the strains shown. Intoxicated cells were incubated for 1 h before cell viability was measured using the CellTiter reagent. Percent cell death 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)** Human neutrophil-like cells were incubated with the strains shown at an MOI of 30, and intracellular bacterial survival was 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. ** $P \leq 0.01$, ,***, $P \leq 0.001$.

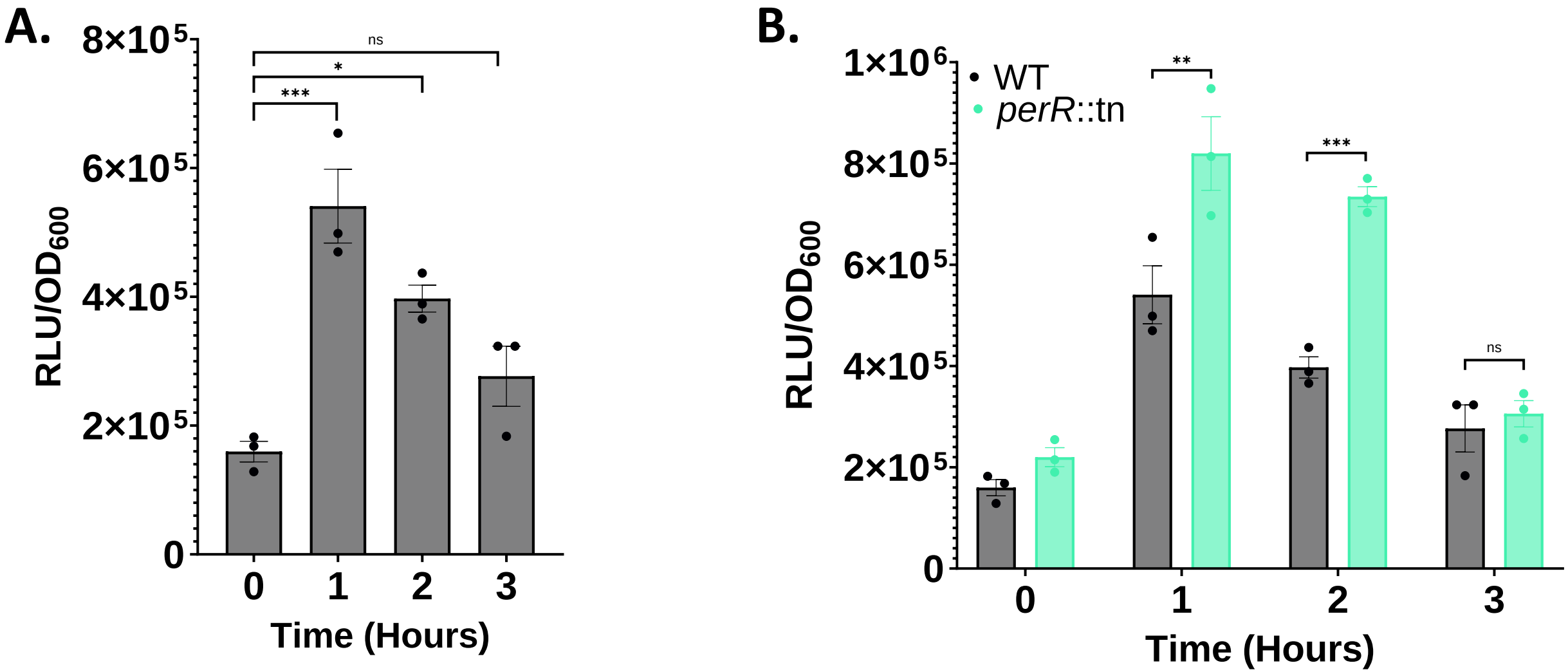

Figure 3. SSR42 is upregulated in response to neutrophils via PerR Derepression. (A) Differentiated HL60 cells were seeded at a concentration of $2x10^5$ before the *S. aureus* WT harboring a P_{SSR42}-lux reporter fusion was added at a final OD_{600} of 0.1. Luminescence and OD_{600} reads were taken 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. * $P \leq 0.05$ ** $P \leq 0.01$, ,***, $P \leq 0.001$.

