Quorum-sensing *agr* system of *Staphylococcus aureus* primes gene expression for protection from lethal oxidative stress

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Abstract (234 words)

The *agr* quorum-sensing system links *Staphylococcus aureus* metabolism to virulence, in part by increasing bacterial survival during exposure to lethal concentrations of H_2O_2, a crucial host defense against *S. aureus*. We now report that protection by *agr* surprisingly extends beyond post-exponential growth to the exit from stationary phase when the *agr* system is no longer turned on. Thus, *agr* can be considered a constitutive protective factor. Deletion of *agr* increased both respiration and fermentation but decreased ATP levels and growth, suggesting that Δ*agr* cells assume a hyperactive metabolic state in response to reduced metabolic efficiency. As expected from increased respiratory gene expression, reactive oxygen species (ROS) accumulated more in the *agr* mutant than in wild-type cells, thereby explaining elevated susceptibility of Δ*agr* strains to lethal H_2O_2 doses. Increased survival of wild-type *agr* cells during H_2O_2 exposure required *sodA*, which detoxifies superoxide. Additionally, pretreatment of *S. aureus* with respiration-reducing menadione protected Δ*agr* cells from killing by H_2O_2. Thus, genetic deletion and pharmacologic experiments indicate that *agr* helps control endogenous ROS, thereby providing resilience against exogenous ROS. The long-lived "memory" of *agr*-mediated protection, which is uncoupled from *agr* activation kinetics, increased hematogenous dissemination to certain tissues during sepsis in ROS-producing, wild-type mice but not ROS-deficient ($Nox2^{-/-}$) mice. These results demonstrate the importance of protection that anticipates impending ROS-mediated immune attack. The ubiquity of quorum sensing suggests that it protects many bacterial species from oxidative damage.

111 **Introduction (main text 4,779 words)**

112 Innate, bactericidal immune defenses and antimicrobials act, at least in part, by stimulating
113 the accumulation of reactive oxygen species (ROS) in bacteria (1, 2). Thus, understanding how
114 *Staphylococcus aureus* and other bacterial pathogens manage ROS-mediated stress has
115 important implications for controlling infections.

116 Knowledge of factors that govern the biology of ROS has advanced considerably in recent
117 years. For example, studies have centered on how specific metabolic features, such as aerobic
118 respiration, affect killing by ROS (3, 4), and small-molecule enhancers of ROS-mediated lethality
119 are emerging (5, 6). Less well characterized is how defense against ROS and metabolism
120 changes integrate with the virulence regulatory network that promotes *S. aureus* pathogenesis.
121 The *agr* quorum-sensing system provides a way to study this dynamic: *agr* is a major virulence
122 regulator that responds to oxidative stress (H_2O_2). The response occurs through a redox sensor in
123 AgrA that attenuates *agr* activity, thereby increasing expression of glutathione peroxidase (BsaA),
124 an enzyme that detoxifies ROS (7). Whether protection from ROS also occurs from positive *agr*
125 action is unknown and likely to be an important issue in development of Agr-targeted therapies
126 (8).

127 In cultured *S. aureus*, *agr* governs the expression of ~200 genes. Its two-part regulatory
128 role is characterized by 1) increased post-exponential-phase production of toxins and
129 exoenzymes that facilitate dissemination of bacteria via tissue invasion, and 2) decreased
130 production of cell surface and other proteins that facilitate adherence, attachment, biofilm
131 production, and evasion of host defenses (9, 10). Thus, *agr* coordinates a switch from an
132 adherent state to an invasive state at elevated bacterial population density. The invasive state
133 would be facilitated by protection from host defense.

134 The *agr* locus consists of two divergent transcription units driven by promoters P2 and P3
135 (11). The P2 operon encodes the quorum-signaling module, which contains four genes, *agrB,*
136 *agrD, agrC*, and *agrA*. AgrC is a receptor histidine kinase, and AgrA is a DNA-binding response
137 regulator. AgrD is an autoinducing, secreted peptide derived from a pro-peptide processed by
138 AgrB. The autoinducing peptide binds to and causes autophosphorylation of the AgrC histidine
139 kinase, which phosphorylates and activates the DNA-binding AgrA response regulator. AgrA then
140 stimulates transcription from the P2 (RNAII) and P3 (RNAIII) promoters. RNAIII is a regulatory
141 RNA that additionally contains the gene for delta-hemolysin (*hld*). The DNA-binding domain of
142 AgrA contains an intramolecular disulfide switch (7). Oxidation leads to dissociation of AgrA from
143 DNA, thereby preventing an AgrA-mediated down-regulation of the BsaA peroxidase.

144 When we used antimicrobials to study bacterial responses to lethal stress involving the
145 accumulation of ROS, we found that inactivation (deletion) of *agr* reduces lethality arising from
146 treatment with antimicrobials, such as fluoroquinolones, in a largely *bsaA*-dependent manner (12).
147 Thus, oxidation sensing appears to be an intrinsic checkpoint that ameliorates the endogenous
148 oxidative burden generated by certain antimicrobials. Surprisingly, deletion of *agr increases* the
149 lethal effects of exogenous H_2O_2 (12), in contrast to the expected expression of the protective
150 *bsaA* system (7). Thus, *agr* must help protect *S. aureus* from exogenous ROS, a principal host
151 defense, through mechanisms other than *bsaA*.
152 In the present work we found that protection by wild-type *agr* against lethal concentrations
153 of H_2O_2 was unexpectedly long-lived and 1) associated with decreased expression of respiration
154 genes, and 2) potentially aided by defense systems that suppress the oxidative surge triggered by
155 subsequent, high-level H_2O_2 exposure. The redox switch in AgrA, plus these additional protective
156 properties, indicate that *agr* increases resilience to oxidative stress in *S. aureus* both when it is
157 present and when it is absent. Thus, *agr* integrates protection from host defense into the
158 regulation of staphylococcal virulence.
159
160 **Results**
161
162 ***agr protects S. aureus from lethal concentrations of H_2O_2 throughout the growth cycle.***
163 Because *agr* is a quorum-sensing regulon, maximal *agr* activity occurs during exponential growth
164 (Figure 1. figure supplement 1) and is followed by a sharp drop during stationary phase (12, 13).
165 Surprisingly, protection from H_2O_2 toxicity by wild-type *agr*, assessed by comparison with an *agr*
166 deletion mutant, was observed throughout the growth cycle (Figure 1A). Indeed, maximal
167 protection occurred shortly after overnight growth, long after induction and expression of *agr*
168 transcripts. Comparison of survival rates of *Δagr* mutant and wild-type cells, following dilution of
169 overnight cultures and regrowth for 1 h prior to challenge with 20 mM H_2O_2, revealed an initial rate
170 of killing that was ~1,000-fold faster for the *Δagr* mutant (Figure 1B). Peroxide concentration
171 dependence was observed up to 10 mM during a 60-min treatment; at that point, mutant survival
172 was about 100-fold lower (Figure 1C). Complementation tests confirmed that the *agr* deletion
173 elevated killing by H_2O_2 (Figure 1D).
174 We also monitored the time required for the wild-type *agr* survival advantage against H_2O_2
175 to manifest itself (Figure 1. figure supplement 2). Overnight cultures were not readily killed by
176 H_2O_2, as expected from previous results with other lethal stressors (14). Following dilution to fresh
177 medium, wild-type survival dropped gradually, while mutant survival, although lower, was constant

178 for 20 min. By 40 min, mutant survival exhibited a precipitous 10-fold drop not seen with wild-type
179 cells (Figure 1. figure supplement 2). This drop in mutant survival correlated temporally with
180 changes in cell density (Figure 1. figure supplement 2); i.e., the first cell division following dilution
181 to fresh medium. Overall, the *agr*-mediated survival advantage during H_2O_2 exposure was absent
182 in stationary-phase cells and small during lag phase (before exponential growth resumes), but it
183 increased markedly during early growth.
184 Lag-time differences between strains were more obvious in experiments using less
185 complex, chemically defined medium (CDM) with highly diluted starting cultures and automated
186 growth analysis (Figure 1. figure supplement 3). In CDM, wild-type cells divided within ~150 min,
187 while the lag times with the *Δagr* mutant were more than 205 min (in Tryptic Soy Broth the lag
188 time is 30 min for both). These observations suggest a novel *agr*-mediated decrease in time to
189 enter exponential growth following dilution of stationary phase cultures. The poor killing of *agr*
190 mutant cells by H_2O_2 early in lag phase is consistent with other work in which cells experiencing
191 long lag times are less readily killed (15), presumably due to remaining longer in a dormant,
192 protected state. To focus on effects during growth, subsequent experiments were performed after
193 incubation of overnight cultures for 1 h in fresh Tryptic Soy Broth unless otherwise specified.
194 The elevating effect of *agr* inactivation on H_2O_2-mediated lethality was observed across a
195 variety of *S. aureus* strains, although differences in wild-type survival were observed (Figure 1.
196 figure supplement 4). Thus, *agr*-mediated protection from H_2O_2 appears to be common among *S.*
197 *aureus* lineages.
198
199 ***Expression of RNAIII and repression of Rot is required for protection from H_2O_2-mediated***
200 ***lethality.*** Δ*rnaIII* and Δ*agr* mutants showed identical loss of protection from H_2O_2-mediated killing
201 (Figure 2A), indicating that protection is RNAIII-dependent. Since RNAIII represses translation of
202 the downstream regulator Rot (16), a transcription factor having a key role in *agr* regulation of
203 staphylococcal virulence, we also examined the effects of *rot* on the protective action of *agr*
204 against H_2O_2. When the wild-type strain, a Δ*agr* mutant, a Δ*rot* mutant, and a Δ*agr* Δ*rot* double
205 mutant were compared for survival following treatment with 20 mM H_2O_2, survival of the Δ*agr* Δ*rot*
206 double mutant phenocopied that of the wild-type strain (Figure 2B): the *rot* deletion reversed the
207 effect of an *agr* deficiency. These data are consistent with *agr* activity allowing induction of *rot*-
208 repressed genes important for protection from peroxide (RNAIII repression of the Rot repressor).
209 When a low-copy-number plasmid expressing *rot* was introduced into a wild-type strain,
210 the transformant was more readily killed by H_2O_2, indicating that expression of *rot* is sufficient for
211 increased lethality (Figs. 2C-D). These data suggest that wild-type Rot down-regulates expression

212 of protective genes. The observed epistatic effect of *agr* and *rot* did not apply to other
213 downstream, potentially epistatic regulators, such as *saeRS*, *mgrA*, and *sigB* (Figure 2—figure
214 supplement 1) (17). Thus, the epistatic relationship between *agr* and protection from H_2O_2
215 appears to be *rot*-specific.
216
217 ***agr-mediated protection from H_2O_2 stress is kinetically uncoupled from agr activation.***
218 Since *agr*-mediated protection from H_2O_2 occurs throughout the growth cycle, it was possible that
219 protection arises from constitutive, low-level *agr* expression rather than from autoinduction and
220 thereby quorum sensing. To test for a requirement of quorum in the *agr*-mediated oxidative-stress
221 phenotypes, we characterized the role of *agr* activation using a mixed culture strategy in which
222 one strain, an in-frame deletion mutant of *agrBD*, is activated *in trans* by AIP produced by a
223 second, Δ*rnaIII* mutant strain (Figure 3A). The AIP-responsive Δ*agrBD* strain carried an intact
224 RNAIII, while the Δ*rnaIII* mutant was wild-type for *agrBD*. As shown in Figure 3B, hemolytic
225 activity (a marker for RNAIII) of the Δ*agrBD* mutant was restored by mixing it with the Δ*rnaIII*
226 mutant strain that secreted AIP into the surrounding medium. This result confirmed that *agrCA*-
227 directed *trans*-activation of RNAIII by AIP remained intact in the Δ*agrBD* mutant.
228 Mixed culture tests using these mutants, scored by differential plating for the presence of
229 an erythromycin resistance marker in the Δ*agrBD* mutant, showed no protection from lethality of
230 H_2O_2 when the two strains were mixed 1:1 immediately prior to growth from stationary phase
231 (Figure 3C). Autoinducer accumulated during subsequent growth, activating *agr* expression and
232 commencing protection from exogenous H_2O_2 (Figs. 3D-E). During H_2O_2 treatment, the
233 percentage of the Δ*agrBD* mutant (*rnaIII*$^+$) increased while the percentage of the Δ*rnaIII* mutant
234 decreased*;* this cis-acting result is consistent with the idea that pathways downstream from
235 RNAIII, such as those regulated by *rot*, are the primary drivers of *agr*-mediated protection from
236 H_2O_2. These results confirm an intimate link between *agr*-mediated protection and the quorum-
237 controlled *agr* gene expression program of late exponential phase. However, after an overnight
238 co-culture of the Δ*rnaIII* and Δ*agrBD* mutant strains, the Δ*agrBD* mutant demonstrated the same
239 degree of protection expected for wild-type cells during exposure to H_2O_2 (Figure 3F-H). Thus,
240 protection by *agr* after overnight co-culture extends to growth resumption from stationary phase,
241 prior to reaching quorum, and therefore protection is uncoupled from the constraint of strict cell-
242 density dependence. These results indicate that protection lasts long after maximal transcription
243 of *agr*, when *agr* expression has largely halted (12, 13). This phenomenon is a critical feature of
244 the *agr* system not appreciated in previous analyses of *agr* activation kinetics.
245

246 ***agr deficiency increases transcription of genes involved in respiration and overflow***
247 ***metabolism in the absence of stress***. To explore mechanisms underlying protection from H_2O_2,
248 we performed RNA-seq with the Δ*agr* and wild-type strains after growth to late exponential growth
249 phase, a point when *agr* expression is maximal. As expected, *agr* up-regulated the transcription of
250 many known virulence genes (Supplementary file 1). The Δ*agr* strain showed elevated expression
251 of genes involved in respiration (*cydA*, *qoxA-D*) and fermentation (18, 19), including *nrdGD,*
252 alcohol dehydrogenases (*adhE* and *adh1*) and lactate dehydrogenases (*ldh*, *ddh*) (Figure 4A and
253 Supplementary file 1). Increased respiration and fermentation are expected to increase energy
254 generation. However, metabolic modeling of transcriptomic data showed a ~30% reduction in
255 tricarboxylic acid (TCA) cycle and lactate flux per unit of glucose taken up by the Δ*agr* mutant
256 (Figure 4B, Supplementary file 2). Additionally, intracellular ATP levels were ~50% lower in the
257 *Δagr* mutant compared to the wild-type control, suggesting reduced metabolic efficiency during
258 exponential growth (Figure 5A). Moreover, although the *agr* deletion has little effect on growth in
259 the rich medium in which RNA-seq was performed (20), analysis in nutrient-constrained medium
260 (CDM) revealed decreased growth rate and yield of the Δ*agr* mutant relative to wild-type *S.*
261 *aureus* (Figure 1—figure supplement 3). Collectively, these data suggest that Δ*agr* increases
262 respiration and fermentation to compensate for low metabolic efficiency. Consistent with this idea,
263 *agr* deficiency also increases ATP-yielding carbon "overflow" pathways, as evidenced by
264 increased acetate production (Figure 5B) (21, 22). The increase in accumulated acetate in the
265 culture medium during exponential growth was largely consumed after 24 h of growth (Figure 5B).
266 Thus, Δ*agr* mutants exhibit TCA cycle proficiency (20) and, despite some expense of efficiency,
267 an increased catabolism of acetate.
268 Differential transcription of selected genes was confirmed by RT-qPCR measurements
269 (Figure 4—figure supplement 1) We also confirmed that respiration levels were lower (15%) in
270 wild-type compared to Δ*agr* (Figure 5E-F). Although the stimulatory effect of the *agr* deletion on
271 production of the fermentation product lactate was not observed in optimally aerated broth
272 cultures after growth to late exponential growth phase, it was confirmed for organisms grown in
273 broth under more metabolically demanding suboptimal aeration conditions (limitations in the rate
274 of respiration when oxygen is limiting are expected to increase overall levels of fermentation)
275 (Figure 5C). Overall, these results are consistent with transcription-level up-regulation of
276 respiratory and fermentative pathways in *agr*-deficient strains.
277 Since respiration and fermentation generally increase NAD^+/NADH ratios and since these
278 activities are increased in *Δagr* strains (Figure 5C and E-F), we expected a higher NAD^+/NADH
279 ratio relative to wild-type cells. However, we observed a decrease in the NAD^+/NADH ratio due to

280 an increase in NADH accompanied by relative stability in NAD^+ compared to wild-type.
281 Collectively, these observations suggest that a surge in NADH accumulation and reductive stress
282 in the Δ*agr* strain induces a burst in respiration, but levels of NADH are saturating, thereby driving
283 fermentation under microaerobic conditions.

284 To help determine the metabolic fate of glucose, we measured glucose consumption and
285 intracellular levels of pyruvate and TCA-cycle metabolites fumarate and citrate in the wild-type
286 and *Δagr* mutant strains. At 4 h of growth to late-exponential phase, intracellular pyruvate and
287 acetyl-CoA levels were increased in the *Δagr* mutant compared to wild-type strain, but levels of
288 fumarate and citrate were similar (Figure 5—figure supplement 1D-E). Glucose was depleted after
289 4 h of growth, but glucose consumption after 3 h of growth (exponential phase) was increased in
290 the *Δagr* mutant compared to the wild-type strain (Figure 5—figure supplement 1A). These
291 observations, together with the decrease in the NAD^+/NADH ratio and increase in acetate and
292 lactate production described above, are consistent with a model in which respiration in Δ*agr*
293 mutants is inadequate for 1) energy production, resulting in an increase in acetogenesis, and 2)
294 maintenance of redox balance, resulting in an increase in fermentative metabolism, lactate
295 production, and conversion of NADH to NAD^+. Increased levels of acetate compared to lactate
296 under optimal aeration conditions suggests that demand for ATP is in excess of demand for NAD^+.