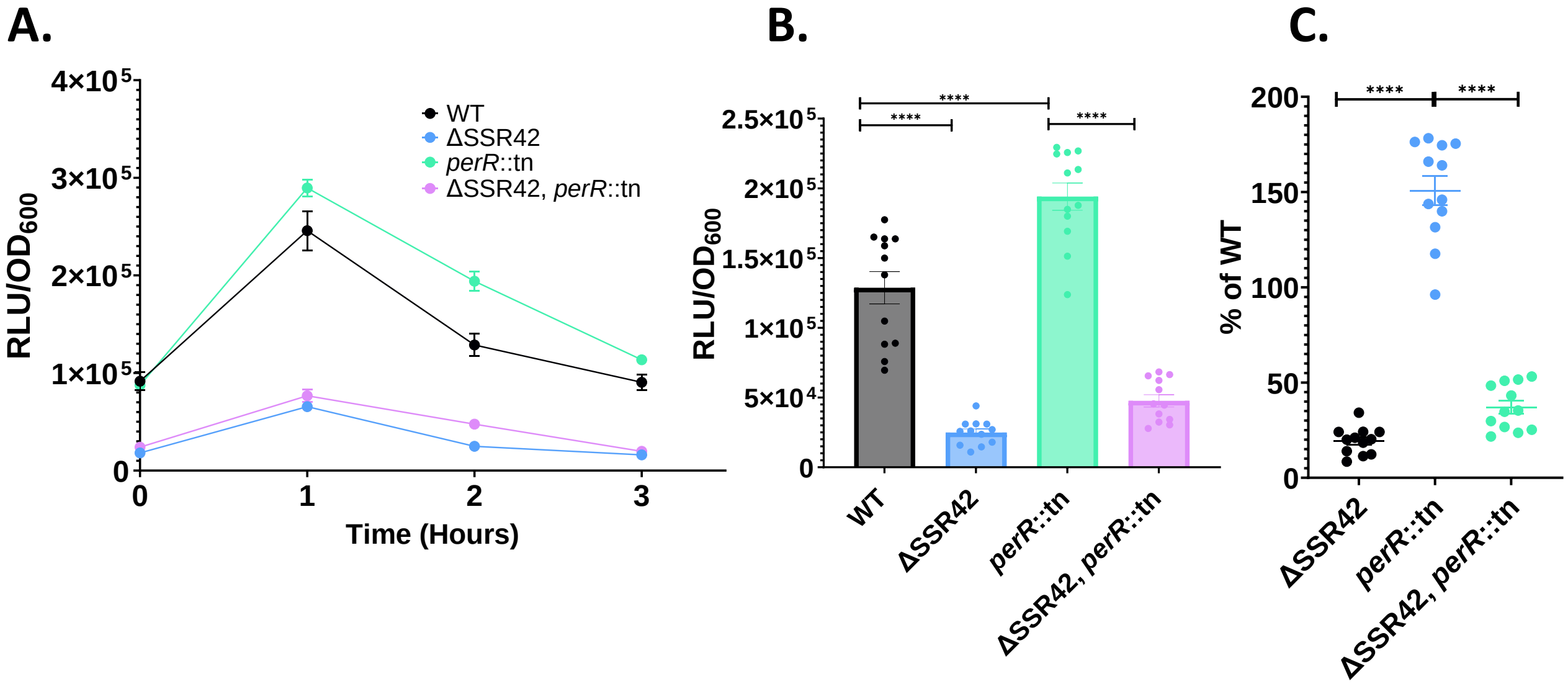

Figure 4. SSR42 controls LukAB expression in response to neutrophils. (A) Differentiated HL60 cells were seeded at a concentration of $2x10^5$ before the *S. aureus* WT harboring a P_{lukAB}-lux reporter fusion was added at a final OD_{600} of 0.1. Luminescence and OD_{600} reads were taken at T=0 and then hourly for 3 hours. Luminescence was normalized to OD_{600}. Data is from biological triplicate samples. **(B)** As in A but 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 student's T-test with Welch's correction was used to determine significance. Error bars are ±SEM. ****, P<0.0001

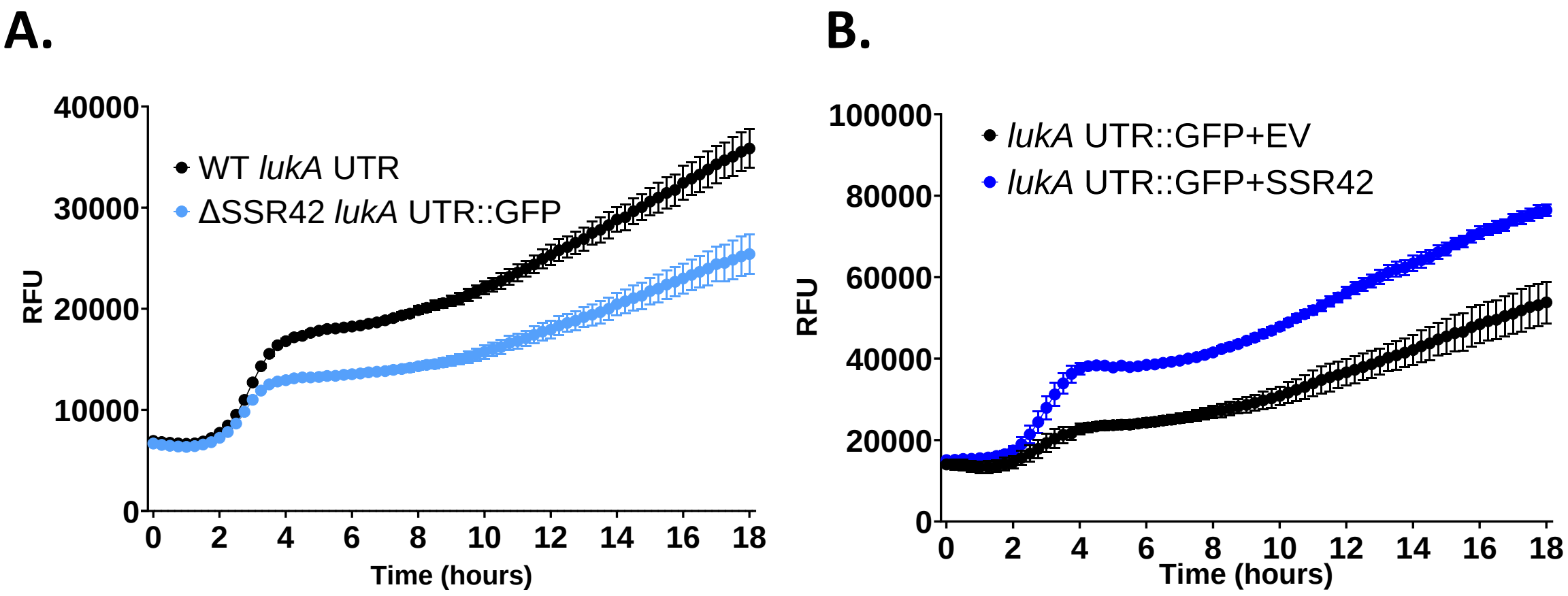

Figure 5. SSR42 post-transcriptionally controls LukAB abundance. (A) A portion of the *lukA* 5' UTR and coding sequence was translationally fused to GFP and was introduced into the wild-type or ΔSSR42 strains **(B)** The *lukA-GFP* reporter fusion plasmid was co-expressed with either an empty vector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