297 Elevated respiratory activity of Δ*agr* is expected to increase endogenous ROS (4). To test
298 this idea, we assessed ROS accumulation in bulk culture by flow cytometry of Δ*agr* and wild-type
299 stains using carboxy-H2DCFDA, a dye that becomes fluorescent in the presence of several forms
300 of ROS. As shown in Figure 6, ROS levels increased with *agr* deficiency, indicating correlation
301 between *agr* activity, lower ROS levels, and increased bacterial survival in response to exogenous
302 H_2O_2. These data help explain elevated lethality of peroxide in the absence of *agr*. Since lower
303 ROS accumulation in wild-type cells correlates with decreased respiration and protection from
304 killing by H_2O_2, the data also support the idea that suppression of endogenous ROS is key to *agr*-
305 mediated protection from exogenous H_2O_2-mediated lethality.
306

307 ***Transcriptional changes due to Δagr mutation are long-lived and result in down-regulation***
308 ***of H_2O_2-stimulated genes relative to those in an agr wild-type.*** We reasoned that the
309 transcriptional changes due to the Δ*agr* mutation likely persist, as does this strain's susceptibility
310 to killing by H_2O_2, after growth from overnight culture. With this in mind, and to determine whether
311 *agr*-mediated changes act through *rot*, we performed RNA-seq experiments after 1-hr growth from
312 overnight cultures of a Δ*agr* Δ*rot* double mutant that phenocopies wild-type with respect to H_2O_2-
313 mediated death and with respect to its parental Δ*agr* strain (Supplementary file 3). Fold-changes

314 and number of genes differentially expressed were lower in the Δ*agr* mutant relative to the wild-
315 type culture, potentially because a significant portion of the population, even after an hour of
316 growth (early exponential phase), still consisted of cells experiencing stationary phase at the time
317 of sampling. Nevertheless, we did observe a shift to expression of fermentation-associated genes
318 (*ilvA, pflAB*, *aldh1*, *ddh*, *lctp2*) in the Δ*agr* strain (Figure 4C and Supplementary file 3). Thus, up-
319 regulation of metabolic genes in the Δ*agr* mutant extends beyond post-exponential growth to the
320 exit from stationary phase and into subsequent cell proliferation, as does the long-lived protection
321 from H_2O_2-mediated killing seen with the wild-type strain.
322 To examine induction of genes by lethal levels of H_2O_2, our gene expression analysis
323 included a comparison between untreated and H_2O_2-treated cells after growth from overnight
324 culture (Supplementary file 3). The Δ*agr* Δ*rot* double mutant that phenocopies wild-type had
325 elevated expression of many genes involved in lowering oxidative stress compared to the Δ*agr*
326 mutant. Those genes are involved in the regulation of misfolded proteins (*mcsA*, *mcsB*, *clpC*,
327 *clpB*), Fe-S cluster repair (*iscS*), DNA protection and repair (*dps*), and genes regulated by the
328 protein-damage repair gene *bshA (fhuB/G, queC-E)* (23) (Figure 7, Figure 7—figure supplement
329 1, and Supplementary file 3). Elevated expression of protective genes suggests that the double
330 mutant survives damage from H_2O_2 better because protective genes are rendered inducible (loss
331 of Rot-mediated repression). Overall, the data show that *agr* wild-type cells assume a long-lived
332 stage after activation at high cell density in which they are primed to express genes (e.g., *clpB/C,*
333 *dps*) that protect against high levels of exogenous oxidative stress.
334
335 ***Endogenous ROS is involved in agr-mediated protection from lethal, exogenous H_2O_2***
336 ***stress.*** We next monitored the effect of reducing respiration and ATP levels by adding
337 subinhibitory doses of the redox cycling agent menadione (24) to cultures of Δ*agr* and wild-type
338 cells prior to lethal levels of H_2O_2. Addition of menadione for 30 min, which induces a burst of
339 ROS that inactivates the TCA cycle and thereby respiration (24), protected the Δ*agr* mutant but
340 had little effect on the wild-type strain (Figure 8A). Menadione's effect on respiration and ATP can
341 be reversed by N-acetyl cysteine (24). Addition of N-acetyl cysteine in the presence of menadione
342 restored H_2O_2 susceptibility to the *agr* mutant (Figure 8A). Thus, blocking endogenous ROS
343 production/accumulation reverses the lethal effect of an *agr* deficiency with respect to a
344 subsequent exogenous challenge with H_2O_2.
345 Rowe et al. (24) showed that menadione exerts its effects on endogenous ROS by
346 inactivating the TCA cycle in *S. aureus*. To determine whether this mechanism can induce
347 protection in the Δ*agr* mutant, we inactivated the TCA cycle gene *acnA* in *agr* wild-type and Δ*agr*

348 strains (Figure 8—figure supplement 2). We found that *ΔacnA* mutation completely protected the
349 Δ*agr* mutant from peroxide killing after growth to late exponential growth phase but had little effect
350 on the wild-type *agr* strain. This finding supports the idea that TCA cycle activity contributes to an
351 imbalance in endogenous ROS homeostasis in the Δ*agr* mutant, and that this shift is a critical
352 factor for Δ*agr* hyperlethality. When we evaluated long-lived protection by comparing survival
353 rates of *Δagr ΔacnA* mutant and *Δagr* cells following dilution of overnight cultures and regrowth
354 prior to challenge with H_2O_2, *ΔacnA* remained protective, but less so (Figure 8—figure supplement
355 2). These partial effects of an *ΔacnA* deficiency suggest that *Δagr* stimulates long-lived lethality
356 for peroxide through both TCA-dependent and TCA-independent pathways.
357 *S. aureus* has multiple enzymes that control the endogenous production and detoxification
358 of ROS. SodA and SodM dismutate superoxide ($O_2^{\bullet-}$) to H_2O_2, and catalase and AhpC then
359 convert H_2O_2 to water, limiting the formation of toxic hydroxyl radical ($OH^{\bullet}$). Accordingly, we asked
360 whether mutations in these pathways affect *agr*-dependent phenotypes with respect to lethal H_2O_2
361 exposure. A deficiency in the *sodA* superoxide dismutase (25) resulted in lower survival of the
362 wild-type strain, similar to that observed with the Δ*agr* mutant (Figure 8B-C). The effect was
363 reversed by complementation with *sodA* on a low-copy-number plasmid (Figure 8D). The Δ*sodA*
364 mutation had no effect on killing with the Δ*agr* strain. Moreover, *sodA* expression (Supplementary
365 file 1) and activity levels (Figure 8E) were similar for wild-type and the Δ*agr* mutant. Together,
366 these observations suggest that the contribution of *sodA* toward protective priming by wild-type
367 involves dismutation of low levels of endogenous superoxide generated by respiration. In contrast,
368 endogenous levels of ROS are saturating for *sodA* in Δ*agr* cells. Inactivation of *sodM*, which is
369 thought to be primarily induced by exogenous oxidative stress (26), had no noticeable effect on
370 the H_2O_2 susceptibility of the wild-type or the Δ*agr* mutant. We conclude that scavenging
371 enzymes, such as SodA, are better able to control the threat posed by endogenous ROS in wild-
372 type than in Δ*agr* cells. They render the former better able to survive a subsequent lethal dose of
373 H_2O_2, a compound that freely enters cells (27) and would add to endogenous ROS levels.
374 Other oxidative-stress-response mutations in genes encoding catalase, thiol-dependent
375 peroxidases, and bacillithiol showed little effect on the relative lethality of H_2O_2 between wild-type
376 and Δ*agr* mutant strains (Figure 8—figure supplement 1). Thus, protection against H_2O_2 lethality
377 by these genes is not *agr*-specific. Paradoxically, a deficiency of *ahpC* (*ahpC::bursa*), which
378 encodes a peroxidase (28), almost completely reversed the elevated killing associated with the
379 Δ*agr* mutation (Figure 8F). An *ahpC* deficiency had no effect on the response of the otherwise
380 wild-type strain. A deficiency in other downstream genes in the *ahpC* operon (*ahpF*,

381 SAUSA300_0377-0378) showed no effect, indicating that the protective behavior of mutant *ahpC*
382 was not caused by polar effects (Figure 8—figure supplement 3).
383 Results with *ahpC* deficiencies were initially surprising, because reduced ROS
384 detoxification should increase rather than decrease killing. Compensatory expression of other
385 protective genes, such as *katA* in the Δ*ahpC* Δ*agr* double mutant (28), might enable cells to better
386 survive damage from subsequent stress-stimulated ROS increases. Indeed, *katA* expression
387 increased > 10-fold in the Δ*ahpC* and Δ*ahpC* Δ*agr* double mutants (Figure 8G). Thus, Δ*katA*
388 overcomes Δ*ahpC*-mediated protection, consistent with the idea expressed previously that *katA* is
389 more protective than *ahpC* against high levels of exogenous oxidative stress (28, 29). We
390 conclude that the protective action of an *ahpC*-deficient mutant is due to a pre-induced,
391 compensatory increase in expression of another protective catalase.
392
393 ***Importance of the long-lived "memory" of agr-mediated protection in a murine***
394 ***intraperitoneal infection model****.* To determine whether long-lived *agr*-mediated protection is
395 important for *S. aureus* pathogenesis, we used the mixed infection strategy (outlined in Figure 3)
396 in which a Δ*agrBD* mutant is "primed" in response to AIP produced by a Δ*rnaIII* mutant after
397 overnight co-culture containing an equal ratio of the two mutant strains (Figure 9A and Figure 9—
398 figure supplement 1). Then mice were infected via intraperitoneal inoculation; 2 h later, we
399 lavaged the peritoneal cavity and harvested organs for determination of colony forming units
400 (CFU). By 2 h after bacterial administration, the number of *S. aureus* cells injected as inoculum
401 had declined by 1000-fold (Figure 9B and Figure 9—figure supplement 1). Mutant proportions,
402 identified by differential plating, demonstrated that Δ*agrBD* cells were enriched by ~30% in both
403 peritoneum and organs compared to the Δ*rnaIII* mutant. The fraction of Δ*agrBD* (*rnaIII*$^{+}$) mutants
404 in sites of bacterial dissemination (heart, kidney, liver, lungs, and spleen) was similar to their
405 elevated fraction in the peritoneum, thereby suggesting that *agr* enhances intraperitoneal infection
406 and access to, rather than entry into extraperitoneal organs. In a control infection in which Δ*agrBD*
407 was "unprimed" by mixing Δ*agrBD* and Δ*rnaIII* mutants immediately before growth from stationary
408 phase, the proportion of Δ*agrBD* bacterial burden was lower at all tissue sites (Figure 9A and
409 Figure 9—figure supplement 1). This drop represented a decline in long-lived *agr* induction of
410 virulence.
411 To study *agr*-ROS effects during infection, we repeated *in vivo* studies using *Nox2*$^{-/-}$ (also
412 known as *Cybb*$^{-/-}$) mice deficient in enzymes associated with host phagocyte production of ROS
413 (the gp91 [phox] component of the phagocyte NADPH oxidase)(30). We found that *agr*-mediated
414 priming (mixing Δ*agrBD* and Δ*rnaIII* before overnight co-culture) failed to increase hematogenous

415 dissemination to lung and spleen tissues following infection of *Nox2*$^{-/-}$ mice (Figure 9). Thus,
416 when the host makes little ROS, long-lived *agr*-mediated protection has little effect in these
417 tissues. The data also indicate that *agr*-mediated protection against ROS enhances fitness in lung
418 and spleen, but it is dispensable for full virulence in other organs. Collectively, the murine
419 experiments indicate that the long-lived "memory" of *agr* induction enhances overall pathogenicity
420 of *S. aureus* during sepsis. They also support data previously published indicating that clearance
421 of disseminating bloodstream pathogens (31) and protection from ROS buildup (32) are tuned to
422 particular sites in the host organism.
423
424 **Discussion**
425 We report that *agr*, a quorum-sensing regulator of virulence in *S. aureus*, provides
426 surprisingly long-lived protection from the lethal action of exogenous H_2O_2. The protection, which
427 is uncoupled from *agr* activation kinetics, arises in part from limiting the accumulation of
428 endogenous ROS. This apparent tolerance to lethal stress derives from an RNAIII-*rot* regulatory
429 connection that couples virulence-factor production to metabolism and thereby to levels of ROS.
430 Collectively, the results suggest that *agr* anticipates and protects the bacterium from increases in
431 ROS expected from the host during *S. aureus* infection.
432 Details of *agr*-mediated protection are sketched in Figure 10. At low levels of ROS, *agr* is
433 activated by a redox sensor in AgrA, RNAIII is expressed and represses the Rot repressor,
434 thereby rendering protective genes (e.g., *clpB/C, dps*) inducible via an unknown mechanism
435 (induction, candidate protective gene(s), and their connection to endogenous ROS levels are
436 being pursued, independent of the current report). Superoxide dismutase and scavenging
437 catalases/peroxidases detoxify superoxide and peroxide, respectively (scavenging deficiencies
438 reduce the protective effect of wild-type). Deletion of *agr* eliminates expression of RNAIII and
439 repression of Rot, resulting in a metabolic instability associated with a 100-fold increase in H_2O_2-
440 mediated death.
441 The *agr* system directly reduces H_2O_2-mediated killing by reducing levels of endogenous
442 ROS, much like intrinsic tolerance to lethal antimicrobial stress (33). However, the protective
443 system we describe is distinct in that it primes cells for induction of genes (e.g., *clpB/C, dps*) that
444 mitigate damage upon subsequent exposure to high levels of ROS. Still unidentified protective
445 genes exist; thus, *agr*-mediated protection may be further shaped by both known (*ahpC*) and
446 unidentified pathways and factors that modify the redox state. Another distinctive feature of *agr*-
447 mediated protection is its manifestation even in early log-phase cultures, long after the maximal

448 transcription of *agr* at high cell density, i.e., quorum. In a sense, *S. aureus* has a "memory" of the
449 *agr*-activated state.
450 Transcriptional profiling during growth from diluted, overnight cultures revealed that the
451 Δ*agr* mutation elevated expression of several respiration and fermentation genes. Acceleration of
452 cellular respiration is likely a source of ROS, as it appears to be for bactericidal antibiotics (3). Our
453 work supports this idea by showing that increased respiration caused by deletion of *agr* is
454 associated with increased ROS-mediated lethality. How *agr* deficiency is connected to the
455 corruption of downstream processes that result in metabolic inefficiency and increased
456 endogenous ROS levels is unknown. Given that Δ*agr* mutants are unable to downregulate surface
457 proteins during stationary phase (34, 35), it is possible that deletion of *agr* perturbs the
458 cytoplasmic membrane or the machinery that sorts proteins across the cell wall. In support of this
459 notion, jamming SecY translocation machinery of *E. coli* results in downstream events shared with
460 antibiotic lethality, including accelerated respiration and accumulation of ROS (36). In this
461 scenario, the formation of a futile macromolecular cycle may accelerate cellular respiration to
462 meet the metabolic demand of unresolvable problems caused by elevated surface sorting.
463 As noted above, *agr* is inactivated by oxidation, which elevates levels of the antioxidant
464 BsaA during exposure to H_2O_2 (7). That would make our finding that H_2O_2-mediated killing is
465 increased in the *Δagr* mutant paradoxical. This apparent inconsistency can be explained by a
466 focus of prior work on growth-related phenotypes (7) rather than on lethality (the underlying
467 mechanisms are distinct (2)). Additionally, we note that *bsaA* was not upregulated in either our
468 RNA-seq experiments (Supplementary file 1) or in previous transcriptional profiling data (37).
469 Thus, an alternative, but not mutually exclusive, hypothesis is that the growth-related effect of
470 *bsaA* on *agr*-mediated responses to stress is strain dependent. Another complexity involves test
471 conditions, as indicated by consideration of previous work in which wild-type cells exhibited
472 greater oxidative stress than the *agr*-deficient mutant due to *agrA*-mediated production of ROS-
473 inducing phenol-soluble modulins (37). The present experiments were performed in highly diluted
474 cultures in which levels of these modulins are likely low (38, 39). The complex relationship
475 between *agr*, ROS-mediated lethality, and physiological state illustrates the importance of
476 understanding *agr* biology before applying therapies that inactivate *agr* (8).
477 We also note that although the absence of *agr* increases killing by high levels of H_2O_2, it
478 has the opposite effect for lethal concentrations of ciprofloxacin (12). In the latter case, the
479 absence of *agr* upregulates expression of *bsaA* in the strain examined; *bsaA* counters
480 endogenous ROS induced by ciprofloxacin (12). The present work shows that excess
481 endogenous ROS is generated during *agr* deficiency. Thus, protection from endogenous lethal

482 stress via *agr* inactivation may not be only through the redox-dependent *bsaA* but also by a
483 second pathway involving increased respiratory metabolism. The present work also supports the
484 idea that exposing bacteria to exogenous H_2O_2 does not fully recapitulate the intracellular
485 environment created by antibiotics and other stresses that act via ROS-mediated cell death (36),
486 emphasizing that inactivation of *agr* can be either destructive or protective, depending on the type
487 of lethal stress. Similar results have been reported with other bacteria: *mazF*, *lepA*, and *cpx* are
488 destructive or protective based on the level of lethal stress (40, 41).
489 The protective activity of *agr* carried over to *in vivo* studies using mice, as it was largely
490 absent if the mice were deficient in host phagocyte production of ROS (*Nox2*$^{-/-}$ mice with a null
491 allele of NADPH oxidase). The benefits of *agr* to *S. aureus* fitness seen with NADPH oxidase-
492 proficient mice were observed largely in lungs, a key host defense niche for neutrophil-mediated
493 clearance of disseminating pathogens (31). The redox switch in AgrA, plus the protective
494 properties associated with *agr* activation, lead to a clinical model in which *agr* links
495 virulence-factor expression to an intrinsic protection against a lethal, H_2O_2-mediated immune
496 response during infection (Figure 11). In this model, *agr* quorum-sensing renders cells better
497 prepared to respond to lethal, exogenous oxidative stress. We note, however, that *agr*-mediated
498 fitness benefits were present in certain tissues even in NADPH oxidase-deficient mice, indicating
499 the existence of long-lived factors other than those that suppress oxidative stress. Thus, such a
500 pre-emptive defense system may apply to many challenges experienced by *S. aureus* during
501 infection, especially during bloodstream dissemination and conditions within inflamed tissues (42,
502 43).
503 In conclusion, uncoupling of *agr*-mediated tolerance from bacterial population density
504 anticipates increases in exogenous ROS expected during *S. aureus*-host interactions, thereby
505 contributing to virulence. The ubiquity of quorum sensing suggests that it protects many bacterial
506 species from oxidative damage. A next step is to find RNA, protein, and/or epigenetic markers
507 underlying the *agr*-mediated "memory" that improves protection against subsequent H_2O_2
508 exposure, since that will provide insights into the role of *agr* in cellular survival and adaptation
509 during infection. Discovering ways to manipulate the lethal stress response, as seen with
510 supplementation of antimicrobials with N-acetyl-cysteine during treatment of *Mycobacterium*
511 *tuberculosis* (44) and development of inhibitors of enzymes that produce protective H_2S (5), could
512 reveal novel approaches for enhancing antimicrobial therapy and host defense systems (45-47).
513

514 **Materials and Methods**
515
516 ***Bacterial strains, plasmids, primers and growth conditions.*** *S. aureus* strains, plasmids, and
517 primers used in the study are described in Tables 1 and 2. Bacterial cells were grown in Tryptic
518 Soy Broth (TSB, glucose concentration at 2.5 g/L) at 37ºC with rotary shaking at 180 rpm. For
519 suboptimal aeration, broth cultures were grown in a closed-capped 15 mL conical tube with 10 mL
520 of TSB. Colony formation was on Tryptic Soy Agar (TSA) with or without defibrinated sheep blood,
521 incubated at 37ºC or 30ºC. Phages 80α and Φ11-mediated transduction was used for strain
522 construction (48); transductants were selected on TSA plates containing the appropriate
523 antimicrobial.
524 For analysis of *in vitro* growth curves, overnight cultures grown in TSB were diluted 1:1000
525 in chemically defined medium (CDM) (49), and growth was monitored at 37°C in 96-well plates
526 (100 µL/well) using an Agilent LogPhase 600 Microbiology Reader (Santa Clara, CA) with 1 mm
527 orbital shaking, measuring OD_{600} at 40-min intervals. The curves represent averaged values from
528 five biological replicates. The exponential phase was used to determine growth rate (μ) from two
529 datapoints, OD_1 and OD_2 flanking the linear portion of the growth curve, following the equation
530 $\ln OD_2 - \ln OD_1 / t_2 - t_1$, as described (50).
531
532 ***Measurement of bioluminescence.*** Overnight cultures were diluted to OD_{600} ~0.05 and grown in
533 TSB at 37ºC with rotary shaking at 180 rpm. Aliquots (100 µL) were inoculated into flat bottom 96-
534 well microtiter plates (Corning, Corning, NY), and bioluminescence was detected using a BioTek
535 Synergy Neo2 plate reader (Agilent, Santa Clara, CA).
536
537 ***Antimicrobials and chemicals.*** Antimicrobials, chemicals, and reagents were obtained from
538 MilliporeSigma (Burlington, MA) or Thermo Fisher Scientific (Waltham, MA).
539
540 ***Construction of mutants.*** Transposon mutants were generated by transducing *Bursa aurealis*
541 insertions, obtained from the University of Nebraska transposon mutant (ΦNE) library (51), into
542 LAC or LAC *agr::tetM* using phages 80α and Φ11.
543 Construction of the Δ*agrBD* mutant: *S. aureus* Δ*agrBD* mutant GAW128 was generated by
544 a chromosomal integration strategy outlined in Chen *et al*. (52) in an *agr*-null background strain,
545 BS687. Plasmid pJC1111, a suicide plasmid containing a cadmium resistance (Cd^R) cassette and
546 the SaPI1 *attS* site that enables single-copy insertion into the corresponding chromosomal SaPI1
547 *att*C site, was used as the backbone vector for the *S. aureus agrBD* construct. pJC1000 contains
548 the RN6734 *agr* locus cloned into pUC18. Inverse PCR of pJC1000 was performed using *agrBD*

549 primers GWO#27 and GWO#28, re-ligated following treatment with polynucleotide kinase, and
550 designated pGAW98. The *Sph*I-*EcoR*I fragment of pGAW98 was ligated into the SaPI1 integration
551 vector pJC1111 and designated pGAW119. Strain RN9011 (RN4220 with pRN7023 [vector (Cm^R)
552 containing SaPI1 integrase]) was electroporated with plasmid pGAW119 and plated on GL agar
553 containing 0.1 mM $CdCl_2$. Phage 80α lysates of Cd^R colonies were used to transduce BS687
554 (RN6734 Δ*agr*::*ermC*, Erm), generating GAW128 (Δ*agrBD*).
555 To construct *agr* mutant BS687*, agr* flanking regions were amplified with primer pairs
556 JCO#339, JCO#340, and JCO#342, JCO#343 and cloned into the *Hinc*II site of pUC18 to
557 generate pJC1527 and pJC1528, respectively. pJC1530 was generated by four-way ligation of the
558 *Kpn*I-*BamH*I fragment of pJC1527 (*agr* left flank), *Xho*I-*Sph*I fragment of pJC1528 (*agr* right flank),
559 and *BamH*I-*Xho*I fragment from pJC1073 (Erm cassette) to *Kpn*I-*Sph*I digested pJC1202
560 (replacement vector). Plasmid pJC1530 was electroporated into strain RN4220 with selection on
561 GL agar containing 10 µg/mL of chloramphenicol at 30°C. Phage 80α lysates of Cm^R colonies
562 were used to transduce strain JCSA18 (*rpsL** mutant of RN6734 that results in streptomycin
563 resistance) and then allelic exchange of the Em^R Sm^S Cm^R colonies was performed as previously
564 described. Phage 80a lysates of Em^R Sm^R Cm^S colonies were then used to transduce RN6734
565 with selection for Em^R, generating BS687.
566 *sodA* complementation: Plasmid PsarA-*sodA*-pJC1111*,* expressing *sodA* under the control
567 of the constitutive promoter P*sarA*, was integrated into the *S. aureus* chromosome at the SaPI1
568 *attC* site of strain LAC (53), LAC *sodA::tetM*, and LAC *agr::ermC*. Complementation plasmid
569 P*sarA*-*sod*RBS-*sodA* was generated by Gibson assembly and inserted into the SaPI1 integration
570 vector pJC1111. Wild-type *sodA* and the *sarA* promoter were amplified from *S. aureus* gDNA
571 using primers MPsodA#1-2 (*sodA* gene and RBS) and MPsodA#3-4 (P*sarA*). Primers MPsodA#5-
572 6 were used to linearize pJC1111. Primers introduced relevant oligonucleotide overlaps that
573 enabled Gibson assembly (6), generating P*sarA*-*sod*RBS-*sodA*. P*sarA*-*sod*RBS-*sodA* was
574 transformed into *E. coli* DH5α for amplification, purification, and sequence validation via primers
575 pJC1111 FOR and pJC1111 REV. Purified P*sarA*-*sod*RBS-*sodA* was electroporated into RN9011
576 and positive chromosomal integrants at the SaPI1 chromosomal attachment (*att*C) site were
577 selected with 0.1 mM $CdCl_2$. Phage 80a lysates of positive integrants were used to transduce
578 BS1422 (LAC *sod*::*tetM*) and BS1348 (LAC *agr*::*tetM*), generating BS1707 and BS1708,
579 respectively.
580
581 ***Survival measurements.*** To measure lethal action, overnight cultures were diluted (OD_{600}~0.05)
582 in fresh medium and grown with shaking to early exponential (OD_{600}~0.15) or late log (OD_{600}~4)

583 phase, conditions when *agr* expression is largely absent (12) or maximally activated, respectively.
584 Early (undiluted) and late exponential phase cultures (diluted into fresh TSB medium to
585 OD_{600}~0.15) were incubated with H_2O_2 under aerobic conditions either at a fixed concentration for
586 one or more time points or at various concentrations for a fixed time. At the end of treatment,
587 aliquots were removed, concentrated by centrifugation and serially diluted in phosphate-buffered
588 saline to remove H_2O_2, and plated for determination of viable counts at 24 h. Percentage of
589 survival was calculated relative to a sample taken at the time of H_2O_2 addition. When menadione
590 and N-acetylcysteine were used to inhibit or potentiate killing by H_2O_2, they were added prior to
591 lethal treatments as described previously (14). For experiments involving menadione
592 pretreatment, cultures were grown for 3.5 h, and menadione (40 mM solution in 96% EtOH, final
593 concentration 80 μM) was added for the last 0.5 h of culture, preceding the H_2O_2 treatment at 4 h.
594 N-acetylcysteine was used to counter the action of menadione; it was added simultaneously with
595 menadione, at a final concentration of 30 mM (640 mM stock in sterile ddH_2O was used). All
596 experiments were repeated at least three times; similar results were obtained from the biological
597 replicates.
598
599 ***Measurement of glucose consumption.*** Overnight cultures were diluted into fresh TSB
600 (OD_{600}~0.05) and grown for 4 hours with shaking at 180 rpm (OD_{600}~4) at 37°C. Glucose was
601 assayed in the supernatant fluids of bacterial cultures following centrifugation at 12,000 x g, using
602 Centricon-10 concentrators (MilliporeSigma, Burlington, MA), and pH adjustment to 6.5-7.0 using
603 NaOH. Cells were assayed and plated hourly for determination of viable counts as indicated in
604 figures. Glucose content was measured from serial dilutions of supernatants using UV method
605 (cat. no. 10-716-251-035) following manufacturer's instructions (R-Biopharm, Darmstadt,
606 Germany). Glucose consumption was expressed as μg of glucose consumed over 3 h of culture
607 per 10^8 bacterial cells. There was no detectable glucose in culture supernatants at 4 h of culture
608 (data not shown).
609
610 ***Measurement of excreted metabolites.*** Excreted metabolites were assayed in the supernatant
611 fluids of bacterial cultures following centrifugation at 12,000 x g for 10 min for late exponential (4
612 h, OD_{600}~4) or multiple time points (acetate), as indicated in figures. Aliquoted supernatants were
613 stored at −80°C and thawed on ice prior to analysis. Cells were plated for determination of viable
614 counts; L(+)-lactate and acetate concentrations were measured using commercially available
615 colorimetric and fluorometric kits (cat. no. MAK065, ab204719), according to manufacturer's
616 recommendations (MilliporeSigma, Burlington, MA and Abcam, Cambridge, UK, respectively).

617 ***Measurement of intracellular metabolites.*** Overnight cultures were diluted into fresh TSB
618 (OD_{600}~0.05), grown for 4 hours at 37°C with shaking at 180 rpm (OD_{600}~4), and plated for
619 determination of viable counts at 4 h. The remaining cells were concentrated by centrifugation at
620 12,000 x g for 10 min, and resuspended in lysis buffer provided by the assay kit. Cells were lysed
621 by repeated homogenization (2 cycles of 45 sec homogenization time at 6 M/s followed by a 5 min
622 pause on ice) using Lysing Matrix B tubes in a FastPrep-24 homogenizer (MP Biomedicals, Irvine,
623 CA). After lysis, cell debris was removed by centrifugation (12,000 × *g*, 10 min) and the
624 supernatant was used for determination of pyruvate, fumarate, citrate and acetyl-CoA levels using
625 colorimetric (pyruvate, fumarate) or flurometric (citrate, acetyl-CoA) assays **(**cat. no. KA1674,
626 ab102516, KA3791 and MAK039, respectively) and a microplate reader (BioTek Synergy Neo2,
627 Agilent, Santa Clara, CA) according to manufacturer's instructions (Abnova, Taipei City, Taiwan
628 and Abcam, Cambridge, UK and MilliporeSigma, Burlington, MA, respectively). Assayed
629 metabolites were measured in µg and normalized to cell count.
630
631 ***Measurement of oxygen consumption.*** Overnight cultures were diluted into fresh TSB
632 (OD_{600}~0.05), grown for 5 hours at 37°C with shaking at 180 rpm (OD_{600}~4), diluted
633 (OD_{600}~0.025) in fresh TSB, and added to a microtiter plate (200 µL/well). Oxygen consumption
634 rate (OCR) was measured using a Seahorse XF HS Mini Analyzer (Agilent, Santa Clara, CA)
635 according to the manufacturer's instructions. The Seahorse XF sensor cartridge was hydrated in a
636 non-CO_2 37°C incubator with sterile water (overnight) and pre-warmed XF calibrant for 1 hour
637 prior to measurement. OCR measurements were recorded in 15 measurement cycles with 3
638 minutes of measurement and 3 minutes of mixing per cycle. CFU were enumerated to confirm
639 equal concentrations of *agr*-deficient mutant and wild-type cells
640
641 ***Measurement of ATP, NAD^+, and NADH.*** For ATP, overnight cultures were diluted into fresh TSB
642 (OD_{600}~0.05), grown for 4 hours at 37°C with shaking at 180 rpm (OD_{600}~4), diluted (OD_{600}~1.0)
643 in fresh TSB, and incubated at room temperature with reagent for determination of ATP using
644 BacTiter-Glo Microbial Cell Viability Assay (cat. no. G8232; Promega, Madison, WI), according to
645 the manufacturer's instructions. Luminescence was detected a BioTek Synergy Neo2 plate reader
646 (Agilent, Santa Clara, CA). The amount of ATP was calculated and normalized to cell count.
647 For NAD^+ and NADH, overnight cultures were diluted into fresh TSB (OD_{600}~0.05), grown
648 for 4 hours at 37°C with shaking at 180 rpm (OD_{600}~4), and plated for viable counts at 4h or
649 concentrated by centrifugation at 12,000 x g for 10 min and resuspended in lysis buffer provided
650 by the assay kit. Cells were lysed by repeated homogenization (2 cycles of 45 sec

651 homogenization time at 6 M/s followed by a 5 min pause on ice) using Lysing Matrix B tubes in a
652 FastPrep-24 homogenizer (MP Biomedicals, Irvine, CA). After lysis, cell debris was removed by
653 centrifugation (12,000 × *g*, 10 min) and the supernatant was used for determination of NAD^+ and
654 NADH levels using a colorimetric assay kit (cat. no. KA1657; Abnova, Taipei City, Taiwan) and
655 microplate reader (BioTek Synergy Neo2, Agilent, Santa Clara, CA) according to the
656 manufacturer's instructions.
657
658 ***Measurement of baseline ROS levels.*** Overnight cultures were diluted into fresh TSB
659 (OD_{600}~0.05), and grown with shaking to early exponential phase (OD_{600}~0.2). 200 µL of culture
660 was removed and cell density was normalized before staining with carboxy-H2DCFDA fluorescent
661 dye (final concentration 10 µM) (Invitrogen, Waltham, MA). Samples were incubated at room
662 temperature for 5 minutes, then 800 µL of PBS + EDTA buffer (100 mM) was added to each
663 sample, and ROS levels were measured by fluorescence-based flow cytometry (BD Fortessa, BD
664 Biosciences, San Jose, CA). All tubes with cultures were wrapped with aluminum foil to avoid
665 light. A sample containing LAC wild-type cells lacking carboxy-H2DCFDA was included as a
666 control for auto-fluorescence. Forward and side scatter parameters were acquired with logarithmic
667 amplification. ROS was detected using the 488 laser and a 530/30nm bandpass filter. Data were
668 analyzed using FlowJo software version 10.8.1 (BD Biosciences, San Jose, CA).
669
670 ***Measurement of superoxide dismutase (SOD) activity.*** Overnight cultures were diluted
671 (OD_{600}~0.05) into fresh TSB, grown to late exponential phase (OD_{600}~4), diluted to OD_{600}=1,
672 centrifuged at 12,000 x g for 5 min, and the cell pellet was homogenized in 300 µL of ice-cold lysis
673 buffer (100 mM Tris-HCl pH 7.4 + 0.5% Triton + 5 mM 2-mercaptoethanol + 0.2 mM PMSF). SOD
674 activity was measured using a commercially available kit (cat. no CS0009-1KT), according to
675 manufacturers' instructions (MilliporeSigma, Burlington, MA). The experiment was repeated three
676 times with similar results.
677
678 ***RNA sequencing and data analysis.*** Overnight cultures were diluted (OD_{600}~0.05) into fresh
679 TSB medium and grown at 37 °C to early exponential phase (OD_{600}~0.5) (*Δagr* single mutant and
680 *Δagr Δrot* double mutant) or late exponential phase (OD_{600}~4) (wild-type and *Δagr* strains).
681 Samples of *Δagr* and *Δagr Δrot* were divided into two 3 mL aliquots, and the aliquots were
682 incubated at 37 °C for another 30 min, with or without treatment with H_2O_2. Peroxide
683 concentrations for *Δagr* and *Δagr Δrot* were normalized to expected killing at the time of harvest
684 (Figure 7—figure supplement 1).