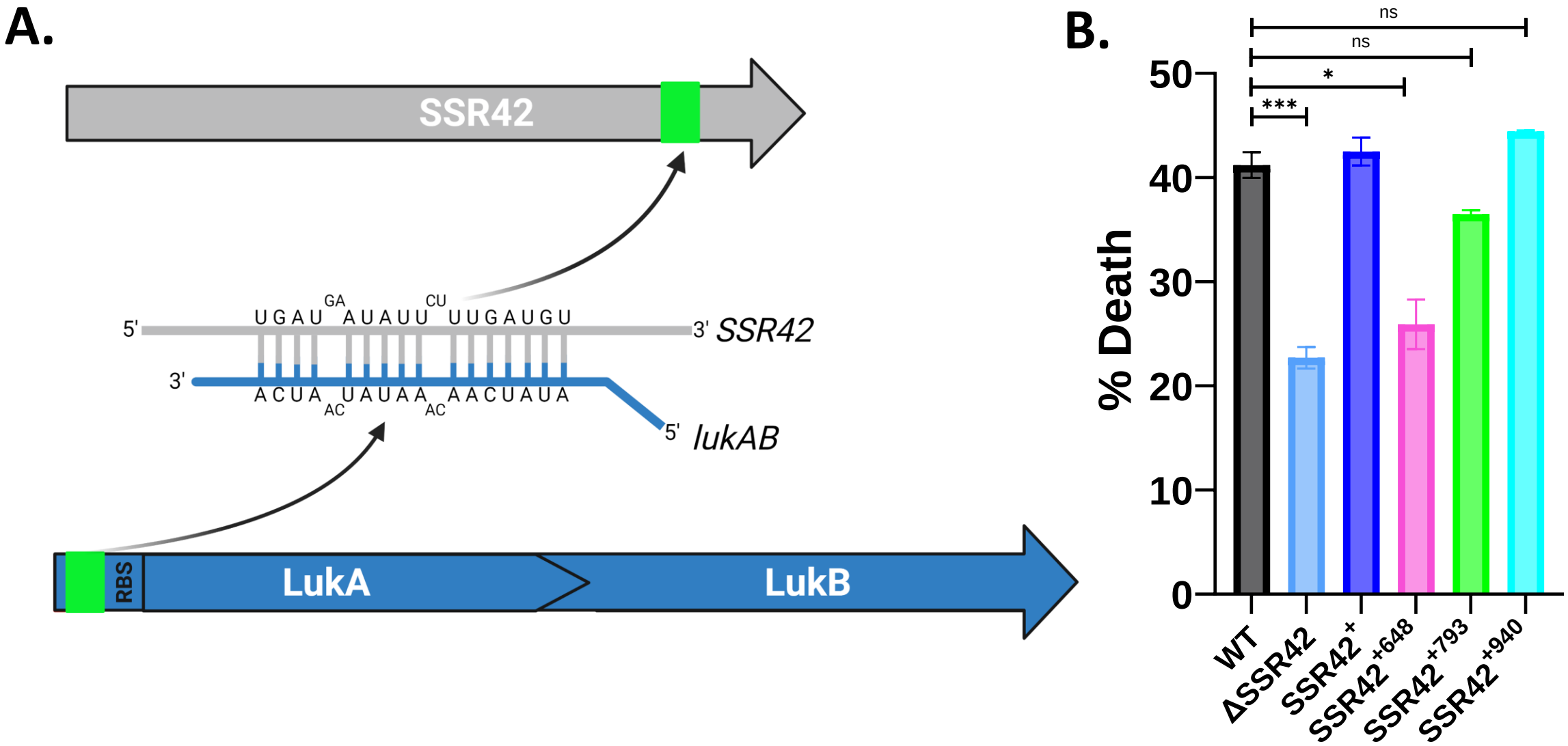

Figure 6. The 3' end of SSR42 mediates binding to the *lukAB 5'* UTR. (A) IntaRNA was used to identify thermodynamically likely regions of base paring between SSR42 and the *lukAB* mRNA, identifying an interaction region between the 3' end of SSR42 and the 5' UTR of *lukAB*.. Created using Biorender.com. **(B)** As in 2A but using the strains noted. Error bars are ±SEM. Student's t test with Welch's correction was used to determine statistical significance relative to wildtype. Experiments performed in biological triplicate. * P≤0.05, ,***, P≤0.001.