685 Three independent cultures for each sample were used for determination of transcriptional
686 profiles. Briefly, cultures were concentrated by centrifugation (12,000 x g for 5 min), and
687 resuspended cells were disrupted using Lysing Matrix B tubes in a FastPrep-24 homogenizer (MP
688 Biomedicals, Irvine, CA) at 6 M/s, for 30s, 3 times (samples were resting on ice between
689 homogenizer runs), and RNAs were extracted from the collected bacterial cells using TRIzol
690 reagent (Thermo Fisher Scientific, Waltham, MA). RNA was isolated using RNeasy (Qiagen,
691 Germantown, MD) mini spin columns. Sequence libraries were generated using the TruSeq
692 Stranded Total RNA Library Prep kit (Illumina, San Diego, CA) following the manufacturer's
693 recommendations. The rRNAs were removed by the Ribo-zero Kit (Illumina) to enrich mRNA,
694 using 13 cycles of PCR amplification of the final library. Amplified libraries were purified using
695 AMPure beads (Beckman Coulter, Brea, CA), quantified by Qubit (Thermo Fisher Scientific,
696 Waltham, MA) and qPCR, and visualized in an Agilent Bioanalyzer (Santa Clara, CA). Pooled
697 libraries were sequenced as paired-end 50-bp reads using an Illumina NovaSeq instrument.
698 Reads were initially trimmed using Trimmomatic version 0.39 (54) to remove adaptors as
699 well as leading or trailing bases with a quality score less than 3, filtering reads with minimum
700 length 36. Reads were mapped to reference strain USA300 FPR3757 (RefSeq identifier
701 GCF_000013465.1) using Bowtie2 version 2.2.5 (55). Using gene annotations from the same
702 assembly, reads mapped to each gene were counted with featureCounts version 2.0.1 (56),
703 producing a counts matrix. Additional analysis was performed in R (R Core Team 2021) using the
704 package DESeq2 version 1.32 (57).
705 Normalization to account for inter-sample library size variation was performed using the
706 built-in normalization function of DESeq2. All RNA-seq heatmaps were colored according to row
707 (gene) z-scores of DESeq2 normalized counts. For differential expression testing, the Wald test
708 was used with a log-2 fold-change threshold of 0.5 and an FDR of 0.1. For simple pairwise
709 comparisons (e.g., the effect of strain under control conditions), datasets were split so that
710 analysis was performed independently for strains used in the comparison. To determine the
711 interaction between strain and condition variables, all samples were included with the
712 experimental design (formula "expression ~ condition + strain + condition:strain", where
713 condition:strain is the interaction between variables).
714
715 ***Metabolic flux prediction.*** The SPOT (Simplified Pearson cOrrelation with Transcriptomic data)
716 computational method (58) was used to analyze the difference in intracellular metabolic fluxes
717 between wild-type LAC and *agr::tetM* mutant grown in TSB to late exponential phase (OD_{600}~4).
718 SPOT is similar to the E-Flux2 method described previously (59), but a recent validation study

719 (60) shows that SPOT generally outperforms E-Flux2. SPOT infers metabolic flux distribution by
720 integrating transcriptomic data in a genome-scale metabolic model of *S. aureus* (61) that was
721 adapted for use with strain LAC. For a list of the metabolic reactions ranked by unit of flux per 100
722 units of glucose uptake flux, see Supplementary file 3.
723
724 ***Real-time qRT-PCR assays.*** Briefly, RNA was purified as described above from late exponential
725 (OD_{600}~4.0) cells, cDNAs were synthesized using Maxima First Strand cDNA Synthesis Kit (Thermo
726 Fisher Scientific, Waltham, MA), and real-time reverse transcription quantitative PCR (qRT-PCR)
727 was performed using QuantiNova™ SYBR Green PCR Kit (Qiagen, Hilden, Germany). Primers
728 were synthesized by IDT Inc. (Coralville, IA). Three independent biological samples were run in
729 triplicate and *rpoB* was used to normalize gene expression. $2^{-\Delta\Delta Ct}$ method was used to calculate the
730 relative fold gene expression (62).
731
732 ***Peritoneal infection of mice.*** C57BL/6 mice and C57BL/6 *Cybb*$^{-/-}$ (also known as
733 gp91phox/nox2) were purchased from the Jackson Laboratory and bred onsite to generate
734 animals for experimentation. Age and gender-matched, 8–10-week-old mice were used. *S. aureus*
735 strains harboring *RNAIII* or *agrBD* deletion in the NCTC 8325 background were grown overnight
736 in TSB (37°C, 180 rpm) separately or mixed at a 1:1 ratio. Overnight cultures were diluted
737 (OD_{600}~0.05) into fresh TSB medium (subcultured separately for the cultures mixed overnight
738 ('primed') or mixed 1:1 for *RNAIII* or *agrBD* mutant single cultures ('unprimed') and grown at 37°C
739 to early exponential phase (OD_{600}~0.5)). Bacteria were washed one time by centrifugation with
740 PBS and adjusted to 10^9 CFU/mL. Twenty C57BL/6 WT mice and 17 *Cybb*$^{-/-}$ mice were injected
741 intraperitoneally with 100 µL of either 'primed' or 'unprimed' inoculum. After 2 h, internal organs,
742 peritoneal lavage and blood were collected. The organs were homogenized in sterile PBS and
743 serial dilutions were plated for viable counts on TS agar. Collected blood was lysed with saponin
744 and plated for viable counts on TSA plates. Peritoneal lavage fluid was serially diluted and plated
745 for viable counts. All animal studies were performed as per an NYU Grossman School of Medicine
746 Institutional Animal Care and Use Committee (IACUC) approved protocol to the Shopsin Lab.
747
748 ***Statistical analysis.*** Prism software (GraphPad, Inc.) was used to perform statistical analyses.
749 Statistical significance was determined using the Student's *t* test, Mann–Whitney *U* test, one-way
750 analysis of variance (ANOVA), or the Kruskal-Wallis test, depending on the data type. Statistical
751 significance was considered to be represented by *P* values of <0.05.
752

753 ***Data availability***. The RNA-seq data are available through the NCBI GEO repository using the
754 accession number GSE207045.
755
756

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Potential competing interests. B.S. has consulted for Basilea Pharmaceutica. V.J.T. has received honoraria from Pfizer and MedImmune, and is an inventor on patents and patent applications filed by New York University, which are currently under commercial license to Janssen Biotech Inc. Janssen Biotech Inc. provides research funding and other payments associated with a licensing agreement. All other authors: no competing interests declared.

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1022 **Figures and Figure supplements**
1023

1024 Quorum-sensing *agr* system of *Staphylococcus aureus* primes gene expression for protection from
1025 lethal oxidative stress
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1027 **Magdalena Podkowik *et al*.**
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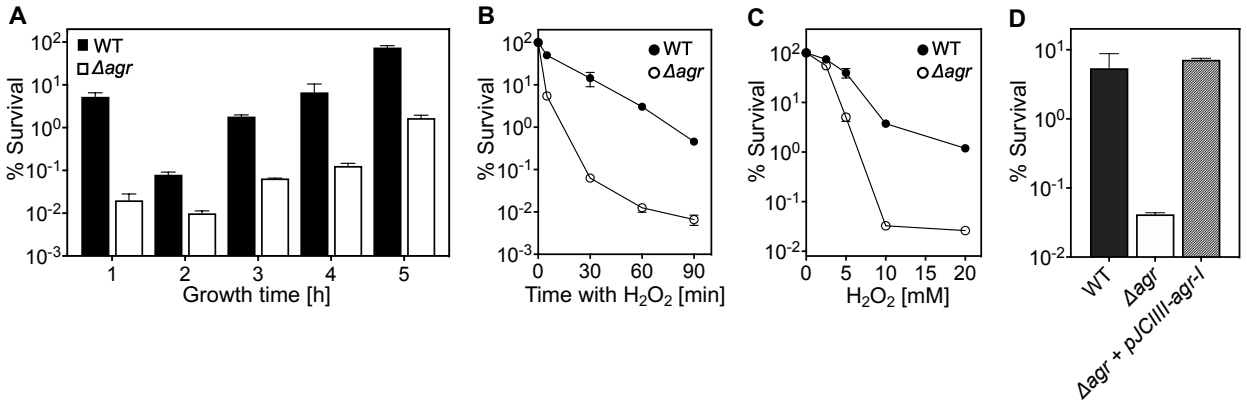
1031 **Figure legends**

Fig. 1.



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1033 **Figure 1. *agr* protects from killing by H_2O_2 throughout the growth cycle.** (A) Effect of culture
1034 growth phase. Overnight cultures of *S. aureus* LAC wild-type (WT, BS819) or *Δagr* (BS1348) were
1035 diluted (OD_{600}~0.05) into fresh TSB medium and grown with shaking from early exponential (1 h,
1036 OD_{600}~0.15) through late log (5 h, OD_{600}~4) phase. At the indicated times, early (undiluted) and
1037 late exponential phase cultures (diluted into fresh TSB medium to OD_{600}~0.15) were treated with
1038 H_2O_2 (20 mM). After 60 min, aliquots were removed, serially diluted, and plated for determination of
1039 viable counts. Percent survival was calculated relative to a sample taken at the time of H_2O_2
1040 addition. (B) Kinetics of killing by H_2O_2. Wild-type and *Δagr* mutant strains were grown to early
1041 exponential (OD_{600}~0.15) and treated with 20 mM H_2O_2 for the times indicated, and percent survival
1042 was determined by plating. (C) Effect of H_2O_2 concentration on survival. Cultures prepared as in
1043 panel B were treated with the indicated peroxide concentrations for 60 min prior to plating and
1044 determination of percent survival. (D) Complementation of *agr* deletion mutation. Cultures of wild-
1045 type (WT) cells (BS819), Δ*agr* mutant (BS1348), and complemented Δ*agr* mutant carrying a
1046 chromosomally integrated wild-type operon (pJC1111-*agrI*) were treated with 20 mM H_2O_2 for 60
1047 min followed by plating to determine percent survival. Data represent the means ± S.D. from
1048 biological replicates (*n* = 3).

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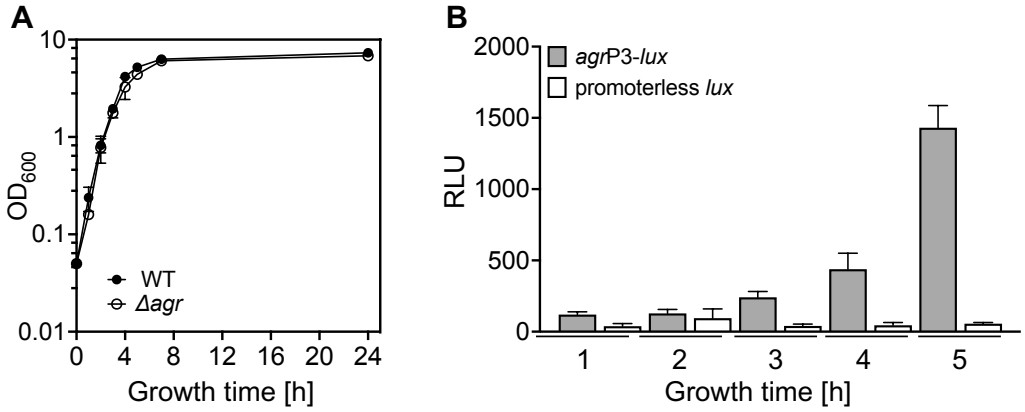

1050

1051 **Figure 1—figure supplement 1. Correlation of growth phase and *agr* expression.** (A) Growth
1052 curves. Overnight cultures of *S. aureus* LAC wild-type (WT, BS819) or *Δagr* mutant (BS1348) were
1053 diluted (OD_{600}~0.05) in fresh TSB medium and growth was monitored by measuring the optical
1054 density at 600 nm (OD_{600}). (B) Tests of *agr*P3 promoter activity. *S. aureus* LAC wild-type (WT,
1055 BS819) containing *agr*P3-*lux* (SaPI1 *att*C::*agr*P3-*lux*; strain BS1222) or control containing a
1056 promoterless *lux* gene within the *att*C site (SaPI1 *att*C::pGYLux, strain BS999) grown as in (A) for
1057 the indicated times. *agr*P3 activity (relative luminescence units [RLU]) was assayed at the indicated
1058 times (see Materials and Methods). Data represent the means ± S.D. from biological replicates (*n*
1059 = 3).

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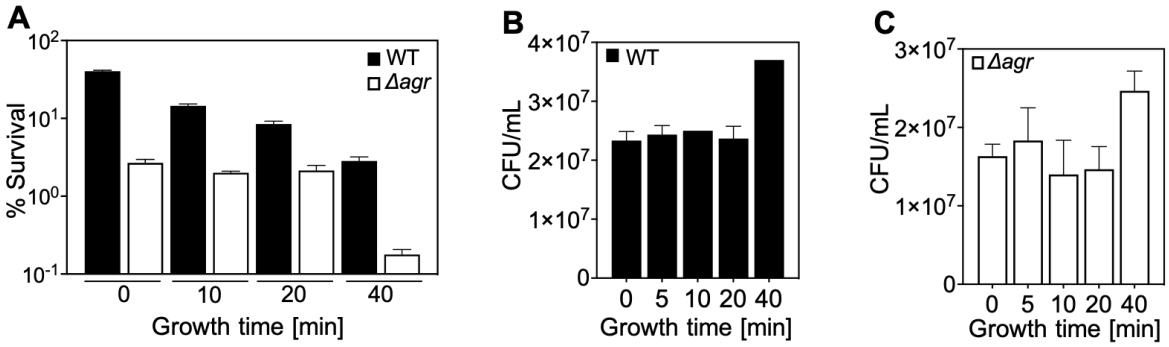

1061

1062 **Figure 1—figure supplement 2. Correlation of lag-time and *agr*-mediated protection from**
1063 **H_2O_2-mediated killing.**

1064 Overnight cultures of *S. aureus* LAC wild-type (WT, BS819) and *Δagr* mutant (BS1348), grown for
1065 the indicated times following dilution to fresh medium, were treated with H_2O_2 (20 mM for 60 min)
1066 (Fig. S1A). Data represent the means ± S.D. from biological replicates (*n* = 3).

1067 Survival of Δ*agr* mutant cells was unchanged up to the 40 min time point, and then it dropped
1068 sharply. The sharp drop coincided with the time to first division (i.e., the lag time), as evidenced by
1069 an increase in colony-forming units (CFUs) at the 40 min time point in the absence of treatment
1070 (Fig. S1B and C). In contrast to results with the Δ*agr* strain, survival of the wild-type strain gradually
1071 decreased throughout the experiment (Fig. S1A). Increased lag-time is associated with tolerance
1072 to lethal stress owing to a delay in growth when switched to a new environment (15). Thus, our
1073 observations suggest that a subpopulation of Δ*agr* mutant cells remains longer in a dormant state,
1074 decreasing the lethality of H_2O_2. The differential effect of the lag time on the wild-type and Δ*agr*
1075 mutant cultures was absent during exponential growth (40 min). These results suggest that *agr*
1076 contributes to at least two forms of protection from H_2O_2-mediated killing: tolerance by a transient
1077 lag state and tolerance during growth phase. To focus on the latter form, assays involving cultures
1078 after overnight growth were grown for ~65 min (OD_{600}~0.15).

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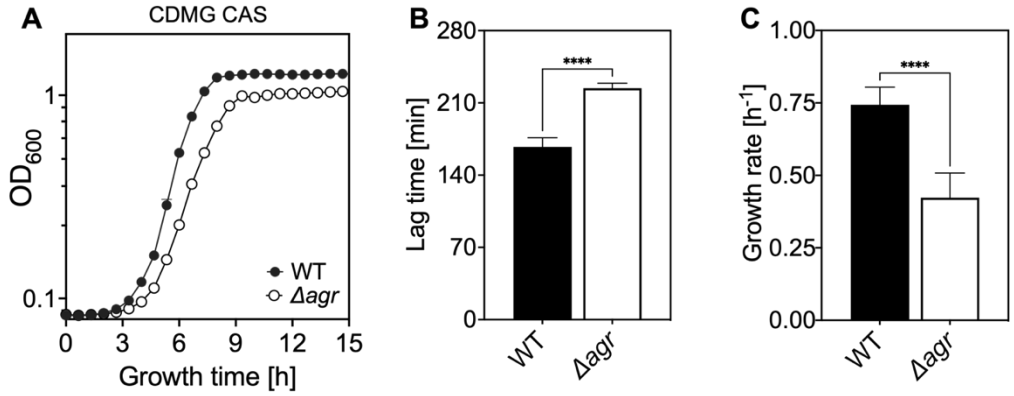
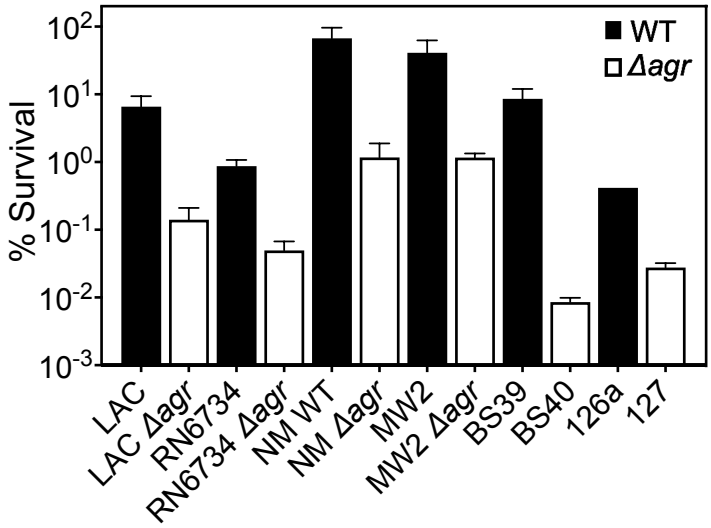


Figure 1—figure supplement 3. Extended lag phase and decreased growth rate and yield of an Δ*agr* mutant. (A) Growth curves. *S. aureus* LAC wild-type (WT, BS819) and *Δagr* mutant (BS1348) cultures were grown in chemically-defined medium supplemented with 0.5% Casamino acids and 14 mM glucose (CDMG CAS) for the indicated times following 1,000-fold dilution of overnight cultures grown in TSB. Growth of diluted cultures was monitored for 15 hours every 40 minutes by measuring the OD_{600} using an Agilent LogPhase 600 Microbiology Reader (Santa Clara, CA). (B) Lag times. Data in panel A were used to determine lag times by extrapolation of the linear portion of the growth curve. Growth rates (μ, h^{-1}) calculated from five biological replicates are displayed in panel (B). Data are means ± S.D. Statistical significance was calculated with Student's two-tailed *t* test (****, $P \leq 0.0001$).

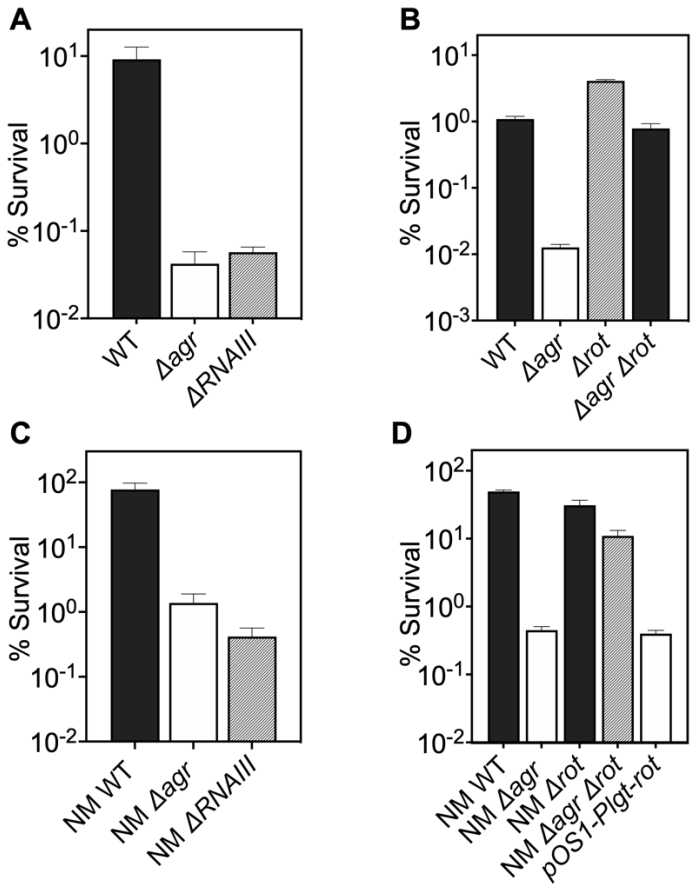


1093

1094 **Figure 1—figure supplement 4. *Agr*-mediated protection from H_2O_2-mediated killing among**
1095 **diverse *S. aureus* strains**. Laboratory strains LAC, RN6734, Newman (NM, BS12), MW2 (BS450),
1096 and clinical isolates BS39 and 126a with *agr* deficient mutant derivatives were compared for survival
1097 following treatment with 20 mM of H_2O_2 for 60 min. In this experiment overnight cultures were diluted
1098 in TSB and grown to early log phase (OD_{600}~0.15). Percent survival was determined relative to
1099 samples taken at the time of peroxide treatment. Some mutants were created by transduction of
1100 marker-disrupted alleles (LAC, RN6734, Newman, MW2) while others were naturally occurring
1101 (BS40, 127) (see Table 1). Data represent the means ± S.D. from biological replicates (n = 3).
1102 The data show that peroxide lethality varies among strains, but in each case deletion of *agr*
1103 increases killing.

1104

Fig. 2.



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1107 **Figure 2. Involvement of *RNAIII* and *rot*-dependent pathways in *agr*-mediated protection from**
1108 **H_2O_2-mediated killing.** Cultures were grown for 1 h following dilution from overnight cultures to
1109 early log phase (OD_{600}~0.15) and then treated with 20 mM H_2O_2 for 60 min before determination of
1110 percent survival by plating and enumeration of colonies. (A) Wild-type LAC (WT, BS819), *Δagr*
1111 mutant (BS1348), and *ΔrnaIII* mutant (GAW183). (B) *Δrot* and *Δagr Δrot* double mutant (BS1302).
1112 (C) Wild-type (WT) strain Newman (NM, BS12), *Δagr* mutant (BS13), and *ΔRNAIII* mutant (BS669).
1113 (D) Overexpression of *rot*. Rot was expressed from a plasmid-borne wild-type *rot* (pOS1-*Plgt-rot*,
1114 strain VJT14.28). Data represent the means ± S.D. from biological replicates (*n* = 3).

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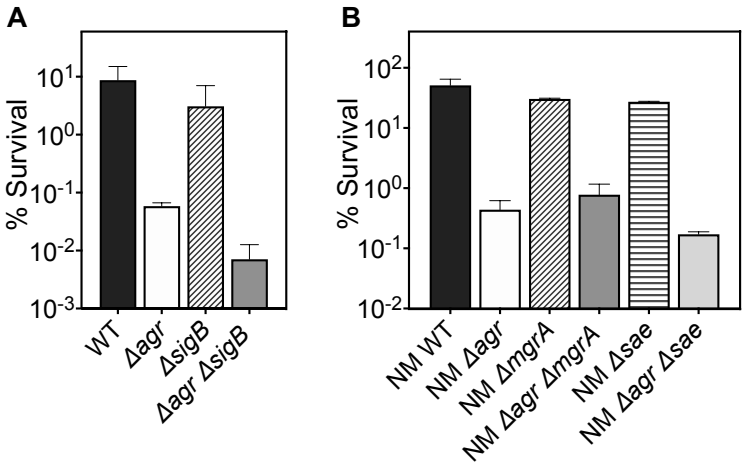

1118

1119 **Figure 2—figure supplement 1. Deficiency of downstream global regulators does not**
1120 **differentially affect *agr*-mediated protection from H_2O_2-mediated cell death**. The effect of (A)
1121 *sigB*, (B) *mgrA* and *sae* on survival in the presence or absence of *agr* during treatment with H_2O_2
1122 was measured. Cells were grown to early log phase (OD_{600}~0.15) and treated with 20 mM of H_2O_2
1123 for 60 min. Data represent the means ± S.D. from biological replicates (*n* = 3). Bacterial strains were
1124 BS819 (LAC) and BS12 (Newman, NM) for WT and BS1348 (LAC) and BS13 (NM) for the *agr*, and
1125 BS BS1435-36, BS1280, BS1282 and BS1246, BS1518 for *sigB*, *sae* and *mgrA* mutants,
1126 respectively.

1127 The genes tested were either part of known two-component systems or SarA protein-family
1128 regulatory circuits involved in virulence gene expression. They are all downstream/epistatic to *agr*-
1129 RNAIII (reviewed in (17)). Mutations in *sigB*, *mgrA*, and *sae* showed little or no effect with respect
1130 to the protective *agr*-mediated phenotype. These results support the idea that *rot* is the primary
1131 regulator pathway that protects the wild-type from H_2O_2-mediated killing.

Fig. 3.

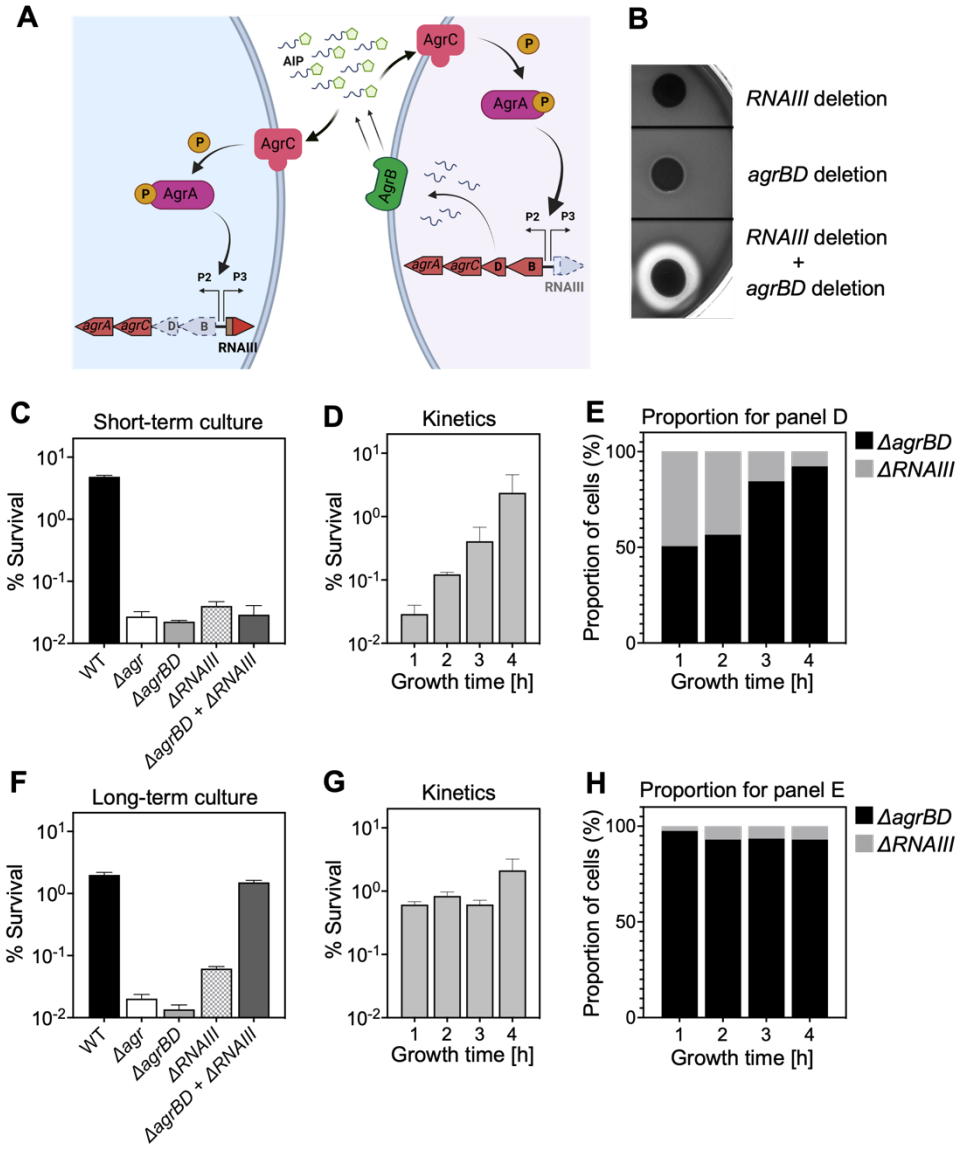

Figure 3. *agr*-mediated protection from H_2O_2 stress is uncoupled from *agr* activation kinetics. (A) Assay design. An Δ*agrBD* deletion mutant (GAW130) was complemented *in trans* by the autoinducing product (AIP) of AgrBD in an *ΔrnaIII* (GAW183) mutant that produces AIP endogenously; AgrC activation in the Δ*agrBD* strain leads to downstream activation of RNAIII*. The agrBD* strain, engineered in-frame to avoid polar effects on downstream genes *agrC* and *agrA*, senses but does not produce autoinducer. The Δ*rnaIII* mutant, constructed by replacement of *rnaIII* with a cadmium resistance cassette (*rnaIII*::*cadA*), produces autoinducer but lacks RNAIII, the effector molecule of *agr*-mediated phenotypes with respect to H_2O_2. The image was created using BioRender (BioRender.com). (B) Trans-activation demonstrated by hemolysin activity on sheep blood agar plates. Bottom of figure shows zone of clearing (hemolysin activity) after mixing 10^8

1143 Δ*agrBD* CFU with an equal number of Δ*rnaIII.* Zone of clearance is a consequence of *AgrC* receptor
1144 activation *in trans* by AIP produced by the Δ*rnaIII* mutant. (C) Absence of trans-activation with short-
1145 term culture. The wild-type strain RN6734 (WT, BS435), Δ*rnaIII* (GAW183), Δ*agrBD* (GAW130),
1146 and Δ*rnaIII* and Δ*agrBD* mutants were mixed 1:1 immediately before growth from overnight culture.
1147 Overnight cultures were diluted (OD_{600}~0.05) into fresh TSB medium, mixed, and grown to early
1148 log phase (OD_{600}~0.15) when they were treated with 20 mM H_2O_2 for 60 min and assayed for
1149 percent survival by plating. (D) Kinetics of killing by H_2O_2. Survival assays employing *ΔrnaIII* and
1150 *ΔagrBD* mixtures, performed as in panel C, but grown from early exponential (1 h, OD_{600}~0.15)
1151 through late log (5 h, OD_{600}~4) phase in TSB. Cultures were treated with H_2O_2 (20 mM for 1 h) at
1152 the indicated time points. (E) Proportion of mixed population for panel D represented by each
1153 mutant after incubation. The Δ*agrBD* mutant contained an erythromycin-resistance marker to
1154 distinguish the strains following plating of serial dilutions on TS agar with or without erythromycin
1155 (5 µg/ml). Data represent the means ± S.D. from biological replicates (*n* = 3). (F) Trans-activation
1156 during long-term culture. The wild-type strain RN6734 (WT, BS435), Δ*rnaIII* (strain GAW183),
1157 Δ*agrBD* (strain GAW130), and Δ*rnaIII* and Δ*agrBD* mutants mixed 1:1 prior to overnight culture.
1158 Survival assays employing Δ*rnaIII* and Δ*agrBD* mixtures, performed as in panel C. (G) Kinetics of
1159 killing by H_2O_2. Survival assays employing Δ*rnaIII* and Δ*agrBD* mixtures, performed as in panel D.
1160 Cultures were treated with H_2O_2 (20 mM for 1 h) at the indicated time points. (H) Proportion of mixed
1161 population for panel G represented by each mutant after incubation, performed as in panel E. Data
1162 represent the means ± S.D. from biological replicates (*n* = 3).
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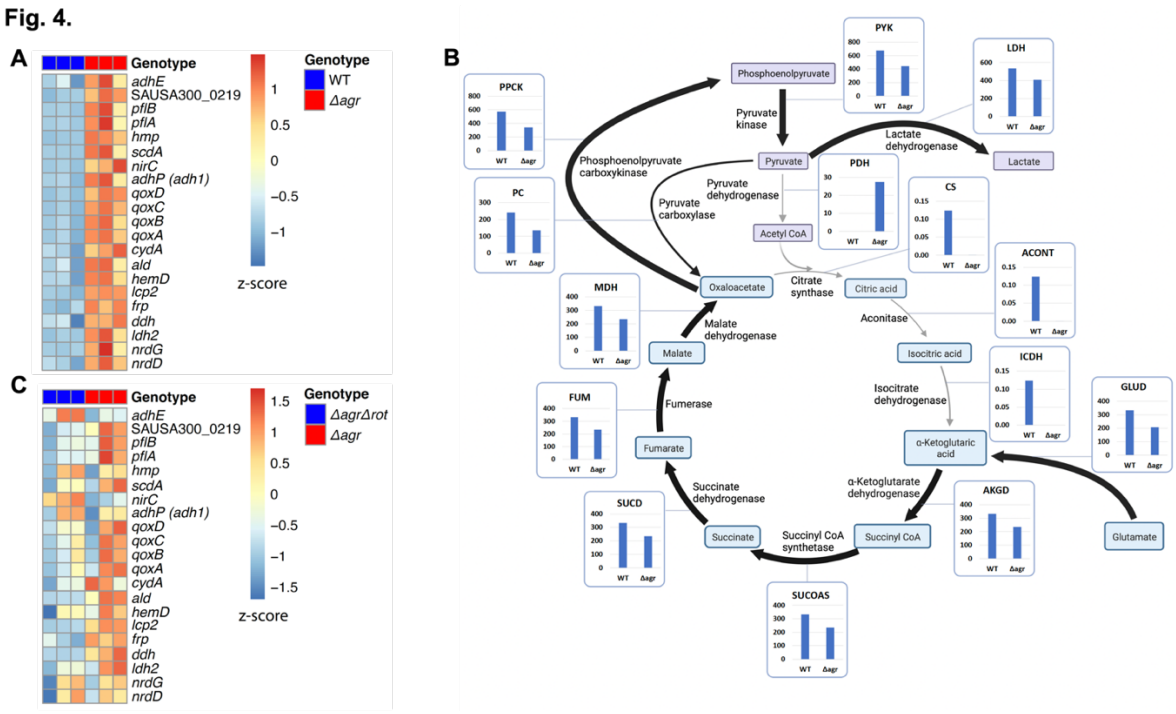
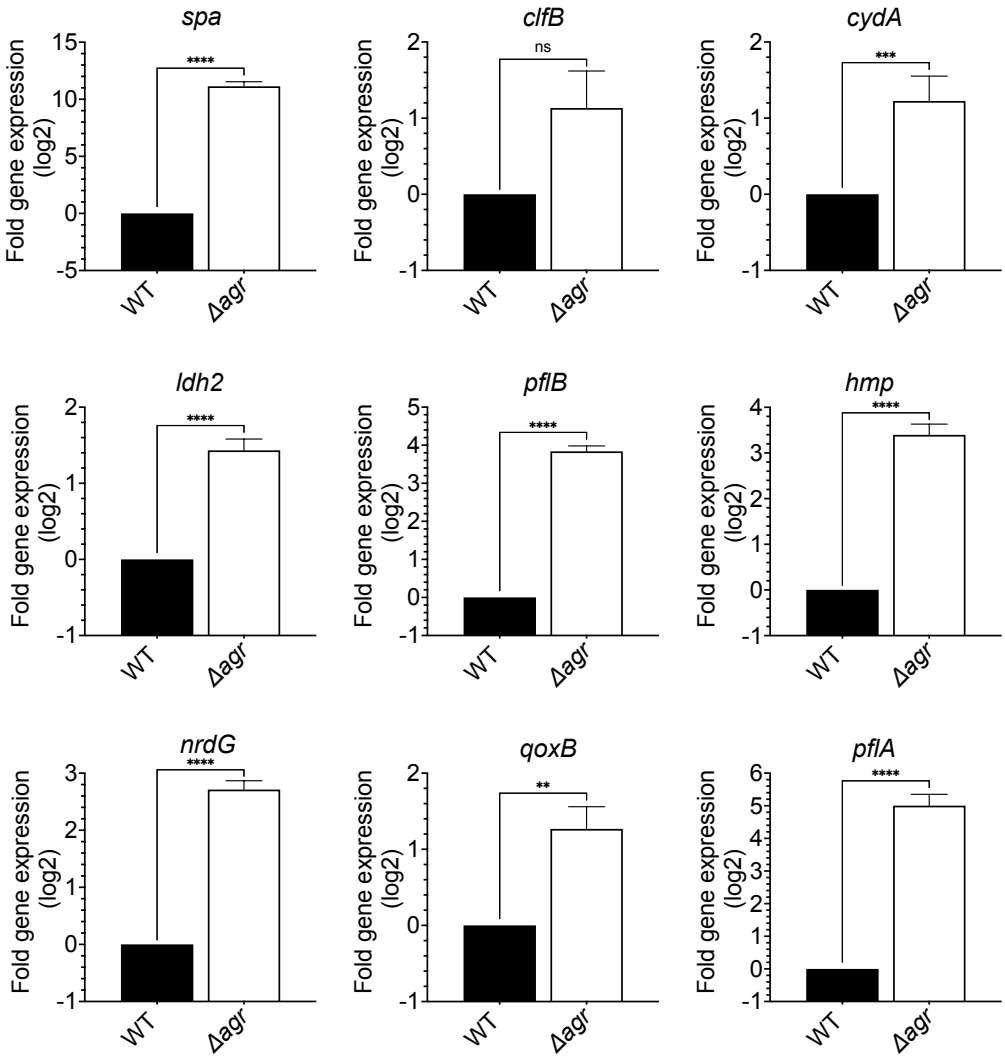