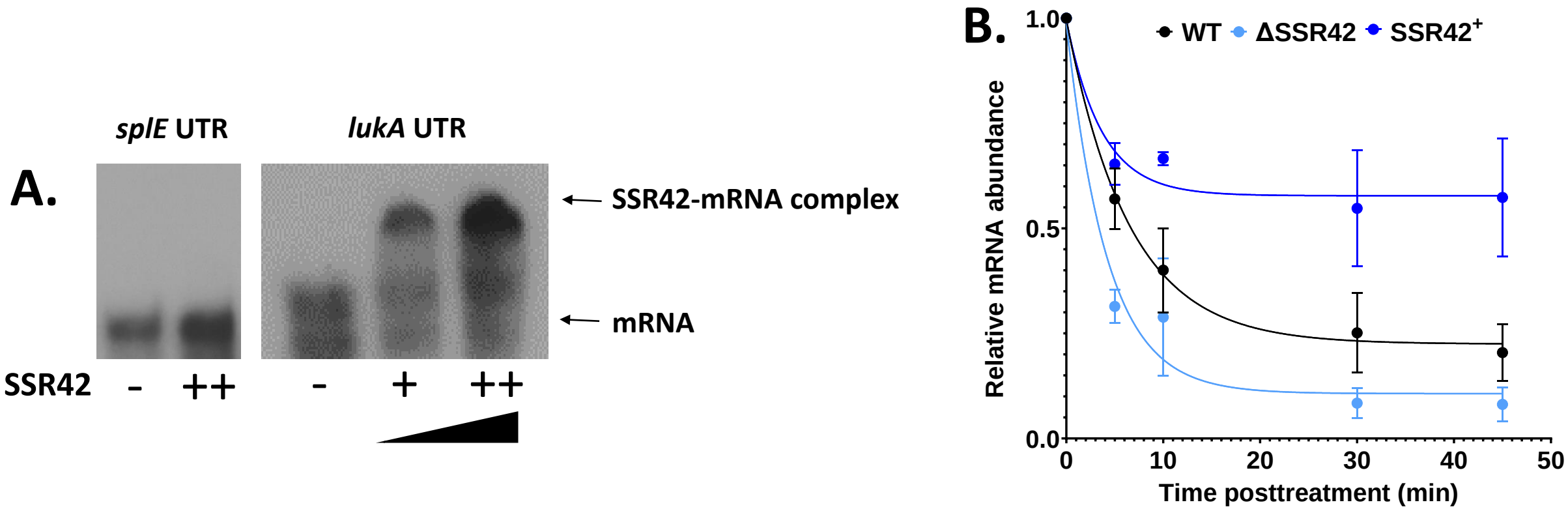

Figure 7. SSR42 directly binds and stabilizes the *lukAB mRNA*. (A) *In vitro* transcribed SSR42 was added in increasing amounts (1,000-fold to 1,500-fold excess) to radiolabeled portions of the *lukAB* mRNA. Samples were heated to 90° C for 2 min and then incubated at room temperature for 30 min before 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.

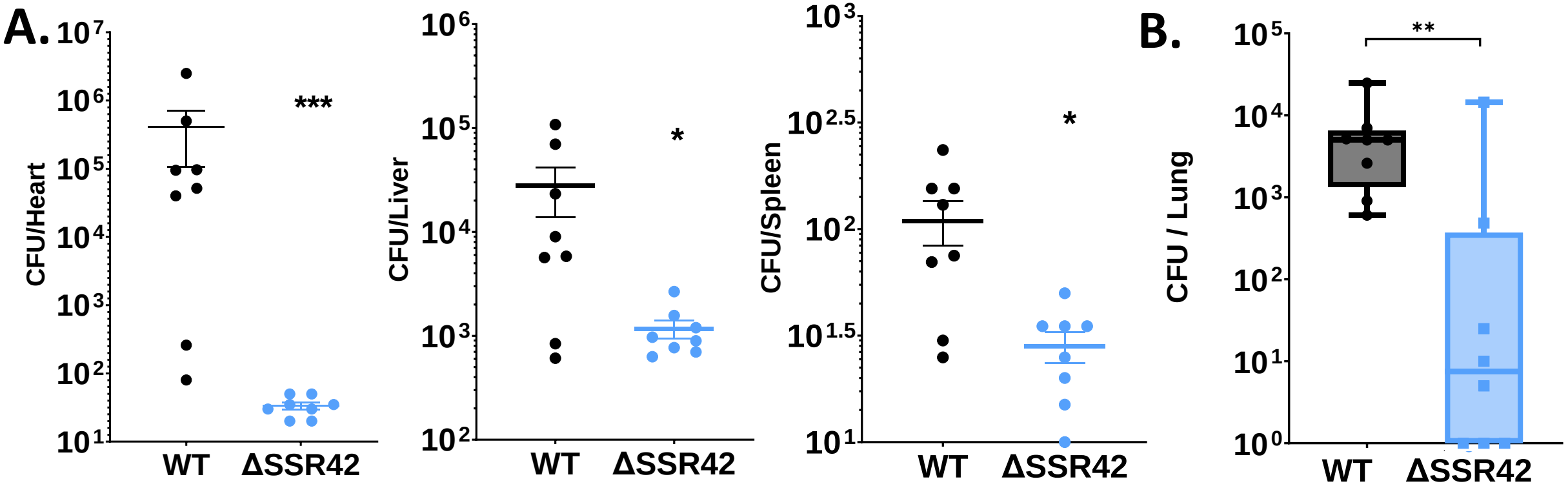

Figure 8. SSR42 is required for full virulence in murine models of invasive disease. (A) CD-1 mice were inoculated with the wild-type or SSR42 mutant at 5 x 10^8 CFU via tail vein injection. Following 7 days of infection, organs were harvested and CFU determined. **(B)** C57BL/6J mice were intranasally inoculated with the wild-type or SSR42 mutant 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.