Figure 4. Association of *agr* deficiency with increased expression of respiration and fermentation genes during aerobic growth. (A) Relative expression of respiration and fermentation genes. RNA-seq comparison of *S. aureus* LAC wild-type (WT, BS819) and Δ*agr* mutant (BS1348) grown to late exponential phase (OD_{600}~4.0). Shown are significantly up-regulated genes in the Δ*agr* mutant (normalized expression values are at least twofold higher than in the wild-type). Heatmap colors indicate expression z-scores. RNA-seq data are from three independent cultures. See Supplementary file 1 for supporting information. (B) Schematic representation of *agr*-induced changes in metabolic flux, inferred from transcriptomic data (Supplementary file 1) by SPOT (Simplified Pearson correlation with Transcriptomic data). Metabolic intermediates and enzymes involved in catalyzing reactions are shown. The magnitude of the flux (units per 100 units of glucose uptake flux) is denoted by arrowhead thickness. Boxed charts indicate relative flux activity levels in wild-type versus Δ*agr* strains. Enzyme names are linked to abbreviations in boxed charts (e.g., lactate dehydrogenase, LDH). See Supplementary file 2 for supporting information. (C) RNA-seq comparison of an Δ*agr* Δ*rot* double mutant (BS1302) with its parental Δ*agr* strain (BS1348). Heatmap colors indicate expression z-scores. Sample preparation and figure labelling as for Figure 4A. See Supplementary file 3 for supporting information.



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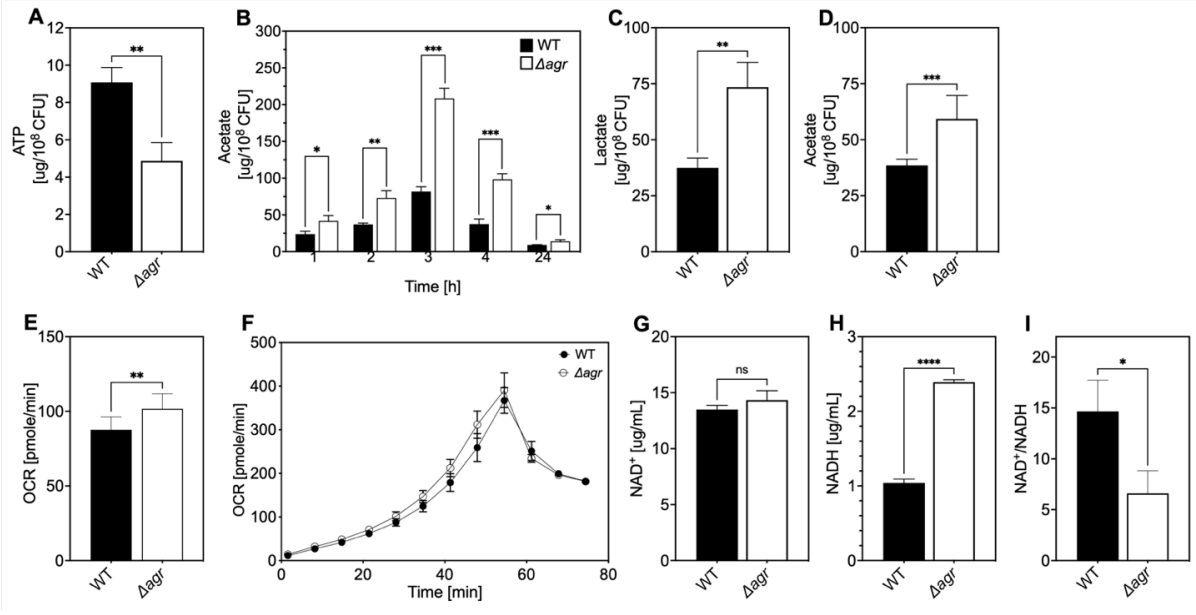
1184 **Figure 4—figure supplement 1**. **Induction of expression of selected fermentive/anaerobic**
1185 **genes stimulated by deletion of *agr***. Total cellular RNA was extracted from late exponential phase
1186 cultures (OD_{600}~4.0) of wild-type (WT, BS819) or Δ*agr* mutant (BS1348), followed by reverse
1187 transcription and PCR amplification of the indicated genes from Fig. 5A, using *rpoB* as an internal
1188 standard. mRNA levels were normalized to those of each gene with an untreated wild-type control.
1189 Data represent the mean ± SEM of three independent experiments. Student's t test was used to
1190 determine statistical differences between samples (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).
1191 In each case the *agr* deletion increased expression, indicating elevated metabolism.
1192

Fig. 5.



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1194 **Fig. 5. Association of *agr* deficiency with a metabolic flux shift toward fermentive metabolism**
1195 **during aerobic growth.** (A) Intracellular ATP levels. Comparison of *S. aureus* LAC wild-type (WT,
1196 BS819) and Δ*agr* mutant (BS1348) strains for ATP expressed as μg/10^8 cells after growth of cultures
1197 in TSB medium to late-exponential phase (OD_{600}~4.0). (B) Extracellular acetate levels. Samples
1198 were taken after 1, 2, 3, 4, and 24 h of growth in TSB medium; strains were wild-type (WT, BS819)
1199 and Δ*agr* mutant (BS1348). (C-D) Extracellular lactate and acetate levels during low oxygen culture.
1200 *S. aureus* LAC wild-type (WT, BS819) and Δ*agr* mutant (BS1348) were grown in TSB medium with
1201 suboptimal aeration to late-exponential phase (4 h, OD_{600}~4.0). (E-F) Oxygen consumption. Strains
1202 LAC wild-type (WT, BS819) and Δ*agr* mutant (BS1348) were compared using Seahorse XFp
1203 analyzer (F), and the rate of oxygen consumption (E) was determined from the linear portion of the
1204 consumption curve. Representative experiments from at least 3 independent assays are shown.
1205 (G-H) NAD^+ and NADH levels. Colorimetric assay of NAD^+ (G) and NADH levels (H) for *S. aureus*
1206 wild-type (WT, BS819) and Δ*agr* mutant (BS1348) after growth of cultures to late-exponential phase
1207 (OD_{600}~4.0). (I) NAD^+/NADH ratio. For all panels, data points are the mean value ± SD (n = 3). *P
1208 < 0.05; ****P < 0.0001, by Student's two-tailed t test. Seahorse statistical significances are
1209 compared to TSB medium.

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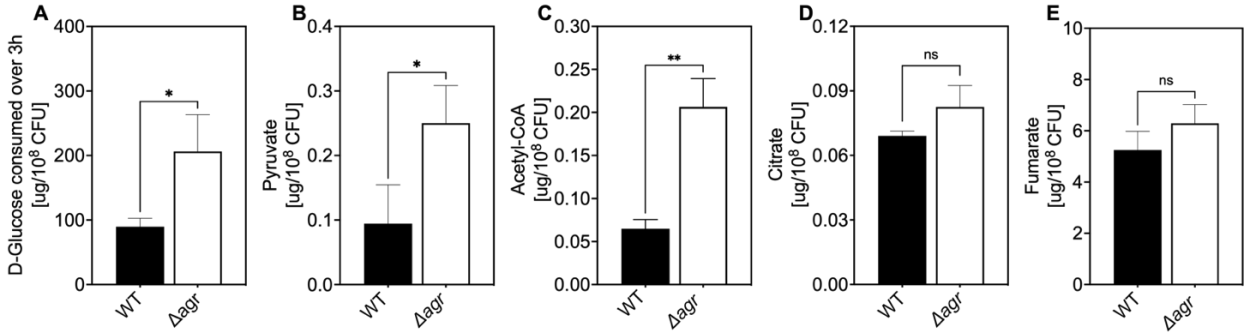

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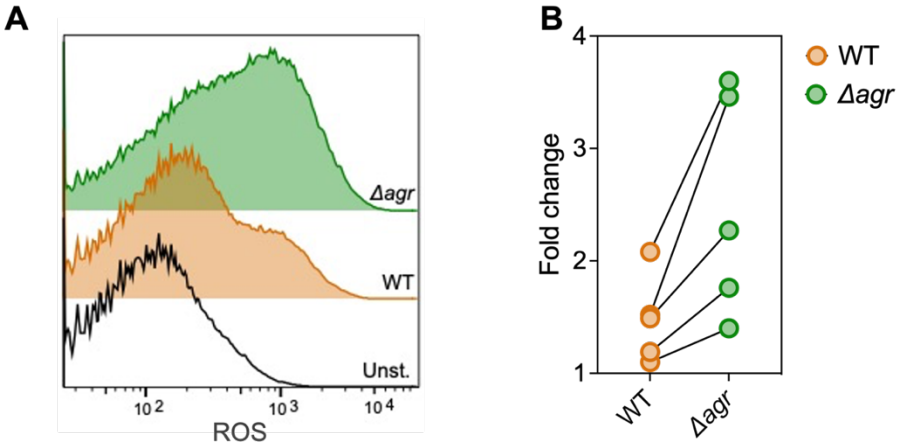
1216 **Figure 5—figure supplement 1. Association of *agr* deficiency with glucose consumption and**
1217 **intracellular levels of pyruvate, acetyl-CoA, and TCA-cycle metabolites.** (A) Extracellular
1218 glucose levels. D-glucose levels per expressed as expressed as μg/10^8 cells of *S. aureus* LAC
1219 wild-type (WT, BS819) or Δ*agr* mutant (BS1348) after growth of cultures in TSB medium to
1220 exponential phase (3 h). (B-E) Intracellular levels of pyruvate, acetyl-CoA, and TCA-cycle
1221 metabolites citrate and fumarate. Levels of the indicated metabolite expressed as expressed as
1222 μg/10^8 cells of *S. aureus* LAC wild-type (WT, BS819) or Δ*agr* mutant (BS1348) after growth in TSB
1223 medium to late-exponential phase (OD_{600}~4.0). For all panels, data points are the mean value ± SD
1224 (n = 3). *P < 0.05; ****P < 0.0001, by Student's two-tailed t test.

Fig. 6.



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1226 **Figure 6. Increase in ROS levels associated with Δ*agr* deficiency.** Flow cytometry
1227 measurements. *S. aureus* LAC wild-type (WT, BS819) and *Δagr* mutant (BS1348) were grown
1228 overnight, diluted, cultured in TSB medium for 1 h, and treated with carboxy-H2DCFDA (10 μM) for
1229 5 min. Relative cell number is on the vertical axis. Unst. indicates samples containing LAC wild-
1230 type cells not treated with carboxy-H2DCFDA. (B) Five replicate experiments gave similar results
1231 ("fold change" indicates the mean wild-type or *Δagr* ROS level divided by the mean
1232 autofluorescence background signal; lines connect results in replicate experiments).

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Fig. 7.

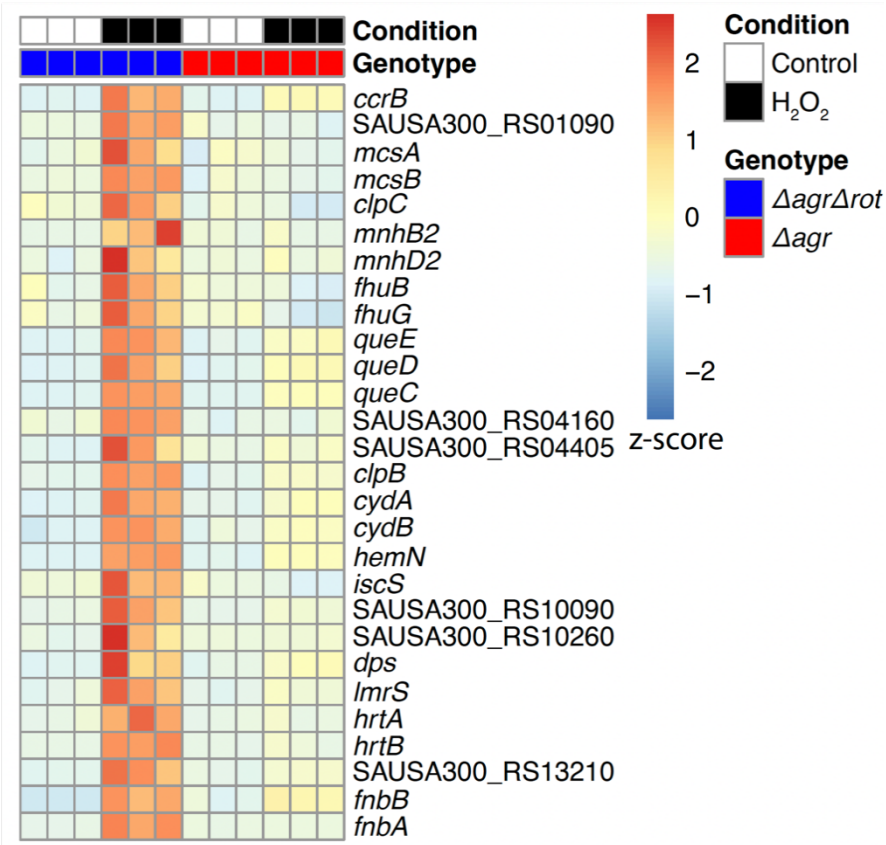

Figure 7. Rot-mediated up-regulation of H_2O_2-stimulated genes relative to those in an *agr* mutant. Genes shown are those up-regulated in a *Δagr Δrot* double mutant (BS1302) relative to that observed with the *Δagr* strain (BS1348). H_2O_2 treatment was for 30 min. Peroxide concentrations for *Δagr* (2.5 mM H_2O_2) and *Δagr Δrot* (10 mM H_2O_2) were determined to achieve ~50% cell survival [see Methods and Figure 7—figure supplement 1]). RNA-seq data are from three independent cultures. Heatmap colors indicate expression z-scores. See Supplementary file 3 for supporting information.

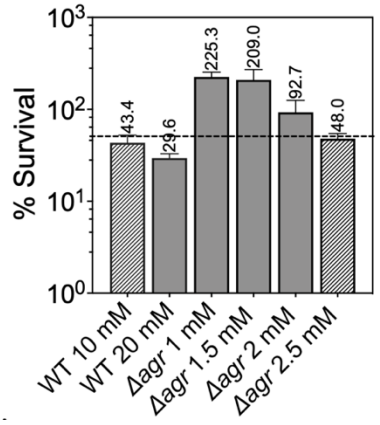

Figure 7—figure supplement 1. Normalization of the lethal concentration of H_2O_2 with wild-type and Δ*agr* strains. Overnight cultures of were diluted into fresh TSB medium and grown to early log phase (OD_{600} = 0.15 to achieve sufficient CFU for RNA-seq). These cultures were treated with the indicated concentrations of H_2O_2 for 30 min prior to measurement of survival by plating. Data represent the means ± S.D. from biological replicates (*n* = 3). Bacterial strains were BS819 for WT and BS1348 for the *agr* mutant.

To focus RNA-seq analysis on lethal rather than cell death responses, we sought to reduce H_2O_2 concentrations and thereby lethality to achieve ~50% (dotted line) cell survival, normalized to wild-type and Δ*agr* mutant strains. Survival of the Δ*agr* mutant with H_2O_2 for 30 min at a concentration of 2.5 mM closely approximated 50% survival of the wild-type with 10 mM H_2O_2, providing a basis for choice of concentrations and treatment time for RNA-seq analysis.

Fig. 8.

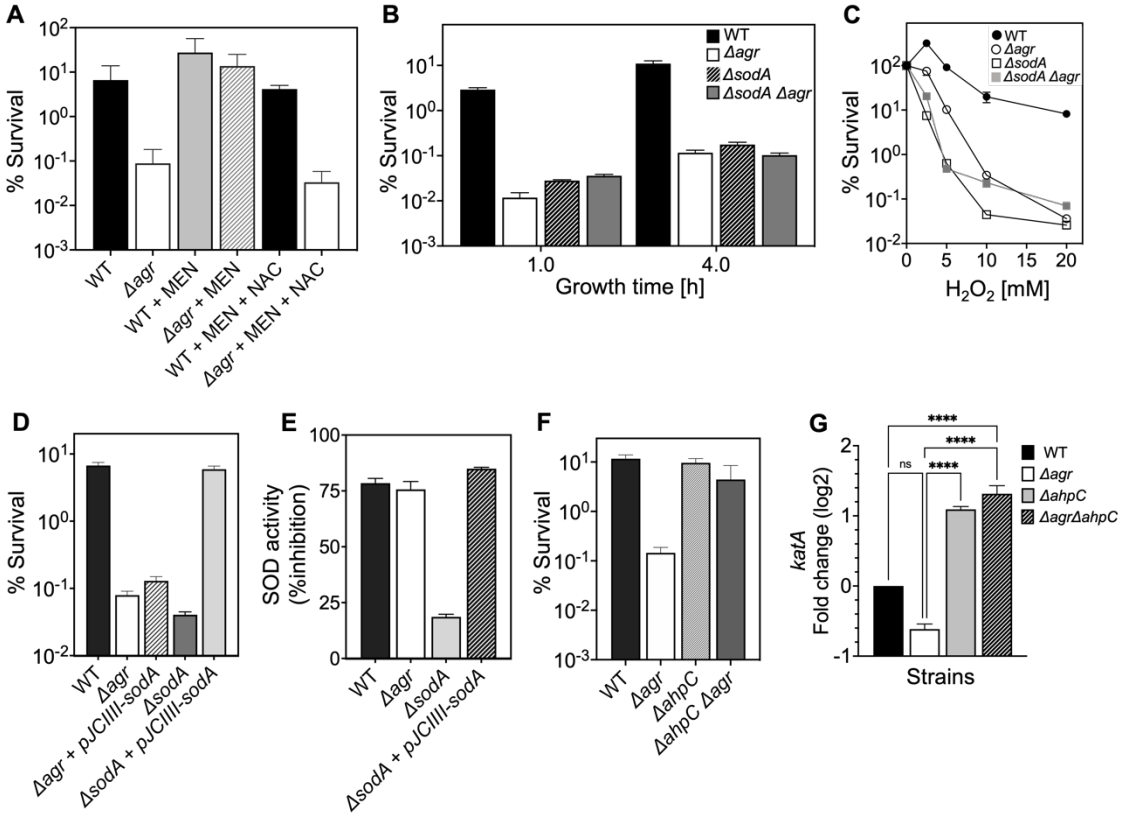

Figure 8. Involvement of endogenous ROS in *agr*-mediated protection from lethal H_2O_2 stress. (A) Protective effect of menadione on survival. *S. aureus* LAC wild type (BS819) and Δ*agr* mutant (BS1348) cultures were grown to late exponential phase (4 h after dilution of overnight cultures), exposed to 80 μM menadione (MD) with or without 4 mM N-acetyl cysteine (NAC) for 30 min prior to treatment with H_2O_2 (20 mM for 1 h) and measurement of survival. (B) Effect of *sodA* deletion on survival. Cultures of wild-type (BS819), Δ*agr* (BS1348), a *sodA::tetM* (BS1422)*, and sodA::tetM-agr* double mutant (BS1423) were grown to early (1 h after dilution, OD_{600}~0.15) or late log (4 h after dilution, OD_{600}~4.0) prior to treatment with 20 mM H_2O_2 for 60 min. (C) Effect of H_2O_2 concentration on survival. Late log (4 h, OD_{600}~4.0) cultures of the wild-type and Δ*agr* mutant strains were treated with indicated concentrations of H_2O_2 for 60 min. (D) Complementation of *sodA* deletion mutation. A plasmid-borne wild-type *sodA* gene was expressed under control of the *sarA* constitutive promoter (pJC1111-*sodA*) in late log-phase (4 h, OD_{600}~4.0) cells treated with 20 mM H_2O_2 for 60 min. (E) SodA activity. Wild-type or the indicated mutants were grown to late-exponential phase (OD_{600}~4.0); Sod activity was measured as in Methods. (F) Effect of *ahpC* deletion on survival. Late log-phase cultures of wild-type (BS819), *Δagr* (BS1348), *ahpC::bursa* (BS1486), and *ΔahpC::bursa-agr* double-mutant (BS1487) cells were treated with 20 mM H_2O_2 for 60 min. (G) Effect of *ahpC* deletion on expression of *katA* in the indicated mutants. Total cellular RNA was

1295 extracted from late exponential-phase cultures (OD_{600}~4.0), followed by reverse transcription and
1296 PCR amplification of the indicated genes, using *rpoB* as an internal standard. mRNA levels were
1297 normalized to those of each gene to wild-type control. Data represent the means ± S.D. from (n =
1298 3) biological replicates. One-way ANOVA was used to determine statistical differences between
1299 samples (****P < 0.0001).
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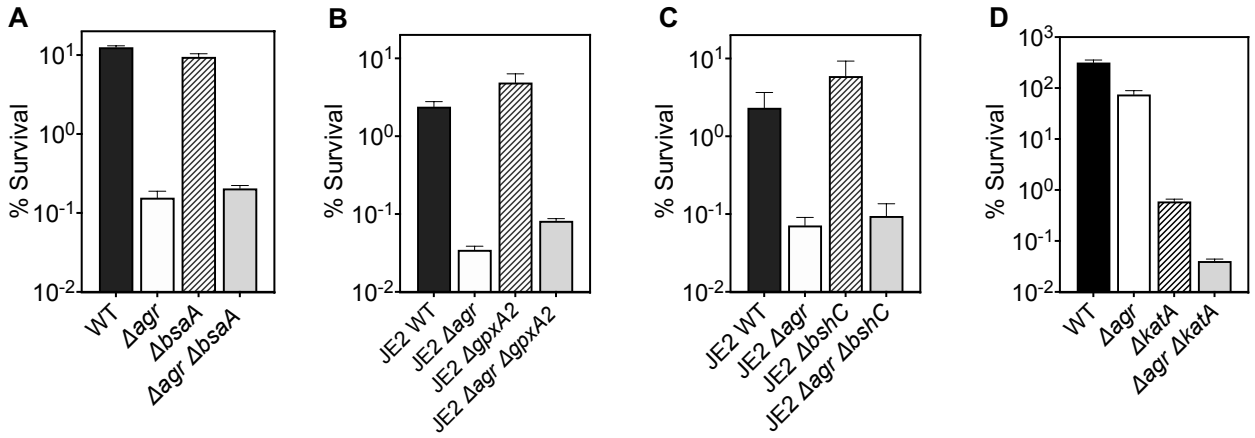
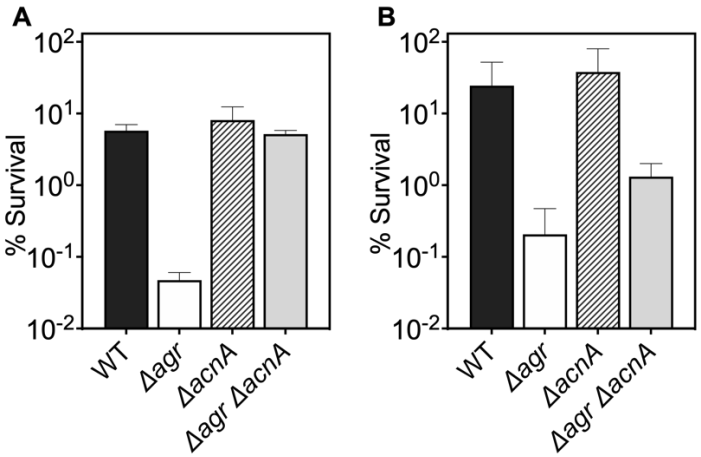


Figure 8—figure supplement 1. **Deficiency in ROS detoxification genes (*katA, bsaA1/gpxA1, bsaA2/gpxA2*, and bacilliothiol (BSH) have no effect on *agr*-mediated protection from H_2O_2-mediated cell death**. Effect of (A) *bsaA* (B) *gpxA2*, (C) *bshC* and (D) *katA* on survival during treatment with H_2O_2. Cells were grown to early log phase (OD_{600}~0.15) and treated with 20 mM of H_2O_2 for 60 min for A-C or with 2 mM of H_2O_2 for 60 min for D. Data represent the means ± S.D. from biological replicates (*n* = 3). Bacterial strains were BS819 (LAC) and BS867 (LAC) for WT, and BS1348 (LAC), BS1010 (JE2), BS1490-91, BS1522-23, BS1527-28 and BS1488-89 for the *agr*, *bsaA*, *gpxA2*, *bshC*, and *katA* mutants, respectively.

Our data with superoxide dismutases (*sodA*) and the peroxiredoxin *ahpC* (Fig. 7) suggest that homeostatic detoxification pathways contribute to *agr*-mediated phenotypes with respect to lethal H_2O_2 stress. Mutations in additional genes involved in H_2O_2 detoxification that included catalase, (*katA*), two thiol-dependent peroxidases (*gpxA1* and *gpxA2*), and the low-molecular-weight thiol bacillithiol (*bshC*) showed no differential effect with respect to *agr*-mediated phenotypes. Notably, *gpxA1,* which is also known as *bsaA1,* was essential for the oxidation-sensing ability of AgrA to confer resistance to H_2O_2-mediated growth inhibition (7). The Δ*katA* mutation was hyperlethal with the wild-type and Δ*agr* mutant, even when otherwise sub-inhibitory concentrations of H_2O_2 were used. Collectively, the data support the idea that *agr*-mediated phenotypes are detoxification pathway-specific.



1320
1321 **Figure 8—figure supplement 2. Deficiency in TCA cycle gene *acnA* reverses the effect of an**
1322 ***agr* deficiency with respect to subsequent challenge with H_2O_2.** (A-B) Effect of *acnA* on survival
1323 during treatment with H_2O_2. Cells were grown to (A) late (OD_{600}~4.0) or (B) early (OD_{600}~0.15) log
1324 phase and treated with 20 mM of H_2O_2 for 60 min. Bacterial strains were *S. aureus* LAC wild-type
1325 (WT, BS819), Δ*agr* mutant (BS1348), *acnA::bursa* (BS1744), and *acnA::bursa-Δagr* double mutant
1326 (BS1745). Data represent the means ± S.D. from biological replicates (*n* = 3).
1327

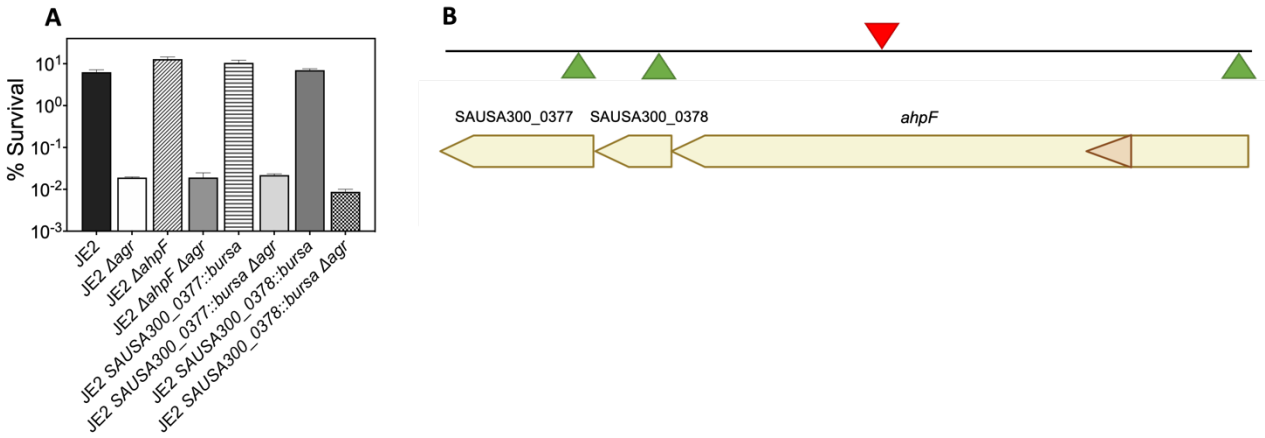
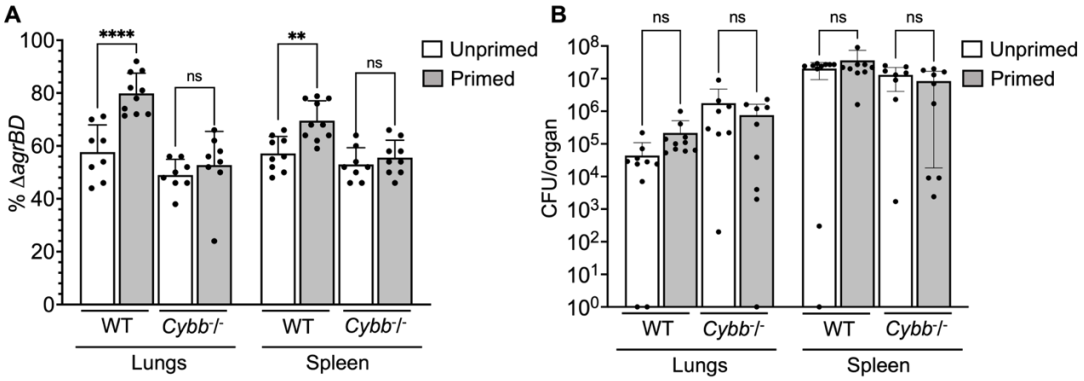


Figure 8—figure supplement 3. Effects of transposon insertion in *ahpC* unexplained by polarity of transposon insertion. (A) Cultures of *S. aureus* wild-type, *Δagr* mutant, and various double mutants were treated with H_2O_2 (20 mM for 60 min) prior to measurement of survival. For strain descriptions, see *SI* Appendix, Table S2. (B) *ahpC* locus map showing the three ORFs located downstream of *ahpC*. The location of four *Bursa aurealis* insertions (NE911, NE1571, NE537, NE725), obtained from The Nebraska Transposon Mutant Library (NTML) (51) used in this study are indicated by triangles. Green triangles, plus-strand insertion; red triangle, minus-strand insertion. Data represent the means ± S.D. from biological replicates (n = 3). Bacterial strains were BS435 for WT and BS1010, BS1494, BS1504, BS1495, BS1501, BS1496 and BS1506 for the *agr*, *ahpF*, SAUSA300_0377, and SAUSA300_0378 mutants, respectively.

Since the *Bursa aurealis* (bursa) transposon insertion in *ahpC* was upstream of several open reading frames (ORFs) in the *ahp*C-F operon, polarity could complicate interpretation of the results. We therefore analyzed the effects of the three bursa mutants in strain JE2 downstream genes: *ahpF*, SAUSA300_0378, SAUSA300_0377. We found that polar effects on downstream elements could not explain the properties of *ahpC*::*bursa*. Thus, *ahpC*::*bursa* could provide insights into the role of *ahpC* in *agr*-mediated phenotypes.

1347

Fig.9.



1348

1349 **Figure 9. Survival advantage of *agr* priming of *S. aureus* absent in phagocyte NADPH-**
1350 **deficient murine infection.** (A) percentage of Δ*agrBD* (AIP-responsive in-frame deletion mutant
1351 carrying an intact RNAIII) cells and (B) bacterial burden in lung or spleen after 2 h of intraperitoneal
1352 infection of wild-type (WT) C57BL/6 mice or phagocyte NADPH oxidase-deficient (*Cybb*$^{-/-}$) mice
1353 (see Figure 9—figure supplement 1 for data with other organs). *ΔagrBD* and *ΔrnaIII* mutant cultures
1354 were grown separately and mixed at a 1:1 ratio either before (primed) or after (unprimed) overnight
1355 growth, as for Figure 3. Both primed and unprimed mixtures were diluted after overnight growth,
1356 grown to early log phase (OD_{600}~0.15), and used as inocula (1 x 10^8 CFU) for intraperitoneal
1357 infection (*n* = 2 groups of 10 mice each). After 2 h, lungs and spleen were harvested and
1358 homogenized; aliquots were diluted and plated to enumerate viable bacteria. Output ratios and total
1359 and mutant CFU from tissue homogenates were determined as for Fig 4E and 4H. A Mann-Whitney
1360 test (panel 9A) or Student's two-tailed *t* test (panel 9B) were used to determine the statistical
1361 significance of the difference between primed and unprimed cultures. Error bars indicate standard
1362 deviation (**$P < 0.01$; ****$P < 0.0001$).