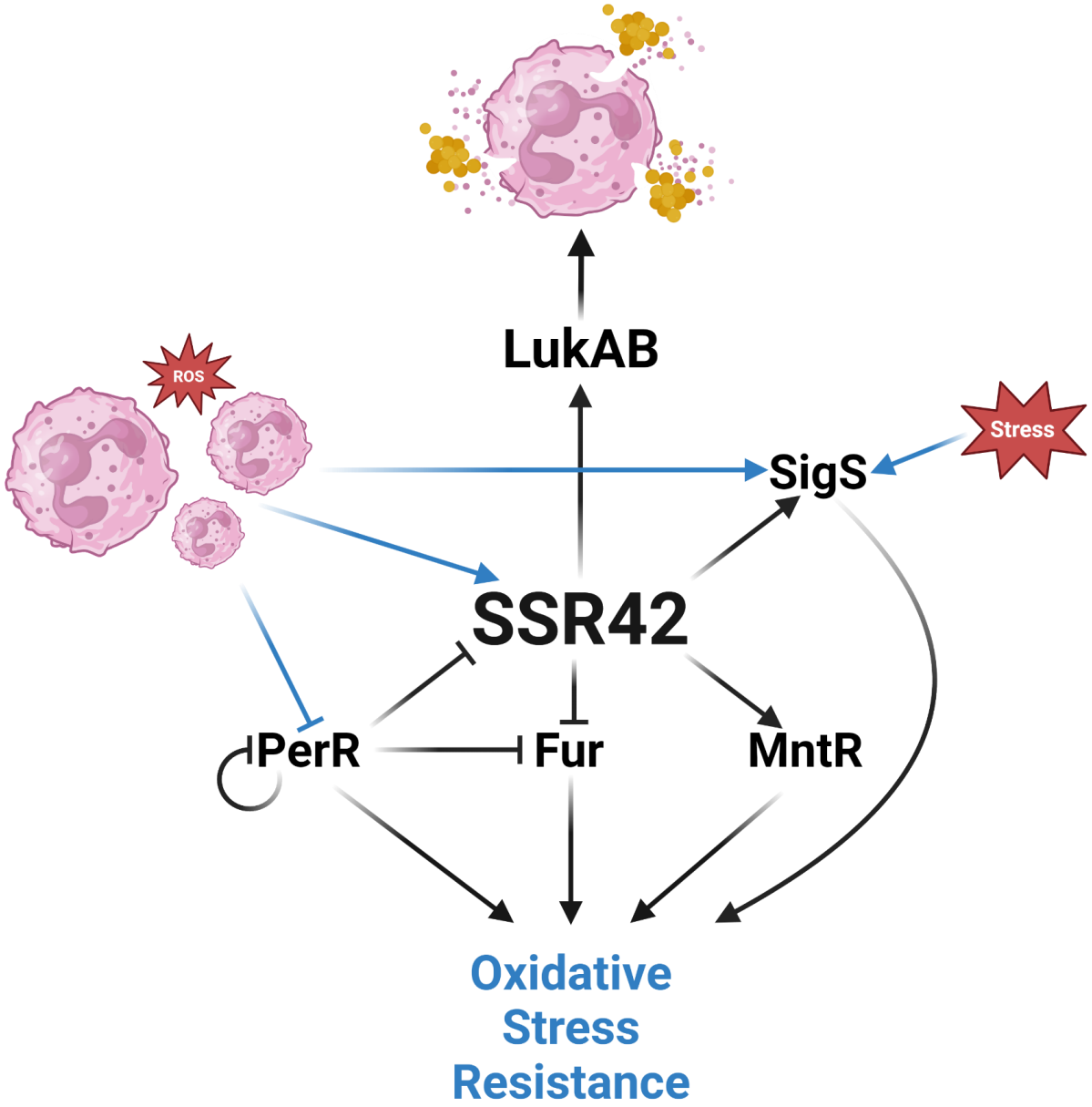

Figure 9. Proposed role of SSR42 in *S. aureus*. The presence of neutrophils and/or reactive oxygen species leads to PerR-mediated derepression of SSR42, which then binds and stabilizes *lukAB* mRNA in order to defend against phagocytosis and/or facilitate escape. The regulatory links between the metalloregulators,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

Table 1. Bacterial strains and plasmids used in this study.