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1364

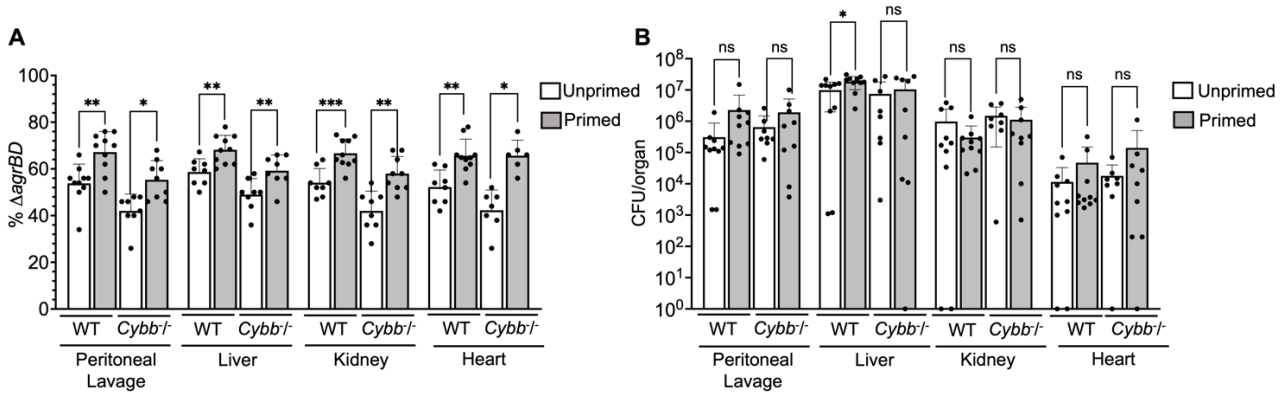
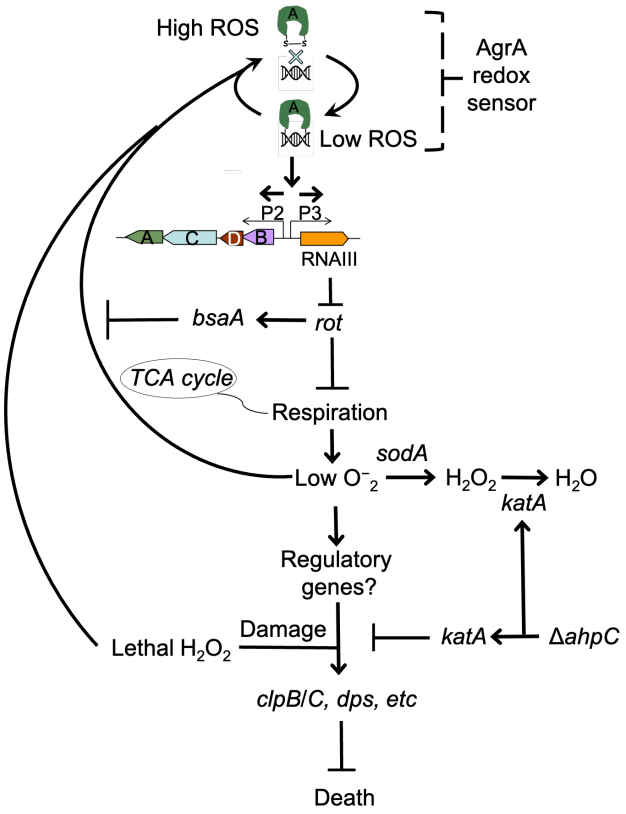


Figure 9—figure supplement 1. Long-lived protection by *agr* increases peritoneal fitness and dissemination to liver, kidney, and heart in both C57BL/6 mice and C57BL/6 *Cybb*$^{-/-}$ (*gp91phox/nox2*) mice. (A) Percentage of Δ*agrBD* or (B) CFU of *S. aureus* RN6734 *ΔrnaIII* (GAW183) and *ΔagrBD* mutant (GAW130) cells in the indicated organ 1 h post intraperitoneal infection of wild-type (WT) C57BL/6 mice or phagocyte NADPH oxidase deficient (*Cybb*$^{-/-}$) mice (see Fig. 9 for data with lung and spleen). Wild-type and mutant strains were grown separately and mixed in a 1:1 ratio either before or after overnight growth, as for Fig. 4. We called wild-type and mutant populations that were mixed prior to or after overnight growth; they were termed "primed" and "unprimed", respectively. Both primed and unprimed mixtures were subsequently diluted, grown to early log phase (OD_{600}~0.15), and used as inoculum for intraperitoneal infection with 1 x 10^8 CFU (*n* = 2 groups of 10 mice each). After 1h, the peritoneum was lavaged and the heart, kidneys, liver, lungs and spleen (Fig. 9) were harvested and homogenized. Samples were then diluted and plated to enumerate viable bacteria. Output ratios and total and mutant CFU from tissue homogenates were determined as for Fig 4E and 4H. A Mann-Whitney test (9A) or Student's two-tailed *t* test (9B) were used to determine the statistical significance of the difference between primed and unprimed cultures. Error bars indicate standard deviation (***P* < 0.05; *****P* < 0.0001).

Long-lived *agr*-mediated functions increased *S. aureus* pathogenesis in both wild-type and mutant mice, indicating a role for long-lived *agr*-mediated functions in pathogenesis other than protection from ROS. Additionally, long-lived *agr*-mediated protection against ROS enhances fitness in lung and spleen (Fig. 9), but it is dispensable for full virulence in other organs; protection is tissue-specific.



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1389

1390 **Figure 10. Schematic representation of *agr*-mediated protection from ROS.** At low levels of
1391 oxidative stress, the redox sensor in AgrA binds to DNA at promoters P2 and P3, activating
1392 expression of the two operons. Expression of RNAIII blocks translation of Rot, which decreases
1393 respiration and production of superoxide. ROS quenchers (*sodA* and *katA*/*ahpC*) suppress
1394 formation of most ROS that would otherwise signal the redox sensor in AgrA to halt stimulation of
1395 RNAIII expression and the production of further superoxide via respiration. This feedback system
1396 regulates respiration thereby limiting the accumulation of ROS in wild-type cells. Wild-type cells are
1397 primed for induction of protective genes (e.g., *clpB/C, dps*) by loss of the *rot* repressor system via
1398 an unknown mechanism when cells experience damage from high levels of oxidative stress
1399 (experimentally introduced as lethal exogenous H_2O_2); *Δagr* cells that experience high levels of
1400 endogenous H_2O_2 fail to induce protective genes. Exogenous H_2O_2 or high levels of endogenous
1401 ROS, for example from extreme stress due to ciprofloxacin (12), lower RNAIII expression and allow
1402 Rot to stimulate *bsaA* expression, which produces a protective antioxidant. The protective action of
1403 an *ahpC* deficiency acts through compensatory expression of *katA*, which results in more effective
1404 scavenging of H_2O_2 produced from increased respiration in Δ*agr* strains and/or exogenous lethal
1405 H_2O_2.

1406

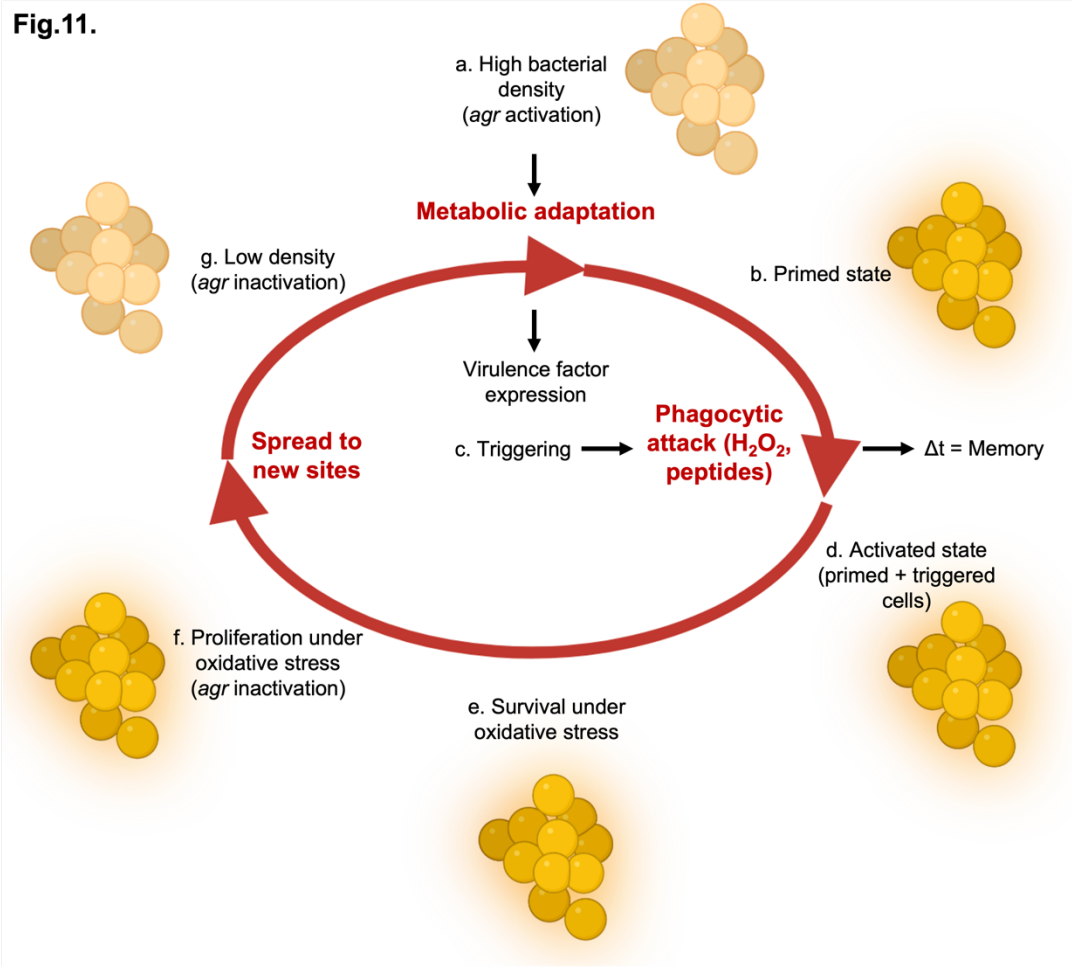

Figure 11. Relationship of *agr* priming and virulence. The ecology of abscess formation and subsequent bacterial dissemination can be described as a cycle. (a) During abscess formation, a hallmark of *S. aureus* disease, *agr* is activated by high bacterial cell density (quorum sensing) (63). (b) The bacterium assumes a primed stage due to repression of the *rot* repressor. (c, d, e) The lethal effects of immune challenge, which is called triggering (64), are survived by the persistence ("memory") of the *agr*-activated state. (f) *agr* expression is inactivated by oxidation, thereby elevating expression of the antioxidant *bsaA* (7), which enables proliferation when oxidative stress is sublethal (7). (g) By surviving damage caused by lethal exogenous oxidative stress, primed *S. aureus* escape from the localized abscess to produce new infectious lesions (bloodstream dissemination) or to infect new hosts, where the cycle would be repeated.

1420 **Table 1. Bacterial strains[a]**
1421

Strain	Background	Relevant Genotype	Reference or Source
BS819	LAC	*agr* group I wild-type (CC8), Erm^S	(65)
BS1348	BS819	*agr::tetM*	(12)
BS820	BS819	*agr::ermC*	(12)
BS821	BS819	*rnaIII::cadA*	(66)
BS12	Newman	*agr* group I wild-type (CC8)	(67)
BS13	BS12	*agr::tetM*	(13)
BS669	BS12	*rnaIII::cadA*	(12)
BS39	BS39 clinical strain	*agr* (+) clinical isolate (CC45)	(68)
BS40	BS40 clinical strain	*agr* (-) clinical isolate (CC45)	(68)
BS867	JE2	*agr* group I wild-type (CC8)	(51)
BS1010	BS867	*agr::cadA* in *S. aureus* JE2	This study
BS1280	BS12	*saeQRS::spec*	(69)
BS1282	BS12	*agr::tetM, saeQRS::spec*	(69)
BS653	*E. coli*	Top10 with pJC1111 (amp^R in *E. coli*; Cd^R in *S. aureus*)	(52)
BS656	RN4220	RN4220 with pRN7023 [shuttle vector (amp^R in *E. coli*; Cm^R in *S. aureus*) containing SaPI1 *int*])	(52)
BS435	RN6734	*agr* group-I prototype strain, derivative of NCTC 8325	(70)
BS688	BS435	*agr*::*cadA*	This study
GAW130	BS435	*agr::cadA*, SaPI1 *att*C::pGAW98 (*agr*-I *ΔagrBD*)	This study
GAW183	BS435	*rnaIII::cad*	This study
BS450	MW2	*agr* group I wild-type (CC1)	(71)
BS451	MW2	*agr::tetM*	This study
BS988	126a	*agr* (+) clinical isolate (CC5)	(68)
BS989	127b	*agr* (-) clinical isolate (CC5)	(68)
BS842	BS819	BS820 with SaPI1-*att*C::*agr*-IpJC1111 (*agr*-I, 8325-4)	(12)
BS1301	BS819	*rot::Tn917*	This study
BS1302	BS819	*agr::tetM, rot::Tn917*	This study
BS1279	BS12	*rot::Tn917*	(69)
BS1281	BS12	*agr::tetM, rot::Tn917*	(69)
VJT14.28	BS12	*pOS1-Plgt-sodARBS-rot*	(69)

BS1486	BS819	*ahpC::bursa* (NE911)	This study, (51)
BS1487	BS819	*agr::tetM, ahpC::bursa*	This study, (51)
BS1488	BS819	*katA::bursa* (NE1366)	This study, (51)
BS1489	BS819	*agr::tetM, katA::bursa*	This study, (51)
BS1399	BS12	*sodA::tetM, sodM::ermC*	(72)
BS1422	BS819	*sodA::tetM*	This study
BS1423	BS819	*agr::ermC, sodA::tetM*	This study
BS1435	BS819	*sigB* clean deletion	(73)
BS1436	BS819	*agr::tetM*, *sigB* clean deletion	(73)
BS1246	BS12	*mgrA::cat*	(74)
BS999	BS819	BS819 with SaPI1 *att*C::pGY*lux* (vector containing promoterless *lux*)	(75)
BS1222	BS819	BS819 with SaPI1 *att*C::P*agr*p3-*lux*	(76)
BS1518	BS12	*agr::tetM*, *mgrA::cat*	This study
BS1527	BS867	*bshC::bursa* (NE230)	(51)
BS1528	BS867	*agr::tetM, bshC::bursa*	This study
BS1522	BS867	*gpxA2::bursa* (NE563)	(51)
BS1523	BS867	*agr::tetM, gpxA2::bursa*	This study
BS1490	BS819	*bsaA::bursa* (NE1730)	This study
BS1491	BS819	*bsaA::bursa, agr::tetM*	This study
BS1707	BS819	BS1422 with SaPI-*att*C::P*sarA*-*sod*RBS-*sodA*	This study
BS1708	BS819	BS1348 with SaPI-*att*C::P*sarA*-*sod*RBS-*sodA*	This study
BS1494	BS867	*ahpF::bursa* (NE1571)	(51)
BS1504	BS867	*agr::tetM, ahpF::bursa*	This study
BS1495	BS867	*SAUSA300_0377::bursa* (NE725)	(51)
BS1501	BS867	*agr::tetM, SAUSA300_0377::bursa*	This study
BS1496	BS867	*SAUSA300_0378::bursa* (NE537)	(51)
BS1502	BS867	*agr::tetM, SAUSA300_0378::bursa*	This study
BS1744	BS819	*acnA::bursa* (NE861)	This study
BS1745	BS1348	*agr::tetM, acnA::bursa*	This study

1422
1423 [a]All bacterial strains are *S. aureus*, unless otherwise indicated. Abbreviations: CC, clonal complex;
1424 NEx, strain designation in the Nebraska Transposon Mutant Library (51).

1425 **Table 2. Oligonucleotides**

1426

#	Name	Gene/Target	Sequence 5'→ 3'	Source
1	pflBRT.1a	*pflB*	AAAAATGGAAGATGGAACAGACAC	(77)
2	pflBRT.1b		TCGATAACTGCATTACTTGTTCC	
3	pflART.1a	*pflA*	TGACAAACATATTAGATTGACAGGAAAGC	(78)
4	pflART.1b		ATCATCAGAATAACCAGGCACAAGG	
5	ldh2RT.1a	*ldh2*	GGATCTGTAGGATCAAGCTATGCC	(42)
6	ldh2RT.2b		TGGTGAAGGACTGTGGACTGTACC	
7	nrdGRT.1a	*nrdG*	CAGTGTTTATGTATCAGGATGTCC	(77)
8	nrdGRT.1b		GTTCGCCACCTAATAGACTTAGCC	
9	qoxB-RT.3A	*qoxB*	GTTGTACTTGGCATGTTCGCC	(79)
10	qoxB-RT.3B		GGCATTATGGTGCATCTTACC	
11	cydA-RT.1A	*cydA*	CATTTCGATACATCTTCCCATGCC	(79)
12	cydA-RT.1B		ATCTGCTAAGAAACTCAATAGTCC	
13	hmp-RT.1A	*hmp*	TGACTTTAGTGAATTTACACCAGG	(79)
14	hmp-RT.1B		CGTTTAACGCCAAAAGTTAAATGG	
15	spaRT1	*spA*	CAAACCTGGTCAAGAACTTGTTGTTG	(80)
16	spaRT2		GCTAATGATAATCCACCAAATACAGTTG	
17	clfB RT1	*clfA*	GGATAGGCAATCATCAAGCACAAG	(80)
18	clfB RT2		GCTATCTACATTCGCACTGTTTGTG	
19	ahp RT For	*ahpC*	CGTAAAAACCCTGGCGAAGTAT	(81)
20	ahp RT Rev		TGCAATGTTTTAGCGCCTTCT	
21	kat RT For	*katA*	TGGTGTTTTTGGGCATCCA	(6)
22	kat RT Rev		CCCTAGGCCCTGCTGTCATA	
23	rpoB F	*rpoB*	GAACATGCAACGTCAAGCAG	(82)
24	rpoB R		AATAGCCGCACCAGAATCAC	

25	MPsodA#1	*sodA*	AGGCGCGCCTTTATTTTGTTGCATTATATAATTCGTCAACTTTTTCCCAG	This study
26	MPsodA#2		GGATGATTATTTATGGCTTTTGAATTACCAAAATTACCATACGC	This study
27	MPsodA#3	*PsarA*	TTCAAAAGCCATAAATAATCATCCTCCTAAGGTACCCGG	This study
28	MPsodA#4		GCGGCCGCTCTGATATTTTTGACTAAACCAAATGCTAACCCAG	This study
29	MPsodA#5	pJC1111	AAAATATCAGAGCGGCCGCCAG	This study
30	MPsodA#6		ACAAAATAAAGGCGCGCCTATTCTAAATG	This study
31	pJC1111 FOR	pJC1111-P*sarA*-*sodRBS*-*sodA*	TGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTC	This study
32	pJC1111 REV		TGATATCAAAATTATACATGTCAACG	This study
33	GWO#27	*agrBD*	CAATTTTACACCACTCTCCTCACTGTCATTATACGATTTAG	This study
34	GWO#28		TAATTTAAATAGAGAGTGTGATAGTAGGTGGAATTATTAAATAG	This study
35	JCO#339	*agr* flanking regions	GGTACCTGAAGCGGGCGAGCGAG	This study
36	JCO#340		GGATCCGATAATAAAGTCAGTTAACGACGTATTCAATTGTAAATCTTGTTGG	This study
37	JCO#342		CTCGAGAAGAAGGGATGAGTTAATCATCATTATGAGAC	This study
38	JCO#343		GCATGCGATCTATCAAGGATGTGATGTTATGAAAGTCCAAATTTATCAATTACCG	This study

1427