Strain Name	Description	Source
E. coli		
DH5α	Cloning strain	(1)
JM109	Cloning strain	Promega pGEM-T Easy Vector Kit
MEJ3834	JM109 pGEM-T::*lukA* UTR	This study
MEJ3845	JM109 pGEM-T::SSR42	This study
S. aureus		
RN4220	Cloning strain, restriction-deficient	Lab stock
TCH1516	USA300 HOU CA-MRSA Erm^s, Wildtype parent strain	(2)
BRT2544	TCH1516 ΔSSR42	This study
NE665	JE2 *perR*::tn	(3)
MEJ4002	TCH1516 *perR*::tn	This study
MEJ4003	TCH1516 ΔSSR42, *perR*::tn	This study
LNS1927	TCH1516 pMK4 EV	This study
BRT2545	TCH1516 ΔSSR42 pMK4 EV	This study
BRT2546	TCH1516 ΔSSR42 pMK4::SSR42	This study
MEJ4000	TCH1516 pXen-1::P_{SSR42}	This study
MEJ4001	TCH1516 *perR*::tn, pXen-1::P_{SSR42}	This study
MEJ3546	TCH1516 pXen-1::P_{lukAB}	This study
MEJ3550	TCH1516 ΔSSR42 pXen-1::P_{lukAB}	This study
MEJ3826	TCH1516 *perR*::tn, pXen-1::P_{lukAB}	This study
MEJ3827	TCH1516 ΔSSR42, *perR*::tn, pXen-1::P_{lukAB}	This study
MEJ3828	TCH1516 ΔSSR42, *perR*::tn, pXen-1::P_{lukAB}, pJB67::PerR	This study
MEJ3831	TCH1516 pCN33::*lukA* UTR	This study
MEJ3832	TCH1516 ΔSSR42 pCN33::*lukA* UTR	This study
MEJ3833	TCH1516 ΔSSR42 pICS3::*SSR42*	This study
MEJ3823	TCH1516 ΔSSR42 pICS3::SSR42-648-nt	This study
MEJ3824	TCH1516 ΔSSR42 pICS3::SSR42-793-nt	This study
MEJ3825	TCH1516 ΔSSR42 pICS3::SSR42-940-nt	This study
Plasmids	**Description**	**Source**
pJB38	Allelic replacement vector, *E. coli* Amp^R, *S. aureus* CM^R	(4)
pMK4	Shuttle vector, *E. coli* Amp^R, *S. aureus* CM^R	(5)
pXen-1	Luciferase reporter plasmid, *E. coli* Amp^R, *S. aureus* CM^R	(6)
pJB67	pCN51 with optimized RBS and Cd-inducible promoter, *E. coli* Amp^R, *S. aureus* Ery^R	(7)
pICS3	RNA expression vector, *E. coli* Amp^R, *S. aureus* CM^R	(8)
pCN33	mRNA target GFP reporter, *E. coli* Amp^R, *S. aureus* Ery^R	(8)
pGEM-T	pGEM-T Easy Vector cloning plasmid	Promega pGEM-T Easy Vector Kit

Table 2. Primers used in this study.

Primer	Sequence*	Description†
OL2939	ATGGGTACCCGTTATCTTGTTGGAAGT	F1 SSR42 deletion
OL3592	TCAGCAATTCATACGCGTGCACCAAATAATTTAATTAGA CTC	R1 SSR42 deletion
OL2389	ATGGAATTCAATCGCTGTAACGGATTCAT	F2 SSR42 deletion
OL3593	ATTATTTGGTGCACGCGTATGAATTGCTGAACTCCAATG	R2 SSR42 deletion
OL2380	GCCACCTGACGTCTAAGAAACC	F MCS pJB38ΔSSR42
OL2381	GCGATTTACATATGAGTTATGC	R MCS pJB38ΔSSR42
OL2888	ACTGGATCCGTGTCTATAATACTTTGACCT	F SSR42 complementation
OL3734	ATGGTCGACGTTCAATATTTACTACAAAGTCGTG	R SSR42 complementation
OL2393	TCGTATGTTGTGTGGAATTG	F MCS pMK4
OL2394	GTGCTGCAAGGCGATTAAG	R MCS pMK4
OL398	TCCTACGGGAGGCAGCAG T	F 16S
OL399	GGACTACCAGGGTATCTA ATCCTGTT	R 16S
OL7625	CAAATAGCTACTCCAAAACGATTAGTTATAA	F RT-qPCR *lukA*
OL7506	CAGGGTTTTCTACAGTAGCAATTC	R RT-qPCR *lukA*
OL2123	GAG TTG TTA TCA ATG GTC AC	F RT-qPCR *sarA*
OL2124	ACT GCT TTA ACA ACT TGT GG	R RT-qPCR *sarA*
OL2487	TAGTGGTCCATCAACAG	F RT-qPCR *lukS*-PV
OL2488	ACGTTCTACTTCACTGATA	R RT-qPCR *lukS*-PV
OL4036	GGCTCTATGAAAGCAGCAGATA	F RT-qPCR *hla*
OL4037	CTGTAGCGAAGTCTGGTGAAA	R RT-qPCR *hla*
OL5048	GGCAGTGGCTCATTCAACTAC	F RT-qPCR *hlgA*
OL5049	CACCTTTAGAGTTCTGACTTTCTAC	R RT-qPCR *hlgA*
OL2170	GGC TAA AGC TGA ACA TAA TG	F RT-qPCR *splE*
OL2171	ATA TTC ACC ATT GGG ATG C	R RT-qPCR *splE*
OL6159	GTCGCACATTCACAAGTTTATC	F RT-qPCR *aur*
OL6160	GCCTGACTGGTCCTTATATTC	R RT-qPCR *aur*
OL4526	ATGGAATTCGTTGGCATGTCATATTTCTCTCTCCTG	F SSR42 promoter for pXen-1
OL7767	ATGGGATCCCGAATATTTTGTAACAGGGCTACTAAG	R SSR42 promoter for pXen-1
OL6607	AATTGAATTCATTAACTGTCTAGTATCAACGATC	F *lukAB* promoter for pXen-1
OL6608	AATTGGATCCAAACACGTTTTTTATTTTTCAT	R *lukAB* promoter for pXen-1
OL7693	CACGCGCGCAATACTACAG	*perR* screening
OL7694	CGCTTACTTATATGAAGTAAGCTAACG	*perR* screening
OL7501	AATT**TAATACGACTCACTATAGGG**TCGGATATGCTTAAT TTTATAGTAAATTGTATG	F *lukA* 5' UTR fragment + T7

		promoter
OL7502	AAATTGCACATGATAATGATGACG	R *lukA* 5' UTR fragment
OL7244	ACTCTGCAGGTGTCTATAATACTTTGACCT	F SSR42 for pICS3
OL7245	ATGGAATTCGTTCAATATTTACTACAAAGTCGTG	R SSR42 for pICS3
OL7337	AATTGTCGACTCTCTTGATGTAAAATCTAAGATGTTTATGC	F SSR42 648-nt truncation
OL7338	AATTGTCGACATGTGGATGCCTGTCTTGATG	F SSR42 793-nt truncation
OL7339	AATTGTCGACATGTAGAAATTGAGTGTGAAAGTTAATAATAGAT	F SSR42 940-nt truncation
OL7499	AATT**TAATACGACTCACTATAGGG**GATTTCAAACCTATGTATTTC	F SSR42 +T7 promoter for pGEM-T
OL7500	AATATGATGTTCAATATTTACTACAAAGTCG	R SSR42 for pGEM-T
OL7501	AATTT**AATACGACTCACTATAGGG**TCGGATATGCTTAATTTTATAGTAAATTGTATG	F *lukA* UTR +T7 promoter for pGEM-T
OL7502	AAATTGCACATGATAATGATGACG	R *lukA* UTR for pGEM-T
OL7523	AATTT**AATACGACTCACTATAGGG**GCAGATAATTTAGATAAATAAATCATCCATCCATA	F *splE* UTR +T7 promoter for pGEM-T
OL7524	TACCCTCAACCACTGTTGT	R *splE* UTR for pGEM-T
OL6163	ATGGAATTCATGCACCAAATAATTTAATTAGACTCTATCG	F *rsp* promoter for pXen-1
OL6164	ATGGGATCCCATGTCATATTTCTCTCTCCTGCC	R *rsp* promoter for pXen-1

*Underline indicates restriction enzyme site, bold indicate T7 promoter.
†F = Forward; R = Reverse; MCS = Multiple Cloning Site

